Springer

Handbook of Marine Biotechnology

Kim

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Springer Handbook of Marine Biotechnology

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List of Abbreviations

Symbols

β-HB	β-hydroxy butyrate
γ-PGA	γ-polyglutamic acid
ω-3	Omega-3 fatty acid
^{1}H NMR	proton nuclear magnetic resonance
(HPLC–DAD)	high pressure liquid chromatography with diode array detection
L-DOPA	L-3,4-dihydroxyphenylalanine
1-D	one-dimensional
12-DS	12-doxylstearic acid
19HF	19′-hexanoyloxy-fucoxanthin
2-D	two-dimensional
2D-DIGE	two-dimensional difference in-gel electrophoresis
3-PGA	3-phosphoglyceric acid
4-DSC	four-disulfide core
8-HPETE	(8*R*)-hydroperoxyeicosa-5,9,11,14-tetraenoic acid

A

A549	human alveolar epithelial cells
A549	human lung adenocarcinoma
AA	arachidonic acid
aa	amino acid
AAPB	aerobic anoxygenic photosynthetic bacteria
AAPH	2,2′-azobis(2-amidino-propane) dihydrochloride
AAR	acyl–acyl carrier protein reductase
ABAP	2,2′-azo-bis-2-amidinopropane
ABC	ATP-binding cassette
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AC	alternating current
ACCase	acetyl-CoA carboxylase
ACE	angiotensin-I-converting enzyme
ACE	angiotensin-converting enzyme
AChE	acetylcholinesterase
ACP	acyl carrier protein
ACP	acyl–acyl carrier protein
ADBAC	alkyldimethylbenzylammonium chloride
ADHD	attention-deficit hyperactivity disorder
ADO	aldehyde-deformylating oxygenase
AF	antifouling
AfDD	acriflavine direct detection
AFLP	amplified fragment length polymorphism
AFM	atomic force microscope
AGC	arginine-graft-chitosan
AgNP	silver (argentum) nanoparticle
AGS	gastric adenocarcinoma
AGS	human gastric cancer cell
AHA	α-hydroxy acids
AHL	acyl-homoserine lactone
AHRE	aromatic hydrocarbon response element
AIDS	acquired immunodeficiency syndrome
AKG	α-ketoglutarate
Akt	nuclear phosphatidylinositol 3,4,5-triphosphate phosphotidyinositol 3-kinase
AL	airlift
ALA	α-linolenic acid
ALBR	airlift bioreactor
ALCL	anaplastic large cell lymphoma
AMD	acid mine drainage
AMIS	anaerobic methane incubation system
amoA	α-subunit
amoA	ammonia-oxidation
AmoB	ammonia mono-oxygenase membrane bound subunit β
AMOP	aquatic microbial oxygenic photoautotroph
AMP	antimicrobial peptide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMS	air membrane surface
anammox	anaerobic ammonium-oxidizing
AnAPB	anaerobic anoxygenic photosynthetic bacteria
AnMBR	anaerobic membrane bioreactor
ANME	anaerobic methanotrophic archaea
anti-MRSA	anti-methicillin-resistant *Staphylococcus aureus*
AOA	ammonia-oxidizing archaea
AOB	ammonia oxidizing bioreactor
AOM	alginate oligosaccharide mixture
AOM	anaerobic oxidation of methane
APTT	activated partial thrombinplastin time
aPTT	activated partial thromboplastin time

ARA	arachidonic acid
Ara-A	arabinofuranosyl adenine or adenine arabinoside
Ara-A	vidarabine
Ara-C	cytarabine
Ara-C	arebinosyl cytosine
ARfD	acute reference doses
ARP	ankyrin repeat protein
ASAP	automated simultaneous analysis phylogenetics
ASC	acid-soluble collagen
ASP	amnesic shellfish poisoning
ASW	artificial seawater
AT	acyltransferase
ATCC	American Type Culture Collection
ATN	autotrophic nitrifier
ATP	adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
AttM	lactonase of *A. tumefaciens*
Au-CNT	gold-coated carbon nanotubes
AuNP	gold (aurum) nanoparticle
AZA	azaspiracid
AZP	azaspiracid shellfish poisoning

B

BAC	bacterial artificial chromosome
BALB/c	albino, laboratory-bred strain of the house mouse
BaP	benzo[*a*]pyrene
BAPD	bis(*p*-aminophenoxy)-dimethylsilane
BC	bubble column
BCBR	bubble column bioreactor
BChl	bacteriochlorophyll
BD	biofilm density
BDL	beyond detectable limit
BDS	bunodosine
BE	bioemulsifer
BF	biofouling
BFR	biofilm reactor
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BLAST	basic local alignment search tool
BMNV	baculoviral midgut gland necrosis virus
BMP	bone morphogenetic protein
BMSC	bone marrow stomal cell
BNR	biological nitrate removal
BP	*Baculoviruspenaei*
BPA	bisphenol A
BPR	back pressure regulator
BPS	back-pressure-system
BS	biosurfactant
Bs	*Bacillus subtilis*
BSA	bovine serum albumin
BSD	blasticidin
BSP	sialoprotein
BTX	brevetoxin
BTX	square wave generator
bvPLA	bee venom, phospholipase AZ

C

C-CTX	Caribbean-CTX
C-g-PEI	chitosan-graft-PEI
C-PE	C-phycoerythrin
C12-TE	*Cinnamomum camphora*
C14-TE	*Umbellularia californica*
Ca-SP	calcium spirulan
CA4	combretastatin
Ca9-22	human gingival carcinoma
CAF	chlorophyll autofluorescence
CAMERA	community cyber infrastructure for advanced marine microbial ecology research and analysis
cAMP	cyclic adenosine monophosphate
Campto	camptothecin
CARD	catalyzed reporter deposition
CARMA	characterizing short read metagenome
CAS	CRISPR-associated
CAS	chemical abstracts service
CAS	chrome azurol S
CAT	chloramphenicol acetyltransferase
CC	column chromatography
CCCP	carbonylcyanide *m*-chlorophenylhydrazone
CCF	conico-cylindrical flask
CCI	chronic constriction injury
CCRF-CEM	human T-cell lymphoblast-like cell line
CCRF-CEM	human Caucasian acute lymphoblastic leukaemia
CD	compact disc
CD	circular dichroism
CD	cluster of differentiation
Cd	cadmium
cDNA	copy DNA
cDNA	complementary DNA
CdO	cadmium oxide
CdS	cadmium sulfide
CEB	cell entrapping bead

CEME	cecropin-medilitin hybrid peptide
CES	collagen and elastin sponge
CF	cystic fibrosis
CFE	cell-free extracts
CFP	ciguatera fish poisoning
Cg-Def	oyster defensin
CGC	cerebellar granule cell
CGN	cerebellar granule neuron
CGRP	calcitonin gene-related peptide
CGS	chitosan-graft-spermine
CH	carbohydrate
CHA	carbonated hydroxyapatite
CharProtDB	Characterized Protein Database
ChAT	choline acetyltransferase
CHD	coronary heart disease
CHL	chlorophyll
CHO	carbohydrate
CHO	Chinese hamster ovary
ChOx	cholesterol oxidase
CI	cyclic imines
CI	cyclic imine
CL	chemiluminescence
CLP	chitosan-linked-PEI
CMC	critical micelle concentration
CMFRI	Central Marine Fisheries Research Institute
CML	chronic myeloid leukemia
CMV	cytomegalovirus mosaic virus
CNN	cloud condensation nuclei
CNS	central nervous system (CNS)
CNS	central nervous system
CO_2-TR	CO_2 transfer rate
CoA	coenzyme A
COC	cyclic olefin co-polymer
COC1	human ovarian adenocarcinoma cisplatin-sensitive cell
COG	Clusters of Orthologous Group
COG0610	restriction enzymes, type I helicase
COG1715	restriction endonuclease
CoML	Census of Marine Life
ConA	concanvalin A
COS	chitooligosaccharide
COS	chitosan oligosaccharides
COS	chitosan chito-oligosaccharide
COSY	correlation spectroscopy
COX	cyclooxygenase
COX-2	cyclooxygenase-2
CPBR	column-type PBR
CPCD	chitosan-graft-PEI-β-cyclodextrin
CPG	consumer packaged good
CPP	cell-penetrating peptide
CPS	capsular polysaccharide
CPU	central processing unit
CR	complement receptor
Cr	chromium
CRISPR	clustered regularly interspersed short palindromic repeat
CRISPR	clustered regularly interspaced short palindromic repeat
CS	chondroitin sulfate
CS-F	Fucosylated CS
CSIR	Council of Scientific Industrial Research
CSMCRI	Central Salt and Marine Chemicals Research Institute
CSP	cell surface protein
CT	computer tomography
CT	collection tank
CTT	conventional thermal treatment
CTX	ciguatoxin
CTX	cholera toxin
CV	cardiovascular
CV	check valve
CyDyes	cyanine dyes
Cys	cysteine
Cytosar-U1	Cytarabine

D

DA	domoic acid
DAE	Diethylaminoethyl
DAG	diacylglycerol
DAN	diaminonaphthalene
DAP	dorsal arm plate
DB	digestion basin
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
DD	degree of deacetylation
DD	decadienal
DEAE	diethylaminoethyl cellulose
delta-DOPA	decarboxy (E)-alpha,beta-dehydro-3,4-dihydroxyphenylalanine
DENV	dengue virus
DF1	fraction 1
DGGE	denaturing gradient gel electrophoresis
DH	dehydratase
DHA	docosahexaenoic acid
DIG	digoxigenin 11-dUTP
DIN	dissolved inorganic nitrogen
DIPS	diffusion induced phase separation
DLD	dihydrolipoamide dehydrogenase

DLS	dynamic light scattering
DMAPP	dimethylallyl pyrophosphate
DMF	dimethylformamide
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DMPO-OH	DMPO-hydroxyl
DMRB	dissimilatory metal reducing bacteria
DMSO	dimethyl sulfoxide
DMXBA	3-(2,4-dimethoxybenzylidene)-anabaseine
DN-BPR	denitrifying biological phosphorus removal
DNA	deoxyribonucleic acid
DNAML	DNA maximum likelihood
DNaseI	deoxyribonuclease
DO	dissolved oxygen
DOC	dissolved organic carbon
DON	dissolved organic nitrogen
DOPA	dihydroxy-L-phenylalanine
DOTUR	defining operational taxonomic units and estimating species richness
DOX	doxorubicin
DP	degree of polymerization
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPPH	2,2-diphenyl-1-picrylhydrazyl
DS	dermatan sulfate
dsDNA	double-stranded DNA
DSLSS	disodium lauryl sulfosuccinate
DSP	diarrhetic shellfish poisoning
DspB	Dispersin B
dsRNA	double-stranded RNA
DTP	diethylenetriamine pentaacetic acid
DTX	dinophysis toxin
DVChl	divinyl-chlorophyll
DVD	digital video disc
DXP	1-deoxy-xylulose-5-phosphate

E

E7389	eribulin mesylate
EBPR	enhanced biological phosphorus removal
ECD	electronic circular dichroism
ECM	extracellular matrix
ECP	extracellular product
ED	electrodialysis
EDC	endocrine disrupting compound
eDNA	environmental DNA
EDP	Entner–Doudoroff pathway
EDS	energy dispersive spectra
EDS	energy dispersive spectroscopy
EDTA	ethylenediaminetetraacetic acid
EDX	energy dispersive X-ray spectroscopy
ee	enantiomeric excess
EF	Erlenmeyer flask
EFC	efficiency of feed conversion
EFSA	European Food Safety Authority
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EMEA	European Medicines Agency
EMEA	European Agency for the Evaluation of Medicinal Products
EMP	Embden–Meyerhof pathway
EMP	Earth Microbiome Project
EPA	environmental protection agency
EPA	eicosapentaenoic acid
epi-DA	epi-domoic acid
EPRE	electrophile response element
EPS	extracellular polysaccharide
EPS	exopolysaccharide
ER	enoyl reductase
ESCRT	endosomal sorting complexes required for transport
ESI	electrospray ionization
ESI-MS	electrospray ionization mass spectrometry
ESR	electron spin resonance
ESSV	extended surface shaken vessel
EST	express sequence tag
ET-743	ecteinascidins
EtOAc	ethyl acetate
ETS	external transcribed spacer
EU	European Union

F

FA	formaldehyde
FA	fatty acid
FACS	fluorescence activated cell sorting
FAEE	fatty acid-ethyl-ester
FAME	fatty acid methyl ester
FAO	Food and Agriculture Organization
FAP	flat alveolar panel
FAP-PBR	flat alveolar panel PBR
FBBR	fluidized bed bioreactor
FcεRI	Fc region of immunoglobulin E
FCA	ferrous ion chelating ability
FCB	free-cell photobioreactor
FCC	face centred cubic
FCF	food conversion factor

FCR	food conversion ratio
FDA	Food and Drug Administration
FD&C	Food, Drug, and Cosmetic Act
FETAX	frog embryo teratogenesis assay-xenopus
FF	functional feed
FISH	fluorescence in situ hybridization
FM	fish meal
FO	fiber optic probe
FOS	fucooligosaccharide
FOSHU	food for specified health uses
FOSMID	F1 origin-based cosmid vector
FPH	fish protein hydrolyzate
FPT	farnesyl protein transferase
FP&LA	Fair Packaging and Labeling Act
FR	fouling-release
FRAP	ferric reducing antioxidant power
FRAP	ferric ion reducing antioxidant power
FRET	fluorescent resonance energy
FRET	fluorescence resonance energy transfer
FTIR	Fourier transform infrared spectroscopy
FXR	farnesoid X-activated receptor

G

G	guluronate
G	guluronic acid
G-CSF	granulocyte colony-stimulating factor
g.b.f.	glass-ball filter
G3–G9	trimer to nonamer of G
GA	glutaraldehyde
GABA	γ-amino-butyric acid
GAG	glycosaminoglycan
GAO	glycogen accumulating organism
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAV	gill-associated virus
GB	glove box
GC	galactosylated chitosan
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography-mass spectrometry
GC-TOF-MS	chromatography-time-of-flight mass spectrometry
GCL	glutamate cysteine ligase
GCS	GC-g-spermine
Gd	gadolinium
gDNA	genomic DNA
GDSL	amino acid motif specific to the esterases
GDSLS	amino acid residues considered as an important motif in the EstHE1 esterase
GEBA	Genomic Encyclopedia of Bacteria and Archaea
GERD	gastroesophageal reflux disease
GFP	expression of signal molecule
GFP	green fluorescent protein
GG	homopolymeric blocks in alginates
GH	glycoside hydrolase
GH	growth hormone
GHF	glycoside hydrolase family
GHG	greenhouse gas
GI	gastrointestinal
GI_{50}	growth inhibition
GLA	γ-linolenic acid
GlcNAc	*N*-acetylglucosamine
GLDC	glutamate decarboxylase
Glu	glutamic acid
Glu-Plg	glutamic plasminogen
Glu-tag	octaglutamic acid-tag
GM	alternating blocks in alginates
GM	genetically modified
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMO	genetically modified organism
GMP	good manufacturing practices
GNO	hydrated gallium nitrate
GOD	glucose oxidase
GOLD	Genomes OnLine Database
GoM	Gulf of Mannar
GoMBR	Gulf of Mannar Marine Biosphere Reserve
GORD	gastroesophageal reflux disease
GOS	Global Ocean Sampling
GPC	gel permeation chromatography
GPCS	galactosylated PEG-chitosan-graft-spermine
GPDH	glycerol-3-phosphate dehydrogenase
GPx	glutathione peroxidase
GRAS	generally recognized as safe
GRPSp	gastrin-releasing peptide, Scylla paramamosain
GSH	glutathione
GST	genome sequence tag
GT	glycosyltransferase
GTA	glycidyl trimethyl ammonium
GulA	α-D-guluronic acid
GWP	green wall panel
GWP-PBR	green wall panel PBR
GYM	gymnodimine
GYM	glucose yeast extract and malt

H

HA	hydroxyapatite
HA	hyaluronic acid
HAB	harmful algal bloom
hADSC	human adipose-derived stem cell
HAP	hydroxyapatite
HBME	human brain microvascular endothelial
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCN	hydrogen cyanide
HCT	human colorectal tumor
HCT116	human colorectal carcinoma cell line
HCV	hepatitis C virus
HDAC	histone deacetylase
HDL	high density lipoprotein
He	helium
HeLa	human cervix carcinoma cells
HeLa	Henrietta Lacks
Hep3B	human liver carcinoma
HepG2	hepatoma cell line
HepG2	Hepatoma Growth2
HF	hydrofluoric
HF	hollow fiber
HF-MBR	hollow-fiber membrane bioreactor
HF-MFBR	hollow-fiber microfiltration bioreactor
HF-sMBR	hollow-fiber submerged MBR
HFAR	hybrid flow-through anaerobic reactor
HFBR	hollow-fiber bioreactor
HGF	hepatocyte growth factor
hGH1	human growth hormone 1
His-tag	hexahistidine-tag
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus
HJ	hydrogen supply
HLE	human leukocyte elastase
hli	high light inducible gene
HM	high methoxy
HMT	histone methyltransferases
HNE	human neutrophil elastase
HP-CI	high-pressure continuous incubation system
HP-MI	high-pressure manifold incubation system
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
HPBBR	high-pressure batch bioreactor
HPBR	high-pressure bioreactor
HPHT	high-pressure/high-temperature
HPHTBR	high-pressure/high-temperature bioreactor
HPLC	high-performance liquid chromatography
HPLC-DAD	high-performance liquid chromatography with diode-array detection
HPTGS	high-pressure thermal gradient system
HPU	high-pressure unit
HPV	hepatopancreatic parvovirus
HPX	hypoxanthine
HR-SEM	high-resolution scanning electron microscopy
HRM	high resolution melting
HRMS	High Resolution Mass Spectrometry
HRP	horseradish peroxidase
HRT	hydraulic retention time
HRTEM	high-resolution transmission electron microscope
HS	heparin sulfate
HS	high-solid
HS	heparan sulfate
HSC70	heat-shock cognate protein 70
HSDH4	17β-hydroxysteroid dehydrogenase type 4
HSV	herpes simplex virus
HT-29	human colon adenocarcinoma
HTHPBR	high-temperature, high-pressure bioreactor
HTLV	T-cell leukaemia virus
HTS	high throughput screening
HUVEC	human umbilical vein endothelial cells

I

I-CTX	Indian-CTX
i.p.	intraperitoneal
I/O	inlet/outlet
IAA	imidazole acetic acid
IAAC	IAA-coupled chitosan
IAC	internal amplification control
IAEA	International Atomic Energy Agency
IAST	ideal adsorbed solution
IBPCS	integrated biomass production conversion system
ICC	intrahepatic cholangiocarcinoma
Ichip	isolation chip
ICP	ion concentration polarization
ICP-MS	inductively coupled plasma mass spectrometry
ICR	imprinting control region
IDO	indoleamine 2,3-dioxygenase

IEMBR	ion exchange membrane bioreactor
IgE	immunoglobulin E
IGF-IR	insulin-like growth factor-I receptor
IgG	immunoglobulin G
IgM	immunoglobulin M
IGR	insect growth regulator
IGS	intergenic spacer
IHHNV	infectious hypodermal and hematopoietic necrosis virus
IHNV	infectious hematopoietic necrosis virus
IISA	integrated in situ analyzer
IISA-Mn	integrated in situ analyzer for Mn
IKK	inhibitor of nuclear factor κ-B kinase subunit beta
IL	ionic liquid
IL	interleukin
IL-6	interleukin-6
IMG	Integrated Microbial Genomes
IMG/M	Integrated Microbial Genomes and Metagenomes System
IMNV	infectious myonecrosis virus
IMO	International Maritime Organization
IMPDH	inosine 5′-monophosphate dehydrogenase
INCI	International Nomenclature of Cosmetic Ingredients
INF	interferon
INF-γ	interferon-γ
InHA	immune inhibitor A precursor
iNOS	inducible nitric oxide synthase
IPCP	integrated pest control programs
IPG	immobilized pH gradient
IPNV	infectious pancreatic necrosis virus
IPP	isopentyl pyrophosphate
IR	Infrared Spectroscopy
IR	infrared
iRFP	near-infrared fluorescent protein
ISH	in situ hybridization
iso-DA A–H	isodomoic acids A, B, C, D, E, F, G, and H
IT	information technology
iTRAQ	isobaric tags relative absolute quantification
ITS	internal transcribed spacer
IUB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
IV	intravenous injection
IV1	injection vessel
IV2	injection vessel

J

JNK	c-Jun N-terminal kinase

K

K562	human chronic myeloid leukemia cells
KB	human epidermoid carcinoma
KC	keratinocyte-derived chemokine
KF	Kahalalide F
KGF	keratinocyte growth factor
KM	kinetic modeling
KR	ketoreductase
KS	ketosynthase
KS	ketoacyl synthase
KSa-KSb-ACP	ketosynthase alpha subunit-ketosyntahase beta subunit-acyl carrier protein
KSR1	kinase suppressor of Ras1

L

L/D	light/dark
LasR	transcriptional activator
LB	Luria-Bertani broth
LB	Luria Bertani
LC	liquid chromatography
LC_{50}	lethal concentration
LC-ESI-MS	liquid chromatography electrospray ionization mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LCA	life cycle
LCB	lactophenol cotton blue
LCFA	long-chain fatty acid
LD_{50}	lethal dose 50
LDL	low-density lipid
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LDPE	low-density polyethylene
LFB	limited filamentous bulking
LHCII	light-harvesting chlorophyll protein complex apoproteins associated with photosystem II
LM	low methoxy
LMA	low methoxy amidated
LN	lymphocytes
LOC	lab-on-a-chip
LOD	limit of detection
LoVo	human colon adenocarcinoma
LOVV	lymphoid organ vacuolization virus

LPS	lipopolysaccharide
LPV	lymphoidalparvo-like virus
LRT	larval rearing tank
LS	low-solid
LS	light source
LSNV	Laem–Singh virus
LSU	long sub-unit
LT	lipophilic toxin
LTA	lipoteichoic acid
LTR	long terminal repeat
LuxR	transcriptional activator
Lys	lysine

M

M	mannuronate
M	mitosis
M	mannuronic acid
M-PBR	membrane photobioreactor
M/G	molar ratio between mannuronic and glucuronic acids in alginates
M3–M9	trimer to nonamer of M
MA	maslinic acid
MAA	mycosporine-like amino acid
mAb	monoclonal antibodies
MABV	marine birnavirus
MAE	microwave-assisted extractions
MALDI	mass spectrometry using assisted laser desorption ionization
ManA	β-D-mannuronic acid
MAP	mussel adhesive protein
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAR	microautorardiography
Mb	met-myoglobin
MBA	mouse bioassay
MBBR	moving bed bioreactor
MBC	minimum bactericidal concentration
Mbp	million base pairs
MBR	membrane bioreactor
MBT	magnetotactic bacteria
MBV	monodon baculovirus
MCF	Michigan Cancer Foundation-7
MCF7	breast carcinoma cell
MCF7	human breast cancer cell line
MCH	melanin-concentrating hormone
MCP	monocyte chemoattractant protein
MCP	marine collagen peptide
MCP	monocyte chemotactic protein
MCP-1	monocyte chemoattractant protein-1
MDA	multiple displacement amplification
MDA-MB-231	human breast carcinoma
MDD	major depressive disorder
MDR	multi-drug resistant
MEGAN	meta genome analyzer
MegDB	Microbial Ecological Genomics DataBase
MeHg	methylmercury
MeHgCy	MeHg-cysteine
MEL-28	human melanoma cell
MEMS	microelectromechanical system
MEMS/NEMS	micro- and nanoelectromechanical systems
MetaBioME	Metagenomic BioMining Engine
MF	microfiltration
MG	heteropolymers of mannuronate and guluronate
MG	alternating blocks in alginates
MG-RAST	meta genomics rapid annotation using subsystem technology
MG-RAST	meta genomics rapid annotation using subsystems technology
MGB	minor groove binder
MHDS	multihead deposition system
MIC	minimum inhibitory concentration
MicroScope	microbial genome annotation & analysis platform
MIP	molecularly imprinted polymer
MIP	macrophage inflammatory protein
MISS	microbially induced sedimentary structures
MLVSS	mixed liquor volatile suspended solids
MM	homopolymeric blocks in alginates
MMAE	monomethyl auristatin E
MMP-9	metalloproteinase-9
MMP2	matrix metalloproteinase 2
MNP	marine natural product
MO	morpholino antisense oligo
MO	morpholino phosphorodiamidate oligonucleotide
MOLT	molybdate uptake transporter
MoMLV	Moloney murine leukemia virus
Mon	manganese
MoV	Mourilyan virus
Mp-TX	*Millepora platyphylla*
MPA	mycophenolic acid
MPBR	membrane photobioreactor
MPL	maximum permitted level
MRBC	Modified roller bottle cultivation
MRE	metal response element
MRI	magnetic resonance imaging

mRNA	messenger RNA
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MS	mass spectroscopy
MS^n	de novo tandem MS
MS/MS	tandem mass spectrometry
MSA	multiple sequence alignment
MSC	mesenchymal stromal cell
MSC	mesenchymal stem cell
MSSA	methicillin-susceptible *Staphylococcus aureus*
MT	shrimp maturation tank
MT	metallothionein
MT	metric ton
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTT	microculture tetrazolium assay
MTX	maitotoxin
MTZ	mass transfer zone
MUF-diNAG	4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside
MUFA	monounsaturated fatty acid
MurNAc	*N*-acetylmuramic acid
MVL	mevalonate
MW	molecular weight
MW	microwave
MWT	microwave treatment

N

NADH	nicotiamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nanoSIMS	nanometer-scale secondary-ion mass spectrometry
NaPDoS	natural product domain seeker
NASBA	nucleic acid sequence-based amplification
NB-L	nitrobenzene with linoleic acid
NBC	nitrifying bacterial consortia
NCBI	National Center for Biotechnology Information
NCE	new chemical entity
NCI	human tumor cell line
NCI	National Cancer Institute
NCI-H460	human lung cancer cell line
NCN-Pt	platinum-dimethylamino pincer complex
NDGA	nordihydroguaiaretic acid
NER	nucleotide excision repair
NF	nuclear factor
NF-κB	nuclear factor-κB
NFT	neurofibrillary tangles
NGS	next generation sequencing
NHase	Nitrile hydratase
NHE	normal hydrogen reference electrode
NHP	*N*-(2-hydroxyphenyl)-2-phenazinamine
Ni	nickel
Ni-NTA	nickel nitrilotriacetic acid
NICD	Notch intracellular domain
NIO	National Institute of Oceanography
nirS	nitrite reductase
nirS	nitrification gene
NK	natural killer
NLase	Nitrilase
NMC-g-PEI	*N*-maleated chitosan-graft-PEI
NMDA	*N*-metyl-D-aspartic acid
NMNP	new marine natural product
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
NO	nitric oxide
NOAA	National Oceanic and Atmospheric Administration
NOB	nitrite-oxidizing bioreactor
NOESY	nuclear Overhauser and exchange spectroscopy
NP	nanoparticle
NP	nonylphenol mixture
NPU	net protein utilization
NRPS	nonribosomal peptide synthase
NRPS	nonribosomal peptide synthetase
NS	neutral sugar
NS2	nonstructural protein-2
NSC-g-PEI	*N*-succinyl chitosan-graft-PEI
NSCLC	non-small cell lung cancer
NSOM	near field scanning optical microscopy
NSP	non-starch polysaccharide
NSP	neurotoxic shellfish poisoning
NTA	nitrilotriacetic acid
NTC	no template controls
NTG	*N*-methyl-*N*′-nitro-*N*-nitroso guanidine
NTS	nontranscribed spacer
NVSCC	N-type voltage-sensitive calcium channel

O

OA	okadaic acid
OA	ocean acidification

OECD	Organization for Economic Cooperation and Development
OHT	overhead tank
OLED	organic light emitting diode
OmpA	outer membrane protein A
OP	organophosphorus
OPCW	Organization for the Prohibition of Chemical Weapons
OPD	organic photodetector
OPF	orange fluorescent protein
OPG	osteoprotegerin
OPH	organophosphorus hydrolase
ORAC	oxygen radical absorbance capacity
ORF	open reading frame
OTAB	octyl-trimethyl ammonium bromide
OTC	over-the-counter

P

P-388	mouse lymphocytic leukemia
P-CTX	Pacific-CTX
PAA	peak antibiotic activity
PAAR	peak activity attainment rate
pAb	polyclonal antibodies
PAFP	photoactivatable fluorescent protein
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PAMA	peak antimicrobial activity
PAMAM	poly(amido amine)
PAN	plane polyacrylonitrile
PAO	phosphorus accumulating organism
PAPS	3′-phosphoadenosine 5′-phosphosulfate
PAR	photosynthetically active radiation
PB-PBR	packed bed photobioreactor
PBBR	packed bed bioreactor
PBEL-ALBR	packed bed external loop airlift bioreactor
PBMC	peripheral blood mononuclear cells
PbNP	lead (plumbum) nanoparticle
PBR	photobioreactor
PBS	phosphate buffered saline
PbTx	brevetoxin
PC	phycocyanin
PCA	principal component analysis
PCD4	programmed cell death 4
PCL	polycaprolactone
PCNA	proliferative cell nuclear antigen
PCP	PEG-graft-chitosan-graft-PEI
PCP-Pd	paradium-diphenylphosphino pincer complex
PCPA	PEG-graft-chitosan-graft-polyarginine
PCR	polymerase chain reaction
PDMS	polydimetylsiloxane
PE	phycoerythrin
PEEK	poly-ether-ether-ketone
PEG	poly(ethylene glycol)
PEI	polyethylene imine
PEI-g-C	PEI-graft-chitosan
PEI-g-SAC	PEI-conjugated stearic acid-graft chitosan
PEP-C	phosphoenolpyruvate carboxylase
PER	protein efficiency ratio
PES	protein expression signature
PET	photoinduced electron transfer
Pfu DNA	thermostable DNA polymerase named after the thermophilic bacterium *Pyrococcus furiosus*
PG	homopolymer of guluronate
PG	propyl gallate
PG	pressure gauge
PGE2	prostaglandin E_2
PGM	personal genome machine
PHA	poly-β-hydoxyalkanoate
PHA	polyhydroxyalkanoate
PHPA	*p*-hydroxyphenyl acetic acid
pI	isoelectric point
PIA	polysaccharide intercellular adhesin
PIA	pyrrole-imidazole alkaloid
PK	polyketide synthase
PKC	protein kinase C
PKD	polycystic kidney domain-like
PKS	polyketide synthase
PLA	polylactide
PLGA/PCL	poly(D,L-lactide-co-glycolide)/polycaprolactone
PlTX	palytoxin
PM	homopolymer of mannuronate
PM	petrosaspongiolide M
PMMA	poly(methyl methacrylate)
PMSF	phenylmethylsulfonyl fluoride
PMT	photomultiplier
PMT	photomultiplier tube
PNAG	poly-*N*-acetyl glucosamine polysaccharide
PnTX	pinnatoxin
PNV	pneumatic valve
POC	particulate organic carbon
POM	poly-oxy-methylene
PON1	paraoxonase
PP	protein phosphatase
PP	piston pump

PP9	perfluoromethyldecalin
PPC	proparacaine
PPE	poly(*para*-phenylene ethynlyene)
ppm	parts per million
PPO	polyphenol oxidases
PPP	Pentose phosphate pathway
ppp-3	palmitoyl pentapeptide-3
PR	proteorhodopsin
Prialt1	ziconotide
ProLuCID	name of a tandem mass spectrabased protein identification program
PRRSV	porcine reproductive and respiratory syndrome virus
PS	polysaccharide
PSC	pepsin-solubilized collagen
PSF	polysulfone
PSI	photosystem I
PSII	photosystem II
PSP	paralytic shellfish poisoning
PST	paralytic shellfish poisoning toxin
PT	prothrombin time
PT	pressure transducer
PTFE	polytetrafluoroethylene
PtOEP	Pt(II)-octaethylporphin
PTP1B	protein tyrosine phosphatase 1B
PtTX	pteriatoxin
PTX	pectenotoxin
PTX	palytoxin
PTX2SA	PTX2 seco acid
PU	polyurethane
PU-g-SP	pullulan-graft-spermine
PUFA	polyunsaturated fatty acid
PV	polyvinyl
PVA	polyvinyl alcohol
PVC	polyvinylchloride
PVDF	polyvinylidene fluoride

Q

QCM-D	quartz crystal microbalance dissipation
qPCR	quantitative PCR
QQ	Quorum Quenching
QS	quorum sensing
QSI	quorum sensing inhibitor

R

r-silicatein	recombinant silicatein
RACE-PCR	rapid amplification of cDNA ends-PCR
RANK	receptor activator of NF-κB
RANKL	ligand of the receptor activator of NF-κB
RANTES	regulated upon activation, normal T-cell expressed and secreted
RAP	RNA III activating protein
RAPD	random amplified polymorphic DNA
RAS	recirculating aquaculture system
RAST	rapid annotation using subsystem technology
RAW	ATCC cell line
RB	Remazol Black B
RBA	Receptor Binding Assay
RBBR	Remazol Brilliant Blue R
RBC	human red blood cell
RBC	rotary biological contactor
RBE	rat brain endothelial
RDBR	rotating disk bioreactor
rDNA	ribosomal DNA
RDP	Ribosomal Database Project
RFLP	restriction fragment length polymorphism
RFP	red fluorescent protein
RGD	arginylglycylaspartic acid
RGY	Remazol Golden Yellow
RHR	resting heart rate
RIA	radioimmunoassay
RIP	RNA III-inhibiting peptide
RiPP	ribosomally synthesized and post-translationally modified peptide
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RO	reverse osmosis
ROS	reactive oxygen species
RP	magnetically driven vapor recirculation pump
RP-HPLC	reversed phase high performance
RPS	relative percent survival
RPS	rhabdovirus of penaeid shrimp
RR	Remazol Red RR
rRNA	ribosomal RNA
RSM	response surface methodology
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
RuBisCo	ribulose-1,5-bisphosphate carboxylase-oxygenase
RuBP	ribulose-1,5-bisphosphate

S

S	synthesis
SAG	single amplified genome
SAHA	suberoylanilide hydroxamic acid
SAM	S-adenosyl-L-methionine
SAR	structure-activity relationship
SAV	surface area volume
SBM	soybean meal
SC	stratum corneum
SCA	Sabouraud chloromphenicol agar
SCG	single-cell genomics
SCIE	science citation index expanded
SCO	single-cell oil
SCUBA	self-contained under water breathing apparatus
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis
Se	selenium
SEAP	secreted form of alkaline phosphatase
Sec-tRNA	Selenocysteine-transfer RNA
SELDI	surface enhanced laser desorption/ionization
SEM	scanning electron microscope
SEM	scanning electron microscopy
SeN	selenoneine
SEQUEST	tandem mass spectrometry data analysis program used for protein identification
SERS	surface enhanced Raman scattering
SF	surfactin
SF-268	human CNS cancer cell line
SFE	supercritical fluid extraction
SFF	solid freeform fabrication
SGC 7901	human gastric cancer cell line
SGNH	a motif representing amino acid residues
SGP	polysaccharide
SGR	specific growth rate
SHRV	snakehead rhabdovirus
SIGEX	substrate-induced gene expression
SIP	stable isotope probing
Sir	strontium
siRNA	small (short) interfering RNA
SIV	subintestinal vessel plexus
SL	level of significance
SLM	St. Lawrence Mesocosm
SLRP	small leucine-rich repeat proteoglycan
SLS	sodium laureth sulfate
SMase 1	sphingomyolinase 1
sMBR	submerged-membrane bioreactor
SMF	submerged fermentation
SMTH	serine hydroxymethyltransferase
SMV	spawner-isolated mortality virus
SNP	single nucleotide polymorphisms
SOC	store-operated Ca^{2+}
SOD	superoxide dismutase
SONAR	sound navigation and ranging
SP	splenocyte
SP	sulfated polysaccharide
SPR	surface plasmon resonance
SPS	sulfated polysaccharide
SPX	spirolide
SR	scavenger receptors
SR	sulfate reduction
SRA	Sequence Read Archive
SRB	sulfate-reducing bacteria
SRT	solids retention time
SSAP	*Sebastes schlegeli*
SSCI	social sciences citation index
SSCP	single stranded conformation polymorphism
SSF	solid-state fermentation
SSR	short sequence repeats
ssRNA	single-stranded RNA
SSSF	semi-solid state fermentation
SSU	short subunit
ST-PBR	stirred-tank photobioreactor
STS	secondarily treated sewage
STX	saxitoxin

T

T-DNA	transfer DNA
t-PA	tissue plasminogen activator
TAG	triacylglyceride
TAG	triacylglycerol
TALE	transcription activator-like effector
TALEN	transcription activator-like effector nuclease
TAN	total ammoniacal nitrogen
Taq DNA	thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus*
TARC	thymus and activation regulated chemokine
TBA	thiobarbituric acid
TBHQ	*tert*-butylhydroquinone
TBT	tri-*n*-butyltin
TCA	tricarboxylic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin

TCP	tricalcium phosphate
TDD	transdermal drug delivery
TDDFT	time-dependent density functional theory
TeBactEn	Text Mining for Bacterial Enzymes
TEM	transmission electron microscope
TEM	transmission electron microscopy
TEOS	tetraethoxysilane
TEOS	tetraethyl orthosilicate
TFA	total fatty acid
TG	triglyceride
TGB	thermal gradient block
TGF-β	transforming growth factor beta
TGI	total growth inhibition
TH	tilapia hepcidin
Th2	T helper 2 cells
Ti(BALDH)	titanium (IV) bis(ammonium lactato) dihydroxide
TLC	thin layer chromatography
TLR	toll-like receptor
TMA	transcription mediated amplification
TMOS	tetramethoxysilane
TMP	transmembrane pressure
TMTD	trimethyltridecanoic acid
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-α
TOCSY	total correlation spectroscopy
TOF	time of flight
TPA	12-O-tetradecanoylphorbol-13-acetate
TPR	tetratricopeptide repeat domain encoding protein
TR-PBR	tubular recycle photobioreactor
TRAP	total radical antioxidant parameter
TRAP	target of RAP
TRSA	tetracycline-resistant *Staphylococcus aureus*
tsa-FISH	tyramide signal amplification FISH
TSV	Taura syndrome virus
TT	thrombin time
TTE	triethylenetetraamine
TTX	11-[^{3}H]-tetrodotoxin

U

u-PA	urokinase plasminogen activator
UA	urocanic acid
UA	uronic acid
UAC	UA-coupled chitosan
UASB	upflow anaerobic sludge blanket
UCP1	uncoupling protein 1
UDP-sugar	nucleoside sugar diphosphate
UF	ultrafiltration
ULS-RDBR	ultralow speed rotating disk bioreactor
USP	ultra-short pulses
UTR	untranslated region
UV	ultraviolet
UVB	ultraviolet B radiation

V

VDA	vascular disrupting agent
VEGF	vascular endothelial growth factor
VFA	volatile fatty acids
VGCC	voltage-gated calcium channel
VGIC	voltage-gated ion channels
VGSC	voltage-gated Na^+ channel
VHSV	viral hemorrhagic septicemia virus
Vira-A1	Vidarabine
VLC	very long-chain
VLDL	very low density lipoprotein
VLP	virus-like particle
VP	viral protein
VRE	vancomycin-resistant *Enterococcus faecium*
VREF	vancomycin-resistant *Enterococcus faecium*
VS	volatile solid
VSV	vesicular stomatitis virus
VTG	estrogen inducible vitellogenin promoter

W

WAP	whey acidic protein
Wap65	warm temperature acclimation-related protein 65
WAT	white adipose tissue
WBC	white blood cell
WGA	whole genome amplification
WHO	World Health Organization
WoRMS	World Register of Marine Species
WSD	white spot disease
WSSV	white spot syndrome virus
ww	wet weight

X

XANES	X-ray adsorption near edge spectroscopy
XOD	xanthine oxidase

Y

YHV	yellow head virus
YTX	yessotoxin

Z

ZFN	zinc finger protein
ZFN	zinc-finger nuclease
ZFP	zinc finger peptide

1. Introduction to Marine Biotechnology

Se-Kwon Kim, Jayachandran Venkatesan

Marine biotechnology is an innovative field of research in science and technology concerning the support of living organisms with marine products and tools. To understand the *omics* of the living species: it is a novel way to produce genetically modified food, drugs, and energy to overcome global demand. The exploitation of biotechnology for drug discovery, including enzymes, antibiotics, and biopolymers, chemical compounds from marine sources are deliberated in this book. The concepts of marine microbiology and molecular biology are explored extensively in the present book. Biomedical applications of marine biomaterials such as tissue engineering, drug delivery, gene delivery, and biosensor areas are thoroughly discussed. Bioenergy from marine sources is a groundbreaking achievement in the field of marine biotechnology and is also covered in this book. Finally, industrial uses of marine-derived products are explored for mankind.

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1.1 Marine Biotechnology – Definition

More than 80% of living organisms on earth are found in the aquatic ecosystem. The largest ecosystem on the planet is the ocean; it can be divided into photic, pelagic, benthic, epipelagic, and aphotic zones. More than 40 000 different kinds of species are present in the marine environment, and they are classified as microorganisms, seagrasses, algae, corals, and animals [1.1]. The marine world is considered as a huge reservoir of various biological active compounds. Marine organisms have the capacity to produce unique compounds due to exposure to exceptionally different oceanic environments, such as temperature, chlorophyll content, salinity, and water quality [1.2, 3]. The oldest known fossils are marine stromatolites, which have been evolving for 3.5 billion years; land fossils are about 450 million years old [1.4, 5]. Although the marine world represents nearly $\frac{3}{4}$ of the earth's surface, it is one of most underutilized biological resources.

Biotechnology is the most powerful tool to discover the many secrets of marine organisms and their compounds. There are numerous definitions and explanations that have been given to marine biotechnology since the day its term was coined [1.6, 7]. According to *Food and Agricultural Organization* (FAO, #8), biotechnology can be defined as [1.8]:

> *any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use.*

J. Grant Burgess suggested that marine biotechnology is [1.4]:

> *biotechnology carried out using biological resources which have come from the marine environment rather than from the terrestrial environment.*

Alternatively, marine biotechnology is also defined as *the industrial use of living organisms or biological techniques developed through basic research*. In another words, The Organization for Economic Cooperation and Development (OECD) defines biotechnology as

> *the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services.*

Thakur et al. defined the Marine biotechnology as [1.9, 10]

> *the application of scientific and engineering principles to the processing of materials by marine biological agents provide good and services.*

Another possibility to define the marine biotechnology is that it might be derived from marine bio (techno) logy.

1.2 Marine Biotechnology – Tools

In recent years, advances in instrumentation and a combination of proteomic and bioinformatics are accelerating our ability to harness biology for commercial improvement [1.4]. The marine biotechnological process has significant capacity to improve human life. Several biotechnological tools have been developed for cost-effective products that can be used for medical, industrial, and environmental applications. A variety of biotechnological methods have been adopted from marine sources such as transgenic methods, genomics, fermentation, gene therapy, bioprocess techniques, bioreactor methods, etc. Marine biotechnology is more often considered in terms of molecular or genomic biological application to generate desirable products. It encompasses the production and application of living organisms and is expected to have numerous impacts on our economy. Marine biotechnology promises breakthroughs in areas such as aquaculture, microbiology, metagenomics, nutraceuticals, pharmaceutical, cosmeceuticals, biomaterials, biomineraliza-

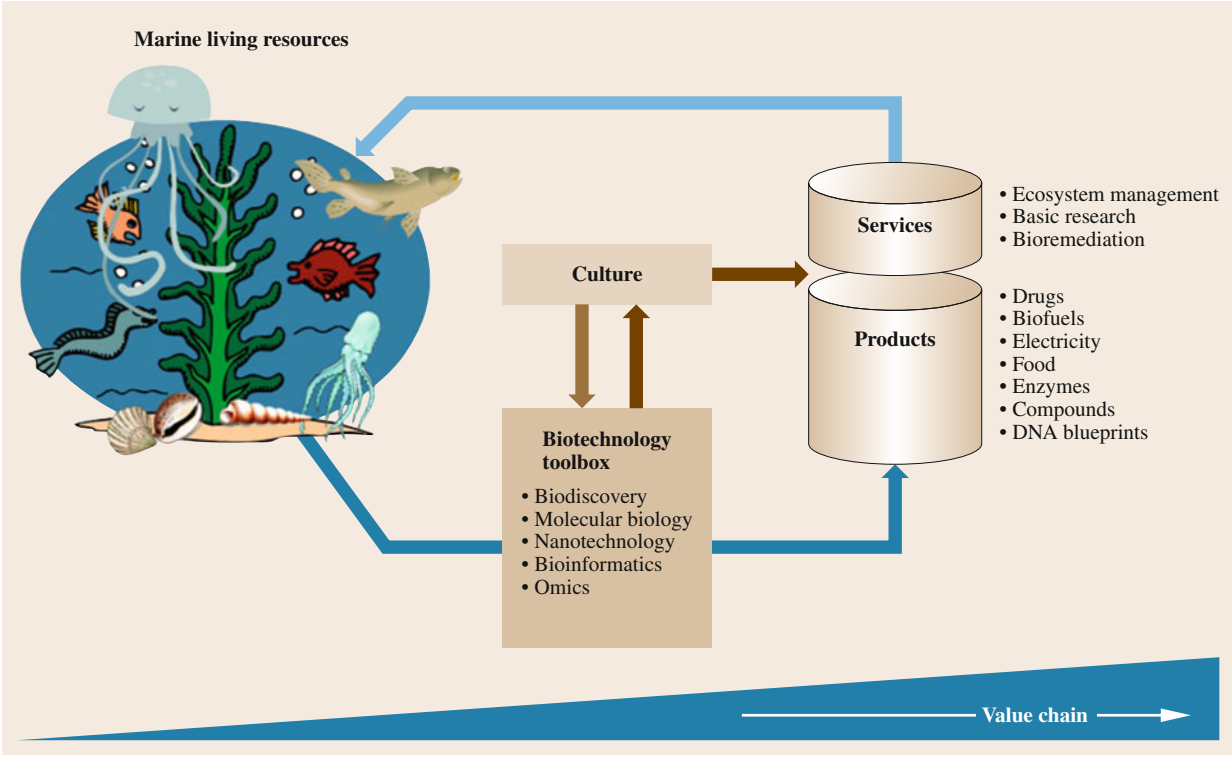

Fig. 1.1 Examples of products and services developed by technological applications using marine bioresources. After [1.11]

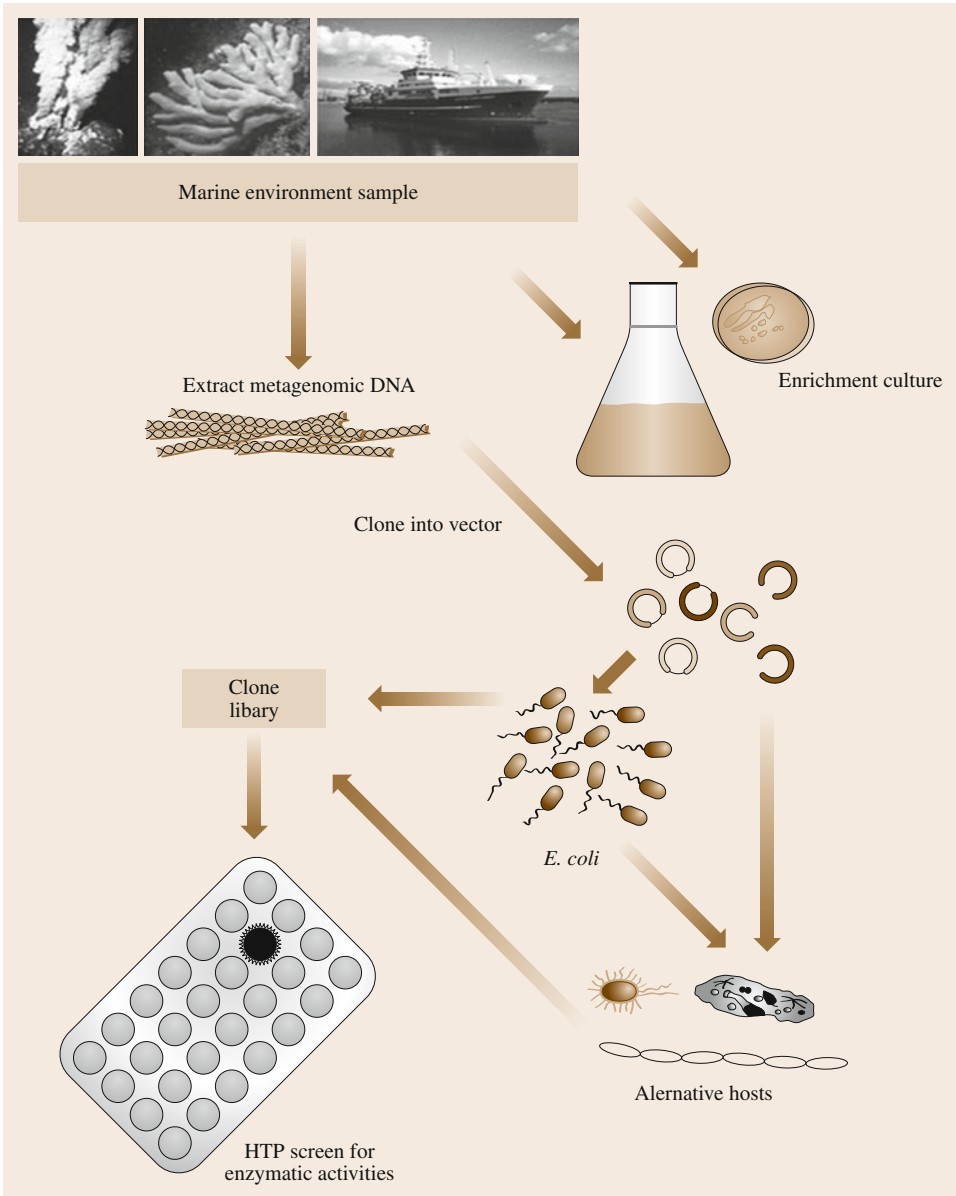

Fig. 1.2 Schematic depiction of functional metagenomic approaches for identification of novel biocatalysts

tion, biofouling, and bioenergy. Products derived by biotechnological methods are commonly more cost efficient with regards to production and also in pure form (Fig. 1.1).

An important consideration in transgenic research is the choice of promoter for regulating the expression of a foreign gene. The discovery of novel processes and techniques in marine biotechnology will create fresh opportunities for the development of innovative materials. The science of biotechnology has given us new tools and tremendous power to create genes and genotypes of plants, animals, and fish. *Lakra* and *Ayyappan* explored the use of synthetic hormones in fish breeding, the production of monosex, uniparental, and polyploid individuals, molecular biology and transgenesis, biotechnology in aquaculture nutrition and health management, gene banking, and marine natural products [1.12].

The biosynthesis and regulation route of many secondary metabolites in marine organisms should be addressed. It is possible, with the recent development of a novel transcriptome profiling methodology that allows for rapid and high-throughput screening of changes in messenger ribonucleic acid (mRNA) sequence pools. The application of genomics-based techniques and the integration of both biochemical and molecular data sets in marine organisms complement ongoing drug discovery efforts [1.13].

Metagenomic-based strategies are powerful tools to isolate and identify enzymes with novel biocatalytic activities from the uncultivable component of microbial communities [1.14]. The recent advances in biotechnological tools such as bioreactors, fermentations, and bioprocessing are useful in the production of functional ingredients, including enzymes that can be used in the food industry [1.15]. Molecular biology is playing a major role in marine biotechnology for an understanding of the genome level. Genomic analysis of marine organisms should be identified to utilize novel genes, proteins, enzymes, and small molecules. The knowledge of metabolic pathways and their genomics is the novel way to understand the mechanism behind the production of the compounds. Metabolic engineering is defined as the optimization of genetic and regulatory pathways to increase the production of compounds by cells (Fig. 1.2).

1.3 Marine Sources and Research Areas

Science and technology continues to move forward in making different technological tools to develop new products from the marine source. Important marine sources in the research are microorganisms, algae, and sponges. Various biotechnological products have been commercialized, ranging from novel drugs, chemicals, and enzymes to bioenergy [1.16–22]. Marine biotechnology plays an important role in the development of various biomaterials, biosensors, seafood safety, aquaculture, bioremediation, and biofouling (Table 1.1). Several drugs are obtained from natural sources, and researchers are still searching for potential organisms from marine sources.

1.4 Applications of Marine Biotechnology

1.4.1 Marine Aquaculture

Marine aquaculture is one of the best examples of marine biotechnology. Fish is one of the most important marine sources for protein supplement in human food. Overfishing and changes in the global environment are contributing to the slow disappearance of this important food resource. By applying marine biotechnological tools, we may be able to provide or improve aquaculture procedures through recombinant technology to develop genetically modified organisms [1.23–26], which could be useful to overcome the global food demand.

1.4.2 Marine Natural Products for Medicine

Marine bioresources are huge reservoirs for various potential biological molecules, which have tremendous potential as human medicines. Natural products are both a fundamental source of a new chemical diversity and an integral component of today's pharmaceutical collection [1.27–33]. Numerous marine compounds are isolated from marine animals, algae, fungi, and bacteria with antibacterial, anticoagulant, antifungal, antimalarial, antiprotozoal, antituberculosis, and antiviral activities. There are now 4 approved products, 13

Table 1.1 Important marine sources and research areas

Research area	Marine source	Aims
Food	Algae, invertebrates, fish	Development of innovative methods, to increase aquaculture production and zero waste recirculation systems
Energy	Algae	Biofuel production, biorefineries
Health	Algae, sponges, microorganisms	To find novel bioactives
Environment	Marine microorganisms	Biosensing technologies for marine environment monitors and non-toxic antifouling technology
Industrial products	Algae	Production of marine biopolymers for food, cosmetics, health

Table 1.2 Examples of market level marine-derived products

Products	Source	Application
Ara-A	Marine sponge	Antiviral
Ara-C	Marine sponge	Anticancer
Okadaic acid	Dinoflagellate	Molecular probe
Manoalide	Marine sponge	Molecular probe
Vent TMA polymerase	Deep-sea hydrothermal vent bacterium	PCR enzyme
Aequorin	Bioluminescent jelly fish	Bioluminescent calcium indicator
Green fluorescent protein	Bioluminescent jelly fish	Reporter gene
Phycoerythrin	Red algae	Conjugated antibodies used in ELISAs and flow cytometry
Cephalosporins	*Cephalosporium* sp., marine fungi	Antibiotic

in clinical trials, and large number of pre-clinical investigations, coming from a wide range of marine sources from many different parts around the world. Prialt ziconotide, a painkiller originally isolated from a Pacific (Philippines) cone snail, Yondelis trabectidin, an anticancer molecule from the Caribbean tunicate *Ecteinascidia turbinata*, and 3-(2,4-dimethoxybenzylidene)-anabaseine (DMXBA) from the ribbon worm *Paranemertes peregrina*, from the Pacific Rim, are a few examples [1.10] (Table 1.2). 59 marine compounds have been reported to affect the cardiovascular, immune, and nervous systems, as well as to possess anti- inflammatory effects. 65 marine metabolites have been shown to bind to a variety of receptors and miscellaneous molecular targets, and thus upon further completion of the mechanism of action studies, will contribute to several pharmacological classes [1.34]. The route to market involves isolation and chemical characterization, followed by synthesis or semi-synthesis of the molecule or an active analog.

Natural product lead compounds from sponges have often been found to be promising pharmaceutical agents. Most of these drugs are used in the treatment of the human immunodeficiency virus (HIV) and the herpes simplex virus (HSV). The most important antiviral lead of marine origin reported thus far is a nucleoside Ara-A (vidarabine), isolated from the sponge *Tethya crypta*. Marine compounds that act on the six hallmarks of cancer presented self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replication, sustained angiogenesis and tissue invasion, and metastasis [1.35–39].

Marine microbes have a huge biochemical diversity and are likely to become a rich source of novel drugs. Marine microbial compounds are an important source for drug development [1.22]. Marine bacteria are one of the important sources for many bioactive compounds, antibiotics, and pharmaceuticals. They are usually found in marine sediments and are also found to be associated with marine organisms [1.40]. Marine fungi are also reported to be a potential source for bioactive compounds. Polyketide synthases are a class of enzymes that are involved in the biosynthesis of secondary metabolites (erythromycin, rapamycin, tetracycline, lovastatin, and resveratrol).

Actinomycetes are one of the most efficient groups of secondary metabolite producers; they exhibit a wide range of biological activities, including antibacterial, antifungal, anticancer, and insecticidal, and enzyme inhibition. Several species have been isolated and screened from the soil in the past decades. Among its various genera, *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora*, and *Actinoplanes* are the major producers of commercially important biomolecules [1.41]. Actinomycetes are virtually unlimited sources of new compounds with many therapeutic applications and hold a prominent position due to their diversity and proven ability to produce novel bioactive compounds; 70% of which are produced by actinomycetes, 20% from fungi, 7% from *Bacillus* sp. and 1−2% by other bacteria [1.42]. Antimicrobial peptides are promising candidates, because their initial interaction with microbes is through binding to lipids [1.43].

Dinoflagellate toxins and bioactives are of increasing interest because of their commercial impact [1.44]. Functional screens to isolate novel cellulases, lipases and esterases, proteases, laccases, oxidoreductases, and biosurfactants have been described [1.45]. Enzyme inhibitors have received increasing attention as useful tools for the study of enzyme structures and their mechanisms. Marine organisms have been documented as a productive source for the enzyme inhibitors. Several commercialized products are shown in Table 1.2. Arebinosyl cytosine (Ara-C) is currently sold by the Pharmacia and Upjohn company under the brand name Cytosar-R [1.10].

1.4.3 Marine Nutraceuticals

Marine nutraceuticals can be derived from a vast array of sources, including marine plants, microorganisms, and sponges. Marine nutraceutical products currently promoted to various countries include fish oil, chitin, chitosan, marine enzymes, chondroitin from shark cartilage, sea cucumbers, and mussels. As mentioned earlier, the marine world represents a largely unexploited reservoir of bioactive substances that can be used for food processing, storage, and protection. Enzymes extracted from fish and marine organisms can provide numerous advantages over traditional enzymes. Fish protein such as collagens and their gelatin derivatives operate at relatively low temperatures and can be used in heat sensitive processes such as gelling and clarifying. Polysaccharides derived from alga, including alginate, carrageenan, and agar types are widely used as thickeners and stabilizers in a variety of food ingredients. In addition, Omega-PUFA (polyunsaturated fatty acid) is an important ingredient in the nutraceutical industry [1.46]. It has been proven that Omega-PUFA, especially eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA) play a significant role in number of aspects of human health [1.47]. The application potential of chitin and chitosan are multidimensional, for example, in food and nutrition, biotechnology, material science, drugs and pharmaceuticals, agriculture and environmental protection, and gene therapy [1.48–52]. Fucoidan is a complex-sulfated polysaccharide, which can be derived from brown algae. Fucoidan has been used in several biological activities and is important for its high bioactive properties, for example, antibacterial, anticoagulant, antiviral, antitumor, etc., and many more yet to be explored [1.53].

1.4.4 Marine Biomaterials

In the recent years, much attention has been paid to marine-derived biomaterials for various biological, biomedical, and environmental applications. A recent report estimates that the global markets for marine biotechnological products might exceed US$ 4.1 billion by 2015, to which the following product segments, marine biomaterials (including food hydrocolloids) could contribute over 40%; marine bioactive substances for healthcare would be the most important and fastest-growing sector. Non-toxic, biocompatible, natural chitin and chitosan from crustaceans have potential use in cosmetics, food, and pharmaceuticals [1.54]. Seaweeds are the abundant source for polysaccharides, which are commercial products (alginate, agar, agarose, and carrageenan) [1.55–61].

1.4.5 Marine Bioenergy

Bioenergy from marine algae is a groundbreaking achievement in the field of marine biotechnology [1.62–67]. Biofuels derived from marine algae are a potential source of sustainable energy that can contribute to future global demands. The realization of this potential will require manipulation of the fundamental biology of algal physiology to increase the efficiency with which solar energy is ultimately converted into usable biomass [1.68]. Anaerobic digestion of microalgae is a necessary step to make microalgae biodiesel and biogas sustainable [1.69, 70]. The potential biomass sources for bioenergy are photosynthetic microalgae and cyanobacteria. There are versatile marine organisms that can be used in the production of biogas, biodiesel, bioethanol, and biohydrogen [1.71–76].

1.4.6 Marine Bioremediation

Bioremediation is also an important area of marine environmental biotechnology. Marine microorganisms have the capacity to degrade the variety of organic pollutants. *Pseudomonas chlororaphis* produces pyoverdin, which catalyzes the degradation of organotin compounds in seawater. Biopolymers and biosurfactants are also applied to environmental waste management and treatment [1.77–81].

1.5 Research Scope

Marine biotechnology plays a vital role in the exploration and study of various marine resources. Marine biotechnology comprises a broad range of subjects: marine bioactive substances, genetics, marine culture, fermentation engineering, and enzyme engineering. The marine biotechnology market is still in the promising stages; during the years 2008 and 2009, the global marine biotechnology market witnessed a slowdown owing to the global economic meltdown. The market gained drive in 2010 with the recovery of the economic situation and is expected to post substantial growth in ensuing years.

Table 1.3 Countries and their marine biotechnology research priorities

Countries		Research priorities
Africa	Mozambique, Nigeria, South Africa, Tunisia and Kenya	Biofuels and bioactives
Central and South America	Brazil, Chile, Argentina, Mexico, Costa Rica	Biodiscovery, bioenergy, bioremediation and biofouling
North America	USA, Canada	Biodiscovery, aquaculture and biofuels
Asia	China, India, South Korea, Japan, Taiwan	Biofuels, biodiscovery for human pharmaceuticals, food, feed, cosmetics
Middle East	Israel	Sponge biotechnology, marine bioactives and biofuels
South East Asia, Indian Islands	Thailand, Vietnam, Indonesia, Malaysia, Singapore, Sri Lanka, the Philippines	Biodiversity for novel bioactives and aquaculture,
Australia Pacific	Australia and New Zealand	Aquaculture and marine bioactives

The research drive on marine biotechnology is high in countries like USA, Brazil, Canada, China, Japan, the Republic of Korea, and Australia, as well as in other countries where activities are grow from a smaller base (Thailand, India, Chile, Argentina, Mexico, and South Africa), and where there are signs that marine biotechnology is increasing in importance as a research priority. It is notable that the major international effort, the Census of Marine Life (CoML), involved 2700 researchers, about 31% from Europe, 44% from USA and Canada, and 25% from the rest of the world, notably Australia, New Zealand, Japan, China, South Africa, India, Indonesia, and Brazil (Table 1.3) [1.82].

The United States is the world leader and represents the single largest region for marine biotechnology worldwide. The marine bioactive substances market is forecasted to register the fastest growth rate of more than 4.0% during the period 2009–2015. Healthcare/biotechnology constitutes the largest, as well as fastest growing, end use for marine biotechnology. Very few countries have initiated national R&D programs to exploit the benefits of biotechnology in the marine sector. However, advances in aquaculture, drug discovery and fisheries are expected to encourage applications of marine biotechnology. The research report titled as *Marine Biotechnology: A Global Strategy Business Report* provides a comprehensive review of the marine biotechnology market, recession on the markets, current market trends, key growth deliverers, introductions of recent products, recent activity in the industry (Aker Bio Marine ASA, CP Kelco US Inc., Cyanotech Corp., Elan Corporation plc, FMC Corp., FMC Biopolymers AS, GlycoMar Ltd., Integrin Advanced Biosystems, International Specialty Products Inc., Lonza Group Ltd., MariCal, Marinova, Martek Biosciences Corp., Mera Pharmaceuticals Inc., New England Biolabs Inc., PharmaMar S.A, PML Applications Ltd., Primex Ltd., Prolume Ltd., Sea Run Holdings Inc., and Tequesta Marine Biosciences), and the profile of major global as well regional market participants. The harnessing of marine resources through biotechnology and development of products and services should be a serious target for any country with significant aquatic biodiversity.

A major task of marine biotechnology is to develop an efficient process for the discovery of novel molecules from the marine environment. The high level of marine biodiversity of marine organisms makes them a prime target for bioprospecting; these are enzymes, bioactive molecules, and biopolymers with varied industrial applications. Biochemical studies of marine organisms are an important task for the discovery of new drug molecules and biological tools and management of biodiversity.

1.6 Organization of the Handbook

This handbook combines the knowledge of sea flora and fauna, biotechnological methods, product development and industrial applications. It is divided into 10 parts. The introduction of the book comprises the definition, history and research scope of marine biotechnology. The first part introduces marine flora and fauna in detail, such as fungi, phototrophs, viruses, microalgae, seaweed, coral, and sponges. In this part, a detailed explanation is given on the production, cultivation, and processing of flora and fauna. The second part of the book introduces the tools and method of marine biotechnology; it covers, bioprocess engineering,

bioinformatics techniques, bioreactors, transgenic technology, quorum sensing, and molecular methods for the detection of invasive species. The third part of the book provides details about marine metagenomics, proteomics, marine metagenomics and supporting technology, microfluidic systems, and genomic mining. The fourth part of the book deals with algal biotechnology, starting with the structure and biological activities of marine algal polysaccharides, two centuries of research on iodine in seaweeds, marine macrophytes, and heavy metal removal by marine algae. The fifth part of the book covers marine microbiology, marine microbial biotechnology, and marine actinomycetes.

The sixth part of the book provides details about marine-derived metabolites, starting with marine natural products, biocatalysts, antimicrobial peptides, marine-derived fungal metabolites, dinoflagellates, carotenoids, Cnidarians, fatty acids, biotoxins, microbial enzymes, and polysaccharides. The seventh part of the book focuses on applications of marine biotechnology, starting with pharmaceuticals, functional food, nutraceuticals and cosmeceuticals. The eighth part of the book covers bioenergy and biofuels; here, the lead authors discuss marine bioenergy, marine algal biotechnology for bioenergy, and biofuels. Biomedical applications are extensively discussed in the ninth part of this book; the topics are marine biomaterials, gene delivery, biosensors, and biomineralization. Finally, the last part of this book focuses on industrial applications of marine biotechnology.

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Marine F Part A

Part A Marine Flora and Fauna

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2. Marine Fungal Diversity and Bioprospecting

Kalaiselvam Murugaiyan

Marine fungi are a large group of eukaryotic organisms. Marine fungi, and particularly wood-inhabiting fungi, have been extensively studied since 1944. These have been termed lignicolous fungi and constitute more than 50% of the total 450 species of obligate marine fungi described so far. Marine fungi occur not only in water and sediment, but also as parasites on plants and animals, as well as symbionts in marine lichens, plants, and algae. A rich pool of fungal species is yet to be discovered and investigated over the coming years. About 150 species have been found exclusively on decaying mangrove wood, aerial roots, and seedlings, and are categorized as *Manglicolous fungi*; most of the species belong to the class of *Ascomycetes*. Fungi in mangroves play a significant role in litter decomposition and nutrient cycling, thereby contributing to the fertility of the environment. Fungal biomass along with detritus contributes significantly to the food chain of detritus-feeding organisms found in mangroves. *Aspergillus* and *Penicillium* are the dominant fungi involved in litter decomposition of mangroves. Fungal endophytes are microfungi; they colonize the internal tissues of vascular plants without producing any apparent disease symptoms and are considered as an important component of biodiversity. The distributions of endophytic mycoflora differ with the host and modify the host plants at genetical, physiological, and ecological levels. These modifications induce profound changes in how plants respond to their environments. The environmental and biological factors such as the availability of substrates or hosts, salinity, hydrostatic pressure, temperature, and the availability of oxygen control the distribution of marine fungi. The adaptation of marine fungi in an extreme environment suggests that they are promising sources for screening natural products.

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2.1 Preamble

Fungi are eukaryotic and non-vascular organisms. The cells of fungi contain nuclei with chromosomes. A fungus is neither a plant nor an animal. The cell walls of fungi are similar to the structure of the cell walls of plants. The only different is in the chemical composition. The fungi's cell wall is composed mainly of chitin, while plant cell walls are mostly made of cellulose. Their cytoplasmic ultrastructure is also similar to that

of plant cells and differs only in the organelles and their structures. Furthermore, fungi are heterotrophic, feeding on other organisms. Unlike animals (heterotrophic) which ingest and then digest food, fungi will first digest food then ingest it.

Most fungi are multicellular; some are unicellular and are composed of filaments called hyphae. Hyphae may contain internal cross walls called septa. Septa may have pores allowing cytoplasmic contents to flow freely from one cell to another, through which they obtain their nutrients from the host. Hyphae may be branched; a dense mass of hyphae is called a mycelium. To maximize nutrient uptake, filamentous fungi form mats of fine hyphae. Fungi reproduces using spores through wind-dissemination. Both types of spores (sexual and asexual) may be reproduced, depending on the species and conditions. They primarily function as recyclers of organic material.

2.2 Diversity of Fungi

The kingdom Fungi is the second largest group after insects and is widely distributed in nature. A conservative estimate of the total number of fungal species thought to exist is 1.5 million [2.1], with only 71 000 having been described so far [2.2]; the vast majority of all extant fungi is yet to be named. Assuming a relatively constant rate at which new species are described, it will take more than 1100 years to catalog and describe all remaining fungi. However, many of these fungi are likely to become extinct before they are ever discovered, given the current rates of habitat and host loss. For example, up to 2% of tropical forests are destroyed globally each year. These habitats are exceedingly rich in fungal species. It should be considered that this estimate is based only on plant parasitic fungi and does not take into account other ecological groups of fungi such as saprotrophs.

2.2.1 Current Status of Marine Fungi

The first scientific report on marine mycology was published in the early 1900s; therein marine fungi are distinct from their terrestrial and freshwater counterparts [2.3, 4]. 24 species of marine and terrestrial fungi have been found on wood-blocks submerged in water from brackish water lakes in Japan [2.5]. Very little scientific information is available on the occurrence and distribution of fungi inhabiting marine environments, including marine mangroves [2.6]. A saprophytic and facultative parasitic fungus was reported in the coastal waters and adjacent pelagic areas of the Hawaiian Islands [2.7]. More than 800 microorganisms have been isolated from marine sediments in Italy [2.8].

Fungal diversity on prop roots, seedlings, and wood of *Rhizophora apiculata*, and wood, roots, and pneumatophores of *Avicennia* spp. are found in the deltaic mangroves of Godavari and Krishna rivers, on the east coast of India [2.9]. Frequency of occurrence and biodiversity of the fungi have been reported from mangroves of the Godavari and Krishna deltas, on the east coast of India [2.10]. The assemblage and diversity of filamentous fungi on leaf and woody litter accumulated on the floor of two mangrove forests (Nethravathi and Udyavara) on the southwest coast of India have been studied [2.11].

The diversity and ecology of fungi colonizing litter of mangroves in the Bay of Bengal region has been studied in the mangroves of the Godavary and Krishna deltas of Andhra Pradesh, Pichavaram of Tamilnadu, Andaman, and Nicobar islands [2.12]. The occurrence of fungi and a checklist of fungi have been prepared based on the pilot study in the Pichavaram mangroves of southeast India [2.13]. After the tsunami on December 26, 2004, the occurrence of filamentous fungi on woody debris by means of short-term (1 month) and long-term (12 months) damp incubation were investigated along five coastal locations on the southeast coast of India [2.14]. Marine fungi associated with decaying wood samples in the brackish water mangrove ecosystem and shoreline ecosystem was reported in south India [2.15]. Filamentous fungal diversity from the sediments of the continental slope has been investigated along the Bay of Bengal [2.16]. Floristic diversity and phorophyte specificity of lichens have been studied along the southeast coast of India, revealing that the latex bearing *Excoecaria agallocha* bears a maximum of lichen diversity followed by *Rhizophora* sp. [2.17, 18]. The above global and Indian scenario of fungal diversity provides unique opportunities for mycologists to explore fungal diversity and exploit the ecological, medicinal, and industrial potentials of fungi.

2.2.2 Major Groups of Fungi

True fungi belong to the Eukaryota kingdom, which has four phyla, 103 orders, 484 families, and 4979 gen-

era. The Deuteromycotina is not accepted as a formal taxonomic category. About 205 new genera have been described from India. Of these, approximately 27 000 species are reported to colonize diversified habitats. The major groups (phyla) of true fungi are *Ascomycota, Basidiomycota, Chytridiomycota*, and *Zygomycota*. Recently, studies have provided support for the recognition of additional phyla, such as *Glomeromycota*, a group of fungi once placed in *Zygomycota*, which form an association with the roots of most plants. A group of parasitic organisms called *Microsporidia* that live inside the cells of animals are also now considered to belong in the fungal kingdom.

A comprehensive classification of the kingdom Fungi was published as the result of collaboration among many fungal taxonomists [2.19]. This classification is used in the *Dictionary of the Fungi* [2.1] and other fungal references and databases. However, the classification system will undergo additional changes as scientists use new methods to study fungi. For example, *Cryptomycota*, a potentially new phylum of organisms within the fungi kingdom has been described. Recent findings suggest that a total of 625 fungi exist on a global scale (278 *Ascomycetes*, 277 anamorphic taxa, 30 *Basidiomycetes*, and 14 *Oomycetes*) and about 150 species of mangrove fungi have been reported from the mangroves of the Indian subcontinent.

Zygomycotina

Zygomycotina undergo asexual reproduction and are ubiquitous in soil and dung. Most of them are saprophytes and very few are parasitic on plants and animals. Trichomycetous fungi colonize the guts of arthropods. More than 1000 fungal species belonging to *Zygomycotina* have been reported from India, which are of high industrial value. For example, *Saksenea vasiformis*, a unique, indigenous fungus, has received special attention in medical mycology.

Ascomycotina

It is estimated that the *Ascomycetes* form approximately 40–45% of total fungi. Ascomycetous fungi include a number of fungi which have been show variations in their morphological diagnostic features (anamorphes and teleomorphs), an ascus-like organizational nature in ascospores, conidiophores and other characters. *Ascomycotina* forms the largest subdivision with 2700 genera and 28 500 species. Yeasts are of significant importance in industrial fermentations, such as brewing and baking. They are vital in decomposition processes to degrade cellulose and other plant polymers. On other hand, they can parasitize plants and can cause serious damage to the crop which leads to economic loss.

Basidiomycotina

Fleshy fungi, which include toadstools, bracket fungi, fairy clubs, puff balls, stinkhorns, earthstars, bird's nest fungi, and jelly fungi, fall into the category of *Basidiomycotina*. Toadstools exist as a symbiont, as well as parasites, where most of them are saprophytic in nature. They can be poisonous, however, the majority is harmless and some of them have higher nutritive values. More than 2000 species of edible mushrooms are reported from different parts of the world.

Mushrooms and Other Macrofungi

Among fungi, especially *Basidiomycetes* has been exploited more owing to its pharmacological properties such as antibiotic, antiviral, phytotoxic and cytostatic activities. Out of the 41 000 species of mushrooms, 850 species are recorded from India. Isolation of antitumor and immunomodulatory compounds of unusual polysaccharides of these macrofungi are being targeted nowadays.

Rust and Smut Fungi

The rust and smut are largest group of plant parasitic fungi in Basidiomycotina, which could cause drastic damage to economically important crop plants like wheat, corn, cereals, legumes, beans, and grasses. More than 160 genera of rusts have been recorded, out of which 46 are monotypic comprising 7000 species. The maximum number of species is seen in temperate and near temperate regions. Rust fungi occur on dicots rather than monocot plants. They parasitize plants that range from ferns to orchids and mints to composites. Several smuts can also cause considerable economic loss to cultivated plants, mainly on angiospermic monocots. Nearly 1450 smut species are distributed in 77 genera and about 3500 synonyms. Teliospore forming smuts (*Ustilaginomycetes*) are parasites on herbaceous, non-woody plants, while those lacking teliospores (*Microstromatales, Exobasidiales*) mostly parasitize woody (*Microstromatales, Exobasidiales*) plants.

Deuteromycotina

Deuteromycetes constitute an artificial group, which represents the asexual phases of *Ascomycotina* and *Basidiomycotina*. The multiplication occurs through the production of mitotic spores or conidia from special-

ized hyphae called conidiophores. Conidial ontogeny forms the basis for identification and segregation of *Fungi imperfecti*. It comprises 1700 genera of *Hyphomycetes*, and 700 genera of *Coelomycetes* that cover some 20 000 known species. They colonize, survive, and multiply in air, litter, soil, and other substrates and contribute extensively towards bio-degradation and recycling of organic matter, enzyme production, and industrial production including antibiotics, immunoregulators, and bio-control agents, in addition to causing profound mycoses, allergies, and plant diseases. About 8000 *Fungi imperfecti* are reported from India. Very few thermophilic fungi are reported from India with growth optima of around 45 °C.

2.3 Habitats of Fungi in the Marine Ecosystem

Different marine habitats support very different fungal communities. Fungi can be found in niches ranging from ocean depths and coastal waters to mangrove swamps and estuaries with low salinity levels. It is more than five decades since the marine mycology has evolved and it evidently states that marine fungi show a great variance not only in their taxonomy but also in their morphological, physiological and ecological adaptation capabilities from both terrestrial and freshwater fungi. Although *marine fungi* is a common term used to refer all fungi that are present in the sea, they are distinctly referred as marine, oceanic, manglicolous, arenicolous, or estuarine, based on their habitat.

2.3.1 Marine Fungi

Marine fungi form an ecological and not a taxonomic group. According to their origin marine fungi are categorized into two types i. e., obligate marine fungi and facultative marine fungi. Obligate marine fungi grow and sporulate exclusively in seawater, and their spores are capable of germinating in seawater. However, facultative marine fungi are those from fresh water or a terrestrial source that have undergone physiological adaptations that allow them to grow and also sporulate in the marine environment [2.4].

Marine fungi comprise an estimated 1500 species, excluding those that form lichens [2.20]. This number is much smaller when compared to the number of named and undescribed terrestrial fungi, which has been estimated to be 250 000 or more [2.4]. So far less than 500 filamentous higher marine fungi have been described and only 79 are associated with algae as parasites or symbionts, and 18 with animal hosts [2.21].

2.3.2 Fungi in Mangroves

Among the tropical marine ecosystems, mangroves and coral reefs are the major habitats in gross productivity [2.22]. Mangrove forests are spread over 181 000 km^2 in 112 countries in the tropics and subtropics [2.23, 24]. Through detritus production and decomposition mangroves support a variety of planktonic, benthic, and fish communities [2.25–27]. Up to 41% of mangroves exist in South East Asia [2.24]. The Indian subcontinent ranks fourth (6700 km^2) after Indonesia, Bangladesh, and Malaysia in mangrove vegetation cover [2.28]. Mangroves are intertidal forested wetland confined to tropical and subtropical regions, and mangrove vegetation is considered to be a dynamic ecotone (or transition zone) between terrestrial and marine habitats [2.29].

Mangrove ecosystems command intensive attention among coastal ecosystems, not only due to their peculiar habitat characteristics but also due to their rich biodiversity. Mangrove forests are biodiversity *hotspots* for marine fungi because the bases of mangrove trunks and aerating roots are permanently or intermittently submerged. Because the upper parts of roots and trunks are rarely or never reached by salt water, terrestrial fungi and lichens occupy the upper part of the trees and marine species occupy the lower part. At the interface, there is an overlap between marine and terrestrial fungi [2.30]. Mangrove fungi constitute the second largest ecological group of marine fungi. The latest estimate of marine fungi is 1500 species, in which 339 are found in mangrove ecosystems [2.31].

The fungi in mangroves play a significant role in litter decomposition and nutrient cycling, thereby contributing to the fertility of the environment. Fungal biomass along with detritus contributes significantly to the food chain of the detritus-feeding organisms found in mangroves. *Aspergillus* and *Penicillium* are dominant fungi involved in litter decomposition of mangroves. Since 1944, marine fungi have been extensively studied, particularly wood-inhabiting fungi [2.3]. About 150 species are found exclusively on decaying mangrove wood, aerial roots, and seedlings, and are categorized as *Manglicolous fungi*; most of the species belong to the class of *Ascomycetes*.

Of the 150 species, more than 30 species of marine fungi are found in the mangroves in the New World. Only a few seem to be host specific [2.4]. For example, *Avicennia germinans* specifically acts as a host for the fungal species *Rhabdosphora avicenniae* and *Mycosphaerella pneumatophorae*, whereas *Rhizophora mangle* forms another host for *Didymosphaeria rhizophora* and *Leptosphaeria australensis*. They are quite abundant in mangroves due to the easy availability of wood as their bait. Large numbers of fungi are found on *Avicennia marina, A. officinalis, Rhizophora mucronata, R. apiculata*, and *Sonneratia alba*. The fungal biodiversity in mangroves may reflect the age of the plants. A well-developed mangrove habitat provides a larger number of fungal species than new mangrove sites.

2.3.3 Fungi as Endophytes

Fungal endophytes are microfungi, mostly belonging to the family *Ascomycetes*. They colonize the internal tissues of vascular plants without producing any apparent disease symptoms. Fungal endophytes are now considered an important component of biodiversity. The distribution of endophytic mycoflora differs with the host. The fungal endophytes can modify the host plants at genetic, physiological, and ecological levels. It has been suggested that the association of fungi as endophytes with mangrove roots would confer protection from adverse environmental conditions and would allow the latter to successfully compete with saprophytic fungi that decompose senescent roots.

Mangroves are a special kind of host plant and also an abundant resource for endophytic fungi diversity. More than 200 species of endophytic fungi have been isolated and identified from mangroves, this being the second largest community of marine fungi. Most endophytic fungi have wide range of hosts, and a few only have a single host. Although several temperate plants have been studied for their endophyte assemblages, very few tropical plants have been screened for endophytes [2.32, 33]. Recently, endophytic fungi were isolated from mature green leaves of *A. officinalis* along the southeast coast of India and it was observed that the occurrences of dominant endophytic fungi are *Rhizopus* sp. (51.7%), *Penicillium lividum* (45%), and *A. ochraceus* (45%). Species richness and diversity are high in the leaves of *R. mucronata*, and the most dominant endophytes are *P. lividum* (45%) and sterile mycelia (40%). *F. moniliforme* (45%), *Alternaria* sp. (46.7%) are the dominant endophytes in leaves of *R. annamalayana* [2.34]. However, the composition and dominant species on each mangrove plant are different and their colonization varies with different parts (leaves, twigs, and stems), the age of the host plants, and with the seasons [2.35].

Scientists have been astonished at the adaptation of mangrove fungi in extreme environments and have suggested that fungi, in particular endophytes, are promising sources for screening new products. Sampling and characterization of endophytic fungal diversity is an emerging challenge and promises to lead to the discovery of new species, novel compounds, and a better understanding of their role in ecosystems.

2.4 Habitat Characteristics and Their Effect on Fungal Diversity

Despite of the insufficiency of data, generalizations have been made on habitat characteristics [2.2]. Five littoral mycogeographic zones such as arctic, temperate, subtropical, tropical, and Antarctic zones, have been proposed based on the sea-surface temperature. Temperature holds the first position among environmental and biological factors controlling the distribution of marine fungi, and then come the availability of substrates or hosts, salinity, hydrostatic pressure, and the availability of oxygen.

2.4.1 Effects of the Substratum

Marine fungi are commonly colonized in different substrata, which includes leaves and drift mangrove woods, *Avicennia alba, Bruguiera cylindrica*, and *Rhizphora apiculata* [2.35–37]. An abundance of the *Hyphomycetes* group of fungi on marine and mangrove substrates has been reported [2.20]. This might be due to the adaptation of their spores to the marine ecosystem by way of production of appendages, which provide buoyancy in water, entrapment, and adherence to the substrates, as reported in mangrove wood driftwood and animal substrates [2.38–40]. In addition to the above, *Hyphomycetes* also possess an enzyme producing ability and hence, they could potentially colonize lignocellulosic woody substrates. Maximum diversity of fungi on the woody litter of *Rhizophora* (64 spp.) and *Avicennia* (55 spp.) are reported from the Godavari and Krishna deltas of the east coast of India [2.41, 42]. The

nutritional features and persistent nature of the wood of *Avicennia* and *Rhizophora* in mangrove habitats might be responsible for yielding the rich mycoflora [2.43]. It is evident from the frequency distribution of fungi from Malaysian mangroves that driftwood supports a greater diversity of fungi than exposed test panels, e.g., 26 species on driftwood, 9 species on test blocks, while 104 species have been recorded on mangrove driftwood [2.44], yet only 77 on exposed test blocks of mangrove wood [2.45].

Differences in fungal counts may be attributed to a rhizosphere effect, which varies with the mangrove species. The effect of root exudation, which includes both promoters (sugars, amino acids, etc.) and inhibitors (phenolic compounds), and the ratio between the two types of compounds influence the fungal growth and multiplication [2.46]. It is also inferred that distributions of fungal species within the mangrove habitat vary with temperature, salinity, humidity, and organic contents [2.47]. Moreover, the frequency of occurrence and relative abundance of marine fungi from various mangrove forests of the world shows variations. This could be attributed to the difference in the species' diversity of the mangrove ecosystem, age, and preference of the host substrate, ecological factors, high temperature, abrasion, desiccation, variation in salinity, and exposure to UV light [2.48]. Mangrove leaf litter is an important substratum colonized by a very different fungal community to that of lignocellulosic materials [2.49, 50]. Higher marine fungi are not common on such leaf material [2.51].

2.4.2 Effects of Salinity

Salinity is an important factor that profoundly influences the abundance and distribution of fungi in the marine environment [2.52]. There seems to be continuous alterations in the intertidal amplitude and salinity, which can considerably affect fungal biodiversity. Earlier physiological studies of marine fungi led to the conclusion that they require sodium chloride at concentrations found in seawater for their growth. In fact, zoosporic fungi such as *Althornia, Haliphthoros* and *Thraustochytrium* species need sodium for growth at the macronutrient level [2.53]. Generally, changes in salinity in brackish water habitats such as estuaries, backwaters, and mangroves are due to the influx of freshwater from land run-off, caused by monsoons or tidal variations. The effects of salinity on fungal growth have been investigated by various authors, who observed only vegetative growth and depicted that most freshwater fungi cannot reproduce at salinities above 30% seawater and suggest that this is the major reason that they do not grow in the sea [2.19, 21, 54]. Further, it is interesting to note that the species diversity is much greater at the mangrove site when compared with the samples from the open ocean site, and this accounts for the larger number of terrestrial fungi recorded there [2.55].

In higher salinity, some species like *Curvularia lunata, Drechslera* sp., *A. terreus, Cladosporium herbarum*, and *Aurobasidium pullulans* are isolated [2.55]. This may be due to the fact that all these species are salt-tolerant fungi, although these species are mostly found in saltpan and seawater zones [2.56]. These species are mostly isolated from marine zones and rarely in fresh water and mangrove zones. These fungal cells employ two main mechanisms for adaptation to salt stress: accumulation of a polyol, glycerol, and maintenance of ion homeostasis [2.57]. When exposed to NaCl, the cells experience both osmotic stress and ion toxicity. To respond to a low external osmotic potential, the accumulating glycerol seemingly compensates for the difference between the extra and intracellular water potential [2.58]. To reduce sodium toxicity, fungal cells have to maintain low cytosolic Na^+ concentrations, and this is achieved by several mechanisms: by restricting Na^+ influx, rapidly extruding Na^+, and/or efficiently compartmentalizing sodium into vacuoles [2.59, 60]. Genetic evidence indicates that both mechanisms are essential for yeast salt tolerance [2.61].

2.4.3 Effects of Temperature

Temperature is the foremost important physical factor influencing the physicochemical characteristics and also the geographical distribution and abundance of mycoflora [2.52]. This may be due to direct solar heating and penetration of the warm surface water from the sea. Generally, the surface water temperature is influenced by the intensity of solar radiation, evaporation, freshwater influx and cooling, and mix up with ebb and flow from adjoining neritic waters. In general, marine fungi need high temperatures (usually between 25–30 °C) to reproduce [2.62]. At higher temperatures, *A. niger, A. terreus* and *Cladosporium herabrum* have been recorded in the marine zone of the Pichavaram mangrove forest [2.55]. The abundance of this group of fungi in the mangrove environment might be due to their spores, which show adaptation by way of pro-

duction of appendages that provide buoyancy in water, entrapment, and adherence to substrates, as reported in mangrove wood [2.63] and driftwood [2.64]. On the contrary, temperatures below 10 °C support the growth of wood fungi, *Digitatispora marina*, but when the temperature reaches 10 °C and above, the fungi stop fruiting on the wood [2.65]. Likewise, a few marine fungi have been recovered from Antarctic waters such as *Thraustochytrium antarticum, Leucosporidium anatartica*, and *Spathulospora antartica*, which leads us to the conclusion that the low temperature of the seawater and the availability of suitable substrata in the Antarctic region are responsible for the lower numbers of marine fungi [2.66].

There is clear evidence that temperature is responsible for sporulation, deposition, and germination of endophytic fungi. It has been demonstrated that endophytes enhance the thermotolerance of temperate plants, augmenting their potential to colonize extreme environments [2.67]. Further, temperature appears to be a major variable affecting the fluctuation of endophyte frequency in plant tissue [2.68]. Recent studies have provided evidence that the population density of leaves is influenced by summer temperatures; however, the number species of endophytic fungi increases with increasing air temperature [2.69]. It is interesting to note that seven isolates of *Corollospora maritime* have been investigated on the 18S gene [2.70], and it was found that five isolates from temperate localities grouped together; those from subtropical collections formed a separate group, while the strain from Aldabra separated from both groups.

2.5 Collection, Isolation, and Identification of Fungi

The water, sediments, substrates, and any solid material from the habitat can be collected in sterile polythene bags for fungal examination. The samples are transferred to the laboratory for further analysis. Isolation of fungi is performed by using a pour plate method with different fungal agar medium plates. The media is weighed out and prepared according to the manufacturer's specifications, with respect to the given instructions and directions. The isolated fungal species are identified up to species level by referring standard mycological manuals and books [2.4, 71–73]. After identification, the fungal species are maintained on a Sabouraud chloromphenicol agar (SCA) medium at 27 °C and they are subcultured at regular intervals using a sterile cork borer. Mycelial disks are cut at random and used for further studies.

2.5.1 Techniques for Sample Collection

Direct examination method. Fungi are directly examined under a dissection microscope for the presence of ascocarps, basidiocarps, or pycnidia. Such fruit bodies are transferred with a needle to a microscopic slide, torn apart in a drop of water to expose the spores, and carefully squeezed under a cover glass. To allow the development of fruit bodies, the litter samples can be incubated in a sterile moist chamber/petridish/polybag at room temperature. The incubated samples are examined under a microscope for fungal fruit bodies.

Another method is called the *wood baiting technique*, where terrestrial wooden logs measuring 6 × 3 × 2 cm are immersed in mangrove waters for certain period and then collected to examine the fungal colonization. To determine the fungal biota in any habitat, grab samples are satisfactory. At the chosen sites, samples are obtained scooping the water, mud, sand, or soil to be examined into the sample container. Plastic vials, soil cans, or other containers may be used to collect the samples. The container should be clean and the sample should be handled aseptically. It is not possible to obtain samples from the bottom of a body of water without water; to obtain samples from the bottom of a body of water deeper than elbow depth any of the available sampling devices can be used. The container is closed with its proper cover, excess sludge, dirt, etc., is wiped or rinsed off after which it is returned to the laboratory. The container should not be completely airtight.

2.5.2 Media Preparation for Isolation of Fungi

Various culture media are used for the isolation of fungi from the samples. The most commonly used media are Sabouraud dextrose agar or potato dextrose agar, along with chloramphenicol to avoid bacterial contamination. The chemical should be added in proper proportion along with agar (for solidification), autoclaved at 121 °C for 15 min, and plated in a sterile Petri dish that is free from contamination. Media used in plating the samples may be prepared the day before the samples are collected. The most convenient method of storage is in 10 ml lots in culture tubes in culture tube

racks in a hot water bath (45–55 °C). Instead of this, the medium may be placed in agar storage bottles and kept in the refrigerator or on the laboratory shelf. If the latter technique is used, the medium should be remelted just before use and pipetted onto the plate with 10 ml wide-mouth pipettes.

2.5.3 Isolation of Fungi

For the best results in the reading of plates, each plate should contain 40 to 60 colonies of fungi. To accomplish this, the sample must be diluted before it is plated. In general, liquids may be diluted only 1 : 10, a fairly rich sewage may be diluted 1 : 100, sludge containing about 4–6% dry matter and relatively poor clayey soils 1 : 1000, and richer, fairly dry materials (dry matter 30–60%), as much as 1 : 10 000. When the sample reaches the laboratory, a series of dilution flasks is prepared. The samples are then serially diluted and inoculated in the medium, and plating is done by various methods as per convenience. Different culture media are available to culture the yeast and other groups of fungi.

Incubation and Subculture of Isolated Fungi

Commonly, fungi can be grown at room temperature. 25 °C is a standard temperature for the growth of fungi and the temperature can be varied in the case of thermophilic fungi when it requires more than 50 °C to grow. For subcultures of fungi, a sterile cork-borer is used. The media block is taken out and inoculated into an another freshly prepared medium, and incubated at a suitable temperature.

2.5.4 Identification of Fungal Isolates

Several techniques are available for the identification of fungi. Because the nature of spore production apparatus and the spores associated with it is fragile, there are two techniques that are usually used. These are described below.

Direct Mounting Technique or LCB Mount

A small portion of mycelium is taken from the medium with a help of teasing needle. The needle must be incinerated and allowed to cool in laboratory conditions. Care must be taken when the fungus is taken out from the medium for mounting because the spores will be diffused into the environment and this may affect the individual dealing with the fungus; the laminar airflow must be in off mode. A drop of lactophenol cotton blue (LCB) solution is placed on the center part of the microscopic slide. The fungal mycelia should be mounted over the LCB. The mycelia should be gently handled as can break into pieces, which results in difficulties during the identification. Later a cover slip is placed over the mount without air bubbles. The slide is placed under a high power objective and the structure of the fungi is observed and identified.

Agar Block Technique

Fungal media are prepared and cut into 1 × 1 cm blocks with a sterile scalpel blade and placed over a slide. A small piece of mycelia is transferred to the agar block over the slide; a cover slip should be placed. The whole setup is placed inside the petri dish and small cotton soaked in water is also placed inside the dish for maintaining the moisture. The Petri dish is incubated at 25 °C for a certain period of time. Once the spores and mycelia have developed, the cover slip is removed aseptically from the agar block placed over the slide mounted with LCB, and observed under a high power objective. Identification is carried out with the help of a standard atlas and monograph using key characters (Table 2.1).

Macroscopic Morphology of Fungi

1. Rate of growth: slow (7–14 days)
   Rapid (2–7 days)
2. Topography: flat, regularly-folded, tangled
3. Texture: creamy, powdery cottony, mucoid, waxy
4. Color: front color of the colony, back color of the colony.

Microscopic Examination of Slide Cultures

This will be done for the following characteristics:

A. Hyphae-pigmentation of hyphal elements, shapes
B. Asexual spores – simple or specialized asexual spores
C. Conidiophores, conidia, macroconidia, and microconidia
D. Sporangiospores
E. Blastospores or chlamydospores
F. Spore size, shape, attachment to the mycelium, unicellular or multicellular, number of compartments
G. Rhizoids, columella.

2.5.5 Molecular Taxonomical Identification

Assembling taxa based primarily on morphological similarities does not necessarily reflect phylogenetic re-

Table 2.1 Key to the classes of fungi

S. No.	Major classes	Key characters
1.	*Zygomycetes*	Hyphal filaments, usually one-celled, rarely septate usually multinucleate; aquatic species propagating by zoospores, terrestrial species by zoospores, conidia, or conidia-like sporangia; sex cells when present forming oospores or zygospores
2.	*Phycomycetes*	Hyphal filaments, when present, multicellular, cell with one, two, or several nuclei, without zoospores, with or without sporangia, usually with conidia; sexual reproduction absent or culminating in the formation of asci or basidia
2a.	*Ascomycetes*	Sexual spores born in asci
2b.	*Basidiomyctes*	Sexual spores born in basidia
2c.	*Fungi imperfecti* (*Deuteromycetes*)	Without a sexual stage in the life cycle, or with sexual stage rare or obscure; spores born on conidiophores, which may produced at random, in clusters, or within pycnidia

lationships but is rather a convenient scheme which hinders exploration of marine fungi that might produce microbial metabolites for therapeutic use. This necessitates the careful identification and selection of species unique to a particular host before the high-throughput screening of metabolites for desired industrial applications. Therefore, taxonomy of fungi is a formidable challenge for most applications.

Fungal taxonomy is a dynamic, progressive discipline that consequently requires changes in nomenclature. The baseline of traditional fungal taxonomy and nomenclature is morphological criteria or their phenotypes. Numerous alternative approaches have been developed, including nutritional and physiological studies, serologic tests, secondary metabolites, and fatty acids. Although some of these are very useful for identifying poorly differentiated fungi such as yeast and black yeast, in most cases they are only complementary tools of morphological data. The fungus as a whole comprises a teleomorph (sexual state) and one or more anamorphs (asexual states). Traditional fungal classification is possible when the fungus is at its teleomorphic stage (asexual spore morphology also helps); however, the snag occurs in cases of fungi where only the anamorphic stage is available. The dual modality of fungal propagation, i. e., sexual and asexual, has led to a dual nomenclature. The anamorph and the teleomorph generally develop at different times and on different substrates. Species identification by morphological traits is often problematic because mycelial pigmentation, and the shape and size of conidia, which are unstable and highly dependent on the composition of media and environmental conditions. Further, subspecies level of identification is usually based upon pathogenic or physiological race reactions on a set of differential cultivars [2.74]. These processes of identification of fungi are time consuming, labor intensive, and subject to varying environmental or cultural growth conditions during the experiments [2.75], and also lead to inappropriate and unreliable application of species [2.76].

Thus methods are needed to distinguish between closely related species that occur in different habitats. Hence, molecular techniques for fungal identification and to investigate genetic variability within species have been increasingly used during the last decade. Molecular techniques based on polymerase chain reaction (PCR) have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal diseases [2.77, 78].

Differentiation of the *Fusarium* species/subspecies based on comparison of deoxyribonucleic acid (DNA) sequences of the ribosomal DNA (rDNA) and internal transcribed spacer (ITS) regions have been reported [2.79, 80]. The sequenced rDNA region of *F. oxysporum* (Accession no. JX 840353) isolated from sediments of mangroves along the southeast Indian coast covered the 18S ribosomal ribonucleic acid (rRNA) gene, partial sequence; internal transcribed spacer1; the 5.8S ribosomal RNA gene and ITS2; complete sequences; and the 28S ribosomal RNA gene, partial sequence. In the constructed phylogenetic tree, the strains *F. oxysporum* (GU205817), *F. oxysporum* (EF495237), *F. oxysporum* (EF495230), and *Fusarium* sp., (JF429684) have 98% similarity with the isolated strain *F. oxysporum* (JX 840353) [2.55] and 97% similarity with *Fusarium* sp., (GU973787). Genotypic identification of the 18S ribosomal RNA gene of endophytic *Fusarium* sp. isolated from leaves of *Rhizophora annamalayana* has been analyzed (accession number JN681281) and the fungus was found to be the closest homolog to *Fusarium moniliforme* [2.34]. Ribosomal RNA genes (rDNA) possess characteristics that are suit-

able for the detection of fungi at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome [2.18]. ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [2.81].

2.6 Bioprospecting of Marine Fungi

The need for new and safe bioactive compounds to provide comfort in all aspect of human life is ever increasing. Due the emergence of new diseases, the development of drug resistant pathogenic microorganisms, the appearance of life threatening viruses, the management of post operative complications in patients with organ transplantations are some of the challenges to scientists. Synthetic antibiotics and drugs are extending antibiotic resistant microbes, however it is essential to investigate a new way to treat diseases [2.82]. This situation has forced scientists to explore different natural sources for safe and potent agents to meet the challenges of the twenty-first century. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources based on their use in traditional medicines or phytomedicines. Due to safety and environmental problems, many synthetic drugs and chemicals have been removed or are currently being targeted for removal from the market, which creates the need to find alternative ways to control farm pests and dermatophytes [2.83–86].

Marine fungi have provided new incentives for research on marine natural products over the past years; this research also continue to be the subject of vigorous chemical investigation [2.87–89]. The diversity of secondary metabolites reported in recent decades is fascinating. This highlights the importance of marine fungi as a source of natural products [2.90–92]. The marine environment is unique in terms of its specific composition in both organic and inorganic substances, as well as temperature ranges and pressure conditions. Ecological niches, e.g., deep-sea hydrothermal vents, mangrove forests, algae, sponge, and fish provide habitats for the evaluation of specific microorganisms.

2.7 Conclusions

Fungi are not only beautiful but play a significant role in the daily life of human beings in addition to their utilization in industry, agriculture, medicine, the food industry, textiles, bioremediation, biodegradation, biogeochemical cycle, biofertilizers, and in many other ways. Fungal biotechnology has become an integral part of human welfare. Fungal biotechnology or *mycotechnology* has advanced considerably in the last five decades. Terrestrial fungi are used in the production of various extracellular enzymes, organic acids, antibiotics, and anticholesterolemic statins. They have been used as expression by hosts, as well as a source of new genes. With modern molecular genetic tools, fungi have been used as *cell factories* for heterologous protein production and human proteins. The focus of future research is oriented towards fungi in special ecological niches, as a basic understanding of the ecology to help reveal the novelty of an organism and its properties. The emphasis is on the following aspects for top priority:

i) Fungi associated with endophytic fungi in marine algae, seagrasses, and mangroves, and the implications.
ii) Fungi associated with marine invertebrates, especially corals and sponges, and their potential for production of bioactive molecules.
iii) Fungi from extreme environments such as the deep sea with elevated hydrostatic pressure and low temperatures, hypersaline waters of the Dead Sea, and anoxic or hypoxic (oxygen deficient) sediments from the marine environment.
iv) Bioremediation of pollutants using salt-tolerant fungi and their salt-tolerant enzymes on a pilot scale and industrial scale.
v) Genomic and proteomic studies with novel organisms such as *Corallochytriumlima cisporum* as a model of animal fungal allies. It is hoped that this will help basic research on evolutionary biology.

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3. Diversity of Marine Phototrophs

Hideaki Miyashita

Phototrophs transform the energy of solar irradiation into chemical-bond energy in organic substances. Those organic substances provide biological energy not only for the activities of life on Earth, but also the formation and maintenance of regional and/or global ecosystems. They also provide biotechnological resources for materials, energy, and so on. Since their energy transformation is the starting point of the energy flow in the marine ecosystem, the understanding of *phototrophs* is important not only for the consideration of the homeostasis of the marine environment, but also for the development of new marine biotechnologies. Traditionally, diatoms and flagellates had been understood to be the major primary producers in the ocean. However, the developments of technologies for phytoplankton research within the past three decades revealed a diversity of phototrophs serving as *primary producers* and contributing to the transformation of light-energy into the chemical energy. The contribution by those diverse phototrophs to the energy flow in the ocean seemed much larger than that by diatoms and flagellates, which indicated the necessity of reconsidering energy flow or primary production. In this paper, the author discusses the diversity of planktonic phototrophs that serve as the *primary producers* (the starting point of the energy flow in the marine environments) based on research results in chronological order.

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3.1 Traditional Understanding of Primary Producers (≈1970s)

Until the mid 1970s, primary production in marine environments was understood to be performed mainly by diatoms and flagellates [3.1]. It was obvious that they contribute to primary production, since they are the major organisms that can be collected by a phytoplankton net (small mesh-size net). Moreover, phytoplankton are distributed in a wide range of water quality, including eutrophic and oligotrophic environments. Dinoflagellates are also widely distributed as a major member of phytoplankton in the oligotrophic ocean. Symbiotic dinoflagellates in corals at tropical reefs play a significant role as the major primary producer in those oligotrophic environments. Thus, diatoms and flagellates were understood to be the major primary producers in marine environments (Fig. 3.1).

The reason why those two microalgae were considered to be the major primary producers was easily speculated from the approach to phytoplankton sampling and the methods for phytoplankton detection at the time. Except for phytoplankton bloom such as red

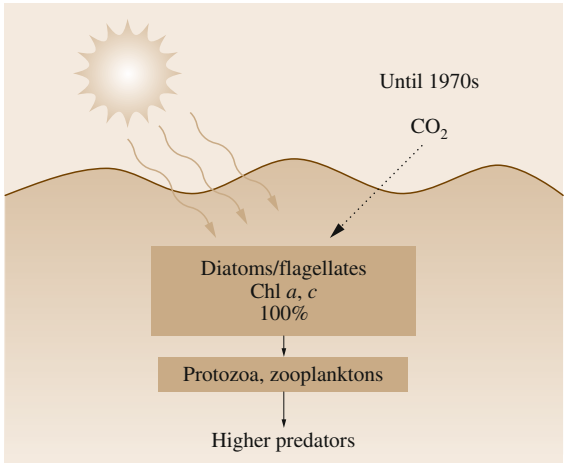

Fig. 3.1 Classical understanding of primary production

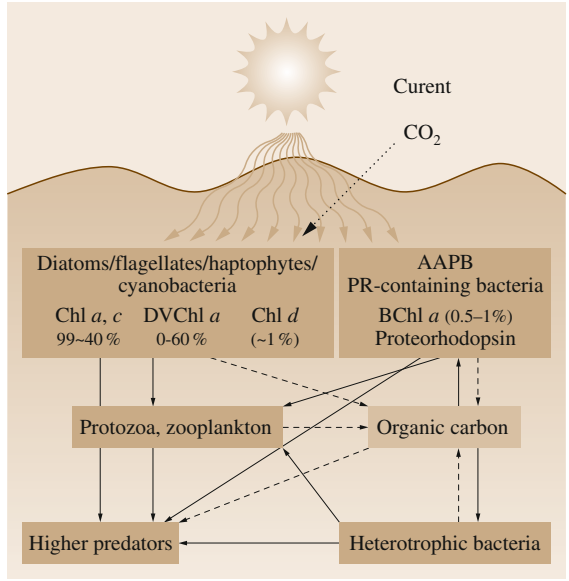

Fig. 3.2 Current understanding of the flow of energy and carbon. AAPB: aerobic anoxygenic photosynthetic bacteria, PR-containing bacteria: proteorhodopsin-containing bacteria

tide or cyanobacterial blooms, plankton nets had been used for phytoplankton sampling. A plankton net is field-equipment used to trap plankton. It is a conical polyethylene net with a defined mesh size and has a collecting bottle attached to the tip end. The mesh size of the net determines the size range of the plankton trapped. In phytoplankton research, nets with mesh sizes of 100 μm (XX13), 70 μm (XX17), and 60 μm (XX25) were used. The result was that only phytoplankton with a cell or colony size of more than 60 μm were used in phytoplankton research until the 1970s (Fig. 3.2).

However recent phytoplankton research using deoxyribonucleic acid (DNA) detection and flow cytometry has shown that the predominant phytoplankton in marine environments is much smaller than those collected by plankton nets. For example, haptophytes including coccolithophores, which are phytoplankton that deposit calcareous plates called coccoliths, are one of the major constituents of primary producers in marine environments. However, most of these pass through a phytoplankton net that is usually used for phytoplankton research, since the cell size of most haptophytes is less than 50 μm. Plankton nets with a smaller mesh size of around 10 μm have been employed for phytoplankton research in recent years. However, the dominant phytoplankton in marine environments has much smaller cell size than the cells that can be caught by those smaller mesh-sized nets.

3.2 Recognition of Picocyanobacteria Dominance (1970s–2000s)

In the late 1970s, the development of observation techniques by fluorescence microscopy and accumulation of knowledge from electron microscopic observations revealed that unicellular cyanobacteria *Synechococcus* spp. are predominantly distributed in the marine environments, especially in tropical and subtropical regions [3.2, 3]. They are very tiny cells with 0.5−2.0 μm in length and 0.5−1.0 μm in diameter, called picocyanobacteria. They play a significant role as a primary producer in those environments. In the late 1980s, the developments of the high performance liquid chromatography (HPLC) technique for pigment analysis and of the cell detection technique using flow cytometry brought new insight in marine picocyanobacteria. Apart from picocyanobacteria, which was recognized as *Synechococcus* spp., it contained divinyl-chlorophyll (DVChl) *a* which had an absorption spectrum slightly different from that of the usual Chl *a* [3.4–6]. Furthermore, those cells did not contain phycobilipigments, which were typical light-harvesting antenna in com-

mon *Synechococcus*. The cyanobacterium named as the genus *Prochlorococcus* is coccoid to ellipsoidal unicellular cells with 0.4–0.6 in diameter and 0.5–0.8 in length. They perform oxygenic photosynthesis using DVChl a/b which can efficiently absorb the blue light dominating in the ocean. They are the dominant picophytoplankton in tropical and subtropical regions between the latitudes of 30° north and 30° south. In several marine regions, the amount of DVChl *a* is three times higher than that of usual Chl *a*, indicating that *Prochlorococcus* play a significant role as a primary producer in those environments.

3.3 Discovery of Ubiquitous Photoheterotrophs (2000–Current Times)

In the early 2000s, two significant findings were reported with respect to the fact that microorganisms other than traditional phytoplankton could possibly contribute significantly to the transformation of light energy into chemical energy in marine environments. One was the wide distribution of aerobic anoxygenic photosynthetic bacteria (AAPB) which contained bacteriochlorophyll (BChl) *a*, that had mainly been recognized as a photosynthetic pigment in anaerobic anoxygenic photosynthetic bacteria (AnAPB) distributed in anaerobic environments [3.7]. The other was the wide distribution of proteorhodopsin-containing bacteria [3.8, 9]. Both bacteria are photoheterotrophs without carbon fixation and had never been recognized as *primary producers*.

APB were first reported in 1979 [3.10]. Phylogenetically, AAPB was derived from non-sulfur purple bacteria [3.11], the anaerobic anoxygenic photosynthetic bacteria (AnAPB) in the α-Proteobacteria. AnAPB had been traditionally understood to distribute in an anaerobic environment and perform photosynthesis without oxygen evolution. They employ bacteriochlorophylls (BChls) for their photosynthesis instead the Chls detected in algae and plants. The amount of primary production by AnAPB reached up to 30% of the total primary production in some specific lakes [3.12]. However, their contribution was negligible on global scale, since such environments were restricted. On the other hand, AAPB grow (photo-)heterotrophycally in an aerobic environment. AAPB are the same with AnAPB on the point that they contain BChl *a*. However, AAPB are aerobic and require molecular oxygen for their growth and BChl *a* synthesis [3.11]. AAPB distributes widely in the euphotic zone of marine environments in amounts with from ranging from 1–24% of the total bacterial count [3.13]. It had been already reported that the phototrophic growth of some APPB was faster than those growing heterotrophically in the dark [3.14]. Moreover, the in situ growth rate of APPB in seawater was faster than that of other heterotrophic bacteria. The detailed physiological role and activity of photosystems in all AAPB have not clarified yet, but AAPB must contribute to the transformation of solar energy in marine environments and act as *primary producer*.

Proteorhodopsin is the rhodopsin-like protein detected in marine Proteobacteria. It is a retial-opsin complex in a member of rhodopsin. Opsin is a bundle of seven transmembrane α-helices. Retinal, vitamin A aldehyde binds to the lysine residue at the central pocket of the opsin. Rhodopsin is well known as a photoreceptor in vertebrates as a light sensor. One rhodopsin-like protein was known to contribute to adenosine triphosphate (ATP) synthesis. Bacteriorhodopsin of *Halobacteria* in *Archea* can transform light energy to transmembrane proton electrochemical gradients ($\Delta\mu H^+$) for ATP synthesis [3.15]. Conformation of retinal molecule changes by absorbing light in synchronization with structure changes of ligand in the central part of opsin. The conformation change lead a serial proton to deliver from cytoplasmic to periplasmic space, and results in a formation of $\Delta\mu H^+$ for ATP synthesis. However, such ATP synthesis using bacteriorhodopsin had been thought to be a special mechanism that is restricted in special Archaean bacterium-distributed extremely salty environments.

However, a large amount and wide variety of rhodopsin-like genes were detected in seawater by metagenomic analysis [3.8, 9]. Moreover the gene was expressed in *E. coli* and acted to form $\Delta\mu H^+$ for ATP synthesis under light [3.16]. The genes of proteorhodopsin are contained in a diversity of bacterial taxa [3.17]. The total amount of proteorhodopsin-containing bacteria is equivalent to 13–80% of the total amount of bacteria and *Archea* in marine environments [3.18, 19]. These results indicate that proteorhodopsin was widely and significantly distributed and play a significant role in light energy transformation in the marine environments. Actually, light-dependent ATP synthesis using proteorhodopsin was

reported in a Flavobacterium strain [3.20]. However, this light-dependent growth was not observed in all proteorhodopsin-containing bacteria. A role of proteorhodopsin other than ATP synthesis was indicated, such as the advantage for the recovery from a starvation situation [3.21]. Detailed research on the roles of proteorhodopsin will clarify its contribution to the light energy conversion process in marine environments.

3.4 Oxygenic Photosynthesis Using Far-Red Light (1990s–2011)

Chl *a* is indispensable for oxygenic photosynthesis, since it plays an essential role in light harvesting, energy transfer, and charge separation in the primary reaction in photosynthesis. This is based on the results from photosynthesis research using land plants and green algae as model organisms. Quantification of the amount of oxygenic phototrophs and the activity of oxygenic photosynthesis are, therefore, estimated based on the amount of Chl *a*. The in situ light absorption range by Chl *a*, from 400 to 700 nm (or from 380 to 710 nm), is defined as photosynthetically active radiation (PAR). PAR is used for the evaluation of light strength for the activity of oxygenic photosynthesis. However, a cyanobacterium *Acaryochloris marina*, which performs oxygenic photosynthesis using Chl *d*, was reported in 1996 [3.22]. Chl *d* is a red-shifted chlorophyll which can absorb far-red light for an almost 30 nm longer wavelength than Chl *a* [3.23]. The cyanobacterium employs Chl *d* not only for the light-harvesting antenna but also for the chlorophyll in the reaction centers [3.24, 25]. As a result, the cyanobacterium can perform oxygenic photosynthesis using far-red light from 700 to 740 nm, which cannot be effectively used by common phototrophs. *Acaryochloris* spp. are widely distributed in coastal areas as epiphytes on seaweed [3.26]. The amount of Chl *d* on the seaweed ranges between about 1−13% versus total Chl *a* content in seaweed. Moreover, chlorophyll was universally detected to be about 1% (versus the Chl *a* amount) in the bottom sediment of coastal areas and lakes in the Antarctic and Arctic [3.26]. The energy transfer efficiency of the photosynthesis using Chl *d* is equivalent to that using Chl *a* [3.27]. Photosynthesis using far-red light, which has not been estimated at all to date, must contribute to primary production in marine environments.

Moreover, in 2010, another red-shifted chlorophyll, Chl *f* was reported from a cyanobacterium isolated from the microbial mat on stromatolite at the coast of Australia [3.28]. Chl *f* has its absorption maximum at around 720 nm and it can absorb far-red light up to around 760 nm in vivo. Because we have to wait for further research to know the contribution of Chl *f* to oxygenic photosynthesis in marine environments, the activity of Chl *f* was not taken into account in the estimation of primary production in marine environments.

These findings on far-red utilization using the red-shifted chlorophylls, Chl *d* or Chl *f*, for oxygenic photosynthesis require reconsideration of the PAR concept and primary production in marine environments.

3.5 Discovery of Picoeukaryotic Phytoplankton (1990s–2011)

In recent years, very diverse eukaryotic picophytoplankton species have been revealed to be widely distributed in significant amounts in marine environments. However, only some dozens of species in chlorophyta, heterokontophyta, and haptophyta were known as eukaryotic phytoplankton until the early 2000s.

Around 2010, several reports on marine picophytoplankton analysis using the techniques of metagenome and flow cytometry were published. These showed that a significant amount of picohaptophytes, including many undescribed species, were widely distributed in marine environments. By pigment composition analysis of marine waters, a carotenoid 19′-hexanoyloxyfucoxanthin (19HF) was predominantly detected in the picoplankton fraction less than 3 μm at a high latitude area from 40° to 60° of both north and south [3.29]. Since picocyanobactria, which is well known as a predominant pico-phytoplankton in tropical and subtropical marine environments, do not contain 19HF, and 19HF is a specific pigment to the algae in the Haptophyta, the amount of picophytoplankton belonging to

Haptophyta accounted for 50% of the total amount of the picophytoplankton fraction [3.29]. A similar estimate was also reported; picohaptophytes formed 5% of the global picophytoplankton biomass and contribute significantly to primary production in the marine environment [3.30].

Furthermore, it was also reported that a significant amount of microalgae in unknown phylum (unisolated phylum to date) also exists in marine environments. In 2007, phytoplankton which did not belong to any known phyla of algae were discovered by metagenomic analysis of a plankton fraction of less than 3 μm in size [3.31]. Its DNA sequence was close to the cryptophycean linage. It was tentatively named *picobiliphytes*. The *picobiliphytes* distribute widely in coastal areas of Europe and the North Atlantic, and the cell number was reaches up to 1.6% of the total eukaryotic phytoplankton. In 2011, the new candidate phylum was also detected by metagenomic analysis targeting plastid DNA. *Rappemonads*, the name tentatively given to the algae in the candidate phylum, were close to haptophytes, but they did not belong to any known phyla phylogenetically [3.32]. *Rappemonads* also widely distribute in coastal areas of Europe, and the Atlantic and Pacific oceans. Since algae in both candidate phyla have not been isolated and cultivated, the characteristics in morphology, physiology, life cycle, photosynthesis, and productivity are not yet known. It might possible to find a further new phylum of photoplankton which has not yet been discovered. The accumulation of knowledge on the diversity of phytoplankton including unknowns might contribute to a better understanding of primary production in marine environments.

3.6 Strange Phototrophic(?) Microorganisms (1990–2011)

The cyanobacterium-like particle UCYN-A was detected by metagenomic analysis of nitrogen-fixing bacteria in marine waters. It was first detected as a cyanobacterium which had a nitrogenase gene for nitrogen fixation in pelagic waters. The wide distribution of UCYN-A indicated that the particle contributes to the nitrogen cycle in pelagic waters. Usual cyanobacteria grow by an oxygenic photosynthesis process photosystems, a respiration system, and genes for a carbon fixation system. However, UCYN-A lacks photosystem II (one of the two photosystems that split water into protons, electrons, and molecular oxygen), RuBisCo (ribulose-1,5-bisphosphate carboxylase-oxygenase), the primary enzyme for fixing CO_2 and the tricarboxylic acid (TCA) cycle for respiration [3.33]. The lacking of these essential genes for free living suggests that UCYN-A might be a symbiont in some organisms. Since it possesses a complete gene set for photosystem I and contains chlorophyll, it must use light energy for some activity of the particle. While it is unclear how the light energy absorbed by photosystem I is used in the particle, it must be contribute to the transformation of light energy into chemical energy.

3.7 Diversity of Light Energy Transformation Systems and Reconsideration of *Photosynthesis*

Organisms that can grow using light as its energy source are called *phototrophs*. Representatives of phototrophs are photosynthetic organisms that can perform *photosynthesis*. *Photosynthesis* is classically the reaction that can reduce carbon dioxide into organic compounds such as sugars using sunlight as a energy source. *Primary producers* are organisms that synthesize organic compounds using inorganic carbon as their carbon source. Their representatives are photosynthetic organisms (such as land plants, algae, cyanobacteria, and some photosynthetic bacteria) and chemoautotrophs. Since the productivity of chemoautotrophs in a general environment is considered to be much smaller than that of photoautotrophs. *Primary producers* has been used synonymously with *photosynthetic organisms*. Similarly, it has been considered that phototrophs and photosynthetic organisms are almost the same so far. However, the recent discoveries of new marine microbes which utilize light energy suggest that the conventional understanding of the terms *phototrophs*, *photosynthetic organisms* and *primary producers* may not necessarily be suitable for

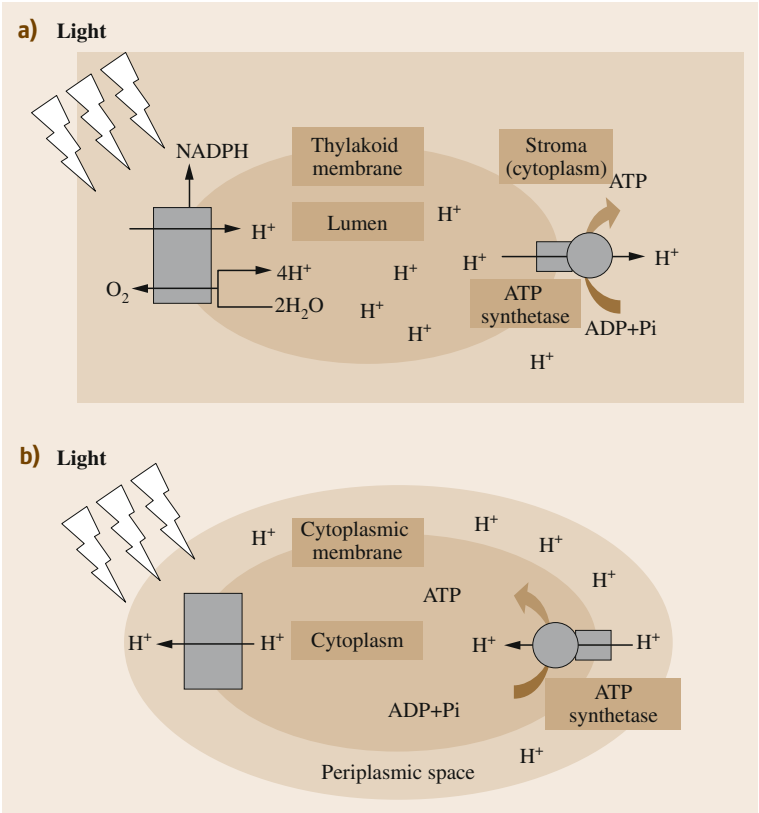

Fig. 3.3a,b Formation of transmembrane electrochemical potential of proton ($\Delta\mu H^+$) using sunlight. **(a)** Cyanobacteria or plastids in algae and land plants; **(b)** photosynthetic bacteria, halorhodopsin and proteorhodopsin-containing bacteria

the understanding of the energy and carbon flow in marine environments.

In this paper, the author defined *phototrophs* as *organisms which can synthesize ATP using light as its energy source*. In another words, phototrophs are

> *the organisms which can synthesize ATP by ATP synthase using the transmembrane electrochemical potential of proton ($\Delta\mu H^+$) by pumping up protons from the membranous one side to another side based on light energy.*

That is, in a chloroplast or cyanobacteria, protons are pumped into thylakoid lumen (the inside of thylakoid) from stroma (cytoplasm in the case of cyanobacteria) (Fig. 3.3a). In cases of non-oxygenic photosynthetic bacteria or proteorhodopsin-containing bacteria, protons are pumped out from the cytoplasmic space to the periplasmic space (Fig. 3.3b). Protons pumped into the thylakoid lumen or pumped out from cytoplasm flow back into the stroma or the cytoplasm, respectively, through ATP synthase, which results in the synthesis of ATP. Here, phototrophs are all organisms that can perform such light-dependent ATP synthesis.

Therefore, photosynthetic organisms are only a part of phototrophs. Primary producers are not only the organisms that can produce organic compounds from inorganic carbon, but also the organisms that can produce ATP using sunlight energy. As a result, phototrophs are almost equal to primary producers in this chapter. Table 3.1 shows primary producers under this concept. Further investigations on how these organisms transform light energy will develop the understanding of energy flows in the marine environment.

Table 3.1 Diversity of phototrophs

Phototrophs	Phylogenetic position	Pigment	Niche	Trophic mode	Carbon source
Anoxygenic phototrophs					
Purple bacteria (including aerobic bacteria)	Bacteria Proteobacteria	Bacteriochlorophyll	Anaerobic ~ Aerobic	Photoheterotroph Photoautotroph	Organic carbon CO_2
Green non-sulfur bacteria (filamentous anoxygenic photosynthetic bacteria)	Bacteria Chloroflexi	Bacteriochlorophyll	Anaerobic ~ Aerobic	Photoheterotroph Photoautotroph	Organic carbon CO_2
Green sulfur bacteria	Bacteria Chlorobi	Bacteriochlorophyll	Obligate anaerobic	Photoautotroph	CO_2
Heliobacteria	Bacteria Firmicutes	Bacteriochlorophyll	Obligate anaerobic	Photoheterotroph	Organic carbon
Chloroacidobacteria	Bacteria Acidobacteria	Bacteriochlorophyll	Aerobic	Photoheterotroph	Organic carbon
Halobacteria	Archaea Euryarchaeota	Bacteriorhodopsin	Aerobic ~ Anaerabic	Heterotroph Photoheterotroph	Organic carbon
PR-containing bacteria	Bacteria (polyphyly)	Proteorhodopsin	Aerobic	Heterotroph Photoheterotroph	Organic carbon
Oxygenic phototrophs					
Cyanobacteria	Bacteria Cyanobacteria	Chlorophyll	Aerobic	Photoautotroph	CO_2
Algae	Eukarya (polyphyly)	Chlorophyll	Aerobic	Photoautotroph Mixotroph	CO_2 Organic carbon
Land plants	Eukarya Streptophyta	Chlorophyll	Aerobic	Photoautotroph	CO_2

3.8 Conclusion

In relation to the food web in marine environments, studies on energy flow are important and relevant to global environment change, preservation and protection of living marine resources, and the diversity of marine microbes. Energy flow in the marine environments has been thought based on the food chain, starting with oxygenic photosynthesis with carbon fixation. However, recent studies have revealed that the energy flow through the microbial loop is very important, in addition to the traditional understanding of the food web, since it is possible that a large amount of bacteria can transform light energy into chemical energy.

To shift to a new paradigm and create a new concept of the energy flow in marine environments, it is required to reveal the diversity of marine phototrophs and their roles in the energy and material flow in marine environments.

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4. Marine Viruses

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Marine viruses are the most abundant *life-forms* in the ocean and exist wherever life is found. The estimated virus count in the ocean is 10^{30}, and every second about 10^{23} viral infections occur in the ocean. These infections are a major source of disease and mortality in organisms ranging from shrimp to whales. Each infection potentially introduces new genetic information into an organism or into progeny viruses. These new genetic changes allow evolution of the host and virus population. Viruses represent the planet's largest pool of genetic diversity. Our understanding of the impact of viruses on global systems, however, is still incomplete. This chapter will present a general review of marine viruses; their impact on marine organisms, such as cyanobacteria, mollusks, and more detailed about shrimp viruses, diagnostics and the control of shrimp viral diseases.

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4.1 Viruses

Viruses are more common in air, soil, and water, including the ocean environment, and exist wherever life is found. They are obligate intracellular parasites and infect all living organisms such as vertebrates, invertebrates, plants, fungi, algae, archaea, and bacteria. The marine environment is the major habitat for many living organisms which have a large virus population, and the interaction between virus and their hosts allow the evolution of both viruses and host organisms. Furthermore, each and every progeny virus has the potential to evolve as a new virus, which may involve a huge impact on living organisms or become an emerging virus [4.1]. The evolved new viral strains change in virulence and transfer viruses between ecosystems. In some circumstances, gene transformation between viruses and their hosts may produce dramatic effects on the hosts. For example, the well-known phage cholera toxin (CTX) gene originated from CTXf or CTXφ bacteriophage through horizontal gene transfer. The bacterium *Vibrio cholera* is normally harmless but could become one of humanity's greatest scourges by incorporating CTX genes of filamentous phage (*Inovirus*) from the family *Inoviridae* [4.2, 3]. Marine viruses are not only the major cause of mortality in marine organisms but are also involved in global biogeochemical cycle changes. Moreover, they are a huge reservoir of the greatest genetic diversity on Earth. The estimated virus count in the ocean is

10^{30}, and every second about 10^{23} viral infections occur in the ocean. Therefore, the monitoring of the virus population in the marine environment and an understanding of the virus replication and host specificity are vital and emerging fields in the study of marine viruses. However, our understanding of the impact of viruses on global systems is still incomplete [4.4].

4.2 General Characteristic Features of Viruses

Viruses consist of genetic materials such as nucleic acids, which are either DNA or RNA, surrounded by a protein coat. They can replicate as obligate intracellular parasites by taking on the biosynthetic functions within a host cell. All forms of cellular life are susceptible to virus infection. Therefore, we can speculate that every type of marine organism is host to at least one type of virus [4.5]. The genome of the DNA virus may be single or double-stranded, and the structure may be linear with free 5′ and 3′ ends or the ends have a covalently closed circular form. RNA viruses can also be categorized based on their genome structure: single-stranded RNA (ssRNA), double-stranded (dsRNA), linear, and circular. Single-stranded genomes can be designated as positive (exactly the same as messenger RNA (mRNA) except thymine replaces uracil in the ssDNA genome) or negative (complementary to the mRNA) sense genomes [4.6]. The genome size of viruses varies from a smaller size of about 1.7 kb (Porcine circovirus (ssDNA) and hepatitis delta virus (ssRNA)) to a larger genome size of approximately 1.2 Mb in mimivirus double-stranded DNA (dsDNA) [4.7]. The virus gene expression differs depending on the viral genome nature. For instance, dsDNA viruses encode genes similarly to cellular organisms such as plants, animals, and bacteria. Nevertheless, ssDNA, ssRNA, and dsRNA viruses following a unique gene expression. Furthermore, most prokaryote, plant, and fungal virus genomes are dsDNA, ssRNA, and dsRNA, respectively [4.6]. The structure and morphology of viruses are also comparable with other virus groups. Some of viruses have a lipid layer or compound which is responsible for the development of an envelope along with proteins on the surface of virions. The envelope involves the attachment and entry of virions into the host cells.

4.3 Host Specificity

Every virus has a host specificity and a particular virus can infect a particular species or cells. However, some viruses can infect multiple organisms or a wide range of species. The wide host range suggests that viruses might be involved in different host shift events. Viruses must adapt to the new genetic and immunologic environment of their hosts in order to replicate [4.8]. Furthermore, the interactions between virus and host are crucial for viral replication. Moreover, the proteins or receptor molecules, which are not uniform for all species for virus attachments, may play an important role in host specificity [4.9, 10]. Thus, viruses must often co-evolve with their hosts, which often leads to a species and host specificity [4.8]. Marine environments contain huge life-forms and many different species living in close proximity, which may allow the possibility of viral host shift events.

4.4 Viral Families in Marine Ecosystems

About 15 DNA viral families persist in the marine environment; however, the majority of the families are of the dsDNA virus family rather than ssDNA viruses. *Microviridae* and *parvoviridae* belong to the ssDNA virus family among the DNA virus families in the marine environment. *Microviridae* is one of the families of bacteriophages with single-stranded circular DNA genome. The virions are nonenveloped, icosahedral with spikes and a size of about 25–27 nm in diameter and lack tails. The genome sizes range

from 4.5 to approximately 6 kb, which can encode about 11 genes, and most of them have overlapping reading frames [4.11]. Most of the viruses and morphology are mentioned in Table 4.1. The most common dsDNA viruses include, *Baculoviridae, Herpesviridae, Iridoviridae, Lipothrixviridae, Nimaviridae, Papovaviridae, Phycodnaviridae, Corticoviridae, Tectiviridae, Myoviridae, Podoviridae*, and *Siphoviridae*. *Baculoviridae, Nimaviridae*, and *Parvoviridae* virus families are responsible for infection in crustaceans. Molluscs or mollusks, a large phylum of invertebrates, are the most predominant host for virus families like *Herpesviridae, Birnaviridae* (dsRNA), *Iridoviridae*, and *Papovaviridae*. *Iridoviridae* is a round, icosahedral-shaped virus, which can also infect fish. *Herpesviridae*, an pleomorphic, icosahedral, and enveloped virus, has a wide range of hosts, including mollusks, fish, turtles, and mammals. *Lipothrixviridae* and *phycodnaviridae* infect archaea and algae, respectively. The lipothrixvirus is an envelope and rod shaped, while phycodnavirus is icosahedrally-shaped. *Phycodnaviridae* is a group of large double-stranded DNA viruses and it can infect important taxa of marine primary producers including toxic bloom formers and macroalgae. A marine brown algae infecting phycodnavirus is the *Ectocarpus siliculosus* virus (EsV-1), which can encode about 231 proteins from a 336 kb size of the genome. EsV-1 is a lysogenic virus and the accumulating evidence indicates that the phycodnaviruses and their genes are ancient [4.12]. RNA viruses are a dynamic, widespread, and persistent component of the marine virus community. Although a vast number of marine RNA viruses have not been isolated, the most recognizable marine RNA viruses are positive-sense ssRNA viruses.

4.5 Marine Phages

Viruses can be arranged in terms of the types of cellular organisms that they infect. Marine phages are viruses that live as obligate parasitic agents in marine bacteria. The concentration of viruses is approximately 10^7 viruses/mL of surface seawater and the majority of the viruses are phages. Marine phages control bacterial abundance and impact the biogeochemical cycle through lysing their host (bacteria). In addition, marine phages are extremely diverse and influence their hosts through selection for resistance, horizontal gene transfer, and manipulation of the bacterial metabolism [4.13]. Sometimes, bacteriophages are responsible for altering the bacterial genome, gene expression, and development as emerging bacterial diseases or virulent strain through viral gene transfer. For instance, a major shrimp pathogen *Vibrio harveyi*, converted a nonvirulent strain into a virulent strain by DNA viruses like *Myoviridae* and *Siphoviridae* by transmission of a toxin gene [4.14]. *Corticoviridae, Tectiviridae, Myoviridae, Podoviridae, Siphoviridae, Microviridae, Cystoviridae*, and *Leviviridae* are common marine phages in the marine environment.

4.5.1 Types of Phage Infection

Phage infections can be categorized into the following types: lytic, lysogenic, chronic, pseudolysogenic, restricted, and abortive. In lytic infection, the produced phages release by destroying or lysing the bacterial cells and only the phage can survive. Lysogeny is a phage infection in which the phage genome can replicate continually without phage virion formation; in this case both bacteria and phages survive. The chronic infection effect is similar to lysogeny infection, both bacteria and phage survive, but it is a productive cycle; the produced mature phages are released from infected bacteria by extrusion or budding [4.15]. Some reports suggest that lysogeny is widespread in marine bacteria, and furthermore, up to 50% of bacterioplankton are lysogenic [4.16, 17]. The pseudolysogenic stage is a latent or quiescent state; the infecting phage genome does not replicate and does not produce phage progeny. This phenomenon is usually induced due to the unfavorable growth condition for bacteria, and this cycle ends with either the lytic or the lysogenic cycle, when it turns into favorable condition. This stage indicates phage and bacteria interactions, which may be crucial for the starving viral genome in the host and may involve the virulence of bacteria [4.18]. The bacterium (host) causes death to the infecting phage via host restriction endonuclease activity, thus there is no chance for phage progeny replication and production; this type of infection is called a restricted infection, and only the host only survives not the phage. Abortive infection involves the death of both host and infecting phage without production of phage progeny [4.15].

Table 4.1 Viruses infecting marine organisms

Virus family	Nucleic acid	Morphology	Size (nm)	Host
Baculoviridae	dsDNA	Enveloped, rods	200–450 × 100–400	Crustacea
Herpesviridae	dsDNA	Enveloped icosahedral	150–200	Mollusks, fish, mammals
Iridoviridae	dsDNA	icosahedral	190–200	Mollusks, fish
Lipothrixviridae	dsDNA	Enveloped, rod-shaped	40 × 400	Archaea
Nimaviridae	dsDNA	Enveloped, rod-shaped with tail-like appendage	120 × 275	Crustacea
Papovaviridae	dsDNA	Round, icosahedral	40–50	Mollusks
Phycodnaviridae	dsDNA	Icosahedral	130–200	Algae
Corticoviridae	dsDNA	Icosahedral with spikes	60–75	Bacteria
Tectiviridae	dsDNA	Icosahedral with spikes	60–75	Bacteria
Myoviridae	dsDNA	icosahedral	80–200	Bacteria
Podoviridae	dsDNA	Icosahedral with noncontractile tail	60	Bacteria
Siphoviridae	dsDNA	Icosahedral with noncontractile tail	60	Bacteria
Microviridae	ssDNA	Icosahedral with spikes	25–27	Bacteria
Parvoviridae	ssDNA	Round, icosahedral	20	Crustacea
Birnaviridae	dsRNA	Round, icosahedral	60	Mollusks, fish
Reoviridae	dsRNA	Icosahedral, some with spikes	50–80	Crustacea (?), mollusks, fish
Cystoviridae	dsRNA	Icosahedral with lipid coat	60–75	Bacteria
Bunyaviridae	ssRNA	Round, enveloped	80–120	Crustacea (?)
Caliciviridae	ssRNA	Round, icosahedral	35–40	Fish, mammals
Coronaviridae	ssRNA	Rod-shaped with projections	200 × 42	Crustacea
Dicistroviridae	ssRNA	Round, icosahedral	30	Crustacea
Leviviridae	ssRNA	Round, icosahedral	26	Bacteria
Marnaviridae	ssRNA	Round, icosahedral	25	Algae
Nodaviridae	ssRNA	Round, icosahedral	30	Fish
Orthomyxoviridae	ssRNA	Round, with spikes	80–120	Fish
Paramyxoviridae	ssRNA	Enveloped, filamentous	60–300 × 1000	Mammals
Picornaviridae	ssRNA	Round, icosahedral	27 × 30	Algae, crustacea (?), thraustochytrids
Rhabdoviridae	ssRNA	Bullet-shaped with projections	45–100 × 100–430	Fish

4.5.2 Cyanophages

Cyanobacteria are photosynthetic prokaryotes; they are one of the largest and most important groups of bacteria on earth. Cyanobacteria play an important role as primary producers in the ocean world. Examples are the cyanobacterial genera *Synechococcus* and *Prochlorococcus*, which together account for about 25% of global photosynthesis [4.19]. However, some groups of viruses infect cyanobacteria and cause mortality, that is, those groups of viruses called cyanophages. At the beginning, the causes of cyanobacteria mortality was poorly understood; it was assumed that it was due to protozoan infection rather than viral infection. However, the recent research data suggests that up to 7% of the heterotrophic bacteria and 5% of the cyanobacteria were associated with phages, and approximately 70% of prokaryotes might be infected by viruses [4.20]. In the 1990s, the first viral infection of marine unicellular cyanobacteria was reported and subsequently the cyanophages were isolated and characterized [4.20, 21].

Horizontal Gene Transfer

The interaction of phages and cyanobacteria may possible for new pathways of carbon and nitrogen cycling in marine food webs through gene transfer between marine organisms [4.20]. Metagenomic analysis of viral fractions reveals that cyanophages are widely distributed in the marine environment and interaction between cyanophages and cyanobacteria have been observed in the ocean [4.22]. Especially, most of the

cyanophage genes are involved in cyanobacteria photosynthesis [4.23]. Photosynthesis genes such as high light inducible genes (*hli*), psbA, and psbD are found in cyanophages. These genes encode the photosystem II (PSII) core reaction-center proteins D1 and D2, respectively. PSII, which catalyzes the light-dependent oxidation of water to molecular oxygen in chloroplasts, is a large pigment–protein complex in the thylakoid membrane. The D1 and D2 proteins of PSII bind the pigments and cofactors necessary for primary photochemistry. PSII is very sensitive to photo inhibition, and the D1 protein of the PSII reaction center is the main target for light-induced damage among the PSII proteins. The damaged D1 proteins are degraded and subsequently replaced with newly synthesized polypeptides in a repair cycle. This efficient repair mechanism is crucial to maintain PSII in a functional state. Cyanophages shut down the most of the host's gene expression during the lytic cycle infection and the proton motive force must be maintained if they are to lyse the host. Therefore, it is necessary to prolong photosynthesis of the hosts during the infection cycle. Thus, it is intended that the phage generates the energy for viral production by encoding *psbA* and other genes involved in photosynthesis. The phylogenetic analysis of the cyanophage *psbA* gene provides evidence that the acquisition of these genes by horizontal gene transfer from their cyanobacterial hosts (*Synechococcus* and *Prochlorococcus*) and gene acquisition were not very recent [4.24].

Types of Cyanophages

Cyanophages have been classified into three-tailed phage families: *Myoviridae, Podoviridae*, and *Siphoviridae*; all of them are dsDNA phages. These viruses can be isolated from both marine and fresh water environments [4.25]. Myoviruses and siphoviruses have broad host ranges and have frequently been isolated from a natural marine ecosystem. However, podoviruses have a very narrow host range with a short noncontractile tail. Myoviruses are nonenveloped, have a head with icosahedral symmetry, and a tail with tubular and helical symmetry, which is separated by a neck. The head diameter is 50–110 nm, while the tail is 16–20 nm, and the capsid is made up of 152 capsomers. Furthermore, morphological evidence supports that marine and freshwater myoviruses are more closely related each other than other bacteriophages. Only six cyanophages (S-PM2, P-SSM2, P-SSM4, P-SSP7, P60, and Syn9) infect cyanobacteria in the marine environment and all of these cyanophage sequences are available in GenBank. Interestingly, all those six cyanophages have been isolated either from *Synechococcus* or *Prochlorococcus*. S-PM2, P-SSM2, and P-SSM4 are more similar in morphology to the *Myoviridae*, However P-SSP7 and P60 belong to the *Podoviridae* family. The genomes of podoviral cyanophages are small and compact compared to myoviral cyanophages. For instance, the genome size of P-SSP7 and P60 are found to be about 44 to 47 kb. Nevertheless, myoviral cyanophages have relatively large genomes; 196, 280 bp, 178, 249 bp, and 252, 401 bp are found in S-PM2, P-SSM4, and P-SSM2, respectively [4.26].

4.5.3 Phage Therapy

Natural bacterial viruses or bacteriophages have been applied to control bacterial diseases. For example, instead of antibiotics, phages are used as a common therapy for human gastrointestinal diseases such as salmonellosis in Russia [4.27]. In 1915, the phage was discovered by the English microbiologist F.W. Twort and subsequently by d'Herelle, who introduced the term bacteriophage [4.28]. Phage research was an important field of research in the 1920s and it was desired to treat bacterial diseases [4.28]. Despite phages having been used as an antibacterial agent in the United States and Europe during the 1920s and 1930s, it has been abandoned in the western countries for various reasons, including the discovery of antibiotics. Although the commercial production of therapeutic phages has ceased in most of the Western world, phages continue to be used therapeutically in Eastern Europe and in the former Soviet Union. Moreover, several institutions in these countries are actively involved in therapeutic phage research and production [4.27].

Antibiotics have been widely used as an antibacterial agent. However, antibiotic drugs allow the development of mutated drug-resistant bacteria. Therefore, an effective alternative therapy is necessary to control the rise of anti-drug-resistant bacteria. Bacteria have the tendency to mutate against antibiotics once in every 10^6 divisions, but the antibiotics are immutable chemicals, which are not effective against the new antibiotic resistant bacterium. Although bacteria are also becoming resistant to phages, the rate of developing resistance against phages is approximately once in every 10^7 divisions, which is approximately tenfold lower compared to antibiotics. Furthermore, phages are living organisms and evolve along with hosts by mutation. Thus mutated phages can overcome the bacterial mutations within either a few days or weeks.

Additionally, phages could be the ideal strain for co-therapy along with antibiotics to prevent the emergence of bacterial resistance to antibiotics. Especially lytic phages are the most suitable candidates for phage therapy, due to the fact that they reproduce rapidly within the bacteria and lyse these in their specific host range [4.29].

Since 2006, the United States Food and Drug Administration and United States Department of Agriculture have approved several bacteriophage products for use on all food products. In marine aquaculture, the use of bacteriophages for the control of bacterial diseases in prawns in Asia has been considered. Phage therapy has been applied to control the bacterial pathogen *Vibrio harveyi*, which causes luminous bacterial disease in shrimp larvae [4.30]. Due to their host specificity, this phage therapy may be applicable to control many bacterial diseases in the future.

4.6 Impact of Marine Viruses on Mollusks

Mollusks are invertebrates and one of the largest marine phyla. These bivalve mollusks are filter feeders, thus marine viruses can be easily accumulated in their tissues and may transfer or infect other species, including higher vertebrates and humans, through the food chain. Although the pathogenic viruses are harmful to other species, it gives serious effect on the bivalve mollusks [4.31]. In the 1960s a major epizootic gill necrosis was observed in the oyster *Crassostrea angulata*, and the first report of viral disease in mollusks was reported at the same time. Most mollusks are associated with many different types of viruses including *Herpesviridae, Papovaviridae, Togaviridae, Retroviridae, Reoviridae, Birnaviridae, Picornaviridae*, and the irido-like virus [4.32]. Marine molluscan viruses and their structure morphology and main hosts are given in Table 4.2. Although many viruses are associated with marine mollusks, herpes-like and birnavirus groups are the major threats to mollusks.

4.6.1 Herpesvirus

Herpes-like virus infections have been identified in various marine mollusks throughout the world. As a result of viral disease, European oyster fisheries were destroyed in the 1970s. Since the first observation of the herpesvirus in *Crassostrea virginica*, many reports suggest that mortality is associated with this virus in *Ostreaedulis* and *C. gigas* in France and New Zealand. Herpes-like viruses were also observed in haemocytes of *O. angasi* adults in Australia and in New Zealand in flat oysters, *Tiostreachilensi*. Ostreid herpesvirus 1 (OsHV1); a herpesvirus has been isolated and reported from several species of bivalve mollusks. Moreover, unlike most herpesviruses, this virus has a wide host range of marine bivalves [4.32]. Sequence analysis results have revealed that it has links with groups of herpesviruses isolated from mammals, birds, and fish [4.33].

4.6.2 Birnavirus

Marine birnaviruses (MABV) are icosahedral, non-enveloped viruses, belonging to *Birnaviridae*. The genome of this virus comprises 2 segments of double-stranded RNA designated A and B [4.34]. MABVs had been responsible for the considerable losses in *Pictada fucata*, a commercially important pearl oyster in Japan [4.35]. MABVs infect a wide range of shellfish and fish, and it has been suspected that the mode of transmission may be due to zooplankton [4.36].

Viral diseases in bivalves are a serious concern without any specific chemotherapies and vaccination. According to the bivalve culture, a routine effective diagnostic tool is needed to monitor virus infection, and it is also important to control viral diseases.

4.7 Marine Viruses and Shrimp Aquaculture

Shrimp comprise a main aquaculture commodity worldwide. Many viruses affect shrimps, causing severe mortality of economically important shrimp species. Approximately 60% of disease losses in shrimp aquaculture is associated with viral pathogens, 20% of bacteria, and 20% by fungi and other pathogens [4.37].

Table 4.2 Viruses associated in marine mollusks

Viruses	Host species	Nucleic acid	Morphology	Symmetry
Herpesviridae	*Crassostrea virginicci*	DNA	90 nm capsid; 200 × 250 nm enveloped virion	Icosahedral
Togaviridae	*Ostrea lurida*	RNA	50 nm, enveloped virion	Icosahedral?
Retroviridae	*Crassostrea virginica*	RNA	100–110 nm, enveloped virion	Anisometric
Reoviridae	*Sepia officinalus*	RNA	75 nm, nonenveloped capsid	Icosahedral
Birnaviridae	*Pictada fucata*	RNA	60 nm, nonenveloped virion	Icosahedral
Iridoviridae	*Octopus vulgaris*	DNA	110–120 nm enveloped	Icosahedral
Papovaviridae	*Crassostrea virginica*	DNA	53 nm, nonenveloped virion	Icosahedral

The shrimp industry has grown rapidly and become a major global enterprise that serves the increasing consumer demand for seafood, and it plays an important role in improving food security, poverty mitigation, employment, and other economic activities in many countries [4.38]. Moreover, shrimp aquaculture has contributed significantly to national economies in Southeast Asia and Central and South America.

Since the early 1990s, shrimp aquaculture has expanded rapidly, reaching a worldwide production of 1 million metric tonnes (MT) in 2002 [4.39]. The global production of cultivated shrimp was about 3.5 million MT in 2009 and it continues to represent a vital source of export income for many countries [4.40]. Despite this success, viral diseases have caused billions of dollars' worth of losses for shrimp farmers.

More than 20 viruses, including white spot syndrome virus (WSSV), yellow head virus (YHV), gill-associated virus (GAV), Taura syndrome virus (TSV), infectious myonecrosis virus (IMNV), *Baculoviruspenaei* (BP), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), Mourilyan virus (MoV), Laem–Singh virus (LSNV), spawner-isolated mortality virus (SMV), baculoviral midgut gland necrosis virus (BMNV), and lymphoid organ vacuolization virus (LOVV), have been reported to infect marine shrimp species [4.41]. With the exception of WSSV, YHV, GAV, MBV, IHHNV, and HPV, the genera of many viruses have not yet been assigned due to the poor level of current virus genome characterization [4.41].

4.7.1 RNA Viruses in Shrimp

Seven ssRNA viruses and one dsRNA shrimp virus have been reported to date (Table 4.3). Among the RNA viruses, YHV and TSV are major threats to shrimp aquaculture, with the YHV virus producing 100% shrimp mortality within 3–7 days of infection. YHV is an enveloped, rod-shaped virus measuring 170 nm × 38 nm that belongs to the *Rhabdoviridae* family; it has a genome size of about 22 kb [4.42].

TSV is a highly infectious disease-producing virus which belongs to the *Picornaviridae* family. TSV is a nonenveloped icosahedron with a diameter of 30–32 nm and an ssRNA genome of approximately 10 kb. Infection with this virus is always associated with large economic losses. TSV has a linear positive-sense ssRNA genome of about 10 kb, excluding the 3′-poly (A) tail, which contains two large open reading frames (ORFs). The viral capsid is made up of one minor and three major structural proteins [4.44].

4.7.2 DNA Viruses of Shrimp

Shrimp-infecting DNA viruses are listed in Table 4.4. Two occluded baculoviruses, BP and MBV, have been reported as serious disease-causing viruses and are responsible for major economic losses in the shrimp farming industry. These are both double-stranded viruses belonging to the *Baculoviridae* family. The BP virion is a rod-shaped nucleocapsid surrounded by a trilaminar envelope. The enveloped virion is 312–320 × 75–87 nm with a nucleocapsid of approximately 306–312 × 62–68 nm containing a circular dsDNA genome. MBV is a highly infectious virus that spreads rapidly and causes high mortality rates in juvenile and larval stages. The virus has an enveloped rod-shaped particle 265–282 × 68–77 nm with a nucleocapsid of 250–269 × 62–68 nm containing a genome of approximately 200 kb [4.44].

4.7.3 White Spot Syndrome Virus (WSSV)

WSSV is also a DNA virus that has been responsible for massive mortality rates among penaeid shrimp cultured on the southern and western coasts of Korea [4.45]. WSSV, which belongs to the *Whispovirus* genus of the *Nimaviridae* family [4.46], is a rapidly replicating virulent pathogen. WSSV consists of an enveloped, rod-

Table 4.3 Known shrimp RNA viruses (after [4.41, 43])

RNA Viruses	Abbreviation	Genome	Taxonomic classification	Known geographic distribution
Yellow head virus	YHV	(+) ssRNA	*Reoiviridae/Okavirus*	Asia, Central America
Taura syndrome virus	TSV	(+) ssRNA	*Picornavirale/Dicistroviridae*	Asia, Americas
Infectious myonecrosis virus	IMNV	(+) ssRNA	Totivirus/Unassigned	Asia, South America
Macrobrachium rosenbergii nodavirus	MrNV	(+) ssRNA	Nodavirus/Unassigned	India, China, Taiwan, Thailand, Australia, Caribbean
Laem-Singh virus	LSNV	(+) dsRNA	Luteovirus-like/Unassigned	South and Southeast Asia
Mourilyan virus	MoV	(−)ssRNA	Bunavirus/Unassigned	Australia, Asia
Gill-associated virus	GAV	(+) ssRNA	*Roniviridae/Okavirus*	Australia, Asia, Pacific
Lymphoid organ vacuolization virus	LOVV	(+) ssRNA?	Togavirus-like?/Unassigned	Americas

Table 4.4 Known shrimp DNA viruses (after [4.41, 43])

DNA Virus	Abbreviation	Genome	Taxonomic classification	Known geographic distribution
Monodonbaculovirus	MBV	dsDNA	Baculoviridae/ Nucleopolyhedrosis virus	Asia, Australia, Americas, Africa
Baculoyiral midgut gland necrosis virus	BMNV	dsDNA	Baciloviridae/Unassigned	Asia, Australia
Whit spot syndrome virus	WSSV	dsDNA	*Nimaviridae/Whispovirus*,	Asia, Americas
Baculoviruspenaei	BP	dsDNA	*Baculoviridae*/Unassigned	Asia
Spawner-isolated mortality virus	SMV	ssDNA	*Parvoviridae*/Unassigned	Australia, Asia
Infectious hypodermal and haematopoietic necrosis virus	IHHNV	ssDNA	*Parvoviridae/ Brevidensovirus*	Asia, Australia, Africa, Americas, Pacific
Hepatopancreatic parvovirus	HPV	ssDNA	Densovirus	Asia, Australia, Africa, Americas

shaped nucleocapsid enclosing a large circular dsDNA genome of approximately 293–300 kb, encompassing 181–184 major ORFs [4.47]. The virion contains five major structural proteins, including two envelope proteins and three nucleocapsid proteins [4.48]. White spot disease commonly causes 80–100% mortality within 1 week of the appearance of clinical signs [4.45, 49].

4.7.4 Shrimp Parvoviruses

Two emerging parvoviruses and one parvo-like virus have been reported in shrimp. Of the parvoviruses in crustaceans, HPV and IHHNV have been studied in detail. A third parvo-like virus, SMV, has been reported from *P. monodon* but has not yet been characterized in detail [4.43]. Another virus, lymphoidalparvo-like virus (LPV), consisting of intranuclear particles 18–20 mm in diameter, has been found only in Australia [4.50]. IHHNV and HPV are nonenveloped icosahedral viruses 22–25 nm in diameter, with ssDNA genomes. The morphology, genome structure, and genome organization of both IHHNV and HPV share similarities with members of the *Parvoviridae* family. These two viruses possess three ORFs and belong to the *Densovirinae* subfamily. However, genome analysis of IHHNV indicated that it is closely related to the mosquito densovirus. Therefore, it has recently been classified as belonging to the genus *Brevidensovirus* and the species' name has been changed to *Penaeus stylirosis densovirus* [4.43]. These two viruses also differ in their infection target organ – HPV infects epithelial cells of the hepatopancreas and midgut, while IHHNV infects multiple organs of ectodermal and mesodermal origin [4.43]. However, the classification of HPV is still uncertain due to the wide range of variation in the VP (viral protein) genes and the existence of many subtypes [4.51].

General Characteristics of HPV

HPV is a small (22–24 nm in diameter) nonenveloped icosahedral shrimp-infecting parvovirus, which was recently proposed as a new member of the *Densovirinae* subfamily of ssDNA viruses in the family *Parvoviridae* [4.52]. The genome of HPV is a linear ssDNA molecule about 6 kb in length, the 5′ and 3′ ends of which have a loop-like self-priming hairpin struc-

ture [4.51]. The genome contains three long ORFs: ORF1 (or left ORF), ORF2 (or mid ORF), and ORF3 (or right ORF). ORF1 encodes a putative nonstructural protein-2 (NS2) of 428 amino acids, the function of which is not yet known. ORF2 encodes nonstructural protein-1 of 579 amino acids, which possesses the most conserved region that includes replication initiator motifs, NTP-binding, and helicase domains. ORF3 encodes a polypeptide of 818 amino acids, which is the major capsid protein (VP) [4.51]. The purified viral proteins were detected as a major band at 57 kDa and a minor band at 54 kDa due to posttranslational modification of the deduced 92-kDa polypeptide. However, the function of the minor band is still unknown [4.51].

Hosts and Geographical Distribution of HPV

HPV was first reported in 1984 in the wild shrimp species *F. chinensis* from Singapore [4.53]. HPV was detected in South Korea in 1985 in *F. chinensis* [4.54] and was subsequently observed in wild and farmed shrimp species in many geographical locations, including Australia, China, Thailand, India, Madagascar, Tanzania, New Caledonia, the Philippines, Indonesia, Malaysia, Kenya, Kuwait, Israel, and South and North America [4.43].

HPV infects epithelial cells of the hepatopancreas and shows basophilic inclusions within enlarged nuclei of tubular epithelial cells. HPV infects a wide range of shrimp species including wild and farmed *P. monodon*, *Penaeusesculentus*, *Penaeusjaponicus*, *F. chinensis*, *Penaeussemisulcatus*, *Penaeusindicus*, *Penaeuspenicillatus*, *Penaeusschmitti*, *L. vannamei*, and *Penaeusstylirostris* [4.43, 44].

HPV Genotypes

Viruses are obligate parasites that are normally present in the environment in stable ecological associations with one or more hosts [4.55]. Viruses should have efficient replication and transmission of infection in the environment or host. The environment is constantly changing because of individual differences in host genetics and the immune response of the host. These environmental variables select for mutants or suitable variant phenotypes through the inherent capacity for evasive behavior [4.41].

The HPV genome has greater genetic diversity than those of other shrimp viruses, such as IHHNV and TSV. In total, eight HPV genomes are currently available in GenBank, and among these, complete genome sequence data are available for only four isolates, including the Korean isolates [4.51, 56–58]. However, incomplete or partial genome sequences are available in GenBank for many isolates. The sequencing results of different isolates suggest that HPV isolates from different shrimp species and/or different geographical regions are genetically different [4.59]. Of the three ORFs of HPV, ORF3 (or right ORF), which encodes the major capsid protein, shows greater variability than the other ORFs. Based on the amino acid sequence of the capsid protein gene, it has been divided into three genotypes: genotype I from Korea, Tanzania, and Madagascar; genotype II from Thailand and Indonesia; and genotype III from Australia and New Caledonia. The Korean strains HPVchin and *Fc*DNV were isolated from the shrimp species *F. chinensis*, Australian (*Pmerg*DNV) and New Caledonian isolates were from the host *P. merguiensis*, and Thai isolate (*Pm*DNV) was from *P. monodon*.

4.7.5 Diagnostic Methods for Shrimp Virus Diseases

Microscopy

Most diagnostic methods applied to shrimp have been adopted from other fields of pathology and are slightly different from those used in fish, veterinary, and human pathology. Diagnostic methods for shrimp pathogens include the traditional methods of morphological pathology (direct light microscopy, histopathology, and electron microscopy), traditional microbiology, and the application of serological methods. Initially, diagnosis of shrimp diseases was dependent on microscopic observations, and characterization viruses isolated from infected tissues. Transmission electron microscopy (TEM) was added to the diagnostic toolkit in the mid-1970s and was used to discover the first *Baculovirus-penaei* in shrimp [4.60]. However, electron microscopic methods are not in common use due to their limited sensitivity as well as the long preparation time, specialized equipment, and highly trained personnel requirements.

Hematology and Clinical Chemistry

Hematology and clinical chemistry are principal diagnostic tools for human and veterinary medicine, but they have also been applied to shrimp pathology by examining the changes in hemolymph parameters (hemocyte count, hemolymph clotting time, glucose, nonprotein nitrogen, ammonia, alkaline phosphatase, and total serum protein levels). However, with the exception of hemocyte count and hemolymph clotting time, these tests have not yet been adopted for routine diagnostic purposes [4.61].

Serological Methods

Several serology-based diagnostic methods have been applied for shrimp virus disease diagnosis. Polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs) have also been virus diagnosis. A fluorescent pAb test for BMNV, the ELISA-based pAb test for BP, and pAb for rhabdovirus of penaeid shrimp (RPS) have been reported, and many attempts have been made to develop mAbs against many other viruses, such as HPV, YHV, WSSV, BP, and IHHNV. However, the development of mAbs involves problems related to specificity. Although mAbs may be specific for a particular virus, under some conditions, they may react nonspecifically to shrimp tissue due to the nature of IgM (immunoglobulin M) antibodies [4.62]. Specific mAb-based diagnostic tools are likely to be used because of their good sensitivity, versatility, low cost, speed, and simplicity.

Western blotting is a specific method for detecting antigens using mAb or pAb developed against viral capsid protein. This method requires the separation and transfer of the antigens prior to antibody treatment. Therefore, the component of the pathogen at a particular molecular weight can be identified with a high degree of accuracy. However, this is a time-consuming process that requires specialized equipment for protein separation and transformation. Therefore, this method is not commonly used for pathogen diagnosis [4.63, 64].

Recently, diagnostic lateral flow strip-based kits have been developed for WSSV and YHV using mAbs to VP28 and structural protein p20 [4.65, 66]. An mAb was raised to *Pm*DNV recombinant VP protein, which was applied to detect HPV infection in Thailand. However, the detection sensitivity of this method is not comparable to sensitive molecular diagnostic tools [4.67]. In this method, a pre-coated mouse antiviral protein antibody is used as a capture antibody at the test line (T), an anti-mouse IgG (immunoglobulin G) is used as the capture antibody at the control line (C), and a colloidal gold-conjugated monoclonal antibody pad is located adjacent to the sample pad. The sample is applied to the sample pad located at one end of the strip and allowed to flow by chromatography through the nitrocellulose membrane from one end to the other. Virus particles in test samples bind to the colloidal gold-conjugated monoclonal antibody and the resulting complex is captured by the antiviral antibody at the test line, yielding a reddish-purple band. Any unbound monoclonal antibody conjugated with colloidal gold moves across the test line and is captured by the IgG to form a band at the control line (C). However, this method is about 500 times less sensitive than a one-step real-time polymerase chain reaction, but is slightly more sensitive than dot blotting.

Molecular Diagnostic Methods

Molecular diagnostic methods include radioactive and nonradioactive labeled genomic probes and DNA amplification methods based on polymerase chain reaction (PCR), and additional PCR-based methods are being applied for the development of efficient diagnostic techniques. The first gene probe introduced into shrimp viral detection involved using a radioactive tag for IHHNV. However, it could only be applied in well-equipped laboratories due to the use of radioactive tags. Therefore, an alternative nonradioactive gene probe was developed using digoxigenin 11-dUTP (DIG). This technology allowed the development of many DIG-labeled probes for viral pathogens, such as IHHNV, HPV, TSV, WSSV, MBV, BP, and the ssRNA virus YHV [4.68]. A commercial kit for making DIG-labeled probes is available for IHHNV [4.68].

Dot blot hybridization is a simplification of the Northern, Southern, and Western blotting methods. In dot blot hybridization, the biomolecules to be detected are not first separated by chromatography. Instead, a mixture containing the molecule to be detected or a homogenized tissue sample is applied directly onto a negatively charged nitrocellulose membrane or a positively charged nylon membrane as a dot.

Dot blotting is a quick, convenient, and relatively cheap method to detect pathogens in hemolymph or tissue homogenate. The nitrocellulose membrane is the most common solid matrix used for sample adsorption. However, the major disadvantage of this method is that the sensitivity is limited to 1000 times less than that of PCR, and in most cases, the sensitivity is insufficient for detecting asymptomatic infection and it is usually associated with high background staining due to nonspecific binding of antibodies to the high concentration of shrimp proteins [4.63]. The background staining resulting from the nonspecific binding of antibodies to various shrimp components usually causes difficulty of interpretation in the case of light infection.

In situ hybridization (ISH) is a unique and powerful hybridization technique that uses a labeled complementary DNA or RNA probe to localize a specific DNA or RNA sequence in a portion or section of tissue. This method has been developed to detect shrimp pathogens using nonradioactive DIG-labeled gene probes [4.61].

Several DNA amplification methods based on PCR have been developed, and PCR has been applied in numerous methodologies for pathogen detection. Small,

often otherwise undetectable amounts of DNA can be amplified by PCR to produce detectable quantities of the target DNA. This is accomplished using specific oligonucleotide primers designed for the target DNA sequence [4.68]. Additional PCR-based methods are being applied for the development of efficient diagnostic methodologies. Multiple viral diseases can be simultaneously detected in a single reaction using more than one set of primers. Real-time PCR is especially attractive as it allows real-time analysis, high specificity, quantitative results, and the detection of low copy numbers (even as low as a single copy of a viral genome).

4.7.6 Factors Responsible for Shrimp Viral Diseases

Poor management in the shrimp farming industry leads to severe pollution in shrimp culture ponds, thereby creating a suitable environment for the development of viral diseases. The emergence of disease in shrimp aquaculture has been attributed to three major factors:

1. High density of cultured shrimp in aquaculture ponds
2. Importation or transportation of brood stock of shrimp from one place to another for culture
3. The introduction of wild brood stock into culture ponds.

The introduction of wild brooders carrying pathogens and inadequate information about disease symptoms have been responsible for the emergence of new diseases in the farming system. For example, HPV originated in the Indo-Pacific region but was later spread to America via importation of live Asian shrimp for aquaculture [4.69]. Similarly, TSV was found in Ecuador and spread to America and Southeast Asia [4.70, 71].

Environmental factors and hosts also play important roles in the emergence of shrimp diseases. Poor sanitation conditions, such as high salinity, pH, and nitrogen levels, in culture ponds can cause stress in shrimp, leading to an increased susceptibility to disease. For example, some viruses such as WSSV are highly lethal stress-related viruses. The temperature also plays an important role in virus infection; e.g., WSSV enters the target cell and replicates at 22 °C and thus leads to 100% mortality within 3 days. However, at a reduced temperature of 16–20 °C, 20–35 days were required to reach 100% mortality in experimentally infected crayfish [4.72]. However, high water temperatures from 27–33 °C inhibit the effects of WSSV in shrimp (*L. vannamei*) at the acute infection stage. However, at the chronic stage, increases in water temperature result in rapid disease progression and mortality in WSSV-infected shrimp [4.73].

4.7.7 Control of Shrimp Viral Disease

The most effective means of control or prevention of viral disease is to destroy the infected animals, decontaminate the ponds, and start again with virus-free brood stocks. Viruses, bacteria, protozoa, and fungi have emerged as major causes of disease in farmed shrimp. Bacterial, fungal, and protozoan diseases are manageable by improving culture practices, routine sanitation, and using probiotics and chemotherapeutic agents. However, management of viral diseases is problematic and has been responsible for the most costly epizootic outbreaks reported to date.

Probiotics

Live bacterial cells, referred to as *probiotics*, have been applied in aquaculture farms to improve water quality or prevent disease. The potential benefits of probiotics in aquaculture ponds include enhanced decomposition of organic matter, reduction of nitrogen and phosphorus concentrations, improved control of algal growth, greater availability of dissolved oxygen, less cyanobacteria (blue-green algae), control of ammonia, nitrite, and hydrogen sulfide, lower incidence of disease and greater survival, and improved levels of shrimp and fish production [4.74]. Few detailed studies on disease control using probiotics have been performed [4.75, 76]. However, properly controlled field tests on probiotics revealed no significant effect on measured water quality parameters [4.74]. The use of probiotics in commercial shrimp farming would be beneficial for control of disease only if positive evidence of efficacy with cost benefit analysis is acquired.

Shrimp–Virus Interactions

Shrimp–virus interactions at the molecular and genetic levels are interesting phenomena that may be useful in disease control. Many new shrimp genes have been discovered, some of which may lead to new products for disease control. A study performed in Japan indicated that Kuruma shrimp (*P. japonicus*) survived in a pond after WSSV injection; it was not protected from infection, but showed resistance to disease. This mechanism was designated as a *quasi-immune* response (immune-like system). Recently, a factor was found in shrimp hemolymph that could prevent shrimp from dying upon

injection of WSSV [4.77]. Another phenomenon has been reported that describes protection against WSD (white spot disease) by persistent IHHNV infection in *P. stylirostris*. However, infection was not prevented, although disease severity was reduced [4.78]. We still know very little about the interaction of shrimp with viral pathogens, and a better understanding of shrimp–virus interactions may lead to the development of better methods for viral disease control.

Shrimp Viral Vaccines

The term *vaccine* is applicable to vertebrates because the vertebrate process involves antibodies. However, antibodies do not occur in shrimp. In addition, *vaccinated* shrimp generally become infected but do not develop disease as a result. Therefore, the term *tolerines* has been recommended to describe agents that could be used in the same way as vaccines in shrimp [4.79].

Two types of tolerine have been studied in shrimp; the first type was developed in Thailand in the mid-1990s and is still commercially available under the brand name SEMBVAC, while the second consists of inactivated whole particles of WSSV. Shrimp acquires some degree of tolerance to WSSV and suffer less from the disease after infection due to ingestion of these products [4.80]. Other types of tolerance have also been reported consisting of individual or mixed protein subunits of viral particles that are administered either by injection or by mixing with shrimp feed [4.81, 82].

RNA Interference (RNAi)

RNA interference (RNAi) is a gene silencing technology and the process by which a gene is post transcriptionally suppressed using dsRNA with sequences that match those of viral genes to destroy their homologous mRNA in a sequence-specific manner [4.83]. This technology was recently used in the laboratory to protect shrimp from viral diseases [4.84, 85]. RNAi was used to suppress the replication of YHV and *Pm*DNV [4.83, 86]. However, application of this technology has disadvantages associated with issues of cost, safety, and public acceptance of genetic engineering techniques.

4.8 Conclusion

Viruses, one of the most rapidly evolving genetic agents among all biological entities are involved noncyclic changes in their genetic characteristics. Furthermore, the abundance of both DNA and RNA viruses in the marine environment, viral diversity, and the interaction between their hosts are ecologically important. A disease caused by marine viruses can cause a huge impact on aquaculture practice and aquatic organisms. In some circumstances, the environment pressure or high density of cultured organisms also allows for stress to the organisms, which facilitate virus infections. Moreover, it is challenging to control virus infection in wild marine organisms and viral host shift events in the ocean. Molecular biology-based methods are rapidly advancing the study of virus genomes and their molecular mechanisms. These advanced studies enable the development of rapid diagnostic tools, which could be useful for identifying viral pathogens in marine organisms and marine water samples. The advantages of molecular diagnostic methods are that they are sensitive and beneficial to preventing the spread of viral diseases to other organisms, due to the diagnosis at an early stage of infections in farmed aquaculture systems. Furthermore, therapeutic studies such as virus–host interactions, phage therapy, and RNAi are tools to control viral diseases. Although it is possible to control viral diseases in farmed aquaculture systems by using modern technology, it is still a big question as to how to control viral disease in the ocean. In addition, we must consider the cost of applying new technologies. Despite the fact that the diagnosis and control of known viral pathogens are quite possible, it is an unresolved problem to diagnose an unidentified or unknown virus population in the marine ecosystem. Even though there are a few drawbacks of viral diagnosis or control of virus impacts in aquaculture organisms, it is necessary to characterize the viruses associated with marine organisms for further studies in the future. Moreover, only a tiny fraction of viruses has been discovered and the potential discovery still seems as vast as the ocean itself. Therefore, further research is still needed to analyze the presence of viruses in the marine environment and the new potential hosts of marine viruses yet to be discovered.

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5. Marine Microalgae

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Marine microalgae, the largest primary producer in the marine system, have been attracting wide attention as potential resources of new metabolites and biofuels. Whole genome sequencing and genetic modifications of microalgae have been rapidly advancing during the last few decades. In this chapter, the diversity of marine microalgae, the microalgal natural components, and the biotechnologies associated with microalgae are reviewed.

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5.1 Overview

Algae are the primary producers of oxygen in the aquatic environment. These microorganisms are widely distributed in marine systems and have a great diversity with respect to size, morphology, life cycle, pigments, and metabolism. About half of the global oxygen production is accomplished by marine microalgae. They play an important role in CO_2 recycling through photosynthesis, which is similar to higher plants in O_2-evolved systems (PSI (photosystem I) and PSII (photosystem II)). In addition to having a long history of use as food and as live feed in aquaculture, microalgae have also been considered as a promising source for high-value added products for pharmaceuticals, cosmetics, and other industrial applications, such as β-carotene, astaxanthin, polyunsaturated fatty acids (PUFAs). The utilization of microalgae as a suitable feedstock for sustainable biofuel production has gained worldwide attention over the last 20 years. In general, microalgal triacylglycerols, hydrocarbons, and polysaccharides are considered as biofuel precursors. Alkanes and short chain fatty acid methyl esters (FAMEs), ranging from C9 to C17, have been considered as the potential alternative jet fuel, while those ranging from C9 to C23 have been mainly recognized as biodiesel. FAMEs are normally generated from the methyl esterification of triacylglycerols. On the other hand, with the degradation and fermentation of microalgal polysaccharides, the generated ethanol can be used as an alternative fuel to gasoline. Compared with higher plants, biofuel from microalgae has two advantages: 1) a relatively higher productivity, and 2) no competition to agriculture.

On the other hand, increased reports of the microalgal whole genome sequence data have been significantly facilitating the better understanding of their evolutionary lineage and the species specificity of the microalgal metabolic pathway. In addition, gene transformation has been achieved in 18 microalgal genera.

In this chapter, we reviewed the fundamental characteristics of marine microalgae, the useful microalgal natural products, as well as the biotechnological aspects of marine microalgae.

5.2 Marine Microalgae

Algae are a very diverse group of photosynthetic organisms other than land plants, which have been classified into many classes, such as *Cyanophyceae*, *Chlorophyceae*, *Rhodophyceae*, *Cryptophyceae*, *Dinophyceae*, *Bacillariophyceae*, *Haptophyceae*, *Euglenophyceae* or *Prasinophyceae*. For convenience, they are referred to as blue-green algae, green algae, brown algae, or red algae due to the difference in composition of photosynthetic pigments. However, it is difficult to make a clear definition of algae because even multicellular eukaryotic microalgae (what is called seaweed) are also included. In this section, some representative marine microalgae are summarized to introduce their biotechnological applications.

Cyanophyceae (cyanobacteria, blue-green algae) are oxygenic photosynthetic prokaryotes that comprise a single taxonomic and phylogenetic group. Chloroplasts in eukaryotes evolved from endosymbiotic cyanobacteria. They show a large diversity in their morphology, physiology, ecology, biochemistry, and other characteristics. Typically, cyanobacteria contain chlorophyll a and phycocyanin. Three genera, i. e., *Prochlorococcus*, *Prochloron*, and *Prochlorothrix*, lack phycocyanin and possess chlorophyll a and b [5.1]. A unicellular cyanobacterium that synthesizes chlorophyll d has also been discovered [5.2]. Marine *Synechococcus* and *Prochlorococcus* contribute largely to global oxygen production. Cyanobacteria have gained attention as a source of bioactive compounds and biopolymers (polyhydroxyalkanoates (PHA)s) [5.3]. Bioactive compounds isolated from marine cyanobacteria were summarized by *Burja* et al. [5.4] and *Takeyama* and *Matsunaga* [5.5]. Several strains of cyanobacteria (*Synechococcus elongates* and *Anabaena variabilis*) have been reported to produce long-chain alkanes and alkenes [5.6]. These findings make cyanobacterial alkane and alkenes a promising source of biofuels [5.7]. Two enzyme families that are responsible for straight-chain hydrocarbon production in cyanobacteria have recently been identified as an acyl–acyl carrier protein reductase (AAR) and an aldehyde-deformylating oxygenase (ADO). These enzymes convert fatty acid intermediates to alkanes and alkenes. This discovery of the cyanobacterial alkane biosynthesis indicates possibilities for optimizing the biodiesel production in cyanobacterial strains with modest gains in alkanes [5.8].

Chlorophyceae, which are one of the classes of green algae, possess chlorophyll a and b, the same predominant photosynthetic pigments as those of land plants. *Chlorophyceae* form starch in the chloroplast as a storage product of photosynthesis. Especially, *Chlamydomonas reinhardtii* has been used as a representative eukaryotic microalgae for biology and molecular biology studies. Chloroplast transformation was firstly achieved in *C. reinhardtii* [5.9]. Some species of *Chlorophyceae* are found in the marine environment. A marine species of *Chlorophyceae*, *Dunaliella* has been cultivated commercially for food supplements and β-carotene production [5.10]. *Chlorella*, which is a genus of single-cell and chlorophyll a/b-containing algae, belongs to the phylum *Chlorophyta*. It has been known as a potential food resource because of its high content of protein and other nutrients. Miura and others [5.11] reported that *Chlorella* sp. NKG 042 401 contains 10% γ-linolenic acid (C18:3), which is present in the cells mainly in the form of galactolipids. In *Euglenophyceae*, the genus *Euglena* is well known. The chloroplast of *Euglena* originated from the eukaryotic green algae and contains chlorophyll a and b. Although most species are found in freshwater environments, some species also occur in marine environments.

Bacillariophyceae (diatoms) possess chlorophyll a and c, and fucoxanthin as the major carotenoid. Diatoms are widely used as feed in mariculture/aquaculture [5.12, 13]. *Chaetoceros calcitrans*, *Chaetoceros gracilis*, *Chaetoceros muelleri*, *Skeletonema costatum*, and *Thalassiosira pseudonana* are commonly used as live feed for all growth stages of bivalve molluscs (e.g., oysters, scallops, clams, and mussels), for crustacean larvae, and for zooplankton used as feed for larvae. The genera *Navicula*, *Nitzschia*, *Cocconeis*, and *Amphora* also are used to feed juvenile abalone. They store energy either as lipids or as chrysolaminarin. Most diatoms have a high content of eicosapentaenoic acid (EPA) 20 : 5 (n-3). *Phaeodactylum tricornutum* and *Nitzschia laevis* have been especially investigated for EPA production. In addition, EPA production by diatoms was reviewed recently by *Lebeau* and *Robert* [5.14, 15]. Recent advances in heterotrophic production of EPA by microalgae were also reviewed by *Wen* and *Chen* [5.16].

The cells of *Haptophyceae* are brownish or yellowish-green and contain chlorophylls a/c and carotenoids such as β-carotene, fucoxanthin, diadinoxanthin, and diatoxanthin. The cells are commonly covered with scales made mainly by carbohydrates or calcium bicarbonate. Many species known as coccol-

ithophorids produce calcified scales called coccoliths. Most are primarily marine species inhabiting tropical seawater. Microalgal biomass of *Haptophyceae* is commonly used as living feed in aquaculture [5.17]. *Isochrysis galbana* and *Pavlova lutheri*, especially, are used as living feed for bivalve molluscs, crustacean larvae, and zooplanktons that in turn are used for crustacean and fish larvae. Some cells can produce PUFAs such as docosahexaenoic acid (DHA), or EPA. In addition, the DHA content in *I. galbana* has been shown to be enhanced by low temperature or incubation of the culture in the dark after reaching the plateau phase growth [5.18]. Furthermore, it was shown that these algae are useful for DHA enrichment of feed such as rotifers for the larvae of several marine fish species [5.19].

In *Dinophyceae*, a genus *Symbiodinium* (dinoflagellate) has been well described. Various marine invertebrates, such as reef-building corals, jellyfish, sea anemones, and bivalves form symbiotic associations with *Symbiodinium*, commonly known as zooxanthellae. *Symbiodinium* strains have been classified into more than three clades using restriction fragment length polymorphism based on 18S rRNA sequence analysis [5.20]. The composition of *Symbiodinium* populations may also play an important role in the tolerance or sensitivity of corals towards bleaching.

5.3 Microalgal Genomes

Sequencing of microbial genomes has become a routine procedure for gene discovery and genetic engineering of microalgae. *Synechocystis* sp. PCC 6803 was the first photosynthetic organism for which the entire genome sequence was determined. Currently, 72 finished cyanobacterial genome sequences are listed in GenBank, and many additional genome analyses are currently in progress. Most cyanobacteria possess a circular chromosome and a small number of additional plasmids. Genome sizes range from a minimum of 1.44 Mb for the marine cyanobacterium UCYN-A [5.21] to a maximum of 11.58 Mb for the *Calothrix* sp. PCC7103 [5.22]. Prokaryotes typically contain a single copy of their chromosome such as *Escherichia coli*, while large differences between cyanobacteria and other prokaryotes have been reported for chromosomal copy numbers. Some cyanobacteria are oligoploid, for example, *Synechocystis* sp. PCC 6803 are highly polyploid, and the motile wild-type strain contains 218 genome copies in exponential phase and 58 genome copies in linear and stationary phases [5.23].

Recently, a comparative genomics-based approach was used to screen cyanobacteria for the direct production of alkanes, the primary hydrocarbon components of gasoline, diesel, and jet fuel [5.8]. Eleven different cyanobacteria with available genome sequences were grown, and their culture extracts were evaluated for hydrocarbon production. Indeed, ten of these strains produced alkanes. The comparison of predicted proteins from these ten genomes against the eleventh finally led to the discovery of two hypothetical proteins as candidates for alkane biosynthesis. This discovery is the first description of genes responsible for alkane biosynthesis and the first example of a single-step conversion of sugar to fuel-grade alkanes by an engineered microorganism. A comparison of the genome sequences of producing and non-producing organisms led to the identification of the responsible genes.

In eukaryotic microalgae genomics, large-scale sequencing has been demonstrated by next-generation sequencing technologies. These have drastically increased the number of bases obtained per sequencing run while at the same time decreasing the costs per base. The first whole genome sequence of *C. merolae* was determined in 2004 [5.26]; this was the first identi-

Table 5.1 Sequenced whole genomes of microalgal strains

Microalgae species	Genome length (Mbp)	References
Ochrophyta		
Phaeodactylum tricornutum	27.4	[5.24]
Thalassiosira pseudonana	32.4	[5.25]
Rhodophyta		
Cyanidioschyzon merolae	16.5	[5.26]
Chlorophyta		
Chlamydomonas reinhardtii	121	[5.27]
Chlorella variabilis	46.2	[5.28]
Micromonas pusilla	21.9	[5.29]
Micromonas sp.	20.9	[5.29]
Volvox carteri	138	[5.30]
Ostreococcus lucimarinus	13.2	[5.31]
Ostreococcus tauri	12.6	[5.32]
Coccomyxa subellipsoidea	48.8	[5.33]

fied eukaryotic microalgal genome. Up until November 2012, the whole genome sequence of 11 strains of microalgae had been sequenced, including 2 diatoms (*Thalassiosira pseudonana* [5.24] and *Phaeodactylum tricornutum* [5.25]), a red alga (*Cyanidioschyzon merolae* [5.26]), and 8 green algae (*Chlamydomonas reinhardtii* [5.27], *Ostreococcus lucimarinus* [5.31], *Ostreococcus tauri* [5.32], *Chlorella variabilis* [5.28], *Volvox carteri* [5.30], *Coccomyxa subellipsoidea* [5.33], *Micromonas pusilla* [5.29], and *Micromonas* sp. [5.29]), see Table 5.1. In addition, the draft genome sequences of 17 strains of microalgae can been found in the NCBI GenBank databases [5.34]. With next generation technology, the draft genome sequence of the biodiesel producing microalga *Nannochloropsis gaditana* strain CCMP526 were also identified recently [5.35]. The identified microalgal whole genome sequences provide a powerful tool for the discovery of genes and metabolic pathways. Even though most of the predicted microalgal pathways have been proved to be similar to corresponding pathways in higher plants, the urea cycle identified from genomes of diatoms is absent in higher plants but present in animals [5.24]. The existence of an animal metabolic pathway in microalgal cells further highlights the importance of genome analysis for microalgae.

5.4 Genetic Engineering of Microalgae

5.4.1 Genetic Transformation Methods

Genetic studies on microalgae have been redirected mainly toward analysis of photosynthesis and metabolic pathways. A limited number of microalgae such as cyanobacteria have been used in biotechnological applications. The development of molecular techniques for physiological analysis and enhancement of biotechnological applications is necessary. Many attempts at gene transfer have been made in eukaryotic and prokaryotic microalgae. Genetic manipulation in prokaryotic microalgae cyanobacteria was studied extensively after several transformable unicellular strains were discovered. First, the freshwater cyanobacterium *Synechococcus* PCC7942 was reported to have the ability to take up DNA. Subsequently, several other naturally transformable freshwater strains were found. Gene transfer has been developed mainly in the freshwater strains *Synechococcus, Synechocystis, Anabaeba*, and *Nostoc* [5.69]. Several marine cyanobacterial strains of the genus *Synechococcus* have been also used for heterogeneous gene expression and other genetic applications [5.70, 71]. There are two commonly used gene transfer procedures: transformation using naturally occurring or artificially competent cells, e.g., conjugation with *Escherichia coli*, or physical methods for gene introduction, e.g., electroporation and particle bombardment. Natural transformation has been reported for *Synechococcus* sp. PCC7002 [5.72]. Other strains have been transformed successfully by electroporation or conjugation. Further, plasmids harvested from several marine microalgal species have been used as vector DNA for gene transfer. Marine plasmids have been found in *Synechococcus* sp. NKBG042902, which has a high phycocyanin content and a rapid growth rate. This strain contains more than three cryptic endogenous plasmids, and one of these, the plasmid pSY10 has the unique replication characteristic that its copy number increases under high salinity conditions [5.73]. Plasmids are maintained at a high copy number in cyanobacteria, which suggests the possibility that they act as a shuttle vector between cyanobacteria and *E. coli*. In fact, a shuttle vector with *E. coli* has been constructed using pSY10. Conjugative gene transfer using a broad-host range vector pKT230 was successful for the marine cyanobacterium *Synechococcus* sp. NKBG 15041C [5.74]. It has been demonstrated that this plasmid is stably maintained in cyanobacterial cells [5.75]. In marine cyanobacteria, in addition to the plasmid vector system, the construction of a phage vector system is also required to enable the cloning of large DNA fragments in specific cyanobacterial hosts. Since cyanophages were first reported by *Safferman* and *Morris* [5.76], various types of cyanophages have been found in seawater [5.77, 78] and characterized according to their genetic diversity and phylogenetic affiliations [5.79].

Due to the advance of genome, proteome, and metabolome analyses of microalgae, many attempts at gene transfer to eukaryotic microalgae have been made to enhance the production of useful compounds and biomass. However, because of the stiff cell wall of microalgae, the introduction of exogenous genes into microalgal cells could be challenging. The additional frustules and coccoliths surrounding some species of microalgae cells further increases the difficulty. Thus,

Table 5.2 Microalgal strains achieved for the stable transformation

Phylum species	Organelle	Transformation methods	Gene knock-down	References
Diatom				
Cyclotella cryptica	Nucleus	Biolistic		[5.36]
Cylindrotheca fusiformis	Nucleus	Biolistic		[5.37]
Chaetoceros sp.	Nucleus	Biolistic		[5.38]
Navicula saprophila	Nucleus	Biolistic		[5.36]
Phaeodactylum tricornutum	Nucleus	Biolistic	✓	[5.39, 40]
Thalassiosira pseudonana	Nucleus	Biolistic		[5.41]
Fistulifera sp.	Nucleus	Biolistic	✓	[5.42]
Chlorophyta				
Chlamydomonas reinhardtii	Nucleus	Biolistic, Electroporation, Glass beads, Agrobacterium	✓	[5.43–48]
	Chloroplast	Biolistic		[5.9]
	Mitochondoria	Biolistic		[5.33]
Chlorella spp.	Nucleus	Biolistic, Electroporation, Agrobacterium		[5.49–53]
Dunaliella spp.	Nucleus	Biolistic, Electroporation , Glass beads	✓	[5.54–58]
Haematococcus pluvialis	Nucleus	Biolistic, Agrobacterium		[5.59, 60]
Volvox carteri	Nucleus	Biolistic		[5.61]
Dinoflagellate				
Amphidinium sp.	Nucleus	Glass beads		[5.62]
Symbiodinium microadriaticum	Nucleus	Glass beads		[5.62]
Rhodophyta				
Cyanidioschyzon merolae	Nucleus	Glass beads	✓	[5.63, 64]
Porphyridium spp.	Chloroplast	Biolistic, Agrobacterium		[5.65, 66]
Euglenophyta				
Euglena gracilis	Chloroplast	Biolistic		[5.67]
Eustigmatophyte				
Nannochloropsis spp.	Nucleus	Electroporation		[5.35, 68]

the optimization of the gene transformation method for each specific species turns out to be important. Depending on the physiological characteristics of microalgal cells, electroporation, glass beads-mediated transformation, agrobacterium-mediated transformation, and biolistics have frequently been used. Moreover, the level of target protein varied due to multiple insertion, random integration, and (or) gene silencing [5.80]. Stable transformants that have already been reported are summarized in Table 5.2.

Biolistics, also referred to as a gene gun that was originally designed for the delivery of nucleic acid through the cell wall of intact plant cells, has been mostly applied for microalgae gene transformation. The payload in this system is a plasmid DNA-coated tungsten particle (particle size: 0.6−1.6 μm), which can be shot with helium gas. After bombardment, the tungsten particles were shot down to the plant organism or the cell culture on the petri dish. Some cells that are not disrupted by the firing may envelope the DNA-coated tungsten particles and the DNA can then migrate to and integrate into the plant chromosome [5.81]. The transformation efficiency of this methodology is not related to the physical property of the host cell but is highly controlled by the gas pressure at the point of firing. Therefore, theoretically, despite the hard cell wall and frustules, gene transformation can be achieved when the gas pressure is high enough.

Electroporation is a phenomenon when the electrical conductivity and permeability of the cell membrane increase by the externally applied electrical field. If the host cells and plasmids are mixed together, the plasmids can be transferred into the host cells through the transient holes in the cell membrane generated by the electronic shock. Electroporation-based gene transformation methodology has been commonly used to

transform mammalian cells with plasmids. The transformation efficiency was significantly decreased for plant cells due to their thick cell walls. Electroporation-based gene transformation has only been achieved with the *Chlamydomonas reinhardtii* cell wall-deficient mutant and *Dunaliella salina* cells, which have no cell wall [5.46, 57, 82]. However, in the studies mentioned above, the transformation efficiency was tenfold higher than the gene gun method applied to the corresponding strains [5.83].

The glass beads method is a relatively simple transformation procedure that has a higher transformation efficiency than biolistics but it is only capable of transforming cells without cell walls. Both the cell-wall deficient *C. reinhardtii* mutant and *D. salina* have been reported to have been successfully transformed by the glass bead method with a higher efficiency than with the gene gun method [5.56].

Agrobacterium-mediated transformation is based on the characteristic of the soil bacterium *Agrobacterium tumefaciens* that it naturally transfers and inserts its genes into plant chromosomes. Exogenous genes can be transferred into plant cells through *Agrobacterium* transformation using target gene inserted agrobacterium transfer DNA (T-DNA). Although reports of microalgae transferred by agrobacterium transformation are few, *Kathiresan* et al. achieved a twofold transformation efficiency with *Haematococcus pluvialis* over the gene gun method [5.60].

In the transformants generated by the methods mentioned above, it is not rare to find the continuous expression of the target genes in the chloroplast and (or) mitochondria due to the insertion of the target genes into their organelle genome. By using a specific vector containing a homologous sequence in the organelle genome, stable chloroplast and (or) mitochondria transformation can be expected. On the other hand, the target genes are usually found to be randomly inserted into the nucleic genome and even homologous recombination occurs. Thus, it is hardly possible to control the insertion site and the number of the target genes inserted into the nucleic genome, which has made gene functional analysis via gene knock-out difficult. With further consideration of the dual nature of the microalgal life cycle as either haploid or diploid, the possibility of complete knock-out dwindled significantly in diploid cells. The homologous recombination has been applied to the transformation of *C. reinhardtii* and *Volvox carteri*, which maintain an asexual haploid zoospore during the life cycle; their recombination efficiency, however, was inferior [5.84, 85]. Recently, highly efficient homologous recombination was reported in *Nannochloropsis* sp., which suggested the possible use in microalgal gene functional analysis [5.68]. For those diploid microalgae, the knock-down of the target gene via RNAi has been reported and considered as the substitute for knock-out [5.40, 44, 55, 64].

So far, six microalgae including *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Volvox carteri*, and *Cyanidioschyzon merolae* have not only obtained stable transformants but also the whole genome sequence. *P. tricornutum* has been widely used for the studies of metabolic engineering towards enhanced lipid production. Yet, most research in this field has focused on the established stable transformant rather than on high oil-producing strains whose transformation method have not been determined.

5.4.2 Metabolic Engineering

Enhanced production of valuable primary or secondary metabolites in microalgae can be rendered possible by high density cultivation and/or application of genetic manipulation. Recent pharmaceutical interest in unsaturated fatty acids has triggered the search for sources of these valuable compounds. Several eukaryotic microalgae are known to produce highly unsaturated fatty acids such as EPA and DHA, which are valuable dietary components [5.16, 19]. Genetic engineering has been applied to produce EPA in the marine cyanobacterium *Synechococcus* sp. [5.71]. Cyanobacteria do not have the biosynthetic pathway to produce them. The EPA synthesis gene cluster (ca. 38 kbp) isolated from a marine bacterium *Shewanella putrefaciens* SCRC-2738 was cloned to the marine cyanobacterium using a broad-host cosmid vector. The content of EPA grown at 2 °C increased to 0.64 mg g^{-1} dry cells after 24 h incubation at 17 °C. Furthermore, EPA production was improved by partial deletion of the EPA gene cluster to stabilize its expression and maintenance in host cyanobacterial cells [5.86].

Genetic engineering of microalgae for industrial purposes has also been performed in freshwater cyanobacteria where the ketocarotenoid astaxanthin, an extremely efficient antioxidant, was synthesized by the introduction of the β-c-4-oxygenase gene (*crt*O) from the green alga *Haematococcus* [5.87]. Ethylene production was also demonstrated in the cyanobacterium *Synechococcus elongates* PCC7942 by chromosomal insertion of an ethylene forming enzyme [5.88]. How-

ever, the reaction catalyzed by the ethylene forming enzyme induced metabolic stress, which was detrimental to the host cell.

Microalgal biodiesel production is expected to be improved though metabolic engineering. Several transformants have been established for the increased oil content of the microalgal cell, enhanced biomass productivity, and improved quality of the lipids. Acetyl CoA carboxylase (ACCase), which catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the primary substrate of fatty acids synthesis, has been overexpressed in diatom cells to elevate the cell lipid content [5.36]. The vector containing the ACCase gene and its 5′ UTR (untranslated region) from the diatom *Cyclotella cryptica* was constructed and introduced into the diatoms *C. cryptica* and *Navicula saprophila*. Stable high ACCase expression transformants were obtained successfully, yet the predicted increase of neutral lipid content was not achieved, which indicates that TAG (triacylglycerol) accumulation in the microalgal cell is much more complex than previously assumed. With the expression of the hexose transporter that transfers the monohexose from the culture medium into the cell, the transformants of green algae *C. reinhardtii* and *V. carteri* as well as diatom *P. tricornutum* have been demonstrated to be capable to grow in the dark in a medium containing glucose [5.89–91]. Especially, the glucose transporter (Glut1) gene transformant of *P. tricornutum* cultured in dark conditions showed an almost threefold higher biomass production than in light conditions [5.91]. On the other hand, the de-regulation of the light-harvesting proteins in *C. reinhardtii* has been demonstrated to be able to elevate the solar energy conversion efficiencies in photosynthesis when the light-harvesting chlorophyll antenna size is minimized [5.92]. This permits a greater photosynthetic productivity under high cell density conditions as well as the possibility of culturing cells under high sunlight conditions. The transformation of *Cinnamomum camphora* (C12-TE) and *Umbellularia californica* (C14-TE) Acyl-ACP thioesterases genes into diatom *P. tricornutum* resulted in an increased lauric (C12:0) and myristic acid (C14:0) accumulation mutant [5.93]. Levels of lauric acid of up to 6.2% of total fatty acids and myristic acid of up to 15% by weight were achieved. Moreover, 75–90% of the shorter chain length fatty acids produced were demonstrated to be incorporated into triacylglycerols.

5.5 Photobioreactors for Marine Microalgae

Microalgae mass cultivation for the production of useful compounds has been widely discussed since the 1950s. Even though large-scale production of astaxanthin, DHA, and EPA from microalgae have been achieved, the industrial production of microalgal biofuel is still under development. Lower cost and higher productivity and efficiency than current bioreactors are necessary due to the extremely low final price of biodiesel (1 dollar L^{-1}) compared with those high value-added microalgal products.

Both the biology and the economics of microalgae mass cultivation are strongly influenced by photobioreactor design. Photosynthetic microalgae can be cultured in photobioreactors as either an open culture system or a closed system. Based on their localization, these photobioreactors can be divided into outdoor culture systems or indoor culture systems.

Outdoor open culture systems are the simplest method of algal cultivation due to the low construction cost and effortless operation. However, the productivity of these systems can be easily affected by several environmental factors such as contamination of other microorganisms, changes of weather conditions, and the disability of transgenic microalgae cultivation. The need to achieve higher productivity and to maintain monoculture of algae led to the development of closed photobioreactors. Despite higher biomass concentration and better control of culture parameters, CO_2 recycling efficiency, energy profit ratio, energy payback time, and cost of production in these enclosed photobioreactors are not better than those achievable in open systems.

The growth rate and maximum biomass yield of microalgal strains are affected by culture parameters (light, temperature, and pH) and nutritional status (CO_2, nitrogen, and phosphate concentration). On the other hand, increasing the density of cultures decreases photon availability to individual cells. Light penetration of microalgal cultures is poor, especially at high cell densities, and such poor photon availability decreases specific growth rates. Higher biomass yields can be expected if sufficient photons are provided in high density cultures of microalgae. Two major types of bioreactors (tubular [5.94, 95] or flat plate [5.96, 97]) are generally applied for the enclosed system (Fig. 5.1).

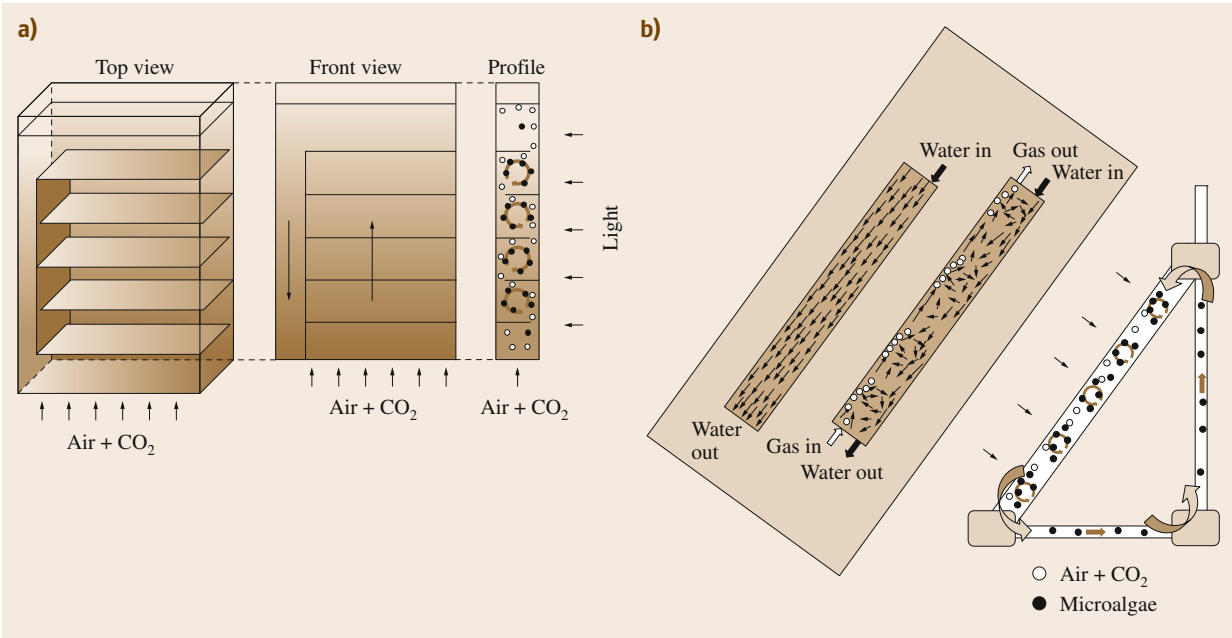

Fig. 5.1a,b Schematics of various photobioreactors. **(a)** After [5.95], **(b)** after [5.96]

Currently these enclosed photobioreactors are industrially applied for biodiesel production, for instance, the outdoor enclosed culture system used in Solix Biofuels Inc. and the indoor enclosed culture system used in Solazyme Inc. In addition, the use of intermittent light instead of continuous illumination can reduce the photoinhibition effect and improve the efficiency of light utilization through the flashing-light effect. On the other hand, the culture medium inside the photobioreactors needs to be stay flowing for mixing the algae and other nutrients. A newly developed flat plate photobioreactor achieved a 1.7-fold increased biomass production for *Chlorella vulgaris* [5.96] by applying the intermittent light from one side of the bioreactor, and the introduction of the air bubbling from the bottom of the bioreactor which generates turbulence inside the container. Recently, based on the flashing-light effect, a triangle bioreactor constructed by three tubular bioreactors with the air bubbling from the hypotenuse has achieved the currently highest volumetric productivity (365 t ha^{-1} a^{-1}) [5.95, 98].

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6. Seaweed Flora of the European North Atlantic and Mediterranean

Leonel Pereira

Algae, like most vegetables, have cells with pigments that allow them to perform the photosynthesis. These organisms have a wide geographical distribution, colonizing various sites, but always linked to the presence of water. They can be found floating in the water, on wet rocks, walls, or in association with other organisms, as in the case of lichens, in association with fungi. They are particularly abundant in lakes, rivers, and seas, occupying the euphotic region (or photic), i. e., up to lower penetration of light effective for the realization of photosynthesis of algae (up to 200 m deep). Those that live in water can be planktonic (microalgae) or benthic (macroalgae). The former are small and live suspended in water (microscopic), whereas the latter remain fixed to a substrate (usually rock), are larger in size (macroscopic), and may reach 50 m in length. Seaweeds inhabit the oceans more than 2000 million years and are used as food by people in Asia since the 17th century. Today, seaweed is used in many countries for several purposes: directly as food, phycocolloids extraction (carrageenan, agar, and alginate) used in the pharmaceutical, cosmetic, and food industries. Algae are also used in the extraction of antiviral, antibacterial compounds, and biofertilizers. The larger or macroscopic algae, called macroalgae or seaweed, are mainly found in three of the algal taxonomic groups: Chlorophyta (green algae); Rhodophyta (red algae); and Phaeophyceae (brown algae). In this chapter, the main European seaweed flora and their biotechnological potential are listed.

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6.1 Marine Macroalgae (Seaweeds)

Algae, like most vegetables, have cells with pigments that allow them to perform the photosynthesis.

Algae have a wide geographical distribution, colonizing various sites, but always linked to the presence of water. They can be found floating in the water, on wet rocks, walls, or in association with other organisms, as in the case of lichens, in association with fungi.

They are particularly abundant in lakes, rivers, and seas, occupying the euphotic region (or photic), i. e.,

up to lower penetration of light effective for the realization of photosynthesis of algae (up to 200 m deep). Those that live in water can be planktonic (microalgae) or benthic (macroalgae). The former are small and remain suspended in water (microscopic), whereas the latter remain fixed to a substrate (usually rock), are larger in size (macroscopic), and may reach 50 m in length [6.1].

6.1.1 Role of Algae in Nature

Algae are primary producers, i. e., they are able to produce oxygen and organic compounds that serve as food for other living beings, through the process of photosynthesis, using sunlight, atmospheric CO_2, and inorganic substances present in water. Algae are, in fact, fundamental to food chains of all aquatic ecosystems.

6.1.2 Main Taxonomic Groups of Benthic Marine Algae

The coloration of seaweed is the visible expression of the combination of different cellular pigments. Thus, the phyla and classes of macroalgae are essentially defined in practice by their particular pigment composition.

Macroalgae are aquatic photosynthetic organisms belonging to the Domain Eukarya and the Kingdoms Plantae (green and red algae) and Chromista (brown algae). Although classification systems vary over time and according to the authors, it is generally accepted to consider that

1. The green algae are included in the Phylum Chlorophyta; their pigmentation is identical to that of terrestrial plants (chlorophyll a, b, and carotenoids);
2. The red algae belong to the phylum Rhodophyta, their photosynthetic pigments are chlorophyll a, phycobilins (R-phycocyanin and R-phycoerythrin), and carotenoids (β-carotene, lutein, and zeaxanthin);
3. The brown algae are included in the phylum Ochrophyta (or Heterokontophyta), class Phaeophyceae; their pigments are the chlorophylls a, c, and carotenoids, dominated by fucoxanthin, responsible for the brownish color [6.1, 2].

6.1.3 Marine Algae Morphology

The diversity of algae is extraordinary; one can find a multitude of morphological types according to their complexity, structure, and environmental adaptations. Macroalgae show a complex degree of morphological organization, with laminar, cylindrical, tubular, or crustose thalli.

6.1.4 Importance of Algae for Mankind

Today, seaweed is used in many countries for very different purposes: directly as food, phycocolloids extraction, extraction of compounds with antiviral, antibacterial, or antitumor activity and as biofertilizers.

About four million tonnes of seaweed are harvested annually worldwide. The major producers are China and Japan, followed by America and Norway. France used to import Japanese seaweed in the 1970s but 10 years later it went on to produce algae for food and biological products users. Contrary to what happens in East Asia, the West is more interested in thickeners and gelling properties of hydrocolloids extracted from seaweeds: carrageenan, agar, and alginate (E407, E406, and E400, respectively) [6.3, 4].

6.1.5 Historical Overview of Algae Use on Health Treatments

Historically, the algae have been used by coastal communities to prepare home medicines to treat various health problems. Such applications are the product of the empirical knowledge of many generations and, in most cases, their mechanisms are unknown. However, the current research, undertaken in order to analyze the components and causes which affect the proper functioning of our body, are already giving their first fruits. So, we know, for example, that the good results obtained with the use of Kelp (*Laminaria* spp.) in the treatment of goiter are due to the fact that the origin of this disease is a diet low in iodine, chemical element present in significant quantities in brown algae [6.3].

Green algae have been used as anthelmintics, astringent, and to treat gout. Brown algae are used in the treatment of rheumatic processes, arteriosclerosis, menstrual disorders, hypertension, gastric ulcers, goiter, skin diseases, syphilis, and as an anticoagulant. Red algae are used as anticoagulants, anthelmintics, and in treating gastritis and diarrhea [6.3, 5].

6.2 The Marine Algae and Their Biotechnological Potential

Over time, the need to find new paths in search of new sources of energy is evident and relevant, especially the methods independent of fossil fuels, because they will run out one day and have an increasingly higher cost of use, both in terms of economic costs but mainly environmental costs.

One way to achieve it is by taking advantage of the biomass from the cultivation of algae in the oceans and seas, which offer large areas available for that purpose, which does not happen on land, where farmland is occupied for the production of food. The algae can produce, for example, biodiesel and ethanol.

On the other hand, in recent years, several marine organisms have been confirmed as an important source of new compounds potentially useful for the development of chemotherapeutic agents. Previous investigations of the production of antibiotic substances by aquatic organisms point to these forms as a rich and varied source of antibacterial and antifungal agents. Over 15 000 novel compounds have been chemically determined. Focusing on bioproducts, recent trends in drug research from natural sources suggest that algae are a promising group to furnish novel biochemically active substances [6.6].

Seaweeds or marine macroalgae are the renewable living resources which are also used as food and fertilizer in many parts of the world. Seaweeds are of nutritional interest as they contain low calorie food but are rich in vitamins, minerals, and dietary fibers [6.7]. In addition to vitamins and minerals, seaweeds are also potentially good sources of proteins, polysaccharides and fibers [6.8]. The lipids, which are present in very small amounts, are unsaturated and afford protection against cardiovascular pathogens.

Seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Compounds with antioxidant, antiviral, antifungal, and antimicrobial activities have been detected in brown, red, and green algae [6.9, 10]. There are numerous reports of compounds derived from macroalgae with a broad range of biological activities, such as antibacterial [6.11], antivirals [6.12], antitumorals [6.13], anticoagulant [6.14], and antifouling [6.15], antihelminthic and antifungal [6.16].

6.3 Taxonomy and Description of Marine Algae with Biotechnological Potential

6.3.1 Domain/Empire Prokaryota, Kingdom Bacteria, Phylum Cyanobacteria (Blue–Green Algae)

- Class: Cyanophyceae
- Order: **Nostocales**

Rivularia bullata (Poir) Berkeley ex Bornet & Flahault

Description. In general, blue-green algae (Cyanobacteria) are not easy to find on the coastline edge (Fig. 6.1). However, one species may be easily confused with a green seaweed (Chlorophyta), whereby described here. This species forms small globular vesicles, dark green, sometimes bluish, gelatinous, and elastic; adherent to exposed rocks, sometimes on *Chthamalus* spp. (Barnacles), together with *Lichina pygmaea* (Lichens), and may attain 5 mm in diameter [6.2, 17].

Habitat. Grows on rocks of the upper littoral zone.

Distribution. NE Atlantic (Ireland and Britain to Portugal); Mediterranean; SW Indian Ocean; SW Pacific; Australia.

Uses and Compounds. Present antibacterial activity [6.18, 19].

6.3.2 Domain/Empire Eukaryota, Kingdom Plantae, Phylum Chlorophyta (Green Algae)

- Class: Bryopsidophyceae
- Order: **Bryopsidales**

Bryopsis hypnoides J.V. Lamouroux

Description. Plants in filamentous tufts, dull or dark green, 10 cm tall, are branching in an irregular, scattered pattern. Primary axes are highly branched. Fronds decrease in diameter with each successive division;

Fig. 6.1 *R. bullata* specimens on *Chthamalus* spp. (Barnacles)

branchlets form irregularly, undifferentiated from axes, constricted at base. Apices rounded; rhizoidal system fibrous, tightly woven [6.20, 21].

Habitat. Common near freshwater and nutrient rich outputs; attaches to hard substrates such as basalt, rocks, or rubble; forms delicate fronds which move with currents.

Worldwide Distribution. Atlantic Ocean, Mediterranean, Caribbean, Indian and Pacific Oceans, Australia.

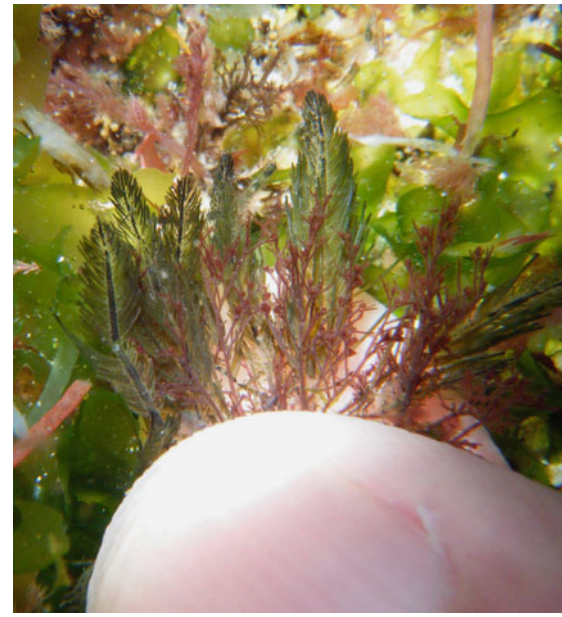

Fig. 6.2 Underwater photo of *Bryopsis plumosa*

Uses and Compounds. The primary structure of bryohealin and of lectin from *Bryopsis hypnoides* had little similarity with any known plant lectin, but rather resembled animal lectins with fucolectin domains [6.20, 22, 23]; Antifouling activity is present [6.24].

Bryopsis pennata J.V. Lamouroux

Description. Thallus filamentous, bushy, in tuft-like mats, to 10 cm high. Fronds feather-like, 8–15 mm wide, pinnately branched; lateral branches of uniform length, constricted at base where joined to main axes. Branchlets in two opposite rows on the upper half of branch, the lower half of branch is bare. The rhizoidal system is fibrous, tightly interwoven. Color is glossy dark green, often with light blue iridescence [6.25].

Habitat. *B. pennata* forms soft, feathery clumps attached to basalt rocks and rubble on shallow reef flats, in tide pools, and in the lower intertidal habitats of coastlines with low wave action.

Distribution. Atlantic Ocean, Mediterranean, Caribbean, Indian and Pacific Oceans, Australia.

Uses and Compounds. *Bryopsis* species are potentially invasive. Like the troublesome *Caulerpa taxifolia*, the genus produces chemical defenses that are toxic to most herbivorous organisms and easily reproduces veg-

etatively from the smallest fragments [6.25, 26]. The extracts of this seaweed have antibacterial [6.27–29], antifungal [6.29], anticancer [6.30], and cardiotonic [6.26] activity.

Bryopsis plumosa (Hudson) C. Agardh

Description. *B. plumosa* is a small, feathery species of green seaweed (Fig. 6.2). The thalli are erect, feather-like, and may reach up to 15 cm in height. It is easily recognized by its delicate branches. All branches are regularly arranged in two rows on opposite sides of the central filament. The lengths of the branches decrease gradually from the base of the plant. It is usually dark to mid-green in color [6.31, 32].

Common Names. Evenly branched mossy feather weed; stoneworts.

Habitat. Not uncommon; in low littoral rock pools and in the sublittoral. Is an epilithic species that is usually found in a deep lower shore pool or subtidally in both sheltered and well-exposed habitats.

Distribution. NE Atlantic (Scandinavia to Mauretania); Mediterranean, Caribbean; SW Atlantic; Indian Ocean; NW Pacific (Japan, China); NE Pacific (Alaska, British Columbia); SE Pacific (Chile); Indo-Pacific (Philippines, Vietnam, Indonesia); Pacific Islands; Australia, New Zealand.

Uses and Compounds. Produces lectins with biological activity [6.23]; present antioxidant [6.33] and antimicrobial [6.34] activity, medicinal and, pharmaceutical uses [6.35].

Caulerpa cupressoides (Vahl) C. Agardh

Description. Erect fronds arise from stolons of up to several diameters in length, anchored to the substratum by rhizoidal structures. Fronds are usually rich forked toward the apex, and at base they are naked (usually three) rows of rounded or flattened, ovoidal to conically pointed, short branchlets with a distinctly pointed tip [6.32, 36].

Habitat. On rocks in the tidal zone at depths of 40 m depth.

Distribution. NE Atlantic (Canary Islands); Caribbean; NW Pacific (Japan, China); West Pacific (Philippines, Indonesia); Pacific Islands; Australia.

Uses and Compounds. This seaweed is reported to be edible, to have antibacterial [6.37–40], antifungal, antioxidant [6.41], anticoagulant [6.41, 42], analgesic [6.43, 44] anti-inflammatory [6.43, 44], and antithrombotic [6.45] properties, and used to treat high blood pressure. However, some *Caulerpa* species produce toxins to protect themselves from browsing fish. This also makes them toxic to humans [6.36].

Caulerpa peltata J.V. Lamouroux

Common Name. Big parasol green seaweed.

Description. This seaweed has structures that look like fleshy umbrellas, with a thick circular portion (about 1−1.5 cm across) on a little stalk. These little umbrellas emerge along the length of a *stem* that creeps over the surface; bright yellowish-green to bluish-green color [6.32, 46].

Habitat. Found on shady rocks or dead corals in infralittoral zones along moderately wave-exposed shorelines. *Caulerpa peltata* grows in tide pools, on lower intertidal coral reefs covered with sand or on soft muddy.

Distribution. Widely distributed in tropical seas; Atlantic Islands.

Uses and Compounds. This seaweed was reported to be edible and used as medicine for its antifungal properties and has ability to lower blood pressure [6.46–48], it is also reported to have larvicidal [6.49, 50], antiplasmodial [6.51], citotoxic [6.52], immunomodulatory [6.53], antioxidant [6.52], antimicrobial [6.40, 54], and anticoagulant [6.55] properties. This species was evaluated for producing biodiesel [6.56].

Caulerpa prolifera (Forsskål) J.V. Lamouroux

Description. Fronds leafy, flat, with a distinct slender stipe and entire margins, at times undulating, oval to longitudinal, spatula-shaped or linear oblong, tapering toward the base, at the apex also narrowing but bluntly rounded (Fig. 6.3). Occasionally, more of these dark-green photosynthetic organs emerge from the stipes as well as from the margins or surfaces of the blades. The sparsely branching, relatively thin (1−2 mm) but wiry stolons are widely spread out, often extensive areas colonizing in this way; rhizoids in intervals of 0.5−2 cm [6.32].

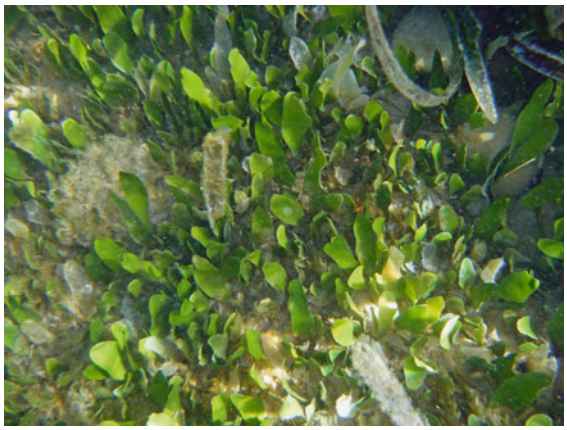

Fig. 6.3 Underwater photo of *Caulerpa prolifera*

Habitat. On sandy or muddy bottoms in sheltered lagoons, only slightly below the low water mark, often among Seagrass; but also on little wave-exposed rocks at depths of several meters.

Distribution. NE Atlantic (S Spain to Canary Islands), Caribbean, Mediterranean; Indian Ocean, SW Pacific (Philippines).

Uses and Compounds. Commonly used in aquaria, to which it is well adapted [6.32, 57], this species produces Caulerpenyne, an acetylenic sesquiterpene, which is specific to *Caulerpa*, as described by *Amico* et al. [6.58, 59] for *C. prolifera*. This seaweed is reported to have antibacterial [6.40, 60, 61], antialgal [6.61], antifungal [6.62], antifouling [6.63], antiproliferative [6.64], antioxidant [6.64, 65], anticoagulant [6.64], and larvicidal activity [6.66].

Caulerpa racemosa (Forsskål) J. Agardh

Common Names. Sea grape; green caviar; grape caulerpa.

Description. It is a bright green seaweed that resembles long skinny vertical bunches of tiny grapes. It can be very similar in appearance to *Caulerpa lentillifera*, though the latter tends to produce denser bunches (though this line can be smudged when *Caulerpa racemosa* grows in wave-exposed waters and develops shorter, stronger branches than normal). *C. racemosa* is quite variable in morphology and has many different growth forms that have been identified and named. A horizontal stolon which is attached to the sediment (usually sand) by descending rhizomes gives rise to erect branches at every few centimeters. These branches can reach as much as 30 cm in height and produce a large number of stalked branchlets which vary in shape from spherical to ovate to disk-shaped, sometimes flattening on top or forming icecream-cone-type shapes. These plants are coenocytic, which means that the entire plant is made up of one giant cell with many nuclei and no cross-walls. It is mainly due to this characteristic, any part of a *C. racemosa* plant that is fragmented, even tiny bits of tissue, can regenerate to form entirely new plants [6.67, 68].

Habitat. Forms intertwined mats in tide pools and on reef flats. Horizontal runners tightly anchor mats to rocks and sand and in calm to moderately heavy surf areas.

Distribution. Eastern Mediterranean; Caribbean, Indian Ocean, NW Pacific (Japan, China), Indo-Pacific (Philippines, Vietnam); Pacific Islands; Australia, New Zealand.

Uses and Compounds. *C. racemosa* is grown commercially in the South Pacific, and harvested wild in other areas [6.67]; edible seaweed, eaten as salad in Polynesia and the Marquesas [6.32]. *C. racemosa*, like *C. lentillifera*, is used commonly in Asian and Island cuisines. It is often used fresh and raw as a salad vegetable and is desired for its peppery flavor which also makes it ideal for use in sauce making [6.69]. This seaweed is common in the Phuket market of Thailand where 10–20 kg of fresh *C. racemosa* is sold per day [6.67].

C. racemosa also contains compounds which function as mild anesthetics, which gives the seaweed clinical value. Traditional medicine of the Philippines uses *C. racemosa* to lower blood pressure and to treat rheumatism [6.67, 68]. This seaweed is reported to have antibacterial [6.39, 60, 70–73], antiviral [6.72, 74, 75], antifungal [6.71], insecticidal [6.76], antioxidant [6.77, 78], anti-inflammatory [6.73, 79], anticoagulant [6.74], analgesic [6.79], hypolipidaemic [6.35, 80], hypoglycemic [6.81] and antitumor [6.77, 82] activity.

Caulerpa taxifolia (M. Vahl) C. Agardh

Description. Plant with widely extending, smooth stolons, about 1 mm in thickness; rhizoid-bearing branches downward and photosynthetic branches growing upward. Erect sections short-stiped and feather-like: the unbranched or sparingly branched axis is covered

Fig. 6.4 Cushion-shaped, dark green, strongly adhered to the rocky substrate (*Codium adhaerens*)

pinnately with 3–6 mm long and 1 mm wide, flattened and upward-bending branchlets. These are compressed, slightly constricted at the base and narrowing toward the mucronate tip [6.32].

Common Names. Killer algae; Aquarium Caulerpa; Folia feathery green seaweed.

Habitat. In sunny locations on sheltered shores, most common in shallow water on sand, mud and rocks, but also in up to 30 m depth.

Distribution. Widely distributed in warmer seas, NE Atlantic (Canary Islands); W Mediterranean; tropical E Atlantic (Ghana); Caribbean; Indian Ocean; NW Pacific (Japan, China); W Pacific and Indo-Pacific (Philippines, Vietnam, Indonesia); Pacific Islands; Australia.

Uses and Compounds. This green seaweed is reported to be edible [6.83], to have antioxidant [6.84], antibacterial [6.83, 85, 86], antiviral [6.87], nematicidal [6.88], and antifungal properties, and used to treat tuberculosis and high blood pressure [6.83]. However, some *Caulerpa* species produce toxins to protect themselves from browsing fish. This also makes them toxic to humans [6.83]; caulerpenyne from *C. taxifolia* which is cytotoxic toward several human cell lines and as such has anticancer, antitumor, and antiproliferative properties [6.35, 89, 90].

Codium adhaerens C. Agardh

Description. Spongy thallus, green light, prostrate, irregularly shaped, and presented with the appearance of a plane carpet firmly fixed to the substrate (Fig. 6.4). It consists of entangled coenocytic filaments and a finished surface by narrow and elongated utricles that are difficult to separate. Firm, gelatinous texture, and smooth to the touch [6.91].

Habitat. Perennial seaweed that can be found on exposed or protected shores under low light conditions, being located mainly in vertical rock faces and gaps.

Distribution. NW Atlantic (common on European bordering countries), Atlantic Islands (Azores, Canary), Africa, Asia, and Australia.

Uses and Compounds. Have vermifuge [6.92], antidiabetic [6.93], antibacterial [6.94], and antiviral [6.95] properties.

Codium bursa (Linnaeus) C. Agardh

Description. Thallus a spongy sphere with a velvety soft, shiny surface; becomes more flattened when increases in size. The internal branched filamentous network becomes looser with increasing size of the sphere, the space fills with water and the surface becomes indented; anchored to substratum by felted filaments [6.32].

Habitat. On rocks, probably prefers sheltered locations; in the infralitoral up to 10 m (−50 m) depth; often in drift.

Distribution. NE Atlantic (Ireland to Canary Islands); Mediterranean.

Uses and Compounds. Have antifungal [6.62], antibacterial [6.96], and antiplasmodial [6.97] activity.

Codium decorticatum (Woodward) M. Howe

Description. Very large, dichotomously branched erect thallus, dark green: from a crustose base rise one to several, up to more than 1 m long ribbons, regularly forking, rounded-flattened (0.5–3 cm wide) and usually visibly compressed at the bifurcations, stretched out like webs, wedge-shaped, and over 6 cm wide at that point [6.32, 98].

Habitat. Inhabits shallow muddy bays and harbors, usually during summer months.

Distribution. NE and E Atlantic (Spain to tropical W Africa), Mediterranean; Caribbean, SW Atlantic (Brazil, Uruguay); Indian Ocean; Hawaii; Antarctica.

Uses and Compounds. Have antibacterial [6.99, 100], antioxidant [6.65, 101, 102], antiviral [6.103], larvicidal [6.104], antifungal [6.105], and antitumor [6.106] activity.

Codium fragile (Suringar) Hariot

Common Names. Dichotomous sponge tang, Forked felt alga, Sea staghorn.

Description. Dark-green alga, ranging from 10 to 40 cm high and consists of repeatedly branching cylindrical segments about 0.5 to 1.0 cm in diameter, and its branches can be as thick as pencil. The segments look like dark green fingers. Its holdfast is a broad, sponge-like cushion of tissue. The tips of segments are blunt and the surface is soft, so it is sometimes mistaken as a sponge. Its body consists of interwoven, filamentous cells with incomplete cross-walls forming the inner part of the branches [6.107, 108].

Habitat. On rocky shores from the upper intertidal rock pools down to the lower intertidal.

Distribution. *C. fragile* is native to the Pacific Ocean in the Sea of Japan, and from Alaska to Baja California, Mexico. Its range has extended southward to South America, on the coasts of Chile and Argentina. It grows along nearly the whole coastline of the eastern North America, from the Gulf of St. Lawrence in Canada to North Carolina. It can be found in the Mediterranean and Adriatic, including the coasts of France, Spain, and Corsica. *C. fragile* ranges from Scandinavia to the Gulf of Gascogne and to the Canaries Islands. It has also been recorded in the Orkneys, Scotland, Ireland, Wales, and England. It can even be found in Australia, Antarctica, and southern Africa [6.107].

Uses and Compounds. *C. fragile* is used for skincare and antiaging products. It is reported to be a regenerating and anti-free radical ingredient, and have rebalancing and energizing properties. Its wealth of macro-elements gives it a remineralizing property [6.109]. One seller claims that *C. fragile* is the ideal ingredient to boost tired and mature skin. It is also used as food in eastern Asia and Chile [6.4, 107, 108, 110]. This species have also antioxidant [6.111, 112], antibacterial [6.112–115], anti-inflammatory [6.116] immunostimulating [6.117], antitumor [6.118], antiangiogenic [6.119], and antifouling [6.115] properties. The bioremediation potential of *C. fragile* has been tested on integrated multitrophic aquaculture (IMTA) [6.118].

Fig. 6.5 Underwater photo of *Codium tomentosum*

Codium tomentosum Stackhouse

Common Names. Spongeweed, Velvet horn.

Description. A small green alga (up to 30 cm long) with a dichotomously branched, cylindrical frond (Fig. 6.5). The frond is solid and spongy with a felt-like touch and has many colorless hairs which can be seen when the plant is immersed in water. The holdfast is disk-like and formed from many fine threads. *C. tomentosum* can be confused with *C. fragile*. However, *C. tomentosum* tends to have a more slender frond with rounded tips whereas *C. fragile* has pointed tips to the frond [6.1, 120, 121].

Habitat. Mainly on rock in the lower shore.

Distribution. *C. tomentosum* is native to the northeast Atlantic Ocean from the British Isles southward to the Azores and Cape Verde. It has also been recorded around the coasts of Africa and in various other parts of the world.

Uses and Compounds. *C. tomentosum* is used in products from the United States, Germany, France, Italy, and the UK. Some of these products are repair and restoration moisturizers, hydration serums, leg and body creams, muds and butters, bath and shower

creams, day creams, night creams, eye creams, anti-aging creams, masks, scrubs, lip balms, and lotions. *C. tomentosum* is a popular food in some parts of Asia [6.120, 122].

This species have antihelminthic and antiprotozoal [6.32, 122, 123], antioxidant [6.124, 125], antigenotoxic [6.124], antitumor [6.126], anticoagulant [6.127–129], antibacterial [6.96, 102, 130, 131] activity.

Halimeda opuntia (Linnaeus) J.V. Lamouroux

Description. Thick, profusely branched clumps of rounded three-lobed or ribbed leaf-like segments, between 10 and 25 cm in height. The branches are numerous and are in different planes, rather than nearly in a single plane as some other species are. This alga can cover larger areas with a dense mat so that individual plants are indistinguishable [6.32, 132].

Habitat. Grow in shallow depressions, cracks and crevices, between hard corals and other somewhat protected areas of the reef, down to 55 m.

Distribution. Mediterranean; Caribbean; SW Pacific and Pacific Islands; Japan; Indian Ocean.

Uses and Compounds. *H. opuntia* show hair-growth-stimulating [6.133], antimicrobial and cytotoxic [6.134–138], antioxidant and hepatoprotective [6.139–141], larvicidal [6.142], antileishmanial [6.143], and antiviral [6.144] activity.

Halimeda tuna (J. Ellis & Solander) J.V. Lamouroux

Description. Thallus calcified, dark green, distinctly segmented with initial branching in one plane; segments disk-like to triangular, up to 2 cm wide. Internodal siphons uncalcified, united in twos or threes, and terminating in pseudodichotomous laterals. Surface cells oppressed to one another in a honeycomb pattern, 25–75 µm in diameter [6.32].

Habitat. Found on hard rocky substratum in shallow water, less than 2 m depth.

Distribution. Globally in warmer seas: NE Atlantic (Morocco, Azores, Canary Islands); Mediterranean; Caribbean; NW Pacific (Japan); SE Pacific (Chile); SW Pacific; Pacific Islands; Indian Ocean; Australia.

Uses and Compounds. This species have antibacterial [6.114, 145, 146], antioxidant [6.65, 147], anticoagulant [6.128], pesticide [6.136, 148], antifungal [6.62], Antitrypanosomal [6.149], cytotoxic and antiproliferative [6.150] activity.

- Class: Dasycladophyceae
- Order: **Dasycladales**

Acetabularia acetabulum (Linnaeus) P.C. Silva

Description. Thallus with a slender stipe, whitish gray or gray-green, merging into a flattened, funnel-shaped cap at the top; this is divided into 20–80 radial rays, navelled, and annulated around navel and below the cap (Fig. 6.6). During growth, delicate filamentous, forking branchlets arise from the uppermost ring and the stipe. These are discarded soon and are therefore lacking in the mature thallus. Only basal parts perennial, plants with fully developed caps between May and August in the Mediterranean [6.32, 151].

Habitat. Mostly forming dense stands on rocks and stones in sheltered bays.

Distribution. NE Atlantic; Mediterranean; Indian Ocean.

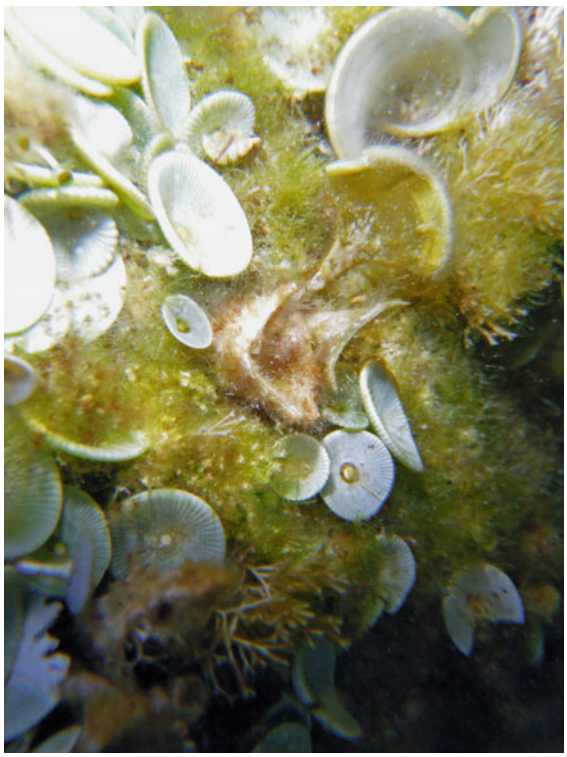

Fig. 6.6 Underwater photo of *Acetabularia acetabulum*

Uses and Compounds. For a time, important researches object in cell biology (cell differentiation, interaction of nucleus and cytoplasm) [6.32].

- Class: Siphonocladophyceae
- Order: **Siphonocladales**

Valonia utricularis (Roth) C. Agardh

Description. Thallus, translucent light- to dark green, primarily consisting of a large (up to 5 mm thick and 20 mm long) bladder- or club- to hose-like *cell*, branching at the base rhizoidally (Fig. 6.7). Later due to outgrowths of this cell cylindrical-clavate branches, often contorted and almost gapless densely packed, thus forming intertwined erect stands [6.32, 152].

Habitat. Plants sessile; at times forming extensive stands beneath other algae. Mostly on rock and reef edges in sheltered locations and shallow water, in up to 2 m depth.

Distribution. Warm NE Atlantic (Portugal to Canary Islands); Mediterranean; Caribbean; Indian Ocean; NW Pacific (Japan, China); Indo-Pacific (Philippines, Vietnam); Pacific Islands; Australia.

Fig. 6.7 *Valonia utricularis* specimen

Uses and Compounds. Show antifungal, antiviral, cytotoxic and atimitotic [6.62] activity.

- Class: Ulvophyceae
- Order: **Cladophorales**

Anadyomene stellata (Wulfen) C. Agardh

Description. *A. stellata* consists of erect, bright green, delicate blades with ruffled edges forming densely packed clumps; blades one cell thick, to 10 cm in height, formed by veins of filaments radiating from base in fan-like branching pattern. Cells between veins arranged in parallel rows. Margins smoothly rounded, formed by small spherical cells; similar to *A. saldanhae* which has cells between veins in random arrangement. Also similar to *A. lacerata* which has lacerated margins formed by elongated vein cells [6.32].

Habitat. Commonly found on rocks or other firm surfaces in the lower intertidal zone.

Distribution. Warmer parts of the NE Atlantic (Azores, Canary Islands), Mediterranean; Caribbean; Indian Ocean; W Pacific (Philippines, Indonesia); Australia.

Uses and Compound. Have anticoagulant [6.153], and antifungal [6.62] activity.

Chaetomorpha aerea (Dillwyn) Kützing

Description. Light green filaments, 10 cm long, growing attached to the substrate, and mostly grouped in tufts. Filament unbranched, growing in flocks or communities attached to sand covered or bare rocky substratum, 2–3 cm in height, yellowish green in color. Cells cylindrical, apical cells 111.2–111.5 µm in length and 110.5–111.6 µm in breadth, middle cells 54.5–62.2 µm in length and 88.8–89.2 µm in breadth, basal cell much longer than the middle and the upper one, showing its length 133.32–144.5 µm and breadth 55.50–58.8 µm, sheath 17–22.5 µm in thickness, sometimes lamellate in mature portion [6.154].

Habitat. Ephemeral species, found in intertidal environment, in both exposed and sheltered stations.

Distribution. Diffused in Mediterranean, Black Sea, NE Atlantic (from Scandinavia to Portugal) NW Atlantic.

Uses and Compounds. Show antibacterial [6.155–157] activity.

Chaetomorpha capillaris (Kützing) Børgesen

Description. Thallus medium green, loose-lying in shade on tidal flats, forming loose, woolly, entangled masses with few if any attachment cells. Filaments unbranched, of similar diameter throughout. Cells (70–) 85–100 (–105) µm in diameter and L/B 1–2, not collapsing on drying; walls 8–12 µm thick; chloroplasts densely reticulate with numerous pyrenoids [6.158].

Habitat. In shade on mangrove pneumatophores or under Samphires on tidal flats.

Distribution. Mediterranean and North Atlantic; Southern Australia and New Zealand.

Uses and Compounds. Have antifungal activity [6.62].

Chaetomorpha linum (O.F. Müller) Kützing

Common Name. Spaghetti algae.

Description. Delicate green seaweed, it grows as a filamentous loosely entangled mass (Fig. 6.8). Plants mostly free floating, sometimes attached to rocks and shells; plant body filamentous, less than 20 cm long, bright green to yellowish green in color, filaments wiry and stiff, unbranched; cell walls thick, appear jointed or articulated with dark green bands; cells constricted at the transverse walls [6.159, 160].

Habitat. Is an intertidal and supralittoral species that can be found in groups of hundreds or thousands of individuals in sandy areas, on rocks or around tide pools.

Distribution. Widespread, including Northern Europe to North Africa Atlantic coasts, North and Baltic; Mediterranean; North American Atlantic Coast (Labrador to Florida); NE Pacific (Alaska to California); SE Pacific (Chile); Indian Ocean, China, Japan, Australia, New Zealand.

Uses and Compounds. Spaghetti algae, though not palatable to many herbivorous species, is popular in reef aquariums for its ability to remove nitrates, assist in buffering pH, uptake carbon dioxide producing oxygen, and assist in balancing trace elements. It also provides hiding spaces for small creatures [6.159]. Possible use on biofuel production [6.161–163], liquid fertilizer [6.164], bioremediation in aquaculture [6.165], and animal feed [6.166]. Extracts of this species have insecticidal [6.167], antimicrobial [6.168–171], antioxidant [6.111, 170], larvicidal [6.171], and antiviral [6.172] activity.

Fig. 6.8 *Chaetomorpha linum* specimens

Cladophora coelothrix Kützing

Description. Thallus medium to dark green, drying brownish, forming dense turfs or cushions to several cm across and to 4 cm high, composed of a basal tangle of branched, often curved, stolon-like filaments giving rise to ascending, more-or-less erect, branch-systems. Growth largely by divisions of conspicuous apical cells, but intercalary cells may divide into shorter cells; feebly to distinctly acropetal at the apices, densely and irregularly branched with many cells (especially lower

ones but often also those near the apices) producing a descending rhizoid from their basal poles, and which attach to the substrate or other filaments with a terminal coralloid holdfast; lateral branches mostly wide-angled (45° or more) arising singly (occasionally two) at or just below a cross wall, with the new wall remaining steeply inclined to the parent cell [6.173].

Habitat. Common in shaded areas or pools near low tide level on rough-water coasts but extending into sheltered areas.

Distribution. Warm temperate to tropical Atlantic coasts of Europe, Africa and America; probably widespread in tropical and warm temperate waters, and in southern Australia.

Uses and Compounds. Have antimicrobial [6.174], and antifungal activity [6.62].

Cladophora fracta (O.F. Müller ex Vahl) Kützing

Description. Filaments branched; attached by rhizoids, pale green, up to 80 cm long, branching irregular or pseudodichotomously divided at wide angles, cells mostly cylindrical; chloroplast parietal net-like with several pyrenoids; main axis up to 85 µm wide, bearing numerous side branches of different lengths; cells of side branches 17–38 µm wide, 5 to 17 times longer than wide, apical cells cylindrical to slightly conical, 16–27 µm wide, 3.5 to 25 times longer than wide [6.175].

Habitat. Frequent in shallow, nutrient-rich ponds and ditches as well as penetrating slightly brackish-water habitats; mostly free floating or unattached and lose lying.

Distribution. NE Atlantic (Sweden to Spain); NW Atlantic; Mediterranean; Australia.

Uses and Compounds. Have antihypertensive [6.176], antimycobacterial [6.177], antioxidant [6.178], antibacterial [6.179, 180], antialgal [6.179], piscidal [6.179] activity, and possible use for biodiesel production [6.181, 182].

Cladophora glomerata (Linnaeus) Kützing

Description. Plants were up to 20 cm high and light to dark green in color. The texture of thallus was soft and slightly mucilage. Plants usually formed in dense tufts well branched. Rhizoids were primary and adventitious and descend from the bases of thallus or from the lower segments of the fronds. Primary branches fused together or not and branched in dichotomous manner. The old branches in the lower portion of the thallus were slight constricted articulations (80–150 µm in width and 160–900 µm in length) and usually branched in dichotomous manner. The branches in the upper portion of thallus were cylindrical (60–120 µm in width and 100–600 µm in length) and branched in dichotomous or trichotomous manner [6.183].

Habitat. Growth of *C. glomerata* usually requires hard substrates for attachment, such as rocks, mussels, and artificial reef structures.

Distribution. Distributed widely around world; NE Atlantic (Sweden to Portugal); NW Atlantic (Canada to Cuba and Mexico); SW Atlantic (Brazil); Mediterranean; Indian Ocean, Pacific.

Uses and Compounds. Used on the treatment of burns [6.184], biosorption of heavy metals [6.185], animal feed [6.186], biodiesel production [6.187], wastewater treatment [6.188], and as food [6.189]. Have antibacterial [6.190–192], antiprotozoal [6.193], antioxidant [6.190, 194], and antifungal activity [6.192, 195].

Cladophora pellucida (Hudson) Kützing

Description. Filaments rigid, erect, setaceous, full dark green, di-tri-chotomous; the axils very acute, the branches erect; articulations many times longer than broad; dissepiments only at the forking of the branches and ramuli [6.196].

Habitat. On the bottoms and sides of deep rock pools, between tide marks, generally near low-water mark.

Distribution. NE Atlantic (from Ireland and Britain to Morocco); Mediterranean; SW Atlantic (Brazil); NE Atlantic (South Africa); Indian Ocean (India); Australia.

Uses and Compounds. Have antitumor [6.197] activity.

Cladophora prolifera (Roth) Kützing

Description. Unattached or basally attached coarse filaments those are usually less than 0.5 mm wide and 3–5 cm long. The filaments are formed of a single row of often swollen cells; if attached then by a discoid base or by rhizoidal outgrowths [6.198].

Fig. 6.9 Herbarium specimen of *Cladophora rupestris* (MACOI no. 2419)

Habitat. Grow fixed to rocks in the upper sublittoral zone.

Distribution. NE and E Atlantic (from Ireland and Britain to Senegal); Mediterranean; NW and SW Atlantic; SE Atlantic; Indo-Pacific Oceans; Australia.

Uses and Compounds. Used on experimental co-culture [6.199]. Have antimicrobial [6.96, 200–203], antifungal [6.200, 204], antiviral [6.103], antioxidant [6.65, 205], and anticoagulant [6.206] activity.

Cladophora rupestris (Linnaeus) Kützing

Description. *C. rupestris* is a densely tufted plant, darkgreen, which grows up to 20 cm in height, with dark green or bluish colored dull fronds. Typical specimens branch profusely upward from the base, in an irregular, whorled or opposite pattern. The stoutness, density, and arrangement of branches give the seaweed a coarse feel (Fig. 6.9).

The morphology of the species is fairly constant over a wide range of habitat conditions and over a wide geographical area. Its morphology is affected by physical damage due to grazing by animals and loss of the apical region on reproduction; both instances are followed by regeneration and proliferation of branches. *C. rupestris* sometimes forms an almost complete cover of stunted growth at high tide level and occasionally in the splash zone where pools are brackish. Filaments are short and branching dense in the most wave exposed locations [6.1, 32].

Habitat. On solid substratum, in all littoral zones, prefers sandy locations in the lower littoral, tolerant to reduced salt content of seawater.

Distribution. NE Atlantic (Scandinavia to N Africa), North Sea and Baltic; Mediterranean; NW Atlantic; NW Pacific (Japan) and SW Pacific; Subarctic.

Uses and Compounds. Contains arabinose, carotenoids, galactose, glucose, polysaccharides, protein, rhamnose, sulfuric acid, and xylose [6.207, 208]. Used for animal feed [6.209], and CO_2 bioremediation [6.210]. Have antimicrobial [6.130, 211], antimycobacterial [6.212], antiprotozoal [6.212], cytotoxic [6.212], and antifouling [6.213] activity.

- Order: **Ulvales**

Ulva clathrata (Roth) C. Agardh

Synonyms. *Enteromorpha ramulosa* (Smith) Carmichael, *Enteromorpha clathrata* (Roth) Greville.

Common Name. Aonori.

Description. Plants less than 30 cm long and soft; thallus repeatedly branched in all directions, cylindrical or compressed with narrow branchlets as much as 40 cm long, light green in color, plants grow first attached to the substratum but later become free floating; cells of the thallus more or less quadrangular in shape with a single cup-shaped chloroplast; pyrenoids 3 to 4.

This species forms tufts, bright green, composed of branched axes, which can reach several centimeters long (20—30 cm). The main axis and branches are covered with conical branchlets very characteristic [6.1].

Habitat. In rocks and stones, from mid-littoral to sublittoral.

Distribution. Entire Atlantic, Mediterranean, Caribbean (Cuba, Mexico), NE and NW Pacific, Australia and New Zealand.

Uses and Compounds. Used as biofilter on shrimp (*Litopenaeus vannamei*) aquaculture [6.214], on shrimp feed [6.215, 216], and for human food [6.4, 217]. Have

antibacterial [6.96, 218–220], anticoagulant [6.221], antifungal [6.217], antiprotozoal [6.123, 218], larvicidal [6.222], analgesic [6.218], anticancer and antiviral [6.217, 218] activity.

Ulva compressa Linnaeus

Synonym. *Enteromorpha compressa* (Linnaeus) Nees.

Common Names. Green nori, Plat darmwier.

Description. *U. compressa* is a green seaweed that can have one of two different growth forms. The first is a flat, narrow sheet with ruffled edges, and the second form (often referred to as *Enteromorpha compressa*) is a hollow tube of tissue, rounded at the top. In both forms the sheets of tissue are very thin; in fact they are exactly one cell thick. Several blades or tubes arise from a common attachment point and can grow up to 200 mm long. *U. compressa* is a shallow water species and is often found in tide pools or on rocks in the intertidal and shallow infra-littoral up to 3 m deep. As a common tidepool species, *U. compressa* is able to withstand great swings in salinity, temperature, and pH [6.223, 224].

Habitat. Marine and estuarine species, rock pools and sandy rocks, particularly in places with widely varying salinities such as upper-shore species.

Distribution. *U. compressa* is widely distributed, found commonly on the NE Atlantic coasts, Pacific coast of North America, throughout the Mediterranean, and also in Africa and Australia.

Uses and Compounds. *U. compressa*, in the same family as *sea lettuce* is a commonly eaten as a sea vegetable, used fresh or dried for both human and animal consumption for its high nutrient levels and good taste; is used dried in cooking, particularly with eggs [6.109, 223, 224]. This seaweed is also utilized as fertilizer to introduce a wide range of minerals to the soil. Many benefits have been associated with *U. compressa* consumption such as cytotoxic [6.225], antimicrobial [6.96, 171, 224, 226–230], antiviral [6.224], and antioxidant [6.224, 225, 231–234] properties. Extracts of *U. compressa* are also added to cosmetics products for a soothing quality that reduces skin itchiness and tautness [6.224].

Ulva fasciata Delile

Description. Thalli thin, sheet-like, up to 50 cm long, consisting of wide blades, 10 – 15 cm wide at base, tapering upward to less that 2.5 cm wide at tip. Basally broadened, but the upper portions divided deeply into many ribbon like segments; margins smooth, often undulate. Holdfast is small without dark rhizoids. Bright grass-green to dark green, gold at margins when reproductive, may be colorless when stressed [6.32, 235].

Fig. 6.10 *Ulva intestinalis* specimens

Habitat. *U. fasciata* is a common species in the intertidal to shallow infra-littoral, often found in tide pools. This species is a quick colonizer and is able to grow very quickly in the right conditions, often reaching bloom levels in the presence of nutrient run-off and fresh water input [6.236].

Distribution. *U. fasciata* has a worldwide distribution in temperate and tropical waters: NE Atlantic; Mediterranean; Caribbean; West coast South Africa; SW Atlantic; entire Pacific; Indiana Ocean; Australia, New Zealand.

Uses and Compounds. *U. fasciata* is a popular edible seaweed in many areas where it grows, such as Hawaii where it is known as *limu palahalala*. *U. fasciata* has a very fine texture and lovely fresh taste and is often chopped into salads or used as a relish, though it can also be cooked and used in soups [6.236]. Green algae extracts are also very nutrient rich and

are a wonderful addition to natural cosmetic products. Have antiviral [6.237, 238], algicidal [6.239, 240] antifungal [6.241] larvicidal [6.242], cytotoxic [6.242], antifouling [6.243], antibacterial [6.241, 242, 244–247], antioxidant [6.246] activity.

Ulva intestinalis Linnaeus

Synonym. *Enteromorpha intestinalis* (Linnaeus) Nees.

Common Name. Gut weed.

Description. *U. intestinalis* is a conspicuous bright grass-green seaweed, consisting of inflated irregularly constricted, tubular fronds that grow from a small discoid base (Fig. 6.10). Fronds are typically unbranched. They may be 10–30 cm or more in length and 6–18 mm in diameter, the tips of which are usually rounded. Like other members of the genus, *U. intestinalis* is a summer annual, decaying and forming masses of bleached white fronds toward the end of the season [6.1, 32].

Habitat. In sheltered as well as exposed locations, on boulders, breakwaters and piers, in pools, also epiphytically; from the upper littoral (also supra-littoral) pools into the sublittoral.

Distribution. More or less globally distributed: entire Atlantic, Mediterranean, Caribbean, NE and NW Pacific, Indian Ocean, Australasia, Antarctica.

Uses and Compounds. This species is used as human food [6.4] and animal feed [6.248, 249], for biomonitoring of heavy metals [6.250, 251], have antimicrobial [6.86, 96, 252, 253], antifungal [6.252], antitumor [6.254, 255], antihemolytic [6.253], larvicidal [6.256], antifouling [6.257], antiplasmodial [6.258], antiprotozoal [6.259], algicidal [6.260], and antioxidant [6.261–263] activity.

Ulva lactuca Linnaeus

Common Names. Sea lettuce, Green laver.

Description. *U. lactuca* is commonly called sea lettuce. Its color can range from light yellowish green to darker green, but is most commonly a vivid green underwater. When exposed at low tide or when washed up on a beach, it is typically darker green. Out of the water the seaweed looks like a rather slimy lime-green mass but in the water the alga actually does look very much like young lettuce leaves. *U. lactuca* is vivid green and cellophane thin (only two cell layers thick), and forms light yellowish green to dark-green translucent sheets. The soft frond grows as a single, irregular, but somewhat round-shaped blade with slightly ruffled edges which are often torn. There can be numerous small holes or perforations scattered throughout. The frond is connected to rocks with a small, almost invisible discoid holdfast, and does not have a stipe. *U. lactuca* may grow to a diameter of 20–30 cm, although it is frequently much smaller, with larger sheets feeling slightly thicker than smaller specimens [6.1, 32, 264].

Habitat. From the tidal zone to the sublittoral, often free floating in pools.

Distribution. *U. lactuca* is ubiquitous, common to most shorelines around the world.

Uses and Compounds. *U. lactuca* is available in different forms from companies in countries which include the UK, Ireland, France, Germany, Vietnam, China, Canada, and the United States. It is sold both in fresh and dried form, in flakes, powders, and salad mixes. It is a delicate seaweed with a mild flavor. *U. lactuca* is sometimes eaten as *green laver*, but it is considered inferior to *purple laver*. It is used as a seasoning by itself and in blends, and can be found in soups and salads [6.32]. This species is also used in animal feed [6.265]. It also a key ingredient in many cosmetic and personal care items such as soap, lotion, toner, lifting cream, eye cream, lip cream, makeup remover, body polish, bath soaks, antiaging products, shaving lotion, shampoo, conditioner, and serums [6.264, 266]. It is also a component in gardening and fertilizer products [6.3].

This species have antioxidant [6.267, 268], antibacterial [6.70, 72, 115, 145, 171, 228, 269–271], antitumor [6.72, 126], anti-inflammatory [6.272], antifouling [6.115, 273], antifungal [6.72, 115], antiviral [6.72, 263], antialgal [6.115] activity.

Ulva linza Linnaeus

Synonym. *Enteromorpha linza* (Linnaeus) J. Agardh.

Common Names. Breed darmwier, Bright grass kelp, Welded green nori.

Description. *U. linza* is a large, ribbon-like species of green seaweed that may reach up to 30 cm in length.

The thalli are unbranched and often have a frilled margin. The thalli taper into a distinct stipe below and are highly compressed. The width of the thallus is greater in the middle than at the base and may reach 5 cm in width. *U. linza* is bright light to dark green in coloration [6.32, 274].

Habitat. Usually found on rocks or in rock pools, usually marine but occasionally found under brackish conditions.

Distribution. *U. linza* is found worldwide in bays and sheltered coasts.

Uses and Compounds. *U. linza* is used as an edible seaweed in many cultures for its high nutrient content and silky texture. Green algae extracts are also very nutrient rich and make a beneficial addition to natural cosmetic products [6.274]. Have antibacterial [6.60, 96, 228, 269, 275, 276], anti-inflammatory [6.277], and antiviral [6.278].

Ulva prolifera O.F. Müller

Synonym. *Enteromorpha prolifera* (O.F. Müller) J. Agardh

Common Name. Green ribbon plant.

Description. The fronds are tubular, though often more or less flattened, little to much branched. The arrangement of the cells, in longitudinal and transverse rows in the central part of the frond, is characteristic of this species, as are the cylindrical chloroplasts seeming to fill the cell and the usually single, central pyrenoids [6.279].

Habitat. A common green alga near the top of the shore, on rocks or other algae, on open coasts or in estuaries and harbors, where it may grow mixed with *U. intestinalis* or other species of the same genus.

Distribution. NE Atlantic (Britain to Senegal); Atlantic Islands, Mediterranean; NW and W Atlantic; Caribbean; SE Atlantic; Pacific; China, Australia.

Uses and Compounds. Used for food [6.280] and animal feed [6.281, 282]; potential use for the production of biofuels [6.283]. Have also antibacterial [6.171, 284], antifouling [6.285], antioxidant [6.101], antifungal [6.226, 286], antitumor [6.126], immunomodulatory [6.287] activity.

Ulva rigida C. Agardh

Common Name. Green laver.

Description. *U. rigida* is a bright green seaweed with a variety of growth forms. The two cell layer thick, sheet-like blades can grow as tiny blades forming carpet-like turfs, or as clumps of several larger blades, or solitarily. In its largest form, *Ulva rigida* can reach up to 10 cm in height, but size and blade shape are both highly variable. Blades may be flat or ruffled, sometimes bearing many small holes (perforations) and sometimes not. However, the defining characteristics of *U. rigida* are common among all its growth forms: the two cell layers of the blades are easily separable, like a deflated balloon, and the holdfasts are composed of many small, tough, dark rhizhoids that are absent in other *Ulva* species. *U. rigida* is common in intertidal and shallow sublittoral waters, often found in tidepools. This seaweed is able to withstand high variation in salinity and water chemistry and actually seems to prefer areas with freshwater input [6.32, 288].

Habitat. Epilithic, in the entire littoral zone to the sublittoral.

Distribution. *U. rigida* has a worldwide distribution in temperate and warm seas.

Uses and Compounds. *U. rigida* is often utilized as a fresh sea vegetable by many island cultures for its high nutrient content and fresh taste [6.288, 289]. This species is used to for animal feed [6.290, 291].

Green algae extracts are also very nutrient-rich and make a beneficial addition to natural cosmetic products. The polysaccharide ulvan is easily extracted from *U. rigida*. It is composed of β-(1,4)-xyloglucan, glucuronan, and cellulose in a linear arrangement. It corresponds to a water-soluble dietary fiber and is resistant to both human digestive tract enzymes and degradation by colonic bacteria. This polysaccharide cannot therefore be considered prebiotic; however, it could potentially be hydrolyzed to bioactive oligosaccharides [6.288, 292].

U. rigida have antigenotoxicity [6.293, 294], antihyperglycemic [6.293], immunomodulating [6.295],

antibacterial [6.201, 296–300], antioxidant [6.300–302], antileishmanial [6.303] activity.

Ulvaria obscura (Kutzing) Gayral
Synonym. *Monostroma obscurum* (Kützing) J. Agardh

Description. Thallus widely bladed, similar to that of *Ulva*, turning brown on dying; monostromatic blade; occurring not often; bipolar species [6.304].

Habitat. In sublittoral, at 3−17 m depth.

Distribution. NE Atlantic (Iceland and Norway to Portugal); Atlantic Islands; NW Atlantic (Alaska and Greenland to Washington); SW Atlantic (Argentina).

Uses and Compounds. *Ulvaria obscura* produces a dopamine that functions as antiherbivore defense [6.305, 306], and flavonoids with protective effect action against free radicals, protecting collagen surrounding of blood vessels. This species have antioxidant [6.307] activity.

- Order: **Ulotrichales**

Gayralia oxysperma (Kützing) K.L. Vinogradova
Synonym. *Monostroma oxyspermum* (Kützing) Doty

Description. Thalli forming leafy monostromatic blades from a few centimeter to a meter or more in length. Cells in the upper portion of blade polygonal and isodiametric, becoming more elongate toward base with long rhizoidal projections in holdfast region. In surface view, cells in groups of two or four; cells uninucleate with parietal chloroplast and single prominent pyrenoid traversed by thylakoids [6.32, 308].

Habitat. On rocks, boulders or on other seaweeds, older free floating; common through the entire littoral to sublittoral boundary, preferably in sheltered sites; tolerant to changing salinity.

Distribution. European coastlines (Atlantic, North Sea and Baltic); Atlantic coasts of North, Central and South America; Caribbean; East Pacific coastlines (Alaska to California), Japan, Mauritius, S Australia.

Uses and Compounds. Consumed as *green vegetable* (SE Asia, China) and used for animal feed [6.32, 109]. Have antiviral activity [6.309].

6.3.3 Domain/Empire Eukaryota, Kingdom Plantae, Phylum Rhodophyta (Red Algae)

- Class: Bangiophyceae
- Order: **Bangiales**

Bangia fuscopurpurea (Dillwyn) Lyngbye
Description. Gelatinous, unbranched, blackish-purple filaments, at first uniseriate later multiseriate, attached by rhizoidal outgrowths from basal and adjacent cells; cells with central star-shaped rhodoplast with pyrenoid [6.310].

Habitat. On rocks, wood, etc., as fleecy mat, especially at high tide level on exposed coasts in autumn and winter, disappearing in spring or early summer, at other times present as sparse filaments.

Distribution. NE Atlantic (Iceland to Senegal)); W Atlantic (Brazil and Argentina); Caribbean; W Pacific (Japan, China); NE Pacific (California, Mexico); Indo-Pacific/Indian Ocean; Australia.

Uses and Compounds. Consumed as *vegetable* [6.310]. Have antioxidant, antibacterial, antiviral, cytotoxic [6.205, 311] activity.

Porphyra leucosticta Thuret
Synonym. *Pyropia leucosticta* (Thuret) Neefus & J. Brodie

Description. Delicate membranous monostromatic reddish-brown fronds, becoming pink on drying, to 150 mm long, with very short stipe from basal holdfast [6.1, 2, 312].

Habitat. Usually epiphytic on larger algae, littoral to shallow sublittoral, spring to autumn, widely distributed, common.

Distribution. NE Atlantic (Norway to Portugal); Mediterranean; NW Atlantic (Canada and USA); SW Atlantic (Brazil); SE Atlantic (Angola).

Uses and Compounds. Produces high percentage of vitamin C and natural carotenoids, and therefore *P. leucosticta* could become a valuable source of raw material to obtain such compounds, which have mul-

tiple uses in the pharmaceutical field, cosmetic and food industry [6.313]; used for food [6.3, 314], and for IMTA aquaculture [6.315]. Extracts have high antioxidant [6.316], and low antiprotozoal, antimycobacterial, and cytotoxic [6.317] activity.

Porphyra linearis Greville

Description. Delicate, linear, membranous, purple-brown fronds, 20–40 mm (–200) mm long and 5–10 (–25) mm broad, usually simple with short stipe from basal holdfast; orange patches when reproductive [6.1, 318].

Habitat. Marine species, zone forming on rock in the upper intertidal and splash zone of semiexposed and exposed shores, generally distributed; a winter and spring annual appearing on semiexposed and exposed.

Distribution. Recorded throughout Northern Europe, from Norway to Portugal, Azores. Canada (Labrador) to USA (at least Connecticut).

Uses and Compounds. Used for food [6.3, 4, 319, 320], and for aquaculture [6.321–323]. Extracts have antimicrobial activity [6.130].

Porphyra umbilicalis (Linnaeus) Kützing

Common Names. Purple laver, Pink laver, Laver, Nori.

Description. A red alga (up to 40 cm across) with a circular (*P. umbilicalis*) or irregularly shaped (*P. leucosticta*), broad frond that is membranous but tough (Fig. 6.11). The plant attaches to rock via a minute discoid hold-fast, is greenish when young gradually changes into purplish red, and has a polythene-like texture. Another species is the *P. linearis*, with narrow stem that attaches the base and appears mainly in winter [6.1, 324, 325].

Habitat. On rocks, mussels, etc., in the littoral to splash zone, generally distributed, abundant, especially on exposed coasts.

Distribution. *P. umbilicalis* occurs in the North Atlantic. In the east, it is found in Iceland and has been recorded from Norway to Portugal and in the Western Mediterranean. In the West, *P. umbilicalis* is found from Labrador in Canada to the mid-Atlantic coast of the United States.

Fig. 6.11 Herbarium specimen of *Porphyra umbilicalis* (MACOI no. 110)

Uses and Compounds. *P. umbilicalis* is rich in protein, vitamins A, C, E, and B, and trace minerals, and also rich in omega-3 polyunsaturated fatty acids (EPA and DHA). It contains special compounds named *mycosporine-like amino acids* (MAAs) which are valuable in certain types of personal care products. Some of the favorable properties associated with compounds from *P. umbilicalis* are that it [6.3, 324, 325]: acts as a natural bio-protector against UVA-induced damage; prevents the formation of *sun burn cells* and premature photo-aging; protects cell structures, especially membrane lipids and DNA from damage by UV-induced radicals; helps in the re-equalization of lipid deficient skins; increases epidermal hydration; protects against *Trans Epidermal Water Loss*; improves intercellular cohesion; reinforces the skin barrier function; restores cell membrane structure after irritant damage; has oxygenating properties that helps revitalize stressed and fatigued skin; able to diminish the appearance of fine lines and wrinkles.

As a result, it is used in many different product applications, such as [6.325, 326]: antioxidant; daily UV protective skin care, sun care; anti-photo-aging care; cares for dry, reactive and sensitive skins; lip care; after sun care.

Specific products identified so far from France, Ireland, Italy, Spain, and United States utilizing *P. umbilicalis* include regenerating face creams and antiaging facial creams, facial masks, and aftershave balm. For food, it is sold in flaked and whole leaf form, as a nori substitute, as laver, and is used as an ingredient in several snack mixes and condiments. It is also used as a pet nutrition supplement [6.3, 4, 320, 325].

- Class: Florideophyceae
- Order: **Ahnfeltiales**

Ahnfeltia plicata (Hudson) E.M. Fries

Description. Perennial red seaweed which forms dense, tangled tufts (Fig. 6.12). The fronds are very fine, tough, and wiry with irregular or dichotomous branching and up to 21 cm in length. The holdfast is disk-like or encrusting, 0.5–2 cm in diameter. The fronds are dark brown when moist and appear almost black when dry. The uppermost branches are often green [6.327].

Habitat. On rocks, mid-littoral to sublittoral, especially common on sand-covered rocks, widely distributed, common.

Distribution. N and NE Atlantic (Greenland to Azores); SW Atlantic (Uruguay); NE Pacific (Alaska to Mexico); NW Pacific (Russia); SE Pacific (Chile); Indian Ocean; Antarctica and the Sub-Antarctic Islands.

Uses and Compounds. A source of so-called *Russian Agar* (agar of high quality and low sulfate content) [6.32, 328, 329]. *A. plicata* is one of the major seaweeds harvested for commercial colloid production as it produces a very high quality, low sulfate and used for many purposes. It forms a major component of Asian cooking, often showing up in soups and jellies. As a nonanimal derived thickening agent, *A. plicata* is also widely used for vegan products (such as marshmallows, gummy bears, cosmetics, etc.) in place of gelatin [6.330].

Fig. 6.12 *Ahnfeltia plicata* specimens

- Order: **Bonnemaisoniales**

Asparagopsis armata Harvey

Common Names. Harpoon weed, Harpoon-alga.

Description. Thallus of the gametophyte tufted, main axes cylindrical, denuded at the base, densely tufted apically, tufts with pyramidal outline, forming lateral brush-like tassels; some lateral branches develop into unbranched, elongated-pointed harpoon-like anchoring structures covered with barbed hooks (a characteristic feature of the species) which are usually pinnately arranged (Fig. 6.13). The tetrasporophyte (*Falkenbergia*

Fig. 6.13 Underwater photo of *Asparagopsis armata*

phase) is small, almost spherical tufts (similar to cotton balls) of multiseriate branching threads (microscope required) [6.32].

Habitat. Both phases readily reproduce vegetatively. Drift specimens of gametophyte readily attach to other algae by barbed branchlets, and produce new shoots; introduced from Southern Hemisphere [6.331].

Distribution. *A. armata* is native to the Southern Hemisphere (Australia and New Zealand) but has been introduced to the Northern Hemisphere, first recorded in Europe in 1925 and since spreading throughout the Channel Islands and all around Great Britain. *A. armata* is now found globally, from the Canary Islands to Morocco and throughout the Pacific and Indian Oceans [6.32, 331].

Uses and Compounds. *A. armata* extract is a powerful antioxidant with antibacterial qualities and is a valued ingredient in many cosmetic products. Present strong cytotoxicity against human cancer cell lines [6.62, 316, 332]. *A. armata* is also harvested or grown for the production of phycocolloid [6.333, 334].

The species has also been examined for its potential as a source of pharmaceutical and bioactive agents since its extracts contain anti-*Leishmania* [6.335], antioxidant [6.336], antiviral [6.337, 338], antifungal [6.62], antimicrobial [6.62, 336, 339, 340] compounds.

Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon

Common Name. Cat's tail red seaweed.

Description. Thallus fluffy, fine, filamentous creeping mats or tufts, to 4 cm high, pale red to gray-pink; branching irregular to alternate. Branches cylindrical, occasionally moniliform (with segments swollen or bead-like), 30–80 µm diameter, central axial filament surrounded by 3 pericentral cells; cells commonly pointed at tips, twice as long as broad, each set rotated approximately 60°; apex with single prominent apical cell cutting off lens-shaped cell basally; holdfast initially disk-like, later becoming branched, tangled, creeping, forming filamentous mass. Tetrasporangia solitary on outer filaments, not in groups or series, formed from one pericentral cell of segment. Fluffy appearance and shaped like a Christmas tree. Grows 3–15 cm high [6.32, 341].

Habitat. A tropical/subtropical species; thalli are epilithic; in shallow sublittoral habitats with heavy water motion.

Distribution. Globally distributed in tropical and subtropical seas: E Atlantic (Azores, Madeira, Canary Islands, W Africa, Cape Verde Islands to Nigeria); W Atlantic (Brazil); Caribbean; W Pacific (Japan, China); NE Pacific (California, Mexico); Pacific Islands (Hawaii); Indo-Pacific/Indian Ocean; Australia, New Zealand.

Uses and Compounds. The species has been examined for its potential as a source of pharmaceutical and bioactive agents since its extracts contain antifouling and anticyanobacterial [6.342], antifungal [6.341], anticoagulant [6.343], and antimicrobial [6.341, 344, 345] compounds.

Used fresh in Hawaii as *limu kohu* (generic name for seaweed) to flavor meat and fish dishes [6.341, 346].

Bonnemaisonia hamifera Hariot

Common Names. Bonnemaison's Hook Weed; Pink cotton wool.

Description. Gametophyte plants occurring from March–June, brownish red, fronds feathery, with a slightly flattened axis to 1 mm wide and 350 mm long, attached to *Cytstoseira* and other algae by crosier-shaped, hook-like modified branches. Tetrasporophyte (*Trailliella* phase) plants occurring all year round, but most obvious in October–March, brownish red, much branched, filamentous, in dense cotton-wool-like tufts to 25 mm in diameter [6.347].

Habitat. Probably introduced from Japan at the end of the last century; gametophyte first found in Europe (Isle of Wight) in 1893, on rocks and other algae, lowest littoral and sublittoral.

Distribution. NE Atlantic (Scandinavia to Canary Islands); SE Atlantic (South Africa); Mediterranean; NW Pacific (Russia, Japan); NE Pacific (California, Mexico).

Uses and Compounds. Extracts of this species have antibacterial [6.348, 349], antioxidant [6.350], and antihypertension [6.351] activity.

- Order: **Ceramiales**

Alsidium helminthochorton (Schwendimann) Kützing

Common Name. Corsican moss.

Description. Upright radially organized polysiphonous thalli, up to 15 cm high, with cylindrical axes and progressively narrower distal branches. Branch apices abruptly tapering; trichoblasts spirally arranged on axis, present on every segment but soon deciduous; parenchymatous cortication occurs to near branch apex 6–8 pericentral cells. Lateral polysiphonous branches originate from trichoblast basal cell [6.352].

Habitat. Found on calm, shallow, photophilic biotopes.

Distribution. Mediterranean.

Uses and Compounds. In Mediterranean, the Greek physician, Stephanopoli, discovered in 1775 that the red seaweed *Alsidium helminthochorton*, found on the rocky shores of Corsica, have an efficient anthelmintic action [6.353–355].

Boergeseniella thuyoides (Harvey) Kylin

Description. Cylindrical, cartilaginous, tufted, deep brownish-purple fronds, to 150 mm high, from creeping rhizoidal base; fronds distichously bi-tripinnate, patent, short, of nearly uniform length giving branches a linear appearance; ramuli short, spinelike; polysiphonous, central siphon with 8–12 pericentral siphons and outer cortication of small, colored cells; articulations as broad as long, barely visible [6.356].

Habitat. On rock and epiphytic in shallow, wave-exposed pools in lower intertidal.

Distribution. NE Atlantic (Ireland and Britain to Morocco).

Uses and Compounds. Extracts of this species have antiviral [6.337, 357], and antibacterial [6.358] activity.

Bornetia secundiflora (J. Agardh) Thuret

Description. *B. secundiflora* is dark red in color, firm and rigid when fresh. The thallus is 5–20 cm high when erect, fan shaped with blunt tips (apices), much branched and tufted, with branches often curved over. The plant has a jelly-like texture. Branches are sparse at the base becoming denser toward the apices [6.359].

Habitat. *B. secundiflora* grows on boulders and bedrock, often under overhangs, from just below extreme low water to 3 m depth. It tolerates sand on rocks and moderately to extreme wave exposure.

Distribution. NE Atlantic (England to the Canary Islands, W Africa); Mediterranean.

Uses and Compounds. Extracts have antifouling activity [6.360, 361].

Bostrychia scorpioides (Hudson) Montagne

Description. Frond flexuous, dull purple, filiform, much branched, inarticulate, dotted, subdichotomous; the branches three or four times pinnated; pinnae and pinnulee patent; apices strongly rolled inward [6.362].

Habitat. On muddy shores near high-water mark, on lower stems of saltmarsh flowering plants, widely distributed, locally abundant.

Distribution. Atlantic shores of Europe, from England to Spain.

Uses and Compounds. Produces Sorbitol; the polyol sorbitol is known to occur naturally in the Rosaceae family especially in *Sorbus aucupria* and in the algae, e.g., *Bostrychia scorpioides*. It is used in the pharmaceutical industry for the treatment against constipation and it known to stimulate vascular contraction as well. Its use in the cosmetics industry, especially in the manufacture of toothpaste is an example of a well established use of carbohydrate and its derivatives in this sector [6.363].

Brongniartella byssoides (Goodenough & Woodward) F. Schmitz

Description. Soft, tufted, deep purplish red fronds, to 300 mm long; main axis well-defined, bi- or tripinnate, bearing alternate, distichous branches. Branches and branchlets articulated, with central siphon and 5–7 pericentral siphons, clothed with short, slender, repeatedly dichotomously branched, monosiphonous ramuli. Its color is light red brown to almost black when dry [6.32, 364].

Habitat. On stones and shells and epiphytic, lower littoral and sublittoral, spring and summer, generally distributed, common in NE Atlantic.

Distribution. NE Atlantic (Scandinavia to Portugal, North Sea, E Baltic Sea), Mediterranean Sea.

Uses and Compounds. Great antioxidant potential; strong cytotoxicity against human cancer cell lines [6.316].

Ceramium virgatum Roth

Synonym. *Ceramium rubrum* C. Agardh.

Common Names. Hornweed, Red hornweed.

Description. Small red seaweed growing up to 30 cm tall. It has a filamentous frond that is irregularly and dichotomously branched, with the branches narrowing toward pincer-like tips. The holdfast is a minute conical disk that extends into a dense mass of rhizoidal filaments. The plant is reddish brown to purple in color and has a banded appearance when viewed closely [6.1, 365].

Habitat. *C. virgatum* is both epilithic and epiphytic, often growing on the stipes and fronds of *Fucus* spp., *Mastocarpus stellatus*, and *Laminaria hyperborea* as well as on the leaves of *Zostera marina*.

Distribution. *C. virgatum* is common worldwide from the Americas to Europe (Atlantic), through the Mediterranean, down to South Africa, Asia, and Antarctica.

Uses and Compounds. *C. virgatum* is used as an extract for cosmetic products [6.313, 366], produces an agar-type polysaccharide [6.367], and have antiviral [6.368], antibacterial [6.276, 369–371], antioxidant [6.372], antialgal [6.373], antiprotozoal, antimycobacterial and cytotoxic activity [6.317].

Chondria capillaris (Hudson) M.J. Wynne

Synonym. *Chondria tenuisssima* (Withering) C. Agardh

Description. Thallus bushy, vivid purple to pale yellowish red, upright cylindrical main axes cartilaginous-firm, with numerous long, softer irregularly spirally arranged lateral axes, branching to the fourth degree; the recent branches also stand rather loosely, spindle-shaped tapering at both ends, the apices pointed and crowned by hair-thin filamentous tufts (magnifying glass required); plants with reproductive bodies appear more densely branched peripherally; discoid holdfast, on prostate axes additional rhizoids [6.32].

Fig. 6.14 *C. coerulescens* specimen (note the blue iridescence in water)

Habitat. On rocks, pebbles, and shells, in shallow water at wave-protected sites, at depths to about 6 m.

Distribution. NE and E Atlantic, Mediterranean, Caribbean, Indian Ocean (India, East Africa, Mauritius).

Uses and Compounds. Extracts have antioxidant and vermifuge [6.335] activity.

Chondria coerulescens (J. Agardh) Falkenberg

Description. *C. coerulescens* has bluish or yellowish fronds with blue iridescence (Fig. 6.14). The fronds are flexible and cartilaginous in texture, turning black when dry. Young axes show a striking turquoise iridescence when alive; the thalli consist of cylindrical erect axes or trailing tufts, and is 3–8 cm high when erect. The distinct main axis is 0.4–0.5 mm in diameter, branching sparsely at irregular intervals in a spiral pattern to 1–3 orders of branching. Branches are linear, often long and curve downward gradually tapering to a slender point, and reattach by secondary holdfast. The morphology shows relatively little variation except that some thalli consist only of inconspicuous isolated erect axes whereas others form dense tufts [6.1, 374].

Habitat. *C. coerulescens* is a sublittoral species, growing on pebbles in mud from extreme low water to 4 m depth.

Distribution. NE Atlantic (Ireland and Britain to Senegal); Mediterranean.

Uses and Compounds. Produces hemagglutinins [6.375].

Chondria dasyphylla (Woodward) C. Agardh

Description. The thallus is 10–21 cm high, reddish brown in color, with the primary erect axis initially arising from a discoid holdfast to 4 mm diameter, subsequently further erect axes arising from the same holdfast giving a clumped appearance. Short creeping branchlets are produced from the lowermost parts of erect axes, and become attached to the substrate by small rhizoidal haptera about 0.5 mm diameter. The thallus branching is irregularly radial, erect-patent with 3–4 orders of branching, ultimate branches arising every two to six (eight) axial cells. Ultimate branchlets are 250–500 µm diameter, the variation being a reflection of the robustness of the plant [6.376].

Habitat. Usually found in mid-littoral pools.

Distribution. NE Atlantic (Ireland to W Africa); Mediterranean, Caribbean, Indian Ocean.

Uses and Compounds. According to the results of *Khanavi* et al. study [6.377], hexane fraction of *Chondria dasyphylla* could contain phytosterols (fucosterol) with cytotoxicity against breast and colon cancer cell line. Extracts of this seaweed have larvicidal [6.378], antibacterial, antifungal, antiprotozoal, antiviral, antifertility, and hypoglycemic [6.218] activity.

Cryptopleura ramosa (Hudson) L. Newton

Description. A short thallus with a stout midrib arises from a discoid holdfast, widening as it branches into flattened red-brown or red-purple fronds to a height of 20 cm (Fig. 6.15). The fronds are thin and membranous, around 2.5 cm in width and may have a slight blue iridescence under water. Repeated branching gives it a bushy tangled appearance with the branches tapering to rounded tips. Morphology is variable and blades can be either erect or prostrate and broadly wedged or strap shaped. Margins may be smooth, undulating, and denticulate or hooked, frequently with a blue iridescence underwater [6.379].

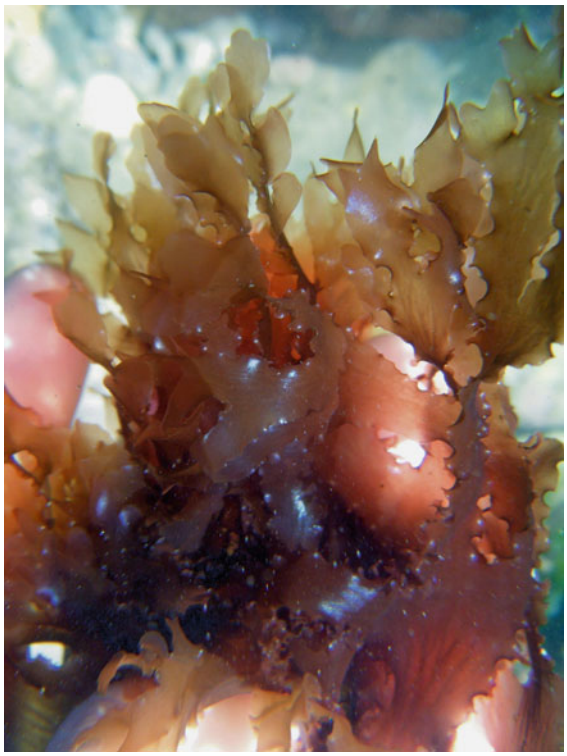

Fig. 6.15 Underwater photo of *C. ramosa*

Habitat. On rocks and often as epiphyte on stipes of *L. hyperborea*, lower intertidal and sublittoral, generally distributed, common.

Distribution. NE Atlantic (Scandinavia to Canary Islands); SW Atlantic (Brazil, Uruguay), Mediterranean Sea.

Uses and Compounds. Tested for biodiesel production [6.380]. Have antifouling [6.373], antiviral [6.381], algicidal [6.382], and antifouling [6.361] activity.

Dasya rigidula (Kützing) Ardissone

Description. Present irregular subdichotomous branching; variable cortication among the thallus; ocellate apex; and stichidia with four tetrasporangia per segment. Traditionally, the species of this genus are diagnosed depending on the branching pattern, presence of ocellate apex, and degree of cortication [6.383, 384].

Habitat. From the mid-littoral to deep waters.

Distribution. NE and E Atlantic (From France to Senegal); Atlantic Islands; West Atlantic; Caribbean; SW Atlantic (Brazil); Mediterranean.

Uses and Compounds. Have antibacterial activity [6.60].

Delesseria sanguinea (Hudson) J.V. Lamouroux

Common Name. Sea beech

Description. Membranous, bright crimson fronds, with cartilaginous, cylindrical, branched stipe, from thickened discoid holdfast, to 300 mm long (Fig. 6.16). Branches bearing spirally arranged, leaflike, ovate-lanceolate blades, each with short stipe and pinnately branched midrib, membranous portion monostromatic, margin undulate (on mature blades), entire; reproductive structures in small oval, stalked blades, borne on midribs in winter [6.385].

Habitat. On rocks, in deep shady lower intertidal pools and in the sublittoral.

Distribution. NE Atlantic (Scandinavia to Portugal, North Sea, Baltic Sea); Mediterranean (sporadically).

Uses and Compounds. *D. sanguinea* is used in the cosmetics industry for its anticoagulant properties and vitamin K content; the active principle being termed *Delesserine* [6.386, 387]. Have also anti-inflammatory and antiskin aging [6.387–389] activity.

Fig. 6.16 Herbarium specimen of *Delesseria sanguinea* (MACOI no. 2549)

Digenea simplex (Wulfen) C. Agardh

Description. Cylindrical axes (1 mm in diameter) rigid, with short secondary branches of the same diameter, carrying dense tufts of thin, straight ramuli; 5–25 cm. Cartilaginous at the apices. Light red (the naked base) to dark red (the branches). The gametophyte and the sporophyte are similar [6.390].

Habitat. Found year round in photophyle sheltered biotopes, from surface down to a few meters; frequently covered by epiphytes.

Distribution. Worldwide distribution in tropical and temperate waters: Mediterranean (with the exception of the Lion Gulf), tropical Atlantic, Red Sea, and Indo-Pacific.

Uses and Compounds. The active agent (Kainic acid), originally isolated in Japan from this species, but now manufactured commercially [6.32], has anti-helminthic activity. The extracts from these red algae have antifungal [6.204] activity.

Halopithys incurva (Hudson) Batters

Description. Tough, cylindrical, cartilaginous, shaggy, dark red fronds, 250 mm long. Main branches alternate or subdichomous, simple or pectinate in lower parts, much branched above, often curved and hooked. Branches with usually double row of short, pointed ramuli on the upper side, ramuli straight, curved or hooked, slightly narrowed at base; axis of 1 central, 5 pericentral siphons, with several layers of cortical cells, outermost small, colored; articulations visible, shorter than broad [6.32].

Habitat. On rocks, mid-littoral pools to subtidal.

Distribution. Warmer NE Atlantic (Ireland to Canary Island); Mediterranean.

Uses and Compounds. Have antibacterial [6.358, 391, 392], antioxidant [6.393], antitumor [6.394], and antiviral [6.357] activity.

Heterosiphonia plumosa (J. Ellis) Batters

Description. *H. plumosa* is a red to deep crimson seaweed which appears black when dried. This species has a flattened, fern-like appearance with a hairy thallus

growing from a discoid holdfast. The fronds are flat or slightly cylindrical up to 20 cm in length and 0.5 cm in diameter at the base, tapering toward the apex. The primary branching from the main frond occurs in a single plane, and is alternately, yet irregularly spaced with up to 1 cm between each branch. Each branch is bare at the base, with the rest bearing an irregular and alternately arranged series of smaller secondary branches. The secondary branches are progressively shorter toward the apex, and each branch bears numerous pointed branchlets giving an overall tufted and feather-like appearance [6.395].

Habitat. On rocks and epiphytic, low intertidal pools and sublittoral, southern and western shores, common in the south, rare in the north.

Distribution. NE Atlantic (Scandinavia to Portugal).

Uses and Compounds. Have high antioxidant activity [6.316].

Hypoglossum hypoglossoides (Stackhouse) F.S. Collins & Hervey

Description. Membranous, rose-pink fronds, 20–200 (300) mm long, arising from a discoid base. Frond linear-lanceolate, with well-marked midrib and thin membranous margins, 1–5 (8) mm wide, repeatedly branched irregularly from midrib; fronds with pointed apices, margins without microscopic veins, monostromatic except in midribs [6.396].

Habitat. On rocks and epiphytic, lower intertidal and sublittoral to 30 m, most abundant in *L. hyperborea* forests.

Distribution. NE and E Atlantic (Scandinavia to Portugal, Azores, Canary Islands, Cap Verde Islands); NW and W Atlantic (North Carolina to West Indies): Mediterranean.

Uses and Compounds. Have antimicrobial activity [6.397].

Laurencia microcladia Kützing

Description. Thallus erect, flexible, soft texture, forming dense tufts up to 15 cm in length. The thallus has greenish-yellow and pinkish apices easily visible. Flaccid consistency does not adhere completely to the herbarium sheet when dry; thalli fully cylindrical with 385–410 µm in diameter; branching irregularly, alternating, dense in the upper two-thirds of the plant, to three branching orders. Adhered to the substrate by rhizoids, from which emerge branches ending in small secondary locking disks [6.398].

Habitat. Present in the mid-littoral rock bottom and up to 1–2 feet deep in the sublittoral exposed coasts. Often found in very beaten but has also been collected in puddles of lower mid-littoral and sublittoral.

Distribution. NE Atlantic (from France to Mauritania); Atlantic Islands (Azores, Canary Islands); Mediterranean; NW Atlantic (USA); Caribbean; SW Atlantic (Venezuela, Brazil).

Uses and Compounds. Have antimitotic and cytotoxic, [6.62, 399–403], antiparasitic [6.402, 404, 405], and antifungal [6.62] activity.

Laurencia obtusa (Hudson) J.V. Lamouroux

Description. Plants 1.5–2.5 cm tall, bushy, with green or yellow axes and rose branchlets, main stems long, which are sparingly alternately branched, 0.75–1.50 cm, but above are increasingly closely paniculately branched and spreading, the smallest branches and the short, truncate, ultimate branchlets opposite or subverticillate, 0.5–0.75 mm diameter; tetrasporangia in a band below the apex of the hardly modified branchlets [6.406].

Habitat. On solid substrata, in extremely shallow locations in calm shallow water, also in exposed locations within the intertidal zone.

Distribution. Worldwide in warm temperate to tropical seas.

Uses and Compounds. Have antibacterial [6.32, 407–410], antimalarial [6.411, 412], antitumor [6.316, 413, 414], antioxidant [6.316, 410, 415], and antifouling [6.416] activity.

Laurencia pyramidalis Bory de Saint-Vincent ex Kützing

Description. Globose tufts of brittle, cartilaginous, narrow, cylindrical, reddish brown to yellowish-red fronds, 150 mm long, from small discoid base (Fig. 6.17). Axis simple, branches patent, often opposite, spirally arranged, shorter toward apex giving regular pyramidal outline [6.417].

Fig. 6.17 Underwater photo of *Laurencia pyramidalis*

Habitat. Usually epiphytic, annual, lower intertidal.

Distribution. NE Atlantic (From Ireland and Britain to Portugal); NW Mediterranean.

Uses and Compounds. Have antioxidant and antimicrobial activity [6.410].

Laurencia viridis Gil-Rodríguez & Haroun

Description. Thallus annual, cartilaginous, mainly greenish with pink tips, with erect axes averaging 4–8 cm (range 2–15 cm) long, having alternate or helicoid branching only in the upper two-third of the thallus; ovoid spermatangia produced in cup-shaped receptacles; cystocarps urceolate, and sessile; tetrasporangia in parallel abaxial rows [6.418].

Habitat. Mainly restricted to exposed low intertidal sites, growing on rocks with strong wave action.

Distribution. Macaronesian region (Azores, Madeira, Selvages, Canaries, and Cape Verde).

Uses and Compounds. Have cytotoxic and antitumor [6.419–423] activity.

Nitophyllum punctatum (Stackhouse) Greville

Description. Delicately membranous, rose-pink fronds with an elongate fan-shaped outline, margins distinctly frilly, to 40 mm or, exceptionally, to 1 m, sessile or shortly stipitate (< 2 mm long); frond veinless, undivided or deeply subdichotomously divided to the base; apices blunt or rounded, often ribbon-like (Fig. 6.18). Gametophyte plants form rounded spots to 5 mm in diameter whilst tetrasporophyte plants form characteristically elongated spots [6.424].

Habitat. In littoral pools in winter and early spring and in the sublittoral (15 m) on cobble or other mobile surfaces and on bedrock in the lower parts of kelp forests from spring to summer.

Distribution. NE Atlantic (Norway to Canary Islands); NW Atlantic (N America); Caribbean, Mediterranean and Indian Ocean (India, Egypt); Sub-Antarctic.

Uses and Compounds. Potential bio-insecticides against mosquito larvae [6.66].

Osmundea hybrida (A.P. de Candolle) K.W. Nam

Description. Cylindrical, cartilaginous, tufted, dark purple to greenish-yellow fronds, 150 mm long; main axis with repeatedly pinnate branching, branches mostly alternate, shorter toward apex giving pyramidal outline; ultimate ramuli short, patent, truncate; axis monosiphonus with elongated pericentral cells and 1–2 outer layers of rounded colored cells; apex con-

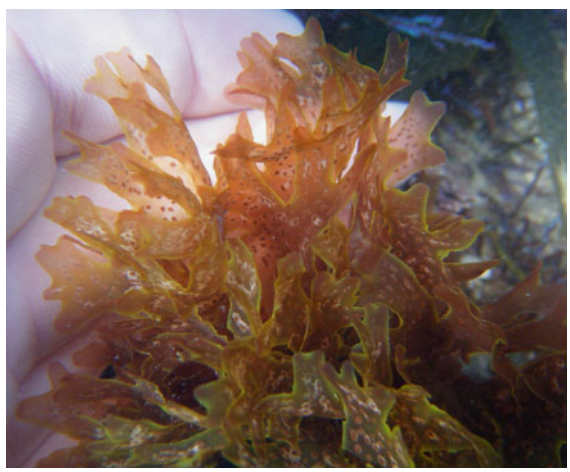

Fig. 6.18 Underwater photo of *N. punctatum*

cave, with ephemerally colorless dichotomous hairs surrounding apical cell [6.425].

Habitat. On stones and shells, sometimes epiphytic, upper intertidal, widely distributed, locally common.

Distribution. NE Atlantic (Britain to Morocco); SW Atlantic (Brazil).

Uses and Compounds. Perform antibacterial activity [6.340, 391].

Osmundea pinnatifida (Hudson) Stackhouse

Common Name. Pepper dulse.

Description. A small red alga (up to 8 cm in length), it is tough and cartilaginous with flattened fronds (Fig. 6.19). Branching is alternate and occurs in one plane only, with branches becoming shorter toward their apex and broadly rounded. The plant is highly variable in size and coloration depending upon its location on the shore. Higher shore plants are generally dwarfed and yellow green in color, owing to exposure to high levels of sunshine while on the lower shore they are reddish brown [6.1, 426].

Habitat. On rocks, perennial, throughout intertidal, often as flattened rosettes covering exposed rocks, also sublittoral, generally distributed, abundant.

Fig. 6.19 *Osmundea pinnatifida* specimen

Distribution. NE Atlantic (Ireland and Britain to Senegal); Atlantic Islands; SW Atlantic (Brazil); Mediterranean (Turkey); SW and East Asia, North America, South America, Australia, and New Zealand.

Uses and Compounds. The marine polyether triterpenoid dehydrothyrsiferol 298, originally isolated from the red alga *Laurencia pinnatifida* was shown to induce apoptosis in estrogen-dependent and independent breast cancer cells [6.419, 420]. Have antibacterial [6.27, 358, 427, 428], antioxidant [6.234, 429], antileishmanial [6.430, 431], anticancer [6.225, 432], antifouling [6.257, 275], insecticidal, and antifungal [6.226] activity.

This aromatic seaweed is dried and used as a pepper- or curry-flavored spice in Scotland, Ireland, and Portugal (Azores Islands) [6.3, 426].

Osmundea truncata (Kützing) K.W. Nam & Maggs

Description. *O. truncata* is much less common than *O. pinnatifida*, is more pinnately branched, generally grows epiphytically, and rapidly degenerates on collection [6.433].

Habitat. On rocks, perennial, throughout intertidal, often as flattened rosettes covering exposed rocks.

Distribution. Mediterranean.

Uses and Compounds. Extracts have antimicrobial activity [6.130].

Pterosiphonia complanata (Clemente) Falkenberg

Description. Thallus with prostate and erect axes, the erect ones flat-compressed and with distichous, alternate pinnate branching (Fig. 6.20); brownish-red color, flexible, and cartilaginous texture; attached by rhizoids which form discoid adhesive structures [6.434].

Habitat. On rocks and as epiphyte on other algae, in the entire intertidal zone and below.

Distribution. Warm to tropical regions of the Atlantic and Mediterranean.

Uses and Compounds. Extracts have antiviral [6.357], and antibacterial [6.358, 435] activity.

Fig. 6.20 *Pterosiphonia complanata* specimen

Ptilota serrata Kützing

Synonym. *Ptilota pectinata* (Gunnerus) Kjellman

Description. *P. serrata* is a typically dark red and less than 5 cm tall seaweed. It is branched in opposite pairs, often with wavy spandrels protruding from the ends of the branches. Simple branchelets with serrated margins on one side stand opposite to composite-pinnately divided lateral branches on the other [6.32].

Habitat. Understory alga in the shallow sublittoral becoming dominant beyond 10 m.

Distribution. North Atlantic (Greenland, Svalbard, Faroe Islands, Norway), and N Pacific (Alaska to Washington).

Uses and Compounds. Has hemagglutinating activity [6.375].

Rhodomela confervoides (Hudson) P.C. Silva

Description. Cylindrical, cartilaginous, bushy, brownish-red fronds, to 300 mm long. Much branched, repeatedly, irregularly. Branches clothed with small, pinnate branchlets and simple, pointed ramuli. In winter, branchlets are shed and frond appears bare, spiky, and very different from summer appearance. Central siphon surrounded by elongated cells with the outer band of small colored cells, articulations not visible [6.436].

Habitat. On rocks and shells, intertidal pools at all levels; widely distributed, common.

Distribution. Warmer NE Atlantic (Ireland to Canary Islands); Mediterranean.

Uses and Compounds. Extracts have antibacterial [6.437], antifungal [6.438], antihyperglycemic [6.439], cytotoxic [6.440], and antioxidant [6.441–443] activity.

Rytiphlaea tinctoria (Clemente) C. Agardh

Description. Thallus red, very dark, cartilaginous, abundantly branched, formed on the principal axes, flattened branches alternate provided in turn with short ramuli ends more or less curved. Structure–uniaxial quickly becoming complex by recloisonnement; in cross section showing the axial cell medulla sometimes indistinct, surrounded by 5 pericentral cells; thallus with apical growth [6.444].

Habitat. In the upper sublittoral, occasionally in the mid-littoral.

Distribution. NE Atlantic (France to Mauritania); Atlantic Islands; Mediterranean.

Uses and Compounds. Extracts are used as colorant [6.445]. Extracts have antimicrobial activity [6.130].

Spyridia filamentosa (Wulfen) Harvey

Common name. Feathery seaweed.

Description. Plants, dull brown to pale pink, usually less than 20 cm tall when in low turf mats, up to 18 cm tall in areas of good water motion; fastened by small discoid holdfast, the lower portions of plants and the lower branches tending to entangle; one to several erect axes, branching irregularly dichotomous or completely irregular, at times also alternate or unilateral; axes crisp, brittle, branching to 4–5 or more orders, the last orders short, becoming matted and giving plants fuzzy appearance; plants often look whitish or lightly calcified, but calcification from crustose coralline algae or sediment, not inherent in plants [6.446].

Habitat. On solid substrates in calm protected areas or adrift; to 8 m deep; common on eroded coral in sandy areas, but occurring in sandy mud as well, in matted intertidal turfs on coral and basalt.

Distribution. NE and E Atlantic (from Ireland and Britain to Sierra Leone); Atlantic Islands; NW and W

Atlantic (USA, Mexico); Caribbean; SW Atlantic; Indian Ocean; SE Pacific; Australia and New Zealand.

Uses and Compounds. Extracts have antibacterial [6.60, 421, 447–449], and antitumor [6.421] activity.

Vertebrata lanosa (Linnaeus) T.A. Christensen

Synonym. *Polysiphonia lanosa* (Linnaeus) Tandy.

Common Names. Wrack siphon weed, Many tubed gable weed.

Description. *V. lanosa* is a filamentous red alga that grows as an obligate epiphyte on *Ascophyllum nodosum*. The short, cylindrical, cartilaginous fronds form beautiful dense reddish purple tufts along the ochre colored thalli of their host. Branches of this alga are dichotomous with pointed tips. Formerly classified as *Polysiphonia lanosa*, it lacks the distinctive banded pattern typical of *Polysiphonia* species. As an obligate epiphyte, *V. lanosa* cannot grow except attached to other seaweed species by tiny structures called rhizoids that grow into the tissue of the algal host. *A. nodosum* is the most frequent host, though *V. lanosa* is sometimes found on *Fucus* species such as *F. vesiculosus* as well, so it is abundant where these species thrive [6.450].

Habitat. Hemi-parasitic on *A. nodosum*, more rarely on *F. vesiculosus*, never on rock (appearances can be deceptive as it can grow on old stalks of both species) in the sheltered mid-littoral, generally distributed, usually abundant where *Ascophyllum* occurs. *Vertebrata* is generally infected with a tiny, more or less colorless parasitic red alga called *Choreocolax polysiphoniae*, to which it is closely related [6.451].

Distribution. *V. lanosa* is fairly common in the N. Atlantic, along the coasts of North America and Europe.

Uses and Compounds. *V. lanosa* is valued for its strong antioxidant and anticancer properties, used in supplements and health and beauty products [6.450]. Extracts of this species have cytotoxic [6.452], antioxidant [6.453], antibacterial [6.454], antifungal [6.455], and antifouling [6.373, 454] activity.

- Order: **Corallinales**

Amphiroa beauvoisii J.V. Lamouroux

Description. More or less erect, sometimes bushy. Size: 2–5 cm high. Branching: dichotomous, occasionally irregular. Intergenicula: tapered toward the base and flattened at the apices (up to 3 mm long, the lower diameter 0.6–0.65 mm, the upper diameter 0.4 mm) apices characteristically knife-like and striped. Geniculа: with 2 (3–4) rows of cells. Color of living specimen: pink, pink-violet. Color of dried specimen: pink-violet to white [6.456].

Habitat. Like that of *A. rigida*, species of sheltered environments, on sublittoral, from surface to 30 m deep.

Distribution. Widely distributed in tropical and subtropical seas.

Uses and Compounds. Extracts have antibacterial activity [6.457].

Amphiroa cryptarthrodia Zanardini

Description. Thallus erect, bushy; endophytic base on other calcareous algae (particularly on *Lithophyllum* species). Size: 2–4 cm high. Dichotomous branching, regular and *geometric* (angle of about 90°); branch junctions usually not coinciding with intergenicula. Branches lying on different planes, slightly swollen apices. Intergenicula cylindrical with rare annular swellings, tapered on the upper part of fronds 1–4 (4.5) mm long, 0.25–0.6 mm in the lower diameter, upper diameter 0.15–0.18 mm. Dark red to pink violet (rare), apart from lighter apices due to annular ridges [6.458].

Habitat. On the rocks in sheltered waters where they often form large lawns, in the same station of *A. rigida* (but less frequently); also reported in tide pools.

Distribution. NE Atlantic (from France to Senegal); Mediterranean.

Uses and Compounds. Used on functional foods and pharmaceuticals [6.205]; extracts have antifungal and antimitotic activity [6.62].

Amphiroa fragilissima

Description. Thalli with dense cushion-like tufted growth, very brittle to strong calcification; branches cylindrical, thin, segmented, rather regularly forking, sometimes also trichotomous, the angles between two fork branches usually rather wide (broadly Y-shaped); the segments are slightly swollen at the endings; yellowish red to whitish pink [6.32].

Habitat. Common in shallow water, especially in seagrass meadows and rock hollows; to 10 m depth.

Distribution. Widely distributed in tropical and subtropical seas.

Uses and Compounds. Used on functional foods [6.109], and the extracts have antiviral [6.237], antibacterial [6.40], cytotoxic and antioxidant [6.459], oxytocic and espamogenic [6.460] activity.

Amphiroa rigida J.V. Lamouroux

Common Name. Twig algae.

Description. Thallus growth lawn-like, segmented branches terete, brittle (calcified), usually forking and simultaneously laterally branching, narrowing toward the tip, segments distally inconspicuous (Fig. 6.21); purple-like conceptacles mostly numerous, especially on peripheral branches [6.32].

Habitat. Species recorded in shallow and deep waters in sheltered and shaded sites; sometimes found on rocky pools in exposed areas; mainly on sublittoral.

Distribution. Cosmopolitan species, present in warmer and tropical seas.

Fig. 6.21 *Amphiroa rigida* specimens

Uses and Compounds. Extracts have antimicrobial [6.391], antifungal, cytotoxic, and antimitotic [6.62] activity.

Corallina elongata J. Ellis & Solander

Description. Whitish pink to reddish lilac, calcified, articulated fronds, fish-bone-like arrangement, to 50 mm high, axis compressed, repeatedly pinnate from discoid base, more abundantly and regularly branched than *C. officinalis*; articulations small [6.1].

Habitat. On rocks, exposed coasts, lower intertidal, southern and western coasts, occasional.

Distribution. NE Atlantic (Ireland and Britain to Senegal); Mediterranean.

Uses and Compounds. Used for R-phycoerythrin extraction and for functional foods [6.461]. Extracts have antimicrobial [6.228], antifungal and antiviral [6.62] activity.

Corallina officinalis Linnaeus

Common Name. Coral weed.

Description. Whitish pink to lilac, calcified, articulated fronds, 60–70 (–120) mm high, axis cylindrical to compressed, repeatedly pinnate from and expanded discoid base, branching often irregular (Fig. 6.22). Growth form very variable often stunted. In unfavorable habitats erect system vestigial, but extensive base may be present [6.1].

Habitat. On rocks, mid-littoral pools and drainage runnels, lower intertidal and shallow sublittoral, widespread and abundant, especially on exposed coasts.

Distribution. *C. officinalis* has been recorded widely in the north Atlantic, from northern Norway to Morocco and from Greenland to Argentina. It is found along the Atlantic coasts of North America from Labrador south to Connecticut and Maryland in the United States. *C. officinalis* has been reported in Japan, China, Australasia, South Africa, and the Arctic Sea.

Uses and Compounds. *C. officinalis* is a very popular ingredient among cosmetics and health and personal care companies. There are known sellers of *C. officinalis*-based products in the United States, China, Italy, France, Switzerland, and Ger-

Fig. 6.22 Underwater photo of *C. officinalis*

many. These are products for men and women, and include toners, moisturizers, cleansers, emulsions, essences, astringents, eye creams, wash gels, shower gels, shave balm, hydration sprays and creams, and masks [6.462]. Extracts have antimicrobial [6.201], anthelmintic [6.354], and antioxidant [6.335, 463] activity.

Haliptilon attenuatum (Kützing) Garbary & H.W. Johansen

Description. Thallus habit: erect, bushy with dense not entangled fronds (D). Size: 2–2.5 cm high. Branching: alternate, dichotomic-pinnate (D-A) (whorled); distal dichotomous branchlets. Intergenicula: cylindrical, tapered near apices, 0.2–0.4 (0.5) mm long, 0.07–0.1 mm in diameter. Genicula: 60–200 µm long. Color of living specimen: from red scarlet to purple. Color of dried specimen: from white to ivory yellow [6.464].

Habitat. Epiphytic species often on *H. virgatum*; mainly on sublittoral.

Distribution. Alboran Sea: Morocco; Middle Western Mediterranean Sea: Italy; North Adriatic Sea: Italy, Croatia.

Uses and Compounds. Extract has antibacterial activity [6.60].

Jania adhaerens J.V. Lamouroux

Description. This species is small (4–5 mm high), heavily calcified, and forms intricately entwined clumps. The branches are pinkish-red color, regularly dichotomous, terete or slightly compressed, and slightly curve downward. Terminal segments are acuminate. This is an epiphytic species, decumbent on other seaweeds such as the species of Sargassaceae, growing in the sublittoral zones along shorelines moderately exposed to water movement [6.465].

Habitat. Growing in the sublittoral zones along shorelines moderately exposed to water movement.

Distribution. Atlantic Islands; Mediterranean; SW and SE Atlantic; Caribbean; Indo-Pacific Oceans; Australia.

Uses and Compounds. Extracts have antigenotoxic [6.466], antifungal, antiviral, and antimicrobial [6.62, 103] activity.

Jania rubens (Linnaeus) J.V. Lamouroux

Description. Slender, rose-pink, articulated, calcified fronds, to 50 mm high; repeatedly dichotomously branched, luxuriant specimens secondarily pinnate (Fig. 6.23). Segments cylindrical, to 120 µm diameter, those bearing branches somewhat compressed, to 180 µm diameter. Fixed by small conical disk,

Fig. 6.23 *Jania rubens* specimens

but spreading vegetatively by developing attachment disks from branches in contact with solid substratum [6.467].

Habitat. Epiphytic, found only growing epiphytically on the brown algae *Cladostephus* and *Cystoseira* lower littoral, often abundant.

Distribution. The range of *J. rubens* stretches from the Baltic Sea and Norway in the north to Portugal, Senegal, East Africa, and the Azores in the south; it is also found in the Mediterranean, the Canary Islands, Indian Ocean, Black Sea, and China Sea, as well as around Brazil.

Uses and Compounds. *J. rubens* has recently starting to be used by cosmetic companies as an extract in natural beauty products. It is prized for its ultra-moisturizing and protective properties due to the high concentration of minerals and trace elements present in its tissue. One provider notes that *J. rubens* is characterized by a concentration of minerals and trace elements 20 000 to 40 000 times greater than that of seawater, thus giving it re-mineralizing properties. It is also used in skin whitening and hydrating products [6.468].

Extracts of this species have antitumor [6.126], bio-insecticide [6.463], antimicrobial [6.469, 470], antihelmintic and cytotoxic [6.354, 471], antifouling [6.472], and antifungal [6.62] activity.

Jania rubens var. *corniculata* (Linnaeus) Yendo

Description. Thallus erect, bushy, attached by a basal crustose disk. Size: 1–4 cm high; dichotomous branching, abundant, and entangled branches. Intergenicula: cylindrical, slightly compressed, bearing basal, sometimes articulated projections (horn-like); becoming wider at distal apex, 0.40–0.75 mm long (times) 0.10 mm in diameter. Genicula: irregular filaments 90–160 µm. Color of living specimen: from violet pink more or less intense. Color of dried specimen: white to yellow ivory [6.473].

Habitat. Epilithic or epiphytic on other algae (particularly *Cladostephus* species), in well-lit sites. Bathymetric

Distribution. From surface to shallow waters (3 m).

Distribution. NE Atlantic (from Ireland and Britain to Morocco); Mediterranean.

Uses and Compounds. Extracts have antioxidant and antimicrobial activity [6.474].

Lithophyllum byssoides (Lamarck) Foslie

Synonym. *Lithophyllum lichenoides* Philippi

Description. *L. byssoides* commonly grows in the intertidal zone. Living specimens, gray–violet in color, form characteristic cushion-like clumps, composed of densely interweaving and anastomosing lamellae which are more or less smooth on the dorsal surface and ridged on the ventral one. Growth form with spiniform lamellae has also been observed. Thalli grow by forming concretions of approximately 10 to 20 cushion-like dumps [6.475].

Habitat. It grows on rocky substrata at mid-littoral.

Distribution. NE Atlantic (Spain, Portugal to Senegal); Mediterranean (Italy).

Uses and Compounds. Extracts have antifungal activity [6.62].

Lithothamnion corallioides (P.L. Crouan & H.M. Crouan) P.L. Crouan & H.M. Crouan

Common Name. Maerl.

Description. An unattached, fragile, alga with a calcareous skeleton. It is very similar to and often confused with *Phymatolithon calcareum*. Its form is very variable but it commonly occurs as highly branched nodules forming a 3D lattice. Individual plants may reach 4–5 cm across and are bright pink in color when alive but white when dead [6.476].

Habitat. Typically found at less than 20 m depth on sand, mud or gravel substrata in areas that are protected from strong wave action but have moderate to high water flow. Usually found as unattached plants.

Distribution. NE Atlantic (Norway to Mauritania); Mediterranean.

Uses and Compounds. Used as fertilizer and as a constituent of pharmaceutical preparations [6.477, 478]. Extracts have antifungal activity [6.62].

Lithothamnion glaciale Kjellman

Common name. Knobby coralline-crust algae.

Description. Bright pink to purplish, minutely white-speckled calcareous crust, becoming very thick, usually with abundant regular or irregular branches, free or attached to substratum [6.479].

Habitat. On rocks, pebbles, shells or free-living, lower intertidal (north-east coasts) and sublittoral to deeper waters.

Distribution. In the NE Atlantic from the British Isles north to Arctic Russia including the Faeroe Isles, Iceland and western Baltic; in the NW Atlantic from Cape Cod north to Arctic Canada and Greenland; also northern Japan and China in the western Pacific.

Uses and Compounds. Commercial extraction for use as a soil conditioner on acidic ground, as an animal food additive, for the filtration of acid drinking water and in pharmaceutical and cosmetic products [6.386, 479]. Extracts promote the induction of metamorphosis of the sea urchin larvae (*Strongylocentrotus droebachiensis*) [6.480].

Mesophyllum lichenoides (J. Ellis) M. Lemoine

Description. Pale to dark purple thin, brittle, leafy calcified fronds, attached at base, margins free, lobed. Fronds semicircular, concentrically banded (Fig. 6.24). Reproduction takes place in winter and spring in small, wart-like conceptacles [6.481].

Habitat. Epiphytic on *C. officinalis* in mid-littoral pools.

Distribution. NE Atlantic (from Ireland to Mauritania); Mediterranean.

Uses and Compounds. Used as a soil conditioner on acidic ground [6.386]. Extracts have antifungal, cytotoxic and antimitotic activity [6.62].

- Order: **Gelidiales**

Gelidiella acerosa

Description. *G. acerosa* is a red algae with yellowish brown, tufted, entangled, erect, cylindrical thalli reaching 6 cm tall. The ends of the fronds are pinnately divided, giving it a feathered appearance. Branch tips of *G. acerosa* terminate in a single apical cell. Short, thick branches attached to the substratum by stoloniferous rhizoids form dense mats along shallow reefs. *G. acerosa* is found on surf-exposed and moderately wave-sheltered rocks and reefs in the lower mid-littoral and the sublittoral zone, and in tide pools at higher levels on the shore [6.482, 483].

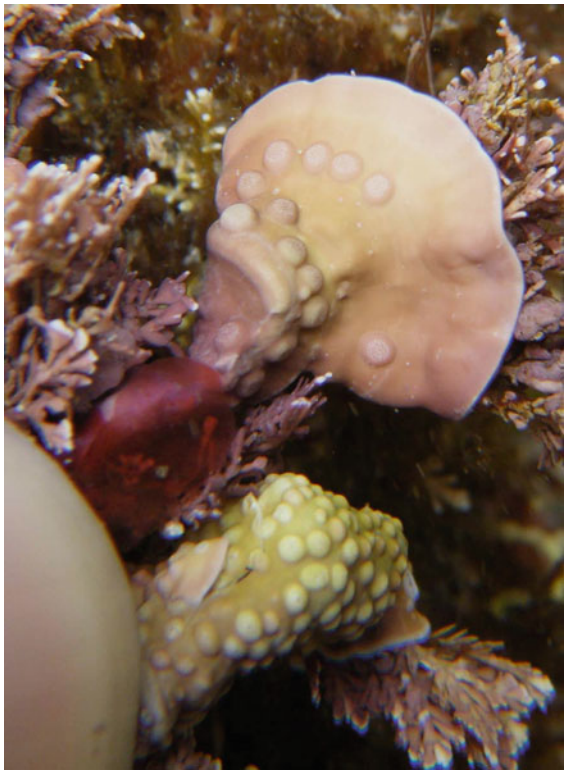

Fig. 6.24 Underwater photo of *Mesophyllum lichenoides*

Habitat. In tide pools, attached to rock reefs at depth of 0–1 m; in association with *Gelidiopsis* and *Gelidium* species.

Distribution. Atlantic Islands: Azores and Cape Verde Islands.

Uses and Compounds. *G. acerosa* is an important commercial species for agar production [6.484–487]. It has also been used traditionally for the preparation of agar-forming hard jellies, or eaten fresh and also prepared as a salad vegetable or cooked and eaten mixed with rice [6.4]. However, over recent years, *G. acerosa* has become a key ingredient in a staggering number of products [6.109]. It is used in nearly 150 hair products, including: hair color and bleaching; shampoos and conditioners; styling mousse, foam, gels, sprays, and lotions; hair relaxers and detanglers; antidandruff hair masks [6.483].

Extracts have antifungal [6.488], contraceptive [6.489], antioxidant [6.490], antifungal, and antibacterial [6.490–493] activity.

Gelidiella ramelosa (Kützing) Feldmann & G. Hamel

Description. Plants occur as turfs to 1–2 cm in height, with prostrate stolons attached by clusters of rhizoids to rock. Erect axes are irregularly pinnately branched and terete to slightly compressed. Structurally, the medulla is pseudoparenchymatous with no discernible central axis, and rhizines are absent. Spermatangia occur in surface sori. Tetrasporangia are borne in irregular whorls in terete, basally constricted stichidia that are lateral on erect axes [6.494].

Habitat. Growing as sparse turf amongst coralline algae.

Distribution. NE Atlantic (from France to Portugal); Mediterranean; Australia.

Uses and Compounds. Extracts have cytotoxic and antimitotic activity [6.62].

Gelidium corneum (Hudson) J.V. Lamouroux

Synonym. *Gelidium sesquipedale* (Clemente) Thuret.

Common Name. Kanteen.

Description. Dark red, hard consistency, cartilaginous, and may reach 30 cm in length thalli. Branches with obtuse apex and attenuated at the base (Fig. 6.25).

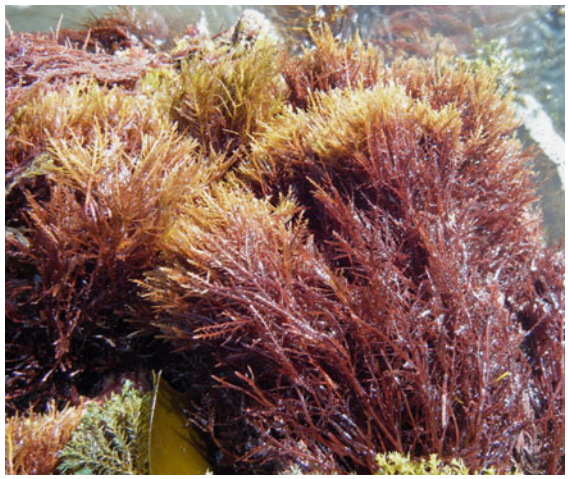

Fig. 6.25 *Gelidium corneum* specimens

This species typically forms dense stands of clumped fronds, often under a kelp canopy [6.1].

Habitat. On rocks and tide pools in exposed areas.

Distribution. E Atlantic (Britain to South Africa); W Atlantic (USA to Brazil); Mediterranean; Indo-Pacific zone; Australia.

Uses and Compounds. One of the main sources of Agar [6.495, 496]. Extracts are used for the production of edible films containing carvacrol for ham packages, and other edible films with antimicrobial effect [6.497–500]. The antimicrobial and antioxidant activity was demonstrated in several studies [6.269, 501, 502].

Gelidium pulchellum (Turner) Kützing

Description. Cartilaginous, regularly or irregularly bipinnate, dark red-brown fronds, 50–100 mm high, arise from a creeping base. Main axes narrow, cylindrical, somewhat flattened above. Ultimate branches short, pointed at first, later ± spathulate, particularly when reproductive; appearance variable with Habitat and time of year [6.503].

Habitat. Abundant in and around tidal pools in mid- and lower littoral in high-light situations; generally epiphytic on *C. officinalis*; widely distributed, common.

Distribution. NE Atlantic (Ireland and Britain to Portugal and Morocco). Australia.

Uses and Compounds. Source of agar [6.3, 503, 504]. Extracts have antibacterial [6.358], and antiviral [6.357] activity.

Gelidium pusillum (Stackhouse) Le Jolis

Description. Cartilaginous, purplish, or blackish red, turf-forming, 2–10 mm high, arising from extensive creeping base and incorporating shell debris and small mollusks; erect fronds flattened and leaf-like and 0.5–2 mm broad [6.505].

Habitat. Marine and estuarine species, on rock pools and sandy rocks, particularly in places with widely varying salinities such as upper shore species.

Distribution. NE and E Atlantic (from Norway to Benin); Mediterranean; EW Atlantic; Caribbean; SE Atlantic; SW Atlantic; Indian Ocean; SW Pacific.

Uses and Compounds. Source of agar [6.505]. Extracts have antibacterial [6.358], and antifungal [6.62] activity.

Gelidium spinosum (S.G. Gmel.) P.C. Silva

Description. Small alga, cartilaginous, crimson to purplish red, 20–60 mm long. Main axes distinctly flattened, often narrower at base, ultimate branches short, often opposite, spine-like or spathulate [6.32].

Habitat. In sheltered locations below the tidal mark.

Distribution. NE Atlantic (Ireland and Britain to Canary Islands); Mediterranean; Indo-Pacific (Indonesia).

Uses and Compounds. Source of Agar [6.506]. Extracts have antiviral, cytotoxic, and antibacterial activity [6.205, 507].

Pterocladiella capillacea (S.G. Gmelin) Santelices and Hommersand

Synonym. *Pterocladia capillacea* (S.G. Gmelin) Bornet.

Common Name. Small agar weed.

Description. Thallus dark brownish red, commonly grows in dense tufts about 4 cm high, and composed of prostrate axes that give rise to the flattened erect axes: branching pinnate to irregularly opposite with uniaxial growth from single apical cells; inner structure pseudoparenchymatous, with long, slender, thick-walled rhizine cells filling the spaces between elongated medullary cells that are surrounded by a three-layered cortex of smaller pigmented cells [6.508].

Fig. 6.26 *Ahnfeltiopsis devoniensis* specimen

Habitat. In large pools and lagoons in the lower littoral and shallow sublittoral, widely distributed, never common.

Distribution. Europe, Atlantic Islands, N America, Caribbean Islands, S America, Africa, SW and SE Asia, Australia and New Zealand, Pacific Islands.

Uses and Compounds. Source of agar [6.509, 510]. Extracts have antioxidant [6.234], antibacterial [6.470], antinociceptive and anti-inflammatory [6.511], anticoagulant [6.512], and antitumor [6.126] activity.

- Order: **Gigartinales**

Ahnfeltiopsis devoniensis (Greville) P.C. Silva & DeCew

Synonym. *Gymnogongrus devoniensis* (Greville) Schotter

Description. *A. devoniensis* is a small red marine alga that grows to only several centimeters in length from a disk-like holdfast (Fig. 6.26). It forms a medium-sized flattened frond with regular dichotomous branching. The branches have parallel sides. The reproductive structures (cystocarps) are internal [6.513].

Habitat. Generally found in the very lower intertidal or shallow sublittoral, in salty areas protected from strong wave action. As far as it is known, it can be found anytime.

Distribution. NE Atlantic (Britain to Portugal).

Uses and Compounds. Source of carrageenan [6.514–516]. Extracts have antihypertensive and antioxidant activity [6.501, 517].

Calliblepharis ciliata (Hudson) Kützing

Common Name. Eyelash weed.

Description. *C. ciliata* is bright to dark red in color. The main blade is erect and forms into a wedge shape

or ovate lobe that expands into a dichotomous or irregularly divided blade up to 7 cm broad and 30 cm long and 350–650 µm thick. Small branches arise from the main blade. It can vary considerably in blade width and degree of subdivision. In southeastern England its blades are often only 10–15 µm thick. The branchlets (proliferations) that arise from the margins of the blade are either pointed or rounded. Patches of encrusting bryozoans are very common on old specimens [6.518, 519].

Habitat. In larger lower intertidal pools and sublittoral (0–25 m) on stones, maerl, and shells, widely distributed, common in south and west, sometimes frequent in drift.

Distribution. NE Atlantic (Ireland and Britain to Mauritania); W Mediterranean.

Uses and Compounds. Source of carrageenan [6.520, 521], and hemagglutinins [6.375].

Calliblepharis jubata (Goodenough & Woodward) Kützing

Description. *C. jubata* is brownish red in color. It has a thallus consisting of a branched holdfast that gives rise to an erect frond that expands into a dichotomous or irregularly divided blade (Fig. 6.27). The outline of the frond is variable but it commonly has a cylindrical or very slightly compressed stipe. Its blades are about 6 mm broad and 30 cm long with narrow branches. The branches appear long and tendril like. Long branchlets (proliferations) arise from the blade surface and margins of the branches [6.1].

Habitat. On rocks and epiphytic, especially on *C. officinalis* in large open pools, mid-littoral to shallow sublittoral (5 m).

Distribution. NE Atlantic (Ireland to Mauritania); W Mediterranean.

Uses and Compounds. Source of carrageenan [6.514–516, 521–523], and hemagglutinins [6.375]. Extracts have antimycobacterial [6.317], anticoagulant [6.524], antiprotozoal, and cytotoxic [6.525] activity.

Caulacanthus ustulatus (Mertens ex Turner) Kützing

Description. Thalli forming small dense entangled tufts of up to 5 cm, reddish-brown color, which blackens

Fig. 6.27 *Calliblepharis jubata* specimen

Fig. 6.28 Underwater photo of *Chondracanthus teedei* var. *lusitanicus*

by desiccation, with rough touch; fixed to the substrate by cylindrical, crawling axes from which derive others, also with circular section, irregularly branched; ramifications with acute apices and behaving small triangular-shaped spines [6.526].

Habitat. It lives on rocks and clams in well-lighted areas with little inclination at mid-littoral zones in semiexposed shores.

Distribution. NE and E Atlantic (Ireland and Britain to Guinea); Mediterranean; NW Atlantic (Canada to Mexico); SW Atlantic (Brazil); SE Atlantic (Angola to South Africa); Indian and Pacific Oceans; Australia.

Uses and Compounds. Produces a kappa/iota-hybrid carrageenan [6.527]. Extracts have antibacterial activity [6.358].

Chondracanthus acicularis (Roth) Fredericq

Description. Cartilaginous, cylindrical, or compressed, purple-red or blackish fronds, sometimes with greenish or whitish spots, to 100 mm long, irregularly bipinnately branched, branches curved, sharply pointed [6.1].

Habitat. On rocks, lower intertidal, generally uncommon but may be locally common on sheltered, silty shores in the lower intertidal.

Distribution. NE and E Atlantic (Ireland and Britain to Cameroon); Mediterranean; NW and SW Atlantic; Caribbean; Indian Ocean; Pacific (Hawaii); E Australia.

Uses and Compounds. Source of carrageenan [6.514–516]. Extracts have antiviral [6.103], antibacterial [6.269, 357], antioxidant [6.429], antifungic [6.204], and anticoagulant [6.512] activity.

Chondracanthus teedei var. *lusitanicus* (Rodrigues) Bárbara & Cremades

Description. The fronds of this alga are cartilaginous-membranous, with purple-violet color that darkens by desiccation, becoming greenish yellow with decay (Fig. 6.28). The main axes of the fronds, as their ramifications are wide, reaching 1 cm in the older portions. This species is confused sometimes with *Calliblepharis jubata* [6.1].

Habitat. On rocks in mid-littoral zone.

Distribution. NE Atlantic (Spain and Portugal).

Uses and Compounds. Produces carrageenan [6.514–516, 528]. Extracts have cytotoxic activity [6.529].

Chondrus crispus Stackhouse

Description. Cartilaginous, dark purplish red, red, yellowish or greenish fronds of 150 mm high, gametophyte plants are often iridescent under water when in good condition; stipe compressed, narrow, expanding gradually to a flat, repeatedly dichotomously branched frond, in tufts from a discoid holdfast. Axils rounded, apices blunt or subacute, frond thicker in center than margins; breadth of segments varies frequently; branching, color, and thickness also vary frequently.

Highly variable (polymorphous) thalli may reach 15 cm long, cartilaginous consistency and reddish-pink or -brown color and iridescent in water. These algae are fixed by a disk whose first unbranched stipe gradually expands into a fan-like blade, repeatedly dichotomously divided, with ends rounded or truncated. Small dilations (2–3 mm in diameter), which are the reproductive structures may appear on the surface of the blades [6.1].

Common Names. Irish moss, Carrageen moss, Jelly moss.

Habitat. On rocks, lower intertidal and shallow sublittoral, in pools in the mid-littoral in some locations; widely distributed in the northwestern and northeastern Atlantic, often abundant.

Distribution. *C. crispus* has a wide distribution. It includes the northwest Atlantic from Labrador and the Maritime Provinces in Canada southward to New Jersey and Delaware in the United States. It can be found to a limited extent in the western Baltic Sea, from northern Russia and Norway to southern Spain, spanning the northeast Atlantic, the North Sea, and the English Channel. It can be found in the Mediterranean, Portugal, the Azores, the Faroes, and West Africa, as well as in the Bering Sea from Russia to Alaska.

Uses and Compounds. *C. crispus* is harvested as a common source of the polysaccharide carrageenan, which is extracted from cell walls and forms a gel that is used commercially as a thickener [6.4, 514–516].

The gelling and thickening properties of carrageenan are used widely in the cosmetics, food, and

pharmaceutical industries. Examples of applications include making ice cream and air fresheners, beer clarification, and treatment for coughs and diarrhea [6.109, 530, 531].

Extracts have antifouling and antibacterial [6.61, 532], antioxidant, and antimicrobial [6.205, 373, 533].

Cystoclonium purpureum (Hudson) Batters

Description. Rather soft, cylindrical, dull-purplish pink fronds, 3 mm wide, to 600 mm long. Branches numerous, alternate, branchlets tapered at both ends; branches sometimes drawn out into long twisting tendrils; multiaxial, medulla a cordlike strand of loosely interwoven, narrow filaments, surrounded by large, rounded cells, with outer layer of small, angular, assimilatory cells; said to have an onion-like smell shortly after collection [6.534].

Habitat. On rocks and stones in mid-littoral and in shallow sublittoral, generally distributed, abundant.

Distribution. North Atlantic (Arctic Canada to Spain); North Sea (Helgoland); Baltic, Mediterranean (Greece), Indian Ocean (Pakistan), Australia (Queensland).

Uses and Compounds. Source of carrageenan [6.32, 521, 535]. Extracts have nematicide [6.536], antibacterial [6.86, 358, 537], and anticoagulant [6.375] activity.

Dilsea carnosa (Schmidel) Kuntze

Common Name. Red rags.

Description. One of the larger red seaweeds, *D. carnosa* is tough and leathery (Fig. 6.29). Several dark reddish brown, flat, blades gradually expand from a single disk-shaped holdfast, and have short cylindrical stipes. The blades do not have a midrib or veins and are simple wedge or spoon shaped. The blades can be 15–30 cm long and 5–20 cm wide. Older blades may be split from the margin toward the base but the rounded oblong (obovate) shape of the blade is never quite lost [6.1, 538].

Habitat. On rocks in shady pools, lower intertidal on rock, and shallow sublittoral to 25 m, usually on rock in kelp forests; widely distributed, common.

Distribution. NE Atlantic (Iceland and Spitsbergen to Portugal).

Fig. 6.29 *Dilsea carnosa* specimens

Uses and Compounds. Produces carrageenan [6.539, 540], and extracts antisettlement [6.257], antiprotozoal [6.525], antifungal [6.541], and antifouling [6.542] activity.

Dumontia contorta (S.G.Gmelin) Ruprecht

Description. Erect thallus, cylindrical when young, compressed when older, tubular-hollow, brownish red to crimson-purple, often yellow-brown at the tips. Thallus with irregular simple lateral branches, tapering at both ends, sometimes slightly swollen, often twisted around the longitudinal axis; soft, gelatinous. Holdfast a small, persistent disk, expanding with age [6.32].

Habitat. On rocks and pebbles, shallow open pools, upper intertidal to shallow sublittoral, common, widely distributed.

Distribution. NE Atlantic (Spitsbergen to Portugal, North Sea); NW Atlantic; NW Pacific (Russia, Japan); NE Pacific (Alaska).

Uses and Compounds. Extracts have antiviral [6.543], and antioxidant [6.429] activity.

Furcellaria lumbricalis (Hudson) J.V. Lamouroux

Common Names. Clawed fork weed, black carrageen.

Description. Cartilaginous, cylindrical, brownish-black fronds, repeatedly dichotomously branched,

fastigiate, to 2 mm diameter and 300 mm long, with acute apices; attached by much-branched rhizoids. Multiaxial, medulla of cylindrical cells interspersed with rhizoids, cortex of irregular filaments, inner cells elliptical, outer cells narrow, elongated, in radial rows [6.544].

Habitat. On rocks, lower intertidal and shallow sublittoral, in pools and runnels, in open situations, often on sandy and muddy shores, tolerating lowered salinities.

Distribution. North Atlantic (Nova Scotia, Greenaland); NE Atlantic (Faroe Islands, Ireland and Britain to Spain), North Sea (Scandinavia, Hegoland), Baltic; W Mediterranean (Spain, Sardinia, Sicily); Indian Ocean (India, Pakistan).

Uses and Compounds. Source of hybrid carrageenan (Furcellaran) [6.32, 545]. The sulfated polysaccharides of this species have immunostimulation activity [6.546].

Gigartina pistillata (S.G. Gmelin) Stackhouse

Description. *G. pistillata* (Fig. 6.30) is the type species of the genus *Gigartina* and their thalli are erect, up to 20 cm tall, dark red or red-brown, cartilaginous, elastic, dichotomously branched, attached to the substrate through a small disk [6.1].

Habitat. On firm substrata in the intertidal zone, to 12 m depth.

Distribution. NE Atlantic (Ireland and Britain to Senegal); SE Atlantic (South Africa).

Uses and Compounds. Source of carrageenan – (female gametophytes) produces a hybrid carrageenan, 48.7 kappa, 44.5 iota (% mol). *G. pistillata* (tetrasporophytes) produces a hybrid carrageenan xi-lambda [6.547]. The sulfated polysaccharides of this species have antitumor [6.548], antiviral [6.549], anti-inflammatory [6.550], and antioxidant [6.429, 551] activity.

Gymnogongrus crenulatus (Turner) J. Agardh

Description. Cartilaginous, fleshy, flattened, dark red fronds, to 100 mm high, with short cylindrical stipe from basal disk; repeatedly dichotomous, branches strap-shaped, apices rounded, often paler than rest of frond. Reproductive structures appear as external, wart-like excrescences [6.552].

Fig. 6.30 Underwater photo of *Gigartina pistillata*

Habitat. Epilithic, lower littoral in pools and emergent, and sublittoral to 13 m, tolerant of sand cover; plants typically encrusted with species of Bryozoa, Foraminifera, and calcareous algae.

Distribution. NE Atlantic (Ireland and Britain to Mauritania, Canary Islands, Cap Verde Islands); NW Atlantic (New Brunswick to Massachusetts); Australia.

Uses and Compounds. *G. crenulatus* produce a hybrid carrageenan (64.1 kappa, 30.8 iota %mol) [6.547]. The sulfated polysaccharides of this species have antibacterial activity [6.130].

Gymnogongrus griffithsiae (Turner) Martius

Description. Cartilaginous, cylindrical to compressed, brownish red to blackish-purple fronds, to 75 mm high, from an expanded discoid base; repeatedly dichotomous, fastigiate, with rounded, somewhat flattened apices [6.553].

Habitat. Epilithic, littoral in pools and emergent, upper sublittoral; tolerant of sand cover. On sand-covered rocks, lower intertidal, rare [6.518].

Distribution. NE and E Atlantic (Ireland to W Africa); Mediterranean; NW to SW Atlantic (North and South Carolina, Brazil, Uruguay); Caribbean; Australia.

Uses and Compounds. Produces sulfated galactans – carrageenans [6.554] with antioxidant [6.205], and antiviral [6.555–557] activity.

Hypnea cornuta (Kützing) J. Agardh

Description. Plants caespitose or loosely intricate-caespitose, up to 30 cm in length, soft in texture and reddish brown in color; axes subterete, up to 1 mm in diameter at the base, pointed at the apex, spirally branched up to the fourth order; branching angle less than 90°; axes and branches loosely clothed with stellate processes; branches up to 550 µm in diameter, and spinous ultimate branchlets, 60–70 µm in diameter, slightly constricted at their base; extensive basal system of prostrate axes attached by secondary discoid holdfasts produced by the cortical cells at any axis level [6.558].

Habitat. In mid-littoral rock pools exhibit the habit of free-floating plants, while in the open sea it throve attached on rocks within −50 cm.

Distribution. W Atlantic (USA to Brazil); Mediterranean; Caribbean (Cuba); Indian Ocean; NW Pacific (Japan); Indonesia; Australia.

Uses and Compounds. Produces carrageenan [6.559].

Hypnea musciformis (Wulfen) J.V. Lamouroux

Common Names. Hook weed, Hooked seaweed, Hypnea.

Description. *H. musciformis* is a red alga with loosely intertwined cylindrical branches that form clumps or masses up to 20 cm tall, though plants are often broken by wave action before they reach full size (Fig. 6.31). Branches are firm and highly irregularly branched, narrowing to broad, flattened, tendril-like hooks. These hooks make *H. musciformis* easily distinguished from all other *Hypnea* species found in similar Habitats. Holdfasts are small or lacking because the plants usually anchor themselves by attaching to other species such as *Sargassum* (for example, *Sargassum muticum*). The tissue is usually a dark reddish brown, though when growing in nutrient poor water color can pale to yellowish brown. It is found abundantly in shallow coastal waters where it prefers calm, shaded sublittoral reef flats, though it also grows in tide pools and rocky intertidal areas. In bloom stage, it may be found free floating. *H. musciformis* is also a highly opportunistic invader [6.560].

Fig. 6.31 Herbarium specimen of *Hypnea musciformis* (MACOI no. 3733)

Habitat. Common on calm intertidal and shallow sublittoral reef flats, tide pools and on rocky intertidal benches. Most often found low intertidal to shallow sublittoral reef flats, attached to sandy flat rocks.

Distribution. NE Atlantic (Spain and Portugal to Canary Islands), E and SE Atlantic (W Africa); NW Atlantic (Georgia, N. Carolina); SW Atlantic (Brazil, Uruguay); Caribbean; Mediterranean; Red Sea, Indian Ocean and Indo-Pacific; Pacific Islands (Hawaii; Fiji); Australia.

Uses and Compounds. In some areas, *H. musciformis* is grown for harvest of kappa carrageenan [6.561, 562]. It also contains a high amount of natural occurring antioxidants, and is nourishing to the skin. *H. musciformis* is used in over 100 hair color and hair care products such as shampoos, conditioners, and styling gels, it is used in more than 20 sunless tanning products, and in many antiaging creams and applications. *H. musciformis* is used in facial treatments, toners, and moisturizers, makeup and cosmetics, and eye treatments. *H. musciformis* is also commonly joined by *Gelidiella acerosa* and *Sargassum filipendula* as an effective seaweed ingredient set for personal care products [6.109, 560].

Extracts have antiviral [6.238], antifungal [6.563, 564], antitumoral [6.421, 565], antihelminthic [6.566], psychotropic and anxiolytic [6.567], antibacterial [6.38, 70, 242, 421, 568, 569], cytotoxic, antioxidant, larvicidal, and antifeed [6.242, 570, 571] activity.

Hypnea spinella (C. Agardh) Kützing

Description. Fragile, upright, or in tangled mats, to 15 cm high, light brown-red, rose-red or bleached; branching in all directions; branches 0.4–1.0 mm diameter, cylindrical. Branchlets spine-, spur-, or tendril-like, numerous, spirally arranged, to 2.5 mm long, occasionally longer; apices tapering, pointed, slightly up-curved. Cortex 1–2 cells thick; cells rounded to irregular, 7.5–25.0 µm diameter, densely pigmented; medullary cells thick-walled, irregular to ovoid, 100–320 µm diameter, surrounding obvious thick-walled central filament (70–80 µm diameter); holdfast initially disk-like. Tetrasporangia ovoid, 10–23 µm diameter, 25–49 µm long, zonately divided, in swollen sori (nemathecia) girdling middle part of lateral branchlet; cystocarps spherical, 100–900 µm diameter, solitary or clustered at base to middle of branchlet [6.572].

Habitat. Attached to small shells or rubble, often entangled in seagrass beds; lower intertidal to 7 m (reported to 27 m) deep.

Distribution. Warm E Atlantic (Atlantic Islands, W coast of Africa); Mediterranean; Caribbean; NW Pacific (Japan, China); Pacific Islands (Hawaii); Indo-Pacific (Philippines, Vietnam); E Pacific (Mexico, Ecuador); Australia.

Uses and Compounds. This is an edible species, being commonly eaten (boiled in coconut milk) in other parts of the Pacific and Asia [6.573]. Extracts have antifungal [6.204], and immunomodulatory [6.394] activity.

Mastocarpus stellatus (Stackhouse) Guiry

Common Name. False Irish moss.

Description. A small red alga (up to 17 cm in length), the fronds are channeled with a thickened edge and widen from a narrow stipe with disk-like holdfast (Fig. 6.32). The channeling is often slight and is most noticeable at the base of the frond. Mature plants have conspicuous growths of short, shout papillae (reproductive bodies) on the fronds. The plant is dark reddish brown to purple in color and may be bleached. The common name false Irish moss is used as it may be confused with *C. crispus* (Irish moss); the main features separating the two species being the channeled frond and appearance of reproductive bodies on mature plants [6.1].

Fig. 6.32 *Mastocarpus stellatus* specimen

Habitat. This alga is found on rocky shores, particularly in very exposed areas where it grows among barnacles and mussels, on less exposed shores it is often abundant under fucoids.

Distribution. NE Atlantic (Scandinavia to Mauritania, North Sea); NW Atlantic (Newfoundland and Nova Scotia to Rhode Island).

Uses and Compounds. Source of carrageenan [6.514–516]. Extracts have antioxidant activity [6.205, 373, 429, 574].

Phyllophora crispa (Hudson) P.S. Dixon

Description. Bright red or pink seaweed. Each plant has a small disk-shaped base and erect fronds. The fronds consist of short cylindrical stipes (stalks) rarely longer than 1 cm long and blades which may be up to 15 cm long and 10 mm wide. The fronds are dichotomously branched, with undulating margins and an indistinct midrib. The tip of each frond is distinctly rounded. The fronds are perennial and in some case five or six new periods of growth can be identified. Regeneration occurs following erosion or animal

grazing. Continual regeneration leads to great variation in the appearance of individual plants as each new growth could come from the end, margin or surface of the blade. Fronds are frequently encrusted with the spiral tube worm *Spirorbis spirorbis* or bryozoans [6.575].

Habitat. Frequent in shady pools in lower intertidal and on rock subtidally to 30 m.

Distribution. NE Atlantic (Scandinavia to Canary Islands, North Sea); W Mediterranean; Black Sea; Free-floating forms in the Baltic Sea.

Uses and Compounds. Raw material for the agar production on the Black Sea [6.32]. Extracts have antifungal activity [6.62].

Polyides rotundus (Hudson) Gaillon

Common Names. Discoid forked weed, Goat tang.

Description. Dark red or blackish seaweed with smooth, cartilaginous, cylindrical fronds, branching dichotomously in roughly the same plane. The fronds rise from a fleshy, discoid holdfast up to 2 cm in diameter. The reproductive bodies occur as oval-shaped swellings along the sides of the branches or occasionally encircling them. The seaweed grows up to 20 cm in length [6.576].

Habitat. On rocks and stones in open sandy pools and runnels, lower intertidal and shallow sublittoral.

Distribution. N and NE Atlantic (Arctic Ocean to Iberian Peninsula, Baltic); NW Atlantic (Arctic Canada to New York).

Uses and Compounds. Extracts have antifouling activity [6.532].

Solieria chordalis (C. Agardh) J. Agardh

Description. Thallus tufted, vividly red, axes cylindrical, thin, only sparsely branching at first, later characteristically with relatively long, unilaterally inserted appendices, height to 20 cm [6.577].

Habitat. In sheltered localities below the mid-littoral zone on rocks.

Distribution. NE Atlantic (from N France to S Morocco), Mediterranean.

Uses and Compounds. Source of carrageenan [6.521, 578–581]. Extracts have hemagglutinating [6.375], and Immunostimulation [6.581] activity.

Sphaerococcus coronopifolius Stackhouse

Common Name. Berry wart.

Description. Narrow, compressed, two-edged, cartilaginous, scarlet fronds, main axes dark brownish red, to 300 mm long; branching abundant, distichous, subdichotomous or alternate, terminal branchlets acute, fringed with short marginal proliferations. Tetrasporophyte is *Haematocelis fissurata*, a thick crust with oily fissures, with which it is sometimes found [6.582].

Habitat. Rarely on rocks in lower littoral, often common in the shallow sublittoral to 15 m.

Distribution. E Atlantic (Ireland and Britain to Canary Islands); Mediterranean, the Black Sea.

Uses and Compounds. Extracts have antifouling [6.583], cytotoxic and antimitotic [6.62, 332, 584, 585], antiviral [6.586], antibacterial and antifungal [6.62, 275], activity.

Stenogramma interruptum (C. Agardh) Montagne

Description. Cartilaginous, flattened, bright-red fronds, to 70 mm high, shortly stipitate from the discoid base. Lamina fan-shaped, ±dichotomously divided, segments about 10 mm wide, with rounded apices, margin usually smooth, rarely proliferous; reproductive structures often occurring in a clearly visible, often discontinuous, *midrib* over the surface of the fronds [6.587].

Habitat. Epilithic, sublittoral to 13 m, in sheltered areas on small stones among gravel and mud [6.518]. On rocks, stones, etc., sublittoral, southern and western coasts, occasional.

Distribution. NE Atlantic (Ireland and Britain to Morocco); West of Atlantic (Canada to Brazil); E and W Pacific (Japan, Mexico, Ecuador, Chile, Peru); Australia and New Zealand.

Uses and Compounds. Produces carrageenan – polysaccharides from tetrasporic plants (lambda-carrageenan) of *S. interruptum* showed higher anticoagulant activity than those isolated from cystocarpic

Fig. 6.33 Underwater photo of *Gracilaria gracilis*

(kappa/iota-carrageenan) plants [6.512, 588]. Extracts have also antiviral activity [6.35, 549].

- Order: **Gracilariales**

Gracilaria bursa-pastoris (S.G. Gmelin) P.C. Silva

Description. Growing in tufts to 30 cm long, much branched usually simply forked or alternate rarely opposite; somewhat compressed, but not flat. Thallus greenish red, yellow greenish to dark brownish red, with cylindrical axes, forking and with lateral branching, tufted, erect, branches gradually tapering off, not narrowing at their origins, the cystocarp-bearing plants are densely covered with hemispherical reproductive structures (cystocarps); texture cartilaginous-meaty, stiff, bristly; discoid holdfast [6.32].

Habitat. Epilithic, on calmer water of the upper sublittoral.

Distribution. NE Atlantic (Ireland to Cape Verde Islands, Kenya); Mediterranean, Indo-Pacific Oceans (India, Sri Lanka, Philippines, Singapore, Japan, China, Hawaiian Islands, Florida, Mexico); Caribbean (Cuba) and SW Atlantic (Brazil).

Uses and Compounds. Source of agar [6.589] and used directly as food and for animal feed [6.32, 290]. Extracts have antibacterial [6.171], anticoagulant [6.375] activity.

Gracilaria gracilis (Stackhouse) Steentoft, L.M. Irvine & Farnham

Common Name. False Ceylon moss.

Description. Cartilaginous, cylindrical, dull-purple fronds, to 500 mm long, one or several are arising from small, fleshy, perennial discoid holdfast (Fig. 6.33). Branching very irregular, sparse or profuse, branches to 2 mm diameter, apices pointed; intertidal tissue of large thin-walled cells with narrow outer cortical zone of small colorless cells [6.2, 590].

Habitat. On rocks and stones, intertidal and sublittoral, especially on sandy shores, generally distributed, common.

Distribution. Temperate-warm to warm NE and E Atlantic (Ireland to Canary Islands, W Africa); SE Atlantic (South Africa); Mediterranean; Caribbean; W Atlantic (Brazil); NW Pacific (Russia, Japan); NE Pacific (Alaska to California, Mexico); Pacific Islands; Indo-Pacific Ocean; Australia; Antarctica.

Uses and Compounds. Source of agar [6.32, 523, 591–593] and used directly for animal feed [6.594]. Extracts have antimicrobial [6.276, 569], and antioxidant [6.350, 595] activity.

Gracilaria multipartita (Clemente) Harvey

Description. *G. multipartita* is a translucent, dull purple or reddish-brown alga which has fronds that measure up to 25 cm long. It is cartilaginous, very brittle, and has a compressed stipe [6.1].

Habitat. Grows on rocks or other hard substrata, tolerating a wide degree of wave exposure. Recorded from the upper sublittoral to a depth of 15 m. *G. multipartita* is also tolerant of sand.

Distribution. NE Atlantic (Britain to Senegal); Atlantic Islands; Mediterranean (Italy, Spain).

Uses and Compounds. Source of agar [6.596]. Extracts have antibacterial [6.269, 358, 597, 598], and antifungal [6.284, 286] activity.

Gracilaria vermiculophylla (Ohmi) Papenfuss

Common Name. Komulkosiraegi.

Description. *G. vermiculophylla* is a red macroalga that is cartilaginous, cylindrical, and up to 50 cm long. It is coarsely branched, often profusely so. It can be found as loose-lying thalli or attached to small stones or shells. Red algae are often found in the vegetative state, and the characterization of reproductive structures is often necessary for correct identification of *Gracilaria* species [6.599–601].

Habitat. Estuarine habitats, marine habitats, mainly in salinity lagoons. It is well adapted to low energy, shallow-bottom bays, lagoons, estuaries, harbors, and inslets [6.602].

Distribution. Native range – NW Pacific ocean including Japan and E Asia [6.601]; Introduced range – N American E and W coasts, Europe, NE Atlantic coast extending from Morocco to SW Sweden, E Pacific [6.603, 604].

Uses and Compounds. *G. vermiculophylla* is widely collected for the production of Agar, which is used extensively in the pharmaceutical and food industries [6.592, 605–607]. Extracts have antibacterial [6.449], and antioxidant [6.335, 608] activity.

Gracilariopsis longissima (S.G. Gmelin) M. Steentoft, L.M. Irvine & W.F. Farnham

Description. Thalli are from almost simple to profuse and irregularly branched, with the slender cylindrical axis throughout the plant. Cystocarps are scattered throughout the thallus, protruding from the thallus surface. Spermatia are formed in near the surface of the thallus. Tetrasporangia are cruciate and are scattered in the cortex [6.609].

Habitat. Usually lives in the sublittoral environment, on loamy or sandy funds shells or gravel.

Distribution. NE Atlantic (Britain to Portugal); SE Atlantic (Namibia, South Africa).

Uses and Compounds. Source of agar [6.592, 605]. Extracts have antibacterial [6.569, 610, 611] activity.

- Order: **Halimeniales**

Grateloupia filicina (J.V. Lamouroux) C. Agardh

Description. Compressed, tufted, dark purplish brown fronds, to 120 mm high, main axis 1–4 mm broad. Once or twice pinnate, axes and branchlets tapered at base and apex. This is a soft limp and slippery smooth seaweed with somewhat flattened branches that can be red green brown or almost black. Size and shape vary greatly from 0.5–5 mm wide to 2–30 cm long, with either few or many branches [6.612].

Habitat. On rocks in pools; mid-littoral to shallow sublittoral, sporadic, often locally common.

Distribution. Worldwide in warm and temperate-warm seas.

Uses and Compounds. Used as food and source of Carrageenan [6.613–615]. Extracts have antioxidant [6.350, 595, 615–617], antimicrobial [6.34], antiviral [6.615, 618, 619], anticoagulation [6.615, 620, 621] activity.

Grateloupia turuturu Yamada

Description. Thallus flat, membranous, with short stipe, the single fronds linear to broad-lanceolate, undivided or irregularly dividing from the base, narrowing toward the base as well as the tip (Fig. 6.34); sometimes proliferating on the margins and the surface; consistency gelatinous-slippery but firm; discoid holdfast; violet- to crimson-red, often greenish at the top thallus [6.32].

Habitat. Epilithic in shallow tide pools and on sand-covered rocks near coast.

Distribution. SE Pacific (Chile), SW Atlantic (Peru), *G. turuturu* is considered native to Japan, China, and Korea, but has spread to the NE Atlantic, the Mediterranean, South America, Australia, and New Zealand.

Uses and Compounds. In Japan, this seaweed is commonly used as a sea vegetable [6.622]. *G. turuturu* is characterized by its richness in dietary fiber (nearly 60% DW) and therefore appears to be a good source of food fiber for human consumption. This is very interesting because the beneficial effect of fiber on health is already well known [6.623, 624]. This seaweed is also rich in proteins, like *Palmaria palmata*, another red alga

Fig. 6.34 Underwater photo of *Grateloupia turuturu*

now authorized in France as a sea vegetable. Its lipid content is low, like all red seaweeds used in human nutrition, and its eicosapentaenoic acid content is similar to those reported for edible red seaweeds such as *Chondrus cripus* or *Gracilaria verrucosa* [6.625].

Extracts have antifouling [6.586], antibacterial [6.626], anticoagulant [6.512], antioxidant [6.627], and antiviral [6.278] activity.

- Order: **Nemaliales**

Galaxaura rugosa (J. Ellis & Solander) J.V. Lamouroux

Synonym. *Galaxaura elongata* J. Agardh.

Description. Thallus bushy, stiff, compact, forming hemispherical mounds, 5–7 (–12) cm high, dark red-brown; branching irregularly dichotomous; calcification moderate. Branches cylindrical, 0.5–1.5 (–3) mm diameter, densely but evenly covered by stiff hair-like filaments; holdfast inconspicuous. Tetrasporangia at apex of surface filaments, occasionally lateral near tip, spherical to oval, to 32 µm diameter, cruciately divided [6.628].

Habitat. On firm substrata in calmer shallow water; found in up to 18 m depth.

Distribution. E Atlantic (Madeira, Canary Islands, Cap Verde to Gabon); W Atlantic (Brazil); Caribbean; Indian Ocean; NW Pacific (Japan, Philippines, Indonesia); Pacific Islands; Australia, New Zealand.

Uses and Compounds. Extracts have anti-inflammatory [6.629], antimicrobial [6.72, 391], antifungal, cytotoxic, and antiviral [6.72] activity.

Ganonema farinosum (J.V. Lamouroux) K.C. Fan & Yung C. Wang

Synonym. *Liagora farinosa* J.V. Lamouroux

Description. This alga is soft, smooth and about 13 cm tall. It is pinkish, lightly calcified, farinose and attached by small discoid holdfasts. The branching pattern is basically dichotomous, with interdichotomal lengths decreasing toward terminal portions of the thallus. The branches are numerous, cylindrical, measuring about 1.5 mm in diameter, with forking apices into very short, acute and terminal branchlets [6.630].

Habitat. *G. farinosum* is found attached to rocky substrate among other algae in the sublittoral and intertidal areas exposed to air during low tides.

Distribution. *G. farinosum* is widely distributed in the tropical waters of the Atlantic, Pacific, and Indian Oceans; Mediterranean; In SE Asia, it has been recorded from Thailand, Vietnam, Malaysia, Indonesia (Irian Jaya) and the Philippines.

Uses and Compounds. Extracts have antibacterial [6.449], antifungal [6.204], cytotoxic, and antiviral [6.72] activity.

Liagora ceranoides J.V. Lamouroux

Description. Thallus to 11 cm high, pale pink to gray, lightly calcified, mucilaginous, irregularly or dichotomously branched every 2–20 mm, with many proliferous lateral branches to 10 mm long; pressed specimens strongly adherent. Main axes to 2.5 mm in diameter basally, tapering to 0.2 mm near apices [6.631].

Habitat. From intertidal pools to 27 m depth, epilithic on *Lobophora variegata*; widespread in tropical seas.

Distribution. NE Atlantic (from France, Spain): Atlantic Islands; Mediterranean; NW Atlantic; Caribbean; SW and SE Atlantic; Indo-Pacific Oceans; Australia.

Uses and Compounds. Extracts have anticoagulant [6.206], and antioxidant [6.65] activity.

Liagora viscida (Forsskål) C. Agardh

Description. Thallus tufted, gray-purple to greenish white or pink, repeated dense branching at almost the same length, branches terete, tapering toward the top (Fig. 6.35); terminal branches usually spreading as wide-angled bifurcations, moderately calcified, texture flexible-firm [6.32].

Habitat. On firm substrata in shallow water to 5 m depth.

Distribution. NE and North Atlantic (France to Cap Verde Islands); Mediterranean; Indian Ocean (India, Sri Lanka); Pacific Islands (Fiji).

Uses and Compounds. Their endophytic fungi have antitumor and antimicrobial activity [6.632, 633].

Nemalion helminthoides (Velley) Batters

Description. Thallus cylindrical, reddish to purple brown, softly gelatinous, simple or sparingly dichotomously branched, to 2 mm wide, to 250 mm long, with discoid holdfast [6.634].

Habitat. On exposed rocky shores, generally growing on barnacles and limpets.

Distribution. NE Atlantic (Scandinavia to Canary Islands, W Africa); SW Atlantic (Brazil, Uruguay); Mediterranean; NW Pacific (Japan); NE Pacific (Alaska to Mexico); Australia, New Zealand.

Uses and Compounds. Used as food [6.634] and with dietary antioxidants [6.65, 205, 429]. *N. helminthoides* showed appreciable antiherpetic activity [6.635].

Scinaia furcellata (Turner) J. Agardh

Common Name. Southern scinà's weed.

Description. Cylindrical, gelatinous but firm, clear pink to purplish-red fronds, 10 mm wide, 250 mm long, dichotomously branched from small discoid base (Fig. 6.36); constrictions at irregular intervals in mature plants [6.32].

Fig. 6.35 *Liagora viscida* specimen

Fig. 6.36 *Scinaia furcellata* specimen

Habitat. On stones, shells, and mal in the sublittoral (30 m), mainly in clear, unpolluted water; not common.

Distribution. NE and E Atlantic (S Norway to Morocco, Canary Islands to Congo); NW Atlantic; Mediterranean; Indian Ocean.

Uses and Compounds. Extracts have cytotoxic [6.636], antibacterial [6.130], and anticoagulant [6.637] activity.

- Order: **Neamatomales**

Schizymenia dubyi (Chauvin ex Duby) J. Agardh

Description. Erect leaf-like flat fronds, brow-red (liver-colored), translucent, gradually widening from the base, simple or irregularly split or lobed, then at a narrow angle, overlapping; often noticeably sickle-shaped, with smooth margin, not proliferating texture soft-slippery; cystocarps very small and sunken; very short stunted stipe, small discoid holdfast [6.32].

Habitat. Pools and on rock mid lower intertidal zone.

Distribution. NE Atlantic (Iceland to Morocco); Mediterranean; NW Pacific (Japan, China, Korea); Australia.

Uses and Compounds. Extracts have antioxidant [6.350], antitumor [6.638], antiviral, and anticoagulant [6.205, 546, 639, 640] activity.

- Order: **Palmariales**

Palmaria palmata (Linnaeus) Weber & Mohr

Common Name. Dulse, Grannogh, Dillisk, Handed focus.

Description. Reddish brown, membranous or leathery, flattened fronds, 50–300 (–1000) mm long, arising from a discoid base, usually with a small stipe expanding gradually to form simple or dichotomously and palmately divided fronds, often with characteristic marginal leaflets (Fig. 6.37); blade very variable in shape, having broadly ovate to narrowly linear segments [6.641].

Habitat. On rocks, mussels, and epiphytic on several algae, intertidal (at all levels but particularly near low water) and shallow sublittoral, especially on the upper part of *L. hyperborea* stip.

Distribution. *P. palmata* grows along the northern coasts of the Atlantic and Pacific oceans, as far north as Arctic Canada and Russia, and as far south as Portugal in Europe, and New Jersey and California in the United States. In the western Pacific, the southern range of *P. palmata* includes Japan and Korea.

Uses and Compounds. Dulse is good to eat, but only after being dried. In a fresh state it is leathery and unpalatable. After sun drying and proper storage, it is a very pleasant plant to chew. It has very little fat and only a small amount of proteins and cellulose, but is very rich in trace elements and vitamins, particularly vitamin A. Dulse contains large amount of several unusual carbohydrates including an unusual short-chained one, floridoside, and this can form up to 30% of the dry weight. This may account for its palatability [6.642].

Fig. 6.37 *Palmaria palmata* specimen

P. palmata is a good source of dietary requirements; it is rich in potassium, iron, iodine and trace elements, and relatively low in sodium. A small amount can provide more than 100% of the daily amount of Vitamin B_6, 66% of Vitamin B_{12}, iron, and fluoride. It has a slight nutty flavor, and has a long tradition in the cuisines of many Northern European cultures. *P. palmata* is also a traditional food in maritime North America, used in condiments, soups, and appetizers. Younger parts of the fronds are considered the most tender and edible. It can be eaten raw. *P. palmata* is also used as fodder for a variety of animals in many countries, as pet supplements, and sometimes as an agricultural fertilizer [6.4, 642, 643].

Extracts of this species have antioxidant [6.533, 644–647], and antitumor [6.432, 644, 645] activity.

Rhodothamniella floridula (Dillwyn) Feldmann

Description. *R. floridula* is perennial brownish-red seaweed found on the lower shore. It usually covers large areas of rock in sandy habitats. At the base of the seaweed, filaments bind with sand to form a spongy, carpet like mass. The filaments are well-spaced and branch out up to 3 cm in length. Upright filaments of the seaweed uncovered by the ebbing tide appear as tufts of hair; when plants dry out they have a purplish tinge [6.648].

Habitat. Turf-forming on sand-covered rocks, mid- and lower intertidal, often under *Fucus serratus*; widely distributed, very common.

Distribution. NE Atlantic (Ireland and Britain to Portugal); SE Atlantic (Namibia, South Africa); SW Atlantic (Argentina).

Uses and Compounds. Used for the production of pharmaceuticals and functional foods [6.205]. Extracts have antioxidant activity [6.205].

- Order: **Plocamiales**

Plocamium cartilagineum (Linnaeus) P.S. Dixon

Common Names. Cock's comb, Kammtang, Kamwier, Red comb weed.

Description. Bright scarlet seaweed up to 30 cm in length with branching fronds (Fig. 6.38). The branching occurs alternately along the fronds and becomes more frequent toward the tips. The general appearance of these seaweeds can greatly vary from very compact and closely branched to a much broader appearance with widely separated branching. The tips are incurving and ultimate branching occurs only to one side, giving a distinctive feathery or comb-like appearance [6.649].

Habitat. Temperate seas throughout the world; found on coasts of strong to moderate wave action and known from depths of 2–26 m. Growing on *L. hyperborea* stipes and on other algae.

Distribution. NE Atlantic (Scandinavia to Senegal, North Sea), SE Atlantic (Namibia); Mediterranean; Indian Ocean (Pakistan, Mauritius); NW Pacific (Japan); Pacific Islands; NE Pacific (Alaska to California); SE-Pacific (Chile); Australia, New Zealand; Antarctica.

Fig. 6.38 Underwater photo of *Plocamium cartilagineum*

Uses and Compounds. Extracts with a red color are said to have been used for cosmetic purposes in ancient Rome [6.32].

P. cartilagineum is harvested commercially as the main raw material for the agar production on the Pacific coast of North America. Agar is widely used not only in laboratories as a growth medium for bacteria and other cultures, but in food and cosmetics as a gelling agent and stabilizer. It is a very pure, natural, firm gel. *P. cartilagineum* extracts are also high in lypolitic (lipid-digesting) sterols and as such is useful as an additive in slimming applications such as creams and massage products where it is able to provoke the release of fatty acids and eliminate surface fat, acting as a skin-firmer [6.650].

Extracts have antimicrobial [6.651], insecticidal [6.652], cytotoxic and antitumor [6.653], antioxidant [6.125], and antiviral [6.357, 549] activity.

- Order: **Rhodymeniales**

Botryocladia botryoides (Wulfen) Feldmann

Description. Plants rose-red to honey pink, erect, up to 11 cm in length, with irregular pseudodichotomous branching. Main axis and branches terete, 9–2.5 mm in diameter, of solid construction, composed of small pigmented, round to oval, cortical cells (5 µm diameter) that gradually increase in size thallus inward; medullary cells larger, hyaline, and ovoid, up to 50 µm. Axis and branches bearing pyriform vesicles [6.654, 655].

Habitat. Epilithic in depths up to 7 m.

Distribution. NE Atlantic (Atlantic Islands), Western Atlantic [6.654].

Uses and Compounds. Extracts have antifungal activity [6.62].

Champia parvula (C. Agardh) Harvey

Description. Soft, gelatinous, pinkish red, much-branched fronds, densely matted, with blunt apices, to 100 mm high. Axes segmented, with nodal diaphragms, segments about as broad as long, filled with watery mucilage [6.656].

Habitat. Epiphytic on smaller algae in lower intertidal pools and sublittoral, South West England and Ireland, Channel Islands, locally common.

Distribution. Worldwide in tropical to subtropical and warmer seas with bordering warm-temperate zones.

Uses and Compounds. Extracts have anticoagulant [6.637], and antiherpetic [6.657] activity.

Chylocladia verticillata (Lightfoot) Bliding

Description. Soft, gelatinous, cylindrical, pinkish to brown-red fronds (often bleached yellow or almost white in sunny Habitats), to 300 mm high, filled with a watery mucilage. Main axis simple, jointed, with nodal diaphragms, very slightly constricted at nodes, 2-several branches per node, themselves often branched, with small beaded ultimate ramuli. Very variable in branching pattern, plants of exposed coasts often simple with small terminal tufts of ramuli [6.658].

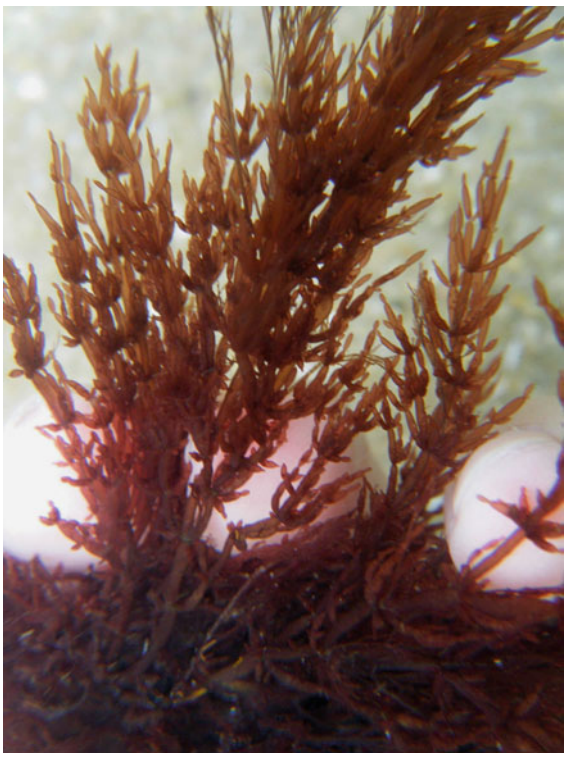

Fig. 6.39 Underwater photo of *Lomentaria articulata*

Habitat. On rocks and stones and epiphytic, lower intertidal and sublittoral, generally distributed, common.

Distribution. NE Atlantic (Norway to Sierra Leone); Mediterranean.

Uses and Compounds. Extracts have cytotoxic activity [6.659].

Lomentaria articulata (Hudson) Lyngbye

Description. A small (up to 20 cm tall, but usually much shorter) dark brown to red alga (Fig. 6.39). The thallus is conspicuously constricted giving a segmented appearance. Starting almost at the base, many branches grow out of each constriction, the bead-like segments becoming gradually smaller toward the tip of the branch. The plant is very shiny and the tips of branches may be bleached pink or orange [6.1].

Habitat. On rocks and stones in pools, lower intertidal and sublittoral, generally distributed, frequent.

Distribution. NE and E Atlantic (Scandinavia to Cameroon), Mediterranean, Black Sea, Indian Ocean (India), W Pacific (Philippines).

Uses and Compounds. Extracts have antioxidant [6.316], and antimicrobial [6.660] activity.

Rhodymenia pseudoplamata (J.V. Lamouroux) P.C. Silva

Description. Flattened, fan shaped, rather stiff, rose-red fronds, to 100 mm high, with long or short stipes arising from a discoidal base. Fronds repeatedly dichotomously lobed, axils wide, apices rounded, margin smooth [6.32].

Habitat. On rocks, shady pools lower intertidal and sublittoral, also epiphytic on *L. hyperborea* stipes.

Distribution. NE Atlantic (Scandinavia to Cap Verde Islands), E Atlantic (W Africa), SE Atlantic

(Namibia, South Africa); NW Atlantic (N and S Carolina), Caribbean, SW Atlantic (Brazil, Uruguay); Mediterranean.

Uses and Compounds. Extracts have antibacterial activity [6.358].

6.3.4 Domain/Empire Eukaryota, Kingdom Chromista, Phylum Ochrophyta; Class Phaeophyceae (Brown Algae)

- Order: **Cutleriales**

Cutleria multifida (Turner) Greville

Description. Only the morphologically more conspicuous *Cutleria*-phase is described: thallus erect, light to yellow-brown, also olive-brown, flattened, membranous, with smooth margins, deeply incised and repeatedly irregular forking, with linear to wedge-shaped sections, gradually narrowing toward the tip and dissected apices, fringed by a tuft of hair-like filaments; without stipe, rhizoidal discoid holdfast [6.32].

Habitat. On rocks, usually isolated, sublittoral to 10 m.

Distribution. NE Atlantic (Scandinavia to Canary Islands); Mediterranean; Indian Ocean; NW Pacific (Japan); SE Pacific (Chile); Pacific Islands; Australia.

Uses and Compounds. Extracts have anticoagulant activity [6.661].

Zanardinia typus (Nardo) P.C. Silva

Common Name. Penny weed.

Description. *Z. typus* is a fairly small brown seaweed with flat fronds that may be either round or fan-like and possibly irregular in shape. The appearance may change with age, varying in color from olive to dark brown or black and in texture from smooth to leathery. The edge of the fronds may be smooth or ruffled, and has a distinct fringe of hairs [6.662].

Habitat. Found mainly on silty boulders or bedrock, rarely on living substrata, on sublittoral from shallow water to about 20 m depth.

Distribution. NE Atlantic (SW Ireland and SW Britain, Canary Islands); Mediterranean (Italy).

Uses and Compounds. Extracts have antifouling [6.663], antioxidant [6.664], antibacterial and antifungal [6.62, 311, 665], antiviral cytotoxic, and antimitotic [6.62, 507].

- Order: **Desmarestiales**

Desmarestia aculeata (Linnaeus) J.V. Lamouroux

Description. This species of brown seaweed looks like a flattened cactus; a flat main axis approximately 2 cm wide with obvious midrib arises from a discoid holdfast. Numerous thinner opposite growing flat side branches are attached to the main axis. *D. ligulata* grows low to sublittoral and reaches 80 cm in length. Other species of flattened *Desmarestia* have been described for our area and there is ongoing discussion about the relationships of these species and their exact number. *D. munda* is described as growing to over 120 cm with side branches as wide as the main axis (4–10 cm), but strictly sublittoral. Beach Watchers have observed individuals that match both descriptions and a combination of the two [6.1].

Habitat. On rocks in pools in the lower intertidal; however, mostly sublittoral, to 15 m depth often abundant.

Distribution. NE Atlantic (Iceland to Morocco); NW Pacific (Japan); NE Pacific (Alaska to California); SE Pacific (Chile); Pacific Islands (Hawaii); Australia, New Zealand.

Uses and Compounds. Source of Laminaran and Alginic acid [6.666].

Desmarestia ligulata (Stackhouse) J.V. Lamouroux

Description. This species of brown seaweed looks like a flattened cactus (Fig. 6.40); a flat main axis approximately 2 cm wide with obvious midrib arises from a discoid holdfast. Numerous thinner opposite growing flat side branches are attached to the main axis. *D. ligulata* grows low to sublittoral and reaches 80 cm in length. Other species of flattened *Desmarestia* have been described for our area and there is ongoing discussion about the relationships of these species and their exact number. *D. munda* is described as growing to over 120 cm with side branches as wide as the main axis

Fig. 6.40 Underwater photo of *Desmarestia ligulata*

(4–10 cm), but strictly sublittoral. Beach Watchers have observed individuals that match both descriptions and a combination of the two [6.1].

Habitat. On rocks in pools in the lower intertidal; however, mostly sublittoral, to 15 m depth often abundant.

Distribution. NE Atlantic (Iceland to Morocco); NW Pacific (Japan); NE Pacific (Alaska to California); SE Pacific (Chile); Pacific Islands (Hawaii); Australia, New Zealand.

Uses and Compounds. The brown algae *D. ligulata* and *D. viridis* accumulate sulfuric acid until their average internal pH is 0.5 – 0.8. A related species, *D. aculeata*, does not accumulate acid [6.667]. Extracts have antimicrobial [6.668], antioxidant, and antitumor [6.316, 421] activity.

- Order: **Dictyotales**

Canistrocarpus cervicornis (Kützing) De Paula & De Clerck

Synonym. *Dictyota cervicornis* Kützing

Description. Thallus tufted, yellow-brown, erect, somewhat intertwined; branches screw-like twisted, narrow ribbons, mostly forking, but asymmetrically branching (one fork longer than the other), widening at the fork-base (to 4 mm), the upper thallus parts narrow (1–2 mm wide), slightly tapering upward, tips acute, margins smooth; anchored to right substrata by a discoidal holdfast [6.669].

Habitat. Grows on rocks and epiphytically in shallow water of warmer seas, but also in up to 20 m depth.

Distribution. Warm E Atlantic; Caribbean; SW Atlantic (Brazil); NW Pacific (Japan, China); Indian Ocean.

Uses and Compounds. Extracts have antisnake [6.670], antifouling [6.671], antifungal [6.204], antiproliferative [6.672], antiviral [6.673], antioxidant and anticoagulant [6.64, 674] activity.

Dictiopteris polypodioides (A.P. De Candolle) J.V. Lamouroux

Synonym. *Dictyopteris membranacea* (Stackhouse) Batters

Description. Thallus flat and leaf-like, to 300 mm long and 20–30 mm broad; fronds olive to yellow-brown, translucent, and ± regularly dichotomously forked with a prominent midrib extending to the apices (Fig. 6.41). Margins sometimes split to the midrib. Initially with an unpleasant smell shortly after collection, and degenerating quickly [6.1].

Habitat. In low-light calm water, to 40 m depth.

Distribution. Cold temperate to warm NE Atlantic (Ireland to Canary Islands); Mediterranean; W Pacific (Philippines); NW Pacific (Japan); Caribbean; Indian Ocean.

Uses and Compounds. Extracts have anti-inflammatory [6.675], antibacterial [6.276, 675], antitumor [6.676], antioxidant [6.675], and cytotoxic [6.62] activity.

Fig. 6.41 Herbarium specimen of *Dictiopteris polypodioides* (MACOI no. 260)

Dictyopteris delicatula J.V. Lamouroux

Description. *D. delicatula* has erect, light-brown, strap-shaped blades attaching to substratum at basal holdfast or to adjacent branches, creating a tangled mass 2–8 cm in height. Dichotomous to irregularly branched bilayered blades, 0.5–5.0 mm wide, have a distinctly raised midrib that may be several cells thick. Cells of blades are arranged in parallel rows at acute angles to midrib. Scattered clusters of hyaline hairs in dense tufts arise along midrib on only one surface of midrib [6.677].

Habitat. Attached to rock-sand coral fragments, also as an epiphyte on larger seaweeds; from the lower sublittoral to ≈ 10 – 30 m depth.

Distribution. Tropical and Sub-tropical Atlantic (Canary Islands), Gulf of Mexico, Caribbean, Indian Ocean, Red Sea, Central Pacific, Costa Rica; Pacific.

Uses and Compounds. Extracts have antifungal [6.204], cytotoxic [6.459], antibacterial [6.449], antiproliferative and antitumor [6.672], antioxidant and anticoagulant [6.459, 678, 679] activity.

Dictyopteris plagiogramma (Montagne) Vickers

Common Name. Limu lipoa

Description. Thalli dark olivaceous green to light tan or almost yellowish, occurring as dense often intertwined tufts of multiple axes arising from a common matted holdfast; fronds 2–8 cm long, 1–8 mm wide, usually crisp in texture and pungently aromatic when fresh; branching of juvenile thalli basically dichotomous, becoming pseudomonopodial at maturity and then highly irregular as wing tissue wears away to leave midribs as several orders of stalks. Blades from apex to base traversed from midrib to margin by regularly spaced microscopic to faintly visible veins that arise at angles of 10–20° and form broad arches bending away from the apices [6.680].

Habitat. Uncommon, on hard substrates, often attached to coral fragments or scattered rocks on deep sand plains; 9–55 m deep.

Distribution. Widespread in the subtropical to tropical waters of the Atlantic, Caribbean, Mediterranean, Indian Oceans, and in the Pacific Ocean.

Uses and Compounds. Produces Laminaran and Alginic acid and is used as food [6.681]. Extracts have antioxidant activity [6.682].

Dictyota ciliolata Sonder ex Kützing

Description. Thallus erect, light brown with yellow green iridescence, sometimes with dark brown striations; branching irregular to dichotomous; branches strap-shaped, margin with teeth, often spirally twisted; medullary cells one layer thick, rectangular [6.683].

Habitat. Attached to rock in seagrass bed, and in intertidal pools in moderately high energy Habitats.

Distribution. Warm E Atlantic (Madeira, Canary Islands, W Africa); Caribbean; SW Atlantic (Brazil); SW Pacific; Indian Ocean; Australia.

Uses and Compounds. Extracts have antifungal [6.204], and antioxidant [6.65] activity.

Dictyota dichotoma (Hudson) J.V. Lamouroux

Description. Thallus flat, homogenous yellow-brown to darker brown, with fairly regular dichotomous branches (Fig. 6.42) with parallel sides to 30 cm long, the tips usually bifid; branches 3 to 12 mm wide, membranous, without a mid-rib [6.1].

Fig. 6.42 Underwater photo of *Dictyota dichotoma*

Habitat. In shallow water on firm substrata, found in >50 m depth.

Distribution. Common and globally distributed.

Uses and Compounds. A chlorine-containing perhydroazulene diterpene, dictyol J 146, was isolated from the brown alga *D. dichotoma* along with two known diterpenes, dictyolactone and sanadaol [6.684, 685]. All three metabolites were algicidal [6.686] to the bloom-forming species *Heterosigma akashiwo* and *Karenia mikimotoi*. Dictyolactone also displayed a moderate activity against the dinoflagellate *Alexandrium catenella*.

Extracts of this seaweed have also anticoagulant [6.512, 637, 687], cytotoxic [6.62, 688, 689], antitumor [6.126, 690], anti-inflammatory [6.691], antifungal [6.62], larvicidal [6.51], antimicrobial [6.18, 40, 692], and antifouling [6.693, 694] activity. Extracts have also used for a liquid fertilizer [6.695].

Dictyota fascida (Roth) J.V. Lamouroux

Synonym. *Dilophus fasciola* (Roth) M.A. Howe

Description. *D. fasciola* has a ribbon-like opaque thallus with a height of 100–150 mm, and width of 1–6 mm. The thalli are flattened, and highly branched in a dichotomous pattern; it is three cells thick and without midrib. The branches are attenuated with forked tips; it has a yellowish-brown coloration [6.696].

Habitat. On firm substrata in the intertidal.

Distribution. Warmer E Atlantic (Madeira, Canary Islands); E Africa); Mediterranean; Indian Ocean.

Uses and Compounds. Extracts have antimicrobial [6.130], antioxidant [6.664], and antifouling [6.697] activity.

Dictyota implexa (Desfontaines) J.V. Lamouroux

Synonyms: *Dictyota dichotoma* var. *implexa* (Desfontaines) S.F. Gray; *Dictyota linearis* (C. Agardh) Greville

Description. Thallus bushy, brown, consisting of intertwined, erect fascicles; thallus regularly forking, width at the base 2–3 mm, abruptly narrowing toward the tipe, then filamentous (< 0.5 mm) [6.32].

Habitat. From 0 to 10 m depth.

Distribution. Atlantic Islands (Azores and Canary Islands); Mediterranean.

Uses and Compounds. Extracts have antibacterial [6.40, 276, 698], antifungal [6.276], and antioxidant [6.112] activity.

Dictyota mertensii (Martius) Kützing

Description. Thallus bushy, brown, often iridescent blue-green under water, erect, robust and stately; the flattened fronds show a branching pattern atypical for the genus: distinct main axes branch repeated-alterning, terminal forkings of the latitudinal axes turn into spur-like, 1–2 mm long, pointed or rounded tips [6.32].

Habitat. On rocks in shallow (about 2 m deep) water, in moderately wave-exposed sites in the intertidal, also in up to approx. 15 m depth.

Distribution. Warm E Atlantic (Canary Islands to Gabon); Caribbean; SW Pacific and Pacific Islands.

Uses and Compounds. Extracts have antibacterial [6.699] and feeding-deterrent [6.700, 701] activity.

Dictyota spiralis Montagne

Synonym. *Dilophus spiralis* (Montagne) G. Hamel

Description. Thallus bushy, erect; lamina segments ribbon-like, regular forking, spiralling around the longitudinal axis, tips straight, pointed. 10–20 cm long, brown, tips a lighter shade [6.702].

Habitat. Near the surface on rocks, also as an epiphyte.

Distribution. NE Atlantic (W Ireland and SW Britain to Mauretania); Mediterranean.

Uses and Compounds. Extracts have antimicrobial activity [6.18, 130, 703].

Lobophora variegata (J.V. Lamouroux) Womersley ex E.C. Oliveira

Description. These algae occur in orange brown to dark brown in colour and bear a leathery feel. The blades may appear as fan shaped, flabellate or as crusts. They are often tightly adhered to the substrate [6.704].

Habitat. Grow in most reef environments, encrusting great areas of shaded, rocky substrates. Especially abundant on undercut wall faces along deep drop-offs.

Distribution. Warm E Atlantic (Madeira, Canary Islands, W Africa); Caribbean (Trinidad); Hawaii; Indian Ocean, Australia.

Uses and Compounds. Lobophorolide was isolated from the common brown alga *L. variegata* and displayed a potent and highly specific activity against the marine filamentous fungi *Dendroyphiella salina* and *Lindra thalassiae* and a potent activity against *C. albicans* and antineoplastic [6.705]. Extracts have also antiprotozoal [6.706, 707], antimicrobial [6.65, 260, 707], anti-inflammatory [6.708], hypoglycemic [6.81], antifungal [6.61, 62], antifouling [6.260], anti-inflammatory, antioxidant, and anticoagulant [6.65, 709, 710] activity.

Padina gymnospora (Kützing) Sonder

Description. Usually the blades are 10–12 cm long and broad, but plants with 15 cm long and to 20 cm broad blades may be found; the frond has three layers of cells except near the enrolled edge where two cell layers occur; the hair lines alternate on both sides of the blade and the dark lines of sporangia are found just above every second hair-line; plants tufted, 5–10 cm tall, the blades 5–20 cm broad rounded or split into narrower portions, the lower parts stalk like and stupose, usually rather moderately calcified on the upper surface [6.711, 712].

Habitat. On rocks and other firm substrata (mangrove-roots), in calm or only moderately wave-exposed locations in the intertidal, found in up to 14 m depth.

Distribution. Warm E and SE Atlantic (Canary Islands to Angola); SW Atlantic (Brazil); Caribbean; W Pacific and Indo-Pacific (Philippines, Vietnam); Pacific Islands; Indian Ocean; Australia.

Uses and Compounds. Extracts have anti-inflammatory [6.713], antibacterial [6.114, 493, 714, 715], antifungal [6.715], anticoagulant [6.716], antioxidant [6.717], neuroprotective [6.718], antiviral [6.172, 719] activity.

Padina pavonica (Linnaeus) Thivy

Common Name. Peacock's tail

Description. The fronds are thin and leafy, flattish and entire when young, but often concave, or almost funnel shaped in mature specimens, with a lactiniate or irregularly lobed margin (Fig. 6.43). The inner (or upper) surface is covered in a thin coating of slime, and the outer (or lower) surface is banded with zones of light brown, dark brown, and olive green. Small, fine hairs form concentric lines, 3–5 mm apart, from the outer margin continuing down the outer (colored) surface of the fronds [6.32].

Habitat. On rocks in sheltered locations near the surface, to 20 m depth.

Distribution. NE Atlantic (S Britain to Canary Islands, W Africa); Mediterranean; Caribbean; W Pacific (Philippines), Pacific Islands; Indian Ocean.

Uses and Compounds. Cosmetic uses for skin antiaging [6.720]. High anticoagulant activity has been reported for purified fucan sulphates from *P. pavonica* (xylofucomannoglucuronan) [6.721]. Extracts have also allelopathic [6.722], antifungal [6.204, 286, 438, 722], antibacterial [6.72, 284, 421, 723, 724], anticoagulant [6.512], antitumor [6.126, 421], antiviral [6.72], cytotoxic, and antimitotic [6.62, 72, 725] activity.

Taonia atomaria (Woodward) J. Agardh

Description. Thallus erect, flat ribbon-shaped, paper-like thin, irregularly branching into wedge-shaped segments and/or deeply dividing into narrower, almost linear bands, the upper margin appearing frayed; without midrib; dark, slightly undulating zones of transverse stripes on both sides (hairs and reproductive structures). Stipe-like at the bottom anchored by a dense rhizoidal felt [6.32].

Fig. 6.43 *Padina pavonica* specimens

Habitat. In pools on rocky and sandy substrata in semiwave-exposed situations up to 10 m depth.

Distribution. NE Atlantic (Britain to Mauretania), Mediterranean.

Uses and Compounds. Used as functional foods ingredient [6.205, 726, 727].

T. atomaria was a source of meroditerpenes atomarianones A 123 and B 124, the cytotoxic agents against the NSCLC-N6 and A-549 cell lines [6.728]. The brown alga *T. atomaria* was also a source of meroditerpenes atomarianones, the cytotoxic agents against some cell lines [6.728].

Sargaquinone were isolated from the brown alga *T. atomaria* and were anti-inflammatory agents by inhibition of leukotriene biosynthesis [6.729].

Extracts have also antioxidant [6.102, 205, 685], antitumor [6.730], and antimicrobial [6.130] activity.

Zonaria tournefortii (J.V. Lamouroux) Montagne

Description. Thallus erect, with flat lobed sections divided into. Wedge-shaped segments, often incised and proliferating; lamina inconspicuously banded by distant, concentric rows of hairs which are parallel to the distal margin; additionally, delicate lines which run radially from the base to the upper margin; margins not enrolled, lamina not calcified; basally thickened, stipe-like, branching, stipe extending like a midrib into the segments; cushion-like rhizoidal network [6.32].

Habitat. In shady locations, from the surface to 40 m depth.

Distribution. Warm NE Atlantic (Madeira, Canary Islands, W Africa); Mediterranean; Caribbean; SW Atlantic (Brazil); SW Indian Ocean (South Africa).

Uses and Compounds. Extracts have antibacterial activity [6.391].

- Order: **Ectocarpales**

Asperococcus bullosus J.V. Lamouroux

Description: Thallus simple, membranous, hollow and irregularly bladder-like bloated; with short stipe which abruptly widens into the hollow inflated thallus body; reproductive structures on the surface are visible as dark dots; discoid holdfast [6.32].

Habitat. Lower intertidal and shallow sublittoral; mainly epiphytic.

Distribution. Widely distributed in temperate seas.

Uses and Compounds. Extracts have antioxidant and antitumor activity [6.205, 731].

Chordaria flagelliformis (O.F. Müller) C. Agardh

Common Name. Black whip weed.

Description. Thallus string-like, with divaricated branches, slippery, solid, dark brown to black, 10–30 cm high; branching irregular, alternate from all sides; branches cylindrical, or slightly compressed, to 3 mm wide, slightly tapering toward the base. Branch apices blunt. Medulla composed of longitudinal filaments of long cylindrical cells to 50–600 µm decreasing toward periphery to 16–18 µm. A layer of assimilative unbranched filaments (of 4–9 cell rows) develop from the peripheral cells [6.732].

Habitat. Growing on stony bottom, in low intertidal to sublittoral, in calm shores and exposed to wave action.

Distribution. NE Atlantic (from Greenland, Scandinavia to Britain, North Sea and Baltic)); NW Atlantic; SE Atlantic; NE Pacific; Antarctica and Sub-Antarctic Islands.

Uses and Compounds. Used as food [6.546, 732], and extracts have antitumor [6.733], anticoagulant and

antithrombotic [6.732, 734], and antioxidant [6.546] activity.

Colpomenia peregrina Sauvageau

Common Name. Oyster thief.

Description. Sometimes regularly spherical (*C. peregrina*) or more or less irregular outline (*C. sinuosa*), yellowish-brown color, fixed to the substrate by filamentous rhizoids. Internally, the thallus is characterized by an outer cortex composed of small colored cells and inner pith composed of large pigmented cells [6.1].

Habitat. On moderately wave-exposed rocks and (mostly) on other algae in the lower intertidal.

Distribution. NE Atlantic (Norway to Canary Islands); W Mediterranean; NW Pacific (Japan); NE Pacific (Alaska to California); Australia, New Zealand.

Uses and Compounds. Extracts have cytotoxic and antibacterial activity [6.735].

Colpomenia sinuosa (Mertens ex Roth) Derbès & Solier

Description. Thallus bladder-like, smooth, slick, hollow, crisp, spherical to sac-like, irregularly expanded or somewhat lobed, to 30 cm diameter, 10 cm high, golden-brown (Fig. 6.44); often covered with fine colorless hairs; reproductive sori as dark raised patches on surface. Membrane 300–500 µm thick, of 4–6 cell layers; medullary cells to 240 µm diameter; cortex 1–2 cells thick; surface cells 3.7–8.0 (–16) µm diameter, darkly pigmented; surface-phaeophycean-hairs transparent, in scattered clusters; holdfast not apparent, attachment at many points [6.1].

Habitat. Firmly attached to hard surfaces or epiphytic on other organisms; lower intertidal to 15 m deep.

Distribution. NE Atlantic (Portugal to Canary Islands); Mediterranean; E and SE Atlantic (W to South Africa); Caribbean; SW Atlantic (Brazil, Uruguay); Indian Ocean; NW Pacific (Japan, China) NE Pacific (California); SE Pacific (Galapagos, Chile); W Pacific and Indo-Pacific (Vietnam, Malaysia, Philippines); Pacific Islands (Hawaii, Polynesia); Australia, New Zealand.

Uses and Compounds. Edible, used as food [6.32], fertilizer, and source of alginic acid [6.109]. Extracts have antibacterial [6.86, 228, 692], antifungal [6.204], antioxidant [6.736, 737], antitumor [6.126], antileukemic, antiprotozoan, and hypolipidemic [6.698] activity.

Fig. 6.44 Underwater photo of *Colpomenia sinuosa*

Ectocarpus siliculosus (Dillwyn) Lyngbye

Description. Plants tufted, often only one to a few cm tall, but in exceptional cases up to 20 cm. Axes freely branched, main axis not distinguishable; filaments up to 30 µm in diameter, tapering toward the apices. Sometimes forming terminal pseudohairs, forms soft beards on larger plants or other firm substrata, and grows up to 2 feet long [6.738].

Habitat. On various firm substrata near the low-tide mark or below, later often free floating; also epiphytic.

Distribution. Almost globally distributed: NE Atlantic (Greenland to Canary Islands; North Sea; Baltic); Mediterranean; NW Atlantic (Canada, USA); SE and SW Atlantic; Caribbean; NW Pacific (Japan, China); NE Pacific, Australia and New Zealand; Sub-Antarctica.

Uses and Compounds. Extracts have antibacterial [6.248, 276, 454], and antioxidant [6.595] activity.

Hydroclathrus clathratus (C. Agardh) M.A. Howe

Common Name. Perforated brown seaweed.

Description. *H. clathratus* is a very interesting brown seaweed, appearing as a 6 – 10 cm yellow-brown clump of very porus, chain-like tissue. The plant

has a very open, sponge-like structure with a complex series of holes perforating narrow, fleshy strips. Its name, *clathratus,* means *latticed,* an apt description. *H. clathratus* is found worldwide in warm seas but is an uncommon species on hard reefs. *H. clathratus* is more often found on in calm, shallow areas where it is anchored in bare sand [6.32, 739].

Habitat. In mid-littoral, often on wave-exposed rocks, more rarely in depths of apertures 1–3 cm, of the bridges 1–3 mm.

Distribution. The worldwide distribution of *H. clathratus* includes Europe, both coasts of Africa, the Pacific Islands, Asia, Australia, North and South America from California through Chile and the Gulf of Mexico.

Uses and Compounds. *H. clathratus* has been used for centuries in traditional cuisine and medicine of island cultures, such as Hawaii. Hydroclathrus clathratus is known to possess anticancer, anti-herpetic, anti-inflammatory, and anticoagulant properties and is now used as a mineral supplement in cosmetics and as a soil-additive (fertilizer) for its high concentration of micronutrients [6.32, 739].

Extracts have antiviral [6.740, 741], antitumor [6.742–747], cytotoxic and antiviral [6.72], and antimicrobial [6.72, 230, 248, 692, 715] activity.

Leathesia marina (Lyngbye) Decaisne

Synonym. *Leathesia difformis*

Common Names. Sea cauliflower, Sea balls.

Description. Thallus round in young stages, light brown, firm-fleshy and slimy-smooth, later hollow, and with an irregularly convoluted surface [6.32].

Habitat. Mostly epiphytically on larger algae or sessile on rocks.

Distribution. Nearly worldwide; NE Atlantic (Iceland to Canary Islands); Mediterranean; NW Atlantic; SE Atlantic (Namibia, South Africa); Indian Ocean (South Africa); NW Pacific (Japan, China); NE Pacific (Alaska to California); SW Pacific (Philippines); Australia; New Zealand, Antarctica and Sub-Antarctica.

Uses and Compounds. Extracts have agricultural bio-control and bio-stimulating [6.748], antifouling [6.749], antiviral [6.750], antioxidant [6.111], and antitumor [6.751] activity.

Petalonia fascia (O.F. Müller) Kuntze

Description. Thallus consists of erect, light to dark brown, dorsoventrally flattened lamina, arising from a holdfast singly or in clusters; linear or broadly lanceolate to almost elliptical, abruptly or gradually narrowing toward the base and merging into a elliptical, abruptly or gradually narrowing toward the base and merging into a short stipe, only slightly narrowing at the tip, rounded and often frayed; thallus undivided, membranous and thin when young, later leathery-tough with smooth, sometimes undulating, margins (Fig. 6.45). The species is very polymorphic especially in relation to thallus width [6.752].

Habitat. Found growing on rock in the mid intertidal to shallow sublittoral from protected to semiexposed Habitats.

Distribution. N NE Atlantic (Greenland to Canary Islands); Mediterranean; NW Atlantic (Arctic to New Jersey; SE Atlantic (Senegal, Namibia, South Africa); SW Atlantic (Brazil, Uruguay); Indian Ocean (Pakistan, South Africa); NW Pacific (Japan, China); NE Pacific (Alaska to California); SE pacific (Chile); Australia, New Zealand; Antarctica, and Sub-Antarctica.

Fig. 6.45 Herbarium specimen of *Petalonia fascia* (MACOI no. 292)

Uses and Compounds. Used as directly food, and the extracts have antimicrobial and antioxidant [6.203, 698] activity.

Punctaria latifolia Greville

Description. Frond oblong or obovate, medium brown, complanate, simple, ovate to ligulate, thickish, gelatinous and tender, usually 5–20 (–30) cm long and (1–) 2–6 cm broad, margin smooth to undulate, with a short, slender, stipe and cuneate base, arising from a small, discoid, rhizoidal holdfast (0.5–) 1–2 (–3) mm across; epiphytic (usually on *Posidonia* or *Heterozostera*) or epilithic; growth diffuse. Structure (2–) 4–6 cells and 80–120 µm thick, with a medulla 2–4 cells thick with few phaeoplasts, and a monostromatic cortex of isodiametric cells mostly of similar size to the medullary cells, 15–25 µm across in surface view, each with several discoid to irregularly shaped phaeoplasts each with a pyrenoid; phaeophycean hairs scattered, single or in small groups, 7–10 µm in diameter [6.753].

Habitat. On rocks in the upper intertidal down to several meters depth.

Distribution. Widely distributed in cooler temperate waters.

Uses and Compounds. Extracts have antibacterial [6.735], antiviral [6.735], cytotoxic, and antitumor [6.102, 735, 751, 754] activity.

Scytosiphon lomentaria (Lyngbye) Link

Common Name. Sausage weed

Description. *S. lomentaria* has cylindrical, shiny, olive brown, unbranched fronds up to 400 mm long. They have short stalks and a large number may arise from a single discoid holdfast. They widen to 3–10 mm and narrow again near the tip. They are hollow and often have irregular constrictions [6.755].

Habitat. It occurs in the littoral zone on wave-exposed shores and rock pools. Small plants are often found growing on limpets and pebbles.

Distribution. Cosmopolitan in temperate and cold seas.

Uses and Compounds. Used directly as food, and the extracts have antioxidant [6.111, 112, 335, 756, 757], antiviral [6.278], antimicrobial [6.421, 698], antitumor [6.421, 758], and antifouling [6.759] activity.

- Order: **Fucales**

Ascophyllum nodosum (Linnaeus) Le Jolis

Common Names. Knotted wrack, Asco, Sea Whistle, Bladderwrack, Egg wrack.

Description. *A. nodosum* is a perennial brown intertidal seaweed species most abundant on sheltered rocky shores in the mid-intertidal zone of the North Atlantic. Olive green in color, *A. nodosum* generally grows upward in the water column anchoring to hard substrates using a disk-shaped holdfast (Fig. 6.46). A single specimen resembles an intertwined mass of shoots and branches. Incoming tides gradually refloat vast stands of *A. nodosum*, until it becomes an expansive swaying undersea forest. Its thallus can measure from 30 to 60 cm, and is flexible to decrease breakage that may result from strong wave action. Its long, thick, leathery, branching strap-like fronds are typically between 0.5 and 2 m in length, and have large egg-shaped air bladders (*pneumatocysts*) at regular intervals along their length which keep the plant floating upright when submerged at high tide, and hang downward, draping over intertidal rocks in a thick, tangled, and glistening mat at low tide. The large air bladders take it toward the light for maximum photosynthesis. The fronds have no midrib.

It is present and grows all year round, and has no resting period. The species grows slowly and can live to be several decades old in wave-sheltered locations of temperate waters, and it has the capability to survive low temperatures. It can eventually grow to three and four meters; individual fronds can become up to 15 years old before breakage. The holdfasts of *A. nodosum* are thought to persist for several decades from which new fronds regenerate. Another interesting attribute of *A. nodosum* is that it repeatedly sloughs its entire outer epidermis, a phenomenon not exhibited by other related seaweeds.

A. nodosum attaches to rocks and boulders on the middle shore in a range of habitats, from estuaries to relatively exposed coasts. These sites can have low, moderate, and high wave action across low-, mid- and upper littoral heights, although some sublittoral populations have been reported. The upper limits of *A. nodosum* distribution are controlled by its ability to resist desiccation and high temperatures [6.1, 760].

Fig. 6.46 *A. nodosum* specimen

Habitat. On rocks in sheltered locations of the mid- to the lower littoral zone.

Distribution. Distribution is confined to the North Atlantic basin, found in the Arctic Ocean, Baltic Sea, Belt Sea, Northern Europe, Gulf of Maine, North Sea, and Northwest Atlantic. Its northern limits are northern Norway and the White Sea in the east, and Baffin Island in the west. Southern distributions extend to northern Portugal and New Jersey.

Uses and Compounds. *A. nodosum* is very effective at accumulating nutrients and minerals from the surrounding seawater, and this is what makes them a valuable resource for human enterprise. This species is harvested for use in items such as food, fertilizer, soil conditioners, animal feed, skin and hair care products, cleaners, degreasers, equestrian products, and nutritional supplements. It is also popular in cosmetology and thalassotherapy. The Industry has more than 200 product types from over 100 companies which include *A. nodosum* as an ingredient [6.3, 4, 760].

Extracts of this species have anticoagulant [6.761], antiviral, and anti-inflammatory [6.762, 763], antibacterial [6.764, 765], antioxidant [6.763, 766], nematicidal [6.767], agricultural biostimulant [6.768], antitumor [6.432], antifouling [6.61], and phytobiotic [6.769] activity.

Bifurcaria bifurcata R. Ross

Common Names. Brown tuning fork weed, Brown forking weed

Description. Up to 30 cm in length; olive-yellow in color, but much darker when dry (Fig. 6.47); holdfast expanded and knobby; frond cylindrical, unbranched near base then branching dichotomously. Elongate reproductive bodies present at ends of branches. Rounded air bladders sometimes present [6.1].

Habitat. Epilithic in intertidal tide pools, occasionally exposed at low tide and extending into the shallow sublittoral.

Distribution. Temperate NE Atlantic (from W Ireland to Morocco).

Uses and Compounds. A linear cytotoxic diterpene bifurcadiol was isolated from the brown alga *B. bifurcata* by *Di Guardia* et al. [6.770] which exhibit cytotoxicity against cultured human tumor

Fig. 6.47 *Bifurcaria bifurcata* specimen

cell lines. Extracts have also antifouling [6.771, 772], antibacterial [6.96, 269, 773], antiprotozoal [6.525], antioxidant [6.429, 731], and antitumoral [6.774] activity.

Cystoseira abies-marina (S.G. Gmelin)

Description. At the base a branching, gnarled stem with teeth-like appendages. Upper thallus tufted, forking or lateral branching at more or less the same length, single branches thin, covered with scattered dark dots, and conspicuous bilateral saw-teeth-like; conceptacles as wart-like swellings in the upper parts of the thallus; without swim bladders [6.32].

Habitat. It is mostly present in the Macronesian islands. Widely spread out in the sublittoral zone, in places subject to wave action.

Distribution. E Atlantic (Azores, Madeira, Canary Islands, Cap Verde Islands); Mediterranean (Spain, Sardinia, Libya, Italy).

Uses and Compounds. This species is used directly for food and as fertilizer [6.775]. Extracts have antioxidant [6.225, 776, 777], antimicrobial [6.778], and cytotoxic [6.225] activity.

Cystoseira baccata (S.G. Gmelin) P.C. Silva

Common Name. Bushy berry wrack

Description. Fronds usually solitary, 1 m or more in length, attached by a thick, conical attaching disk (Fig. 6.48). Axis simple or branched, up to 1 m in length, flattened, about 1×0.4 cm in transverse section; apex smooth and surrounded during periods of active growth by incurred young laterals. Lateral branch systems distichous, alternate, radially symmetrical, and profusely branched in a repeatedly pinnate fashion and bearing sparse, filiform, occasionally bifurcate appendages on the branches of higher orders; deciduous, leaving decurrent bases which give an irregular, zigzag outline to the axis. Aerocysts present in axes of branches of higher order, sometimes in chains; seasonal, particularly numerous in Autumn. Receptacles 1–5 cm long, formed from axes of ultimate ramuli, irregularly nodose and bearing simple, filiform appendages [6.779].

Habitat. Lower intertidal in large sandy pools or lagoons, mostly in persistent stands.

Fig. 6.48 Underwater photo of *Cystoseira baccata*

Distribution. Widely distributed in the NE Atlantic (Baltic, Netherlands, Belgium, Ireland, Britain, N France, N Spain, Portugal, Mauretania, and Canary Islands).

Uses and Compounds. Extracts have antifouling [6.780], antibiotic [6.781, 782], antiplasmodial, and cytotoxic [6.783] activity.

Cystoseira barbata (Stackhouse) C. Agardh

Description. The thallus is 15–20 cm tall-each branch ends with a conic foot (sole); in most cases the basement branches are joined together in a common base. The stem is narrow enough, 3–5 mm thick, cylindrical, the final side is smooth and unequal. The main branches are alternatively or chaotically branched from the stem, very long, cylindrical shape, plenty of small branches whose number gradually decreases, leaving a few singular cylindrical small branches, much shorter than the initial ones; the small branches are uni-

formly distributed along the main branches or come together as brooms nearest their tops. In winter and spring, there are many (air) bladders on branches. Cryptostoma are numerous on the surface of the branches and (air) bladders. The receptacles are spare shaded or cylindrical 0.2−1 cm long, strongly stressed on the scaphidia surface and with thready sterile tops; gathered in dense installments on the lateral surface of the branches: often in the receptacles the (air) bladders are metamorphosed and are distributed close the top branches [6.784].

Habitat. Rocky bottoms on pebbly grounds in sublittoral areas at 0.5−10 m depths.

Distribution. NE Atlantic (France to Portugal); Mediterranean, India and Russia.

Uses and Compounds. Source of alginic acid and the extracts have antioxidant, antibacterial, antifungal, anti-HIV, and hypoglycemic activity [6.130, 192, 201, 784–788].

Cystoseira brachycarpa J. Agardh

Synonym. *Cystoseira balearica* Sauvageau

Description. Plant caespitose, to 20−25 cm in height, attached to the substratum by a more or less compact discoid base formed of haptera; axes numerous, 2 –6 with 8 cm high; apices of the axes not very prominent, flattened and smooth; tophules absent. Primary branches cylindrical, seasonally either with smooth bases or covered with small spinose; conical appendages, some of witch can give rise to smaller branches; secondary and tertiary branches also cylindrical and covered with spinose appendages [6.789].

Habitat. Occurs in the upper sublittoral zone, from the surface to several meters depth, in moderately exposed and high light-intensity places.

Distribution. Mediterranean.

Uses and Compounds. The linear diterpenes eleganolone and elegandiol, isolated from *C. brachycarpa*, inhibit contractile activities of acetylcholine and histamine on ileum musculature of guinea pigs [6.790, 791]. Extracts have antimicrobial and antiviral [6.62, 136, 792, 793], antifungal, cytotoxic, and antimitotic [6.62] activity.

Cystoseira compressa (Esper) Gerloff & Nizamuddin

Description. *C. compressa* has a discoid base with several spined axes; the axes have denticulate margins. It is irregularly branched with compressed primary ramifications, and compact crawling receptacles; the axes have a height of 10−100 mm, and a thickness of 2−5 mm. This alga lacks any leaves, tophules, and air vesicles; *C. compressa* has an olive-brown coloration [6.794].

Habitat. On rocky substrata, especially in the shallow sublittoral of light exposed, sheltered locations, to 30 m depth.

Distribution. NE Atlantic (Azores, Canary Islands); Mediterranean.

Uses and Compounds. Extracts have anti-inflammatory and antiproliferative [6.795], antimicrobial [6.72, 248], antiviral, and cytotoxic [6.72] activity.

Cystoseira crinita Duby

Description. *C. crinita* has an upright, bushy thallus with smooth axes; it reaches a height of 50−350 mm, and a width of 200−500 mm. The main branches are rough, thin, and alternate; the upper section is particularly densely branched. The secondary ramifications are lacking spines, and the terminal receptacles are long, and compact. This alga lacks tophules and the gas vacuoles are either missing or few. *C. crinita* has a light-brown to blackish coloration [6.796].

Habitat. Rocky bottoms on pebbly grounds in sublittoral areas.

Distribution. Mediterranean.

Uses and Compounds. Extracts have antibacterial [6.114, 474, 735], antioxidant [6.717, 797, 798], antiviral and cytotoxic [6.735], anti-inflammatory, and antiproliferative [6.798] activity.

Cystoseira foeniculacea (Linnaeus) Greville

Description. Plant caespitose, to 30 cm, attached to the substratum by a wide and irregular basal disk. Axes to 10 cm high, with a circular cross-section (to 5 mm), normally spinose and with small scars from dehisced primary branches; apices of the axes not very prominent, with small spines [6.799].

Habitat. Occurs in mid-littoral pools and sheltered places.

Distribution. NE Atlantic (Scotland and Ireland to Senegal, Cap Verde Islands); Mediterranean.

Uses and Compounds. Extracts have antibacterial activity [6.60].

Cystoseira humilis Schousboe ex Kützing

Description. Characterized by highly differentiated basal and apical regions and the presence of catenate pneumatocysts (air vesicles). In old plants they have an elongated main axis, and in time the primary laterals become proportionally elongated. Their lower parts are strongly flattened into *foliar expansions* or basal leaves. Fertile regions which bear conceptacles are known as receptacles. These are normally found at the tips of the branches. Their basal and apical regions are highly differentiated. They have catenated pneumatocysts (air vesicles). The aerocyst or air vesicles keep the organism erect, by causing it to float in strong currents [6.800].

Habitat. Occurs in mid-littoral pools and sheltered places.

Distribution. One of the most widely distributed genera of the Fucales order and provides an essential habitat for many epiphytes, invertebrates, and fish. Found mostly in temperate regions of the Northern Hemisphere, such as the Atlantic, Mediterranean, Indian, and Pacific Oceans.

Uses and Compounds. Extracts have antibacterial activity [6.96, 171, 269].

Cystoseira mediterranea Sauvageau

Description. *C. mediterranea* has an upright thallus, with a single cylindrical main axis; irregular ramifications are present with the alga reaching a height of 400 mm. The receptacles are short and cylindrical, and whilst it has gas-filled vesicles, it is lacking in tophules. The main axis has a relatively soft texture, whilst the outer extremities are rough, almost spiny. It is a brown to olive green color and displays a bluish green iridescence when submerged [6.801].

Habitat. Grows in wave-exposed or moderately exposed places, in the upper sublittoral zone, always near the surface.

Distribution. Mediterranean.

Uses and Compounds. Extracts have antitumor [6.62, 421], antimicrobial [6.96, 276, 284, 421], andantifungal [6.62, 276] activity.

Cystoseira nodicaulis (Withering) M. Roberts

Common Name. Bushy noduled wrack.

Description. Thallus to 1 m long, usually solitary, attached by a irregular conical disk. Axis cylindrical usually branched, with smooth, rounded apex immersed between bases or tophules of developing laterals. Lateral branch systems (below) radial or distichous, with greenish-blue iridescence when first formed, about 50 cm long, repeatedly branched in a pinnate manner, either regularly or irregularly, with infrequent cryptostomata and bearing spine-like appendages; deciduous in summer; first-formed laterals of the season with tophules, later without; tophules ovoid, to 15 mm long, smooth or covered with small tubercles, persistent on axis after rest of lateral has been shed. Receptacles formed in the ultimate branchlets, simple or branched, nodose, usually bearing spine-like appendages; air vesicles inconspicuous, dilations of ultimate branchlets, solitary, in series or confluent; sometimes absent [6.802].

Habitat. Found in large intertidal rock pools and lagoons, often with *C. tamariscifolia* and *C. baccata*.

Distribution. Southern and western shores of Britain and Ireland, north to the Argyll in Scotland; Atlantic France and Spain, Portugal, Canary Islands, Morocco, south to Mauritania.

Uses and Compounds. Extracts have antileukemia activity [6.803].

Cystoseira spinosa Sauvageau

Description. Plant with a single axis, to 30–40 cm in height. Axis simple, to 20–30 cm hight: Apex of the axis not prominent and with spines; tophules spinose and oblong; branches both cylindrical and completely flattened, the latter with a pronouced midrib. Spinose appendages more abundantly present in the cylindrical than in the flattened branches; aerocysts absent [6.799].

Habitat. Occurs in the lower sublittoral zone.

Distribution. Mediterranean.

Uses and Compounds. Extracts have antifungal and antimicrobial activity [6.62, 804].

Cystoseira tamariscifolia (Hudson) Papenfuss

Common Name. Rainbow wrack

Description. *C. tamariscifolia* is a bushy seaweed, up to 60 cm in length but usually 30–45 cm. It has a cylindrical frond and branches irregularly (Fig. 6.49). The reproductive bodies on the end of branches are long, oval and spiny. Small air bladders are usually found below the reproductive bodies. *C. tamariscifolia* is olive green in color, almost black when dry. When the plant is seen underwater it has a blue-green iridescence [6.805].

Habitat. Found in rock pools and on the lower shore; grows on both rocky shores and gravelly flats.

Distribution. NE Atlantic (United Kingdom to Mauritania); Mediterranean (France to Turkey).

Uses and Compounds. Extracts have antibacterial and antifungal [6.269, 438, 806–808], antioxidant and antitumor [6.731], and cytotoxic [6.317] activity.

Fig. 6.49 Underwater photo of *C. tamariscifolia*

Cystoseira usneoides (Linnaeus) M. Roberts

Description. Large sized thallus, formed by a central axis and lateral branches with secondary branches – the ones which are closer from basis are flattened and have a foliaceous aspect (Fig. 6.50); presents numerous vesicles [6.809].

Habitat. Is present in relatively deep rocky areas crossed by currents.

Distribution. NE and E Atlantic (France, Spain, Portugal, Morocco, Senegal); Mediterranean (France, Spain, Algeria, Italy).

Uses and Compounds. Extracts have hypoglycemic and hypocholesterolemic [6.810], neuropharmacological [6.811, 812], anticancer, antioxidant, and anti-inflammatory [6.813] activity.

Cystoseira zosteroides C. Agardh

Description. Plant with a single axis, to 30 cm in height, attached to the substratum by simple or branched unfused haptera; axis branched or simple, to 10 cm high; apex of axis smooth and prominent; tophules cylindrical or ovoid, completely smooth, blackish or yellowish brown, sessile or with a sort peduncle; primary branches cylindrical or slightly flattened; secondary and tertiary branches foliaceous, several centimeters long and 1–1.5 mm wide, with an unpronounced midrib and with flattened and triangular spinose appendages, 1 mm long; aerocysts absent [6.799].

Fig. 6.50 *Cystoseira usneoides* specimen

Habitat. Occurs in the lower sublittoral zone.

Distribution. Mediterranean.

Uses and Compounds. Extracts have antifungal and antimitotic activity [6.62].

Fucus ceranoides Linnaeus

Common Name. Horned wrack

Description. Large brown intertidal seaweed, restricted to growing in estuaries or near freshwater streams on the shore. *F. ceranoides* does not have airbladders, but the side of the fronds is often inflated. Frond thin with smooth margin, fan shaped with prominent midrib, without air bladders but frond on either side may be inflated, reproductive bodies' narrow, pointed fronds at ends of branches [6.814].

Habitat. On estuaries or near freshwater streams on the shore, it attaches to stones, rocks, or gravel.

Distribution. *F. ceranoides* is widely distributed in NE Europe but is only common in brackish water. It is characteristic of estuaries and is often abundant where freshwater streams run onto the shore.

Uses and Compounds. Extracts have antioxidant and antitumor activity [6.205].

Fucus distichus Linnaeus

Common Name. Rock weeds

Description. A small tufted brown alga. It has narrow fronds without airbladders and short receptacles. The species has a life span of 3 years and grows up to 30 cm long [6.815].

Habitat. Occurs in rock pools and on rock faces in the upper mid-littoral at wave exposed locations.

Distribution. NE Atlantic (Arctic to Britain and Ireland); NE Pacific (Alaska); Japan.

Uses and Compounds. Extracts have antimicrobial activity [6.816].

Fucus evanescens C. Agardh

Common Name. Arctic wrack

Description. Brown to olive green in color, broad blades, obvious midrib, with Y-shaped forks. Can grow up to 3–90 cm and can be difficult or to distinguish from *F. vesiculosus*, sometimes indistinguishable [6.817].

Habitat. Occurs in rock pools and on rock faces in the upper mid-littoral at wave exposed locations.

Distribution. NE Atlantic (Arctic to Britain and Ireland); NE Pacific (Alaska); Japan.

Uses and Compounds. Source of alginic acid, fucoidan, and used for animal feed [6.817, 818]. Extracts have antiviral [6.819, 820], antibacterial [6.821], antitrypanosomal [6.822], anti-inflammatory, antiangiogenic and antiadhesive [6.823], antithrombotic and hemorrhagic [6.824], anticoagulant [6.823, 825], antitumor [6.826–828], and immunostimulating [6.829–831] activity.

Fucus serratus Linnaeus

Common Names. Toothed wrack, Black wrack, Blackweed

Description. *F. serratus* is a robust, olive-brown shrubby seaweed. It can grow in high densities low on the shore, forming dense mats of long ribbons up to 1 meter long and 2–5 cm across. It attaches to rocks via a discoid holdfast about 3 cm in diameter. Though technically a brown alga, it can vary in color from olive green through reddish brown (though it often has a greenish tint). It typically grows up to 70 cm but has been recorded at over 2 m in length in very sheltered environments. The flat, strap-like fronds have a forward-pointing serrated edge, a distinct midrib, and grow from a short stipe. The fronds are bifurcating (splitting in two repeatedly) [6.832].

Habitat. On firm substrata in the lower mid-littoral; often zone forming.

Distribution. *F. serratus* is a seaweed of the North Atlantic Ocean. It extends from the Canary Islands and northern Portugal along the Atlantic coast of France, into the British Isles and North Sea coasts, and into the W Baltic. It is found in Scandinavia up to Novaya Zemlya, around Iceland, and over to the Gulf of St. Lawrence in the NW Atlantic, ranging from Chaleur Bay and the tip of Cape Breton to Bar Harbor, Maine.

Uses and Compounds. *F. serratus* survives by filtering the ocean for nutrients and as a result, amasses

a huge amount of minerals and vitamins. Used for hundreds of years in seaweed baths, the oils from this seaweed have positive effects on skin, hair and body. The antioxidant [6.766] compounds found naturally in this seaweed have long been known to have pronounced antiaging, skin conditioning, and repair and hydrating effects. This seaweed is used as a food, and is harvested for cosmetics, and it is harvested to make fertilizer. Its edible properties are very similar to that of bladderwrack and interest in this plant is growing, as, being a thyroid stimulant it might could counter obesity by increasing the metabolic rate. It is also known to help women with abnormal menstrual cycling patterns and/or menstrual-related disease histories. It can be stored dried to make a nutritious tea as well as for use in soups and stews as a flavoring [6.833].

This species is collected, dried, and used as a soil additive as well. *F. serratus* is used by over a dozen businesses across the United States, Australia, France, Italy, Ireland, and the United Kingdom in a wide variety of products, such as: antiaging and eye serums, facial moisturizers and sunscreens, dental care compound, revitalizing compound used in skin and hair products, hair shampoos and conditioners, bath soak products, cleansing lotions, body buffs and gels, hand creams, facial, washes, cleansers, and toners, and fertilizers [6.833].

Fucus spiralis Linnaeus

Common Name. Spiral wrack

Description. Well-grown fronds are usually easily recognizable by the flattened, twisted, dichotomously branched thallus, lacking bladders, and the large, oval receptacles at the frond tips, each receptacle being surrounded by a narrow rim of vegetative frond. Nevertheless, younger plants are not always so easy to identify, and even mature plants can be confused with *F. ceranoides* or with bladderless forms of *F. vesiculosus*. Both of these species, however, have narrower, more pointed, rimless receptacles [6.834].

Habitat. Attaches to rocky substrata on sheltered to moderately exposed shores.

Distribution. *F. spiralis* is common on the coasts all around the British Isles, Iceland, W coasts of Europe, Canary Islands, Azores and Northeast North America from New Jersey to Nova Scotia. There are also isolated reports in the N Pacific.

Uses and Compounds. *F. spiralis* has been used historically for treatment of obesity, gout, goiter, and corns, and also in weight reducing and revitalizing bath treatments. It has been used for cattle feed, and as an organic manure. This alga, regularly exposed to sun radiation and its oxidative consequences, has developed optimal bioelectronics characters. It has a high concentration of phloroglucinol derivatives, including phenol acid, and in turn has been used in products from companies in France and the UK such as nutritional supplements, skin serum, body lotion, and compounds and extracts used as ingredients in other skin and hair products [6.835].

Extracts of this species have also antifouling [6.275], antimicrobial [6.269], antioxidant [6.225, 836, 837], anticoagulant [6.55], and antiproliferative [6.225] activity.

Fucus vesiculosus Linnaeus

Common name. Bladder wrack

Description. *F. vesiculosus* varies in color from olive green to olive brown to reddish brown to almost black and is typically about 40 cm in length (although fronds can grow longer). It attaches to rocky substrates by means of a small disk-shaped holdfast. *F. vesiculosus* is characterized by the small nearly spherical gas-filled vesicles (bladders) which look like bubblewrap and occur in pairs one on either side of an obvious central midrib running along the center of the strap-like frond (Fig. 6.51). The flattened, branching fronds can grow from one to two meters in length, and the air-filled bladders which keep the seaweed floating upright in its rocky anchorages increase its ability to photosynthesize. Typically it grows gregariously, forming dense mats of long ribbons up to one meter long and five centimeters across. The appearance of *F. vesiculosus* varies depending on the environmental conditions in which it occurs; in more sheltered areas there are many air bladders, whereas there are fewer in more exposed conditions. Also, in small plants, air bladders may be entirely absent. In exposed areas, it is beneficial for *F. vesiculosus* to lack bladders, as this decreases the potential for severe damage, and minimizes the risk of it being detached and swept away [6.1, 838].

Habitat. On rocks and stones in the mid-littoral; often zone forming.

Distribution. *F. vesiculosus* is found on the coasts of the North Sea, the western Baltic Sea, and the Atlantic

Fig. 6.51 *F. vesiculosus* specimens

and Pacific Oceans. It occurs around the coastline of Greenland, Britain, Ireland, Norway, the Atlantic coast of France, Spain and Morocco, and the Atlantic coasts of Canada and the United States from Hudson Bay to North Carolina.

Uses and Compounds. Primary chemical constituents of *F. vesiculosus* include mucilage, alginic acid, mannitol, beta-carotene, zeaxanthin, iodine and iodine salts, bromine, potassium, volatile oils, and many other minerals, as well as polysaccharides. When used in hot seawater baths or steamed the plants are said to release certain substances that promote good skin, the lower blood pressure and ease arthritic and rheumatic pains. *F. vesiculosus* has been shown to help women with abnormal menstrual cycling patterns and menstrual-related disease histories. A popular use of *F. vesiculosus* in herbal medicine is as a source of iodine (it was the original source of iodine, discovered in 1811), an essential nutrient for the thyroid gland; it can be used in the treatment of underactive thyroid glands (hypothyroidism) and goiter, a swelling of the thyroid gland related to iodine deficiency [6.3, 4, 838].

Extracts of this species have genotoxic and antigenotoxic [6.839], antioxidant [6.717, 766, 840, 841], antithrombotic [6.842], antibacterial and antifouling [6.275], antiviral [6.843], antitumor [6.844, 845], and anticoagulant [6.512] activity.

Halidrys siliquosa (Linnaeus) Lyngbye

Common Name. Sea oak, Pod weed

Description. A large sturdy brown alga 0.3–1 m in length (occasionally up to 2 m) rising from a strong, flattened cone shaped holdfast. The main stem is flattened and branches alternately to give a distinctly zigzag appearance. The stem bears a few, flattened ribbon-like *leafy* fronds. The ends of some branches bear characteristic pod-shaped air bladders (about 0.5 cm wide by 1–4 cm long) that are divided by transverse septa into 10 or 12 compartments. The branches also bear reproductive bodies that appear similar to the bladders but lack the septa. Young plants are olive-green in color while older specimens are dark brown and leathery. This species is perennial [6.846].

Habitat. Distinctive and common rock pool seaweed from the middle to the lower shore (may be found in the upper mid-littoral but only in rock pools). It may also form a zone in the sublittoral below the lower limit.

Distribution. NE Atlantic (from Faroe Islands to Portugal, North Sea, Baltic).

Uses and Compounds. Source of alginic acid. Extracts have antibiotic [6.847, 848], antifouling [6.849], antioxidant, and antitumor [6.421, 731] activity.

Himanthalia elongata (Linnaeus) S.F. Gray

Common Names. Sea spaghetti, Sea thong

Description. Common brown seaweed, which has a two stage morphology. Small button-like thalli are first produced, from which long strap-like reproductive fronds (receptacles) are formed in autumn (Fig. 6.52). The strap-like reproductive fronds grow quickly between February and May, reaching a length of up to 3 m. The plant releases gametes from June until the winter when it starts to decay. Plants commonly live for 2–3 years and reproduce once before dying [6.1].

Habitat. *H. elongata* is common on gently shelving rocky shores attached to hard substrata such as bedrock, or large to very large boulders. It is found on the lower

Fig. 6.52 *Himanthalia elongata* specimen

shore flourishing at the low tide limit with moderate wave exposure where its thallus is typically always submerged, and is considered a sublittoral species.

Distribution. *H. elongata* can be found from the Arctic Ocean south to the Iberian Peninsula, in the waters of the Baltic Sea, the North Sea and the northeast Atlantic Ocean. It is seen on the shores of Norway, the Faroes, Britain, Ireland, the western coast of France, northern Spain, and Portugal.

Uses and Compounds. *H. elongata* is known to provide high levels of Vitamins A, C, and E along with essential amino acids and other natural minerals. It has several properties which make it attractive as an ingredient in personal care and cosmetic products, such as that it is absorbent, viscosity controlling, skin protecting, and can be used as a binding agent. It has a natural ability to help restore balance to skin's moisture levels. It is used in dozens of products made in Ireland, France, Spain, Italy, Canada, and the United States [6.3, 4, 850].

H. elongata is used in shampoos and hair treatments, facial cleansers and skin care products, and also as a fertilizer. It can be sold and eaten fresh when in season, but it is typically sold dried or pickled, and is eaten most commonly in France and Ireland. It is used as an alternative to both traditional semolina spaghetti and green beans. It is found in popular tartar (sauce), *tahini*, pâté and cream products, and also in sea vegetable mixes [6.3, 4, 850].

Extracts of this species have hypoglycemic [6.851], antibiotic [6.852, 853], neuropharmacological [6.854, 855], antimicrobial, and antioxidant [6.429, 533, 765, 766, 856, 857] activity.

Pelvetia canaliculata (Linnaeus) Decaisne & Thuret

Description. *P. canaliculata*, often called channeled wrack, is a very common brown alga (Phaeophyceae) of Europe. It is the only species remaining in the monotypic genus *Pelvetia*. It is relatively small, not growing longer than 15 cm. When viewed underwater, its color can be light olive green, and sometimes yellowish brown (Fig. 6.53). *P. canaliculata*'s color can range from dark brown to dark olive to very dark or blackish green when dried. Its extremities can appear swollen and with orange during spring and summer. These bumpy, irregularly v-shaped swellings with forked tips at the ends of the fronds are its reproductive structures. It appears bushy and grows in dense tufts. Each frond is curled longitudinally (rolled lengthwise) to form a distinct channel. It is irregularly dichotomously branched; each tough and thick branch is of uniform width up to 1 cm, lacks a midrib, and lacks air vesicles or bladders. *P. canaliculata* is a perennial species; it is at least two years old before it reaches maturity, and has a life span of up to 4 or 5 years, growing 3 – 4 cm per year [6.1, 858].

Habitat. *P. canaliculata* grows attached to hard substrata with a small basal disk on the upper shore, and forms the uppermost zone of algae growing at or above high water mark. It needs periods of exposure to the air, and sometimes grows so high up a beach that coarse grass and other long-shore angiosperms grow among it. *P. canaliculata* is found right at the top of the beach in sheltered to moderately exposed areas where it can dry out completely to become black and crispy.

Fig. 6.53 *Pelvetia canaliculata* specimens

Distribution. One can find *P. canaliculata* in the northeast Atlantic, from the Arctic Ocean to the Iberian Peninsula, in the English Channel, and in the North Sea. It is common on the Atlantic shores of Europe from Iceland to Spain, including Norway, Ireland, the UK, the Netherlands, France, and Portugal (southern limit of this species).

Uses and Compounds. *P. canaliculata* was historically harvested for use as animal fodder, and in certain areas there are still animals which graze on the growing plants. It has also been collected historically as a source of food for people. It is included in dozens of products from Italy, France, United Kingdom, and the United States. According to one leading cosmetics producer, *P. canaliculata* stimulates the synthesis of collagens and proteoglycans, which are responsible for giving connective tissue its elastic properties. Using it as a compound can increase the skin's firmness and reduce the appearance of lines and wrinkles. It is reported from another leading cosmetics company that it increases microcirculation and can help reduce fat and cellulite. *P. canaliculata* can be found on ingredient labels for moisturizing lotions, masks and creams, and in body creams, day creams, and night creams. It is found in quality restorative and hydrating serums, and in compounds and extracts used in different products. For food, it is sold in different dried forms, and as a seasoning mix [6.858].

Source of alginic acid and fucoidan [6.859], and the extracts of this species have antioxidant [6.860, 861], anticoagulant [6.862], antifungal [6.863], antifouling [6.275], and antibiotic [6.864] activity.

Sargassum filipendula C. Agardh

Description. The habit of *S. filipendula* is so like that of other species which have been described that it needs but slight attention. This species grows attached to rocks below low water mark, and therefore, unlike *Fucus* and A*scophyllum*, is never exposed to the air. Vegetative plants and reproductive plants bearing all stages of conceptacles are plentiful in summer. Sporelings are abundant also and easily collected, for the discharged eggs and their products, the sporelings, remain attached for some time by mucilage to the surface of reproductive branches near the parent conceptacles. The stem arises from a small disk-shaped holdfast and passes into long cylindrical branches which bear spirally arranged leaves, berry-like floats, which seem to be modified portions of leaves, as generally stated, and short reproductive branches. This form may attain a height of 60 cm, but is commonly shorter [6.865].

Habitat. On firm substrata in the shallow sublittoral, to 6 (−30) m depth.

Distribution. NE Atlantic (Spain and Portugal to Gabon), Caribbean (Cuba), SW Atlantic (Mexico to Brazil).

Uses and Compounds. Extracts have antitumor [6.866–869], antioxidant [6.679, 866, 868, 869], and antifungal [6.863, 870] activity.

Sargassum hornschuchii C. Agardh

Description. Differs from the *S.vulgare* for the shape of the *leaves*: laminated in *S. vulgare* and triangular section in *S. hornschuchii* [6.871].

Habitat. On firm substrata in the shallow sublittoral, to 6 (−30) m depth.

Distribution. Mediterranean.

Uses and Compounds. Extracts have antifungal [6.62], cytotoxic, and antitumor [6.197] activity.

S. muticum (Yendo) Fensholt

Common Names. Japanese seaweed, Japanese brown alga, Japweed, Wire weed, Strangle weed.

Description. *S. muticum* is a large brown seaweed, varying in color from dark brown to pale, yellowish brown depending on the season and the growing conditions. *S. muticum* has regularly alternating lateral shoots or branches, on a central perennial stem (Fig. 6.54). It attaches to the substrate with a disk-shaped holdfast. It has numerous small 2–3 mm round or pear-shaped air-bladders which sit on small stems and cause the alga to stand upright in the water or float if parts of the alga are detached from the basal stem. *S. muticum* has a frond which may be 75–120 cm long in its native range, but normally reaches a length of 1.5–2 m in Swedish waters, 6–7 m in French waters, and up to 8.5 m in Norwegian waters. Lateral branches detach in the summer or autumn, leaving a short perennial basal stem to overwinter [6.872]. During the summer, cigar-shaped reproductive receptacles develop in the areas where the annual shoot or *branch* attaches to the stem, but may also sit on top of the branch [6.1, 873].

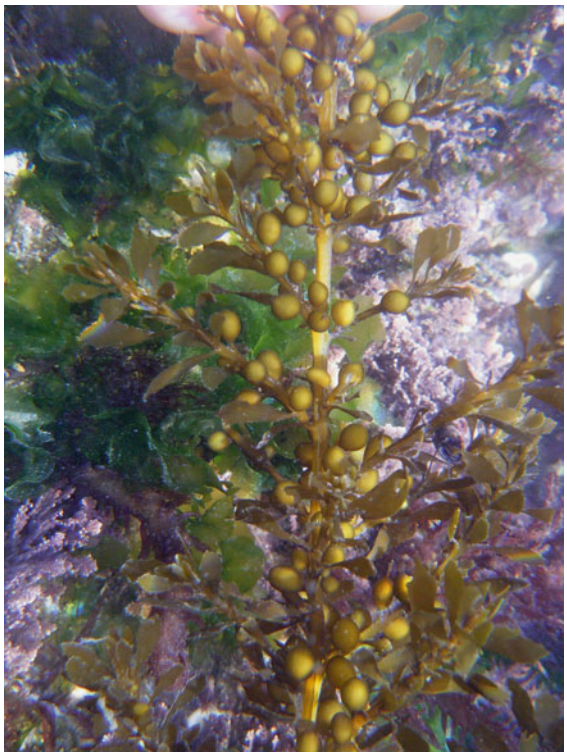

Fig. 6.54 Underwater photo of *S. muticum*

Habitat. In the mid-littoral and to below the tidal mark (3–5 m).

Distribution. Highly invasive: originally from Japan, it now colonizes large parts of the NE Atlantic (Norway to Portugal, North Sea), the W Mediterranean, and the N Pacific: NW Pacific (Japan), NE Pacific (Alaska to Mexico).

Uses and Compounds. Source of Laminaran [6.873], and extracts have antitumor and antibacterial [6.113], antioxidant [6.874], antialgal [6.257], antifungal [6.863], antibiotic [6.875], and antifouling [6.876–880] activity.

Sargassum natans (Linnaeus) Gaillon

Description. *S. natans*, unlike the many benthic *Sargassum* species (i. e., those that live attached to the sea bottom), is a pelagic (free-floating) brown seaweed that occurs mainly far out in the North Central Atlantic Ocean, but washes ashore regularly. It is often associated with the Sargasso Sea, a very large region situated offshore from the southeastern United States, approximately south and east of Bermuda and seaward from the Gulf Stream. The Sargasso Sea, which accumulates large masses of *Sargassum*, results from a ring of prevailing ocean currents that enclose an enormous eddy (about 5.2 million km^2) which rotates clockwise as a result of the Earth's eastward rotation. *S. natans* apparently reproduces only asexually, by fragmentation. *Gower* and *King* [6.881, 882] used satellite imagery to track the origin, distribution, and fate of floating *Sargassum*. Using this approach, they analyzed data from 2002 to 2008 and were able to present the first mapping of the full distribution and movement of pelagic *Sargassum* in the Gulf of Mexico and western Atlantic. Their results revealed a seasonal pattern in which *Sargassum* typically shows strong growth in the northwest Gulf of Mexico in the spring of each year, is transported into the Atlantic in about July, appearing east of Cape Hatteras as a *Sargassum jet*, and ends up northeast of the Bahamas in February of the following year.

Habitat. On firm substrata in the shallow sublittoral, to 6 (–30) m depth.

Distribution. NE Atlantic (Spain); Atlantic Islands; NW Atlantic; Caribbean; SW Atlantic; Pacific Ocean (Indonesia); Australia.

Uses and Compounds. Source of alginic acid [6.883] and extracts of this species have antimicrobial [6.136, 884, 885], antifouling [6.275], and antitrypanosomal [6.822] activity.

Sargassum vulgare C. Agardh

Description. *S. vulgare* has a bush-like thalli reaching 15–70 cm high; the fronds are oval, flattened, olive-green to brown and possess a central rib and undulated edge. The base of the fronds has hollow, spherical vesicles, of 5–8 mm, and clusters of reproductive bodies; these are held in place by a pedicle. The alga is attached to the substrate with irregular rhizoidal branches. *S. vulgare* has an olive brown to dark brown coloration [6.886].

Habitat. On firm substrata in the shallow sublittoral, to 6 (–30)m depth.

Distribution. NE Atlantic (Portugal, S Spain to Canary Islands); Mediterranean; Caribbean; SE Atlantic (W Africa); W Pacific (Philippines); Indian Ocean.

Uses and Compounds. Source of alginic acid [6.765, 886, 887] and extracts of this species have antihelminthic [6.886], antioxidant, antimicrobial and antifungal [6.336, 888, 889], antitumor [6.765, 890, 891], antilipemic [6.892], antifouling [6.893, 894], antimicrobial [6.228], anticoagulant, antithrombotic, and anti-inflammatory [6.889] activity.

- Order: **Laminariales**

Alaria esculenta (Linnaeus) Greville

Common Name. Dabberlocks.

Description. Fronds with olive or yellow-brown fronds to 4 m long and 25 cm wide. Attached by a root-like holdfast at the base from which a narrow flexible stipe arises which continues into the leafy part of the plant as a distinct mid-rib (Fig. 6.55). The reproductive structures, apparent as dark-brown areas, are confined to unbranched leafy appendages borne on the stipe, usually in two rows. This is the only kelp-like plant in Ireland and Britain with a distinct midrib and is the only one with sporangia borne at the base of the frond in special leaflets called sporophylls [6.895, 896].

Fig. 6.55 Herbarium specimen of *Alaria esculenta* (MA-COI no. 2664)

Habitat. Generally grows on rock in very exposed places, often forming a band at low water and in the shallow sublittoral, but also occurs in tidal pools in the lower shore.

Distribution. Coasts of the North Atlantic (France, Scotland, Ireland, Greenland, Iceland, northeastern United States, northeastern Canada), the N Sea (England, Norway, Netherlands), Novaya Zemlya to the N Pacific (Bering Sea and Sea of Japan).

Uses and Compounds. *A. esculenta* can be used for a variety of purposes from human consumption and alginate production to fodder and body-care products. It is rapidly gaining popularity in the natural foods market. It can be ordered from many sellers as whole, flaked, milled, or powdered. It is used

for antiaging body creams, foot creams, bath soaks, body and face masks, body polish, UV-protecting facial moisturizers, self-tanning lotions, lip balm, day- and night-creams, and nutritional supplements to name a few. In Canada and the United States, this seaweed is sometimes sold as *Atlantic Wakame* and is presented as an alternative to traditional Japanese Wakame (*Undaria pinnatifida*). *A. esculenta* also has potential as a foodstuff in aquaculture for herbivorous molluscs such as abalone. Over 20 sellers with over 70 *A. esculenta*-based products have been identified. These sellers are from Germany, Italy, France, Ireland, United Kingdom, Canada, and the United States [6.4, 896, 897].

Chorda filum (Linnaeus) Stackhouse

Common Names. Sea lace; dead man's rope; mermaid's tresses; Cat gut.

Description. *C. filum* is a brown seaweed with long cord-like fronds, only 5 mm thick in diameter. The fronds are hollow, slippery, unbranched, and grow up to 8 m long. The species is attached to the substratum using a small discoid holdfast. It is an annual species, disappearing in winter [6.898, 899].

Habitat. It is found in rock pools on the low shore and in the sublittoral down to 5 m. It is most commonly found in the sheltered bay.

Distribution. Abundant throughout the North Atlantic, on the shores of Europe and America, coast of Brazil, and also in the North Pacific, at Sitka, Unalaschka, and Kamtschatka.

Uses and Compounds. *C. filum* is used fresh as a foodstuff, and for animal feed [6.898]. This species produces alginate and fucoidan, and have antimicrobial [6.474, 546, 844, 898, 900], algicidal [6.257], anticoagulant [6.524], and antioxidant [6.474] activity.

Laminaria digitata (Hudson) J.V. Lamouroux

Description. A large conspicuous kelp growing up to 2 m in length commonly found at low water during spring tides on rocky shores. The frond is broad and digitate, glossy and dark brown in color and lacks a midrib. The stipe is oval in cross section, smooth and flexible and is usually free of epiphytes, although old stipes which has become slightly roughened may support a few epiphytes, notably *Palmaria palmata*. The kelp is attached to freely branched haptera, which spread out to form a shallow dome-shaped holdfast. *L. digitata* may be confused with young *L. hyperborea* plants. However, the stipe of *L. hyperborea* is circular in cross section, is stiff, and snaps easily when bent (although you would not see that in younger fronds) [6.901, 902].

Habitat. On rocks from the low water spring tide level to the shallow sublittoral. In some locations, *L. digitata* may be found in deeper water.

Distribution. *L. digitata* is a North Atlantic Arctic-cold-temperate species which does not occur in the North Pacific. It is found along both coasts of the English Channel; the southernmost occurrence of this species in European waters is on the southern coasts of Brittany. It grows along most coasts of Britain and Ireland, and along the North Sea coasts of Scandinavia. Its northerly range includes into the Barents Sea and the western shores of Novaya Zemlya, and has been reported to occur in the Svarlbard Archipelago. *L. digitata* also grows in Iceland, the Faeroes, southern Greenland, and the eastern coasts of North America, as far south as Cape Cod.

Uses and Compounds. *L. digitata* plants contain minerals, vitamins, and trace elements. These include iodine, calcium, potassium, iron, carotene, alginic acid, laminaran, fucoidan, mannitol, protein, carotene, niacin, phosphorus, the B complex vitamins, vitamin C, and many other trace elements. This species stores flavor-enhancing glutamic acid, or sodium glutamate, which imparts a mellow, silky taste to dishes. The slight sweet background is mannitol, a natural sugar. In terms of relatives, *L. digitata* is closely related to the five species (*Saccharina latissima*, *S. japonica*, *L. angustata*, *L. longissima*, and *L. ochotensis*) typically harvested as *Kombu* in Japan, and is frequently harvested and sold as Kombu in North America. Many recipes calling for Kombu could be made with this form of kelp [6.901–906].

Extracts of this species have antibacterial [6.371], and antioxidant [6.766] activity.

L. hyperborea (Gunnerus) Foslie

Common Name. Oarweed.

Description. *L. hyperborea* is often difficult to distinguish from *L. digitata*, particularly when plants are young (Fig. 6.56). However, the stipe of *L. digitata* is darker and usually oval in cross section instead of cylindrical, is not thicker at the base, does not snap easily,

Fig. 6.56 *Saccorhiza polyschides* (*left*) and *L. hyperborea* (*right*)

and does not have epiphytes on the stipe. Also, the belt of *L. hyperborea* is nearly always below (in deeper water) any belt of *L. digitata*. *L. ochroleuca* is a similar species, but has a smooth stipe (not rough like *L. hyperborea*), and its fronds are typically more golden or yellow. *Saccorhiza polyschides*, another commercially utilized kelp, appears similar to *L. hyperborea* from the frond, but has a twisted stipe near a bulbous haptera, which is very different from *L. hyperborea*'s bird claw holdfast [6.1, 907].

Habitat. Found on bedrock or other stable substrata from extreme low water to depths dependent on light penetration and sea urchin grazing (typically about 8 m depth in coastal waters to 30 m in clear coastal).

Distribution. *L. hyperborea* is a European North Atlantic cold-temperate species which does not extend into areas influenced by Arctic waters; its range is the NE Atlantic Ocean, from Scandinavia south to Spain and the Canary Islands, the Baltic Sea, and the North Sea.

Uses and Compounds. In Europe, *L. hyperborea* is one of the two kelp species commercially exploited by the hydrocolloid industry, the other being *L. digitata*. *L. hyperborea* is also utilized by the cosmetic and agrochemical industries and for biotechnological applications, and by the food industry for emulsifiers and gelling agents. Drift kelp has long been collected as an agricultural fertilizer and soil conditioner. *L. hyperborea* is still harvested and used in popular kelp meal fertilizer products. *L. hyperborea* is a source of laminarin and mannitol, which are used in industrial and other applications. Because of its ability to absorb and retain water, it has been used in wound dressings to prevent adhesions, and has also been used to help dilate the cervix during childbirth [6.3, 907].

Extracts have antifungal [6.863], anticoalgulant [6.512], antibacterial [6.765], antioxidant [6.860], and anticoagulant [6.524] activity.

Laminaria ochroleuca Bachelot de la Pylaie

Common Names. Kelp, Kombu.

Description. *L. ochroleuca* is a glossy, yellow-brown kelp that is prevalent along the intertidal zones. This kelp is quite conspicuous as it grows quite large under the right conditions. The maximum length recorded is 4 m long, but this length is rarely attained and occurs only in specific areas. Under normal conditions *L. ochroleuca* is more likely to reach a maximum length of about 2 m. It has a large heavy holdfast made up of thick haptera (up to 18 cm in diameter) that support the plant and anchor it to rock. This holdfast gives rise to a fairly long, rigid, round, epiphyte-free stipe that tapers somewhat as it approaches the blade. This stipe is so strong and stiff that it stands erect when the plant is out of the water. The blade of this kelp is large, flat, and leathery, and is divided into 5–20 strap-like digits. This kelp is easily distinguished by the distinct yellow area at the junction of the stipe and the blade. The entire plant actually has a very lovely yellowish hue to its smooth, bright, glossy tissue. *L. ochroleuca* is a perennial kelp that retains its stipe and holdfast year long but regenerates a new blade each year [6.1, 908].

Habitat. This species is found on rocks from the low water spring tide level to the shallow sublittoral. In some locations *L. ochroleuca* may be found in deeper water.

Distribution. *L. ochroleuca* is a warm-temperate species of kelp, and is most common in the NE Atlantic from the British Isles to the Sahara and the Atlantic zones of the Mediterranean.

Uses and Compounds. Extracts of *L. ochroleuca* have been found to act as a central nervous system depressant with a slight analgesic activity. It is also able to effectively guard DNA against UV rays and premature aging. With these properties, it is becoming more widely used in cosmetics and in natural therapeutic medicine. Applied topically, *L. ochroleuca* helps reduce inflammation and, like many forms of seaweed, has some amount of moisture-binding properties due to its sterol content. It is used in products from Spain, Germany, France, and the United Kingdom; these include rescue balms, repairing and antiaging moisturizers, as well as dried and packaged foods [6.3, 4, 908, 909].

Extracts have also antimicrobial [6.224, 269, 910–913], antifungal [6.913], and antialgal [6.257] activity.

Saccharina latissima (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders

Synonym. *Laminaria saccharina* (Linnaeus) J.V. Lamouroux

Common Names. Sugar kelp, Sea belt, Sugar wrack.

Description. Just like most kelps, *S. latissima* has blades (lamina), stipes, and holdfasts, which is attached to substrates. The sporophytes of *S. latissima* have a rich medium brown color, a long undivided frond without a midrib and a profusely branched holdfast (Fig. 6.57). Mucilage ducts are absent from stipe; the blade often has two rows of bullations formed in two longitudinal rows parallel to the central axis. The frond of *S. latissima* has a distinctive frilly undulating margin. The stipe of *S. latissima* may be up to 50 cm long [6.1, 914].

Habitat. *S. latissima* is usually found from the sublittoral fringe down to a depth of 30 m. More rarely, it occurs in rock pools. The species usually occurs in sheltered conditions and may be attached to unstable substrata such as boulders and cobbles.

Distribution. N and NE Atlantic (Greenland to Portugal, North Sea, Baltic); NW Atlantic (Canadian Arctic to Massachusetts); NE Pacific (Alaska to California).

Uses and Compounds. Kelp harvesting is also an important industry around the world. Kelp products, alginic acid and fucoidan, are used for different purposes [6.915]. Alginic acid is a common polysaccharide constituent of kelp cell walls and it can be used to form gels. Alginic acid is used as a stabilizer in the food, cosmetic, and pharmaceutical industries: yogurts, shampoos, skin creams, lotions as well as ice creams. Alginic acid is also being used in many dietary products because human body cannot absorb it. In lots of biotechnological research experiments, alginic acid is used to separate substances [6.3, 4].

Fig. 6.57 *Saccharina latissima* specimen

People eat Sugar kelp (also called Kombu in Japanese dishes), which contains appreciable amount of vitamin C and is high in iodine, protein, and calcium. The blade is usually chopped and cooked as a savory garnish (Tororo Konbu) for rice and other dishes, as a vegetable, and as snacks (such

as Tsukudani). Kombu is also used to add flavor to broths (Dashi stock) and stews but it removes itself removed from the liquid at the end of cooking and discarded. It can be used to soften beans during cooking, and to help convert indigestible sugars [6.4].

Extracts of this species have anticoagulant [6.512, 524], antifouling [6.916], antimicrobial [6.533, 816, 917], and antioxidant [6.429, 533, 860] activity.

Saccharina longicruris (Bachelot de la Pylaie) Kuntze

Description. *S. longicruris* is a particularly tall species of kelp. The hollow, cylindrical stipe itself can reach up to 10 m long, plus the frond which adds another 1–2 m. The large branched holdfast grips firmly to the rocky substrate allowing a single long, thin, olive-brown, leafy blade to float near the surface. The midsection of these blades is somewhat thicker, but the edges spread and thin and become wide and ruffled. *S. longicruris* is limited to the sublittoral zone, but can be found either in shallow or in much deeper waters, often preferring areas with a strong flow. In this environment, it is a dominant part of the seaweed community and forms dense multistory forests with older plants forming a canopy of fronds floating on the surface and younger plants protected underneath [6.918].

Habitat. Horizontal distribution is determined to a large extent by substratum type and salinity. In very sheltered sites the species can occur on an unstable substratum of gravel and small stones.

Distribution. The species occurs widely throughout the NW Atlantic and eastern Arctic Oceans, mostly within Canadian territorial waters, and along the west coast of Greenland north of 62° N. The species is rare on the east coast of Greenland, but has been recorded from Iceland, the Faeroes and as far south as Long Sound on the Shetland Islands at the latitude of 60° N. *S. longicruris* has not been recorded from the European mainland.

Uses and Compounds. *S. longicruris* is an edible species of kelp that is related to traditional Japanese Kombu, but is thinner, tenderer, and cooked fastly. It is high in minerals and micronutrients and is particularly delicious as it contains naturally occurring monosodium glutamate – a little known feature of many kelp species [6.918, 919].

Extracts have immunostimulatory [6.920], and skin antiaging [6.921] activity.

Saccorhiza polyschides (Lightfoot) Batters

Common Name. Furbelows.

Description. *S. polyschides* is kelp species with a distinctive large warty holdfast and a flattened stipe with a frilly margin (Fig. 6.56). The stipe is twisted at the base and widens to form a large flat lamina, which is divided into ribbon-like sections. The species is an annual, and very fast growing. It is opportunistic and colonizes available hard substrata in the sublittoral [6.1, 922].

Habitat. In the lower littoral, in calm shallow water to 19 m.

Distribution. NE Atlantic (Norway to Morocco), W Mediterranean.

Uses and Compounds. Source of alginic acid, and possible source of biofuel [6.380]. Extracts of this species have hypoglycemic [6.923], antisettlement [6.360], cytotoxic, and antiplasmodial [6.924] activity.

U. pinnatifida (Harvey) Suringar

Common Name. Wakame.

Description. *U. pinnatifida* belongs to the order Laminariales, with three clearly recognizable parts comprising its visible thallus: blade, stipe, and holdfast. The thallus can reach 1–3 m in length. The blade is lanceolate and broad with a prominent midrib, and translucent with color ranging from green to yellowish brown to dark brown. The blade could also be described as triangular and lobed (Fig. 6.58). The appearance of the blade evolves and changes over time; it is initially simple, flattened, and broad with a pronounced or distinct midrib; older plants have thicker blade tissue which splits horizontally down to the midrib to form fingers or straps, becoming more transversally lobed, and becomes pinnate with age. The margins of the blade can also be described as wavy. The distal portion of the blade and the straps eventually become tattered. The stipe of *U. pinnatifida* is wavy or corrugated above the holdfast. The stipe is also usually short (10–30 cm) in length and up to 1 cm in diameter) and in mature plants bears convoluted wing-like reproductive outgrowths or frills (sporophylls). The stipe is

Fig. 6.58 *U. pinnatifida* specimen

Fig. 6.59 Herbarium specimen of *Cladostephus spongiosus* (MACOI no. 3509)

also flattened, and transitions into the midrib which extends through the middle of the length of the blade. *U. pinnatifida* is attached to its substrate by a branched holdfast comprised of haptera. It may be confused with *A. esculenta*, as it also has a prominent midrib, but the corrugated stipe of *U. pinnatifida* (which contains its sporophylls) is distinctive. Also, *A. esculenta* grows in more exposed and wave-battered locations than *U. pinnatifida* [6.1, 4, 925].

Habitat. Wakame is economically important as a food crop but is also a fouling organism. It is able to compete with native Kelp species in the shallow sublittoral zone.

Distribution. Worldwide distribution.

Uses and Compounds. *U. pinnatifida* is economically important as a food crop, next to Nori, on the Japanese menu, and is eaten both dried and fresh. In East Asian countries the seaweed is known as Wakame and is treated as a delicacy, often added to miso soup. It can be considered a sea vegetable, or edible seaweed. *Wakame* fronds are green and have a subtly sweet flavor and slippery texture. In Asia and Europe, Wakame is distributed either dried or salted, and used in soups (particularly miso soup), and salads (such as tofu salad), or often as a side dish to tofu and a salad vegetable like cucumber. Goma wakame, also known as seaweed salad, is a popular side dish at some sushi restaurants; literally translated, it means *sesame seaweed*. In Korea, *U. pinnatifida* is used in salads or soup such as Miyeokguk. Many women consume *U. pinnatifida* during pregnancy, and *Miyeokguk* is popularly consumed by women after giving birth as Miyeok contains a high content of calcium and iodine, nutrients that are important for nursing new mothers. It is also traditionally eaten on birthdays for this reason, a reminder of the first food that the mother has eaten and passed on to her newborn through her milk, thus bringing good fortune for the rest of the year. After the species was accidentally introduced in 1971 in the Mediterranean via farming of Japanese oysters (*Crassostrea gigas*), *U. pinnatifida* was grown in the French Bretagne as food, which increased exposure of this seaweed to Europeans. *U. pinnatifida* is also used in a wide variety of topical beauty treatments and personal care products, due to its high polysaccharide content and ability to provide moisture [6.3, 4, 925].

Extracts of this species have antihypertensive [6.926], immunomodulating [6.927], antidiabetic [6.928], antiviral [6.929, 930], cytotoxic [6.931], antioxidant [6.932], antitumor [6.933, 934], antiedema

[6.935], antiplasmodial [6.936], anti-osteoporotic [6.937], anti-inflammatory [6.938], antiobesity [6.939, 940], antihypertensive [6.941], and antithrombotic [6.942] activity.

- Order: **Ralfsiales**

Ralfsia verrucosa (J.E. Areschoug) J.E. Areschoug

Description. *R. verrucosa* is an olive brown to khaki encrusting brown seaweed that occurs abundantly in low shore tide pools. Here it often forms large, flat, smooth expanses giving the impression that someone had accidentally dropped khaki paint into the tide pools. *R. verrucosa* can occur in high abundance because of its relatively fast growth rate and also because it produces a chemical which seems to deter most grazers [6.943].

Habitat. Saxicolous, mid-littoral.

Distribution. NE Atlantic (Ireland and Britain to Morocco); SE Atlantic (South Africa); NW Atlantic (Canada, USA); SW Atlantic (Argentina); Mediterranean; Indo-Pacific; Australia.

Uses and Compounds. Extracts have antibacterial and antifouling activity [6.944].

- Order: **Sphacelariales**

Cladostephus spongiosus (Hudson) C. Agardh

Description. Fairly stiffly branched fronds growing from a crust-like discoid holdfast, covered with small branchlets arranged in whorls (Fig. 6.59); maximum length is usually about 15 cm [6.945].

Habitat. On firm substrata in the intertidal zone.

Distribution. NE Atlantic (Iceland to Morocco); Mediterranean, Indian Ocean; Australasia; Antarctica, Sub-Antarctic Islands.

Uses and Compounds. Extracts have antibacterial activity [6.171, 201].

Halopteris filicina (Grateloup) Kützing

Description. Compressed, tufted, dark purplish brown fronds, to 120 mm high, main axis 1–4 mm broad. Once or twice pinnate, axes and branchlets tapered at base and apex [6.946].

Habitat. On rocks in pools, mid-littoral to shallow sublittoral, sporadic, often locally common.

Distribution. NE Atlantic (Ireland and Britain to Morocco); Mediterranean; NW Atlantic (USA); SW Atlantic (Brazil); NE Pacific (South Korea, Japan).

Uses and Compounds. Extracts have antihelminthic [6.946], antimicrobial [6.40, 130, 201, 203], antifouling [6.947], antifungal and antimitotic [6.62] activity.

Stypocaulon scoparium (Linnaeus) Kützing

Synonym. *Halopteris scoparia* (Linnaeus) Sauvageau.

Common Name. Sea broom.

Description. *S. scoparium*, previously known as *H. scoparia*, is a dark brown algae that forms beautiful fluffy clumps in shallow rocky-bottomed water. Growing only up to 15 cm in length, *S. scoparium* has a main axis with alternate plumed branches which are more or less fan shaped when flat, though when buoyed up by water they form inverted cone-shaped tufts with a very delicate appearance due to the many filamentous branches. Plants are usually attached to rocks with small to extensive disks often obscured by many matted rhizoids; though these are lacking in free-living plants. These plants are characterized by pure, sheltered waters with high light levels. *S. scoparium* often forms clumps on cobble in the shallow sublittoral zone, though they are just as likely to be found in shallow tide pools or sandy-bottomed areas [6.32, 948].

Habitat. On rocks and epiphytically on other seaweeds; in sheltered zones of the upper littoral and in up to 5 m depth.

Distribution. NE and E Atlantic (Scandinavia to Cap Verde Islands); Mediterranean.

Uses and Compounds. *S. scoparium* is known to be an ingredient in compounds used in personal care products; it contains growth substances (phytohormones) that include auxins, gibberellins, cytokinins, abscissic acid, and betaines [6.948].

Extracts of this species have antiprotozoal [6.193], antifungal and antimitotic [6.62], antioxidant [6.949], antileukemia [6.950], and antimicrobial [6.296] activity.

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7. Corals

Mohammad Kazem Khalesi

Almost 50% of cytotoxic compounds are isolated from marine organisms such as sponges and corals. Symbiotic corals, mainly soft corals, are potential sources of many unique metabolites including cytotoxic and anticancer compounds. This review focuses on secondary biochemicals with potential biomedical properties extracted from both soft and hard coral species. The coral-derived compounds and extracts, for which biological activities have been reported, are discussed in this review, which covers the years from 1975 to late 2012. For distinction, members of each coral family, and the relevant genera and species have been surveyed separately. A total of 244 compounds (excluding crude extracts) have been introduced from 15 and 6 families each with 139 and 16 species of soft and hard corals, respectively. The majority of introduced compounds are promising as potential cancer therapeutics.

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7.1 Background

The marine environment represents a treasure of useful products that await discovery to used for the treatment of infectious diseases [7.1]. Medicines derived from marine organisms, specifically invertebrates, as a source of novel compounds have been investigated because of their potential for producing new drugs (e.g., toxins with high potency and low solubility), which makes them particularly suitable for consideration as sources of antineoplastic agents [7.2]. Almost 50% of cytotoxic compounds have been isolated from marine organisms such as sponges and corals (reviewed in [7.3]). In comparison with the other life-forms, bioactive compounds have been detected frequently, albeit after sponges, in symbiotic corals, mainly soft corals.

There are two subclasses in *Anthozoa*, namely, *Hexacorallia* (zoantharia) and *Octocorallia*. Soft corals (octocorals: phylum *Cnidaria* (formerly *Coelenterata*), class *Anthoza*, subclass *Octocorallia*) are multicellular

marine animals, most of which form colonies mainly in tropical waters composed of hundreds or thousands of polyps; polyps cover a soft skeleton composed of a protein/calcium carbonate material (> 60% of organic matter). *Cnidaria*, one of the largest phyla consists of three classes, among which *Anthozoa* is the largest [7.4, 5]. Among *Cnidaria*, 21% of the species contain potential marine biomedical compounds [7.6]. Almost 50% of octocorals as members of *Cnidaria* have been reported to produce toxins; about 60% of their extracts are bioactive molecules with medicinal potential [7.7–10]. Published records state that 11–17% of potential new drugs undergoing preclinical stage from 1998 to 2002, originate from octocorals. From the soft coral species investigated up to 2008, more than 30% exhibited cytotoxic activity [7.11], suggesting production of potential medicinals. A similar percentage (30%) was presented in [7.12], where crude extracts were screened from 20 octocorals displaying cytotoxic and antimycobacterial activities (60%, Figs. 7.1–7.10).

7.2 Potential Pharmaceuticals from Soft Corals

Octocorals lack toxic stinging nematocysts, as well as a rigid protective skeleton common to scleractinians, but possess allelopathic capabilities [7.13]. Using allelopathic chemicals, sessile soft corals compete for space, reduce their palatability by producing terpenoids, and reduce fouling by chemical substances and mucus [7.14]. These allelopathic secondary metabolites, especially terpenes, also protect against predators, infections and injuries, and play a range of physiological roles [7.5, 15].

Structurally diverse compounds including diterpenes, prostaglandins, and steroids described in octocorals [7.16–18] render these animals rich sources of steroids, terpenoids, and other types of secondary metabolites, including a large variety of sesquiterpenoids and diterpenoids [7.8, 19]. Octocorals, unlike sponges, are capable of rapidly synthesizing terpenes de novo [7.19]. Similarly, it was reported in [7.20] and [7.21] that toxins are secreted more or less continuously in several species of soft corals.

In a comprehensive study, the antimicrobial activity of extracts of several of the most dominant stony (scleractinian) and soft (alcyonacean) coral species from the coral reef of Eilat (northern Red Sea) was compared against indigenous bacteria. The results revealed substantial variability in antimicrobial activity of the extracts from hard and soft corals. Five out of six (83%) Red Sea alcyonacean soft corals showed considerable antimicrobial activity against the test bacteria, whereas none of the extracts of the six stony corals tested inhibited the test bacteria. It, therefore, appears that soft and hard corals have developed different means to combat potential microbial infections. Antibiotic compounds in alcyonacean soft corals are used to combat microbial attack, whereas stony corals seem to rely on other means [7.22]. Nonetheless, further research on hard (scleractinian) corals revealed bioactive compounds and the biological role of terpenes in hard corals as well. Hence, in this chapter, the pharmaceutical potential of soft and hard coral species will be described separately.

De novo biosynthesis of sterols has also been stated as an important mechanism in octocorals [7.23]. Until early 2009, 561 new polar steroids had been reported from 16 families of octocoral species. Soft corals contribute predominantly as containers of the largest number of marine polar steroids; *unusual sterols* are the only extensive products of a large number of coral species. Particularly, the Alcyonarian corals of the genera *Sarcophyton*, *Sinularia*, and *Lobophytum* are very rich in sterols [7.24].

Soft (octo)corals (order *Alcyonacea*) and gorgonians (order *Gorgonacea*), with fewer examples from zoanthids (order *Zoantharia*) and sea pens (order *Pennatulacea*), have presented the majority of interesting metabolites. Of the six orders in the subclass *Octocorallia*, the *Alcyonacea*, which are fleshy soft corals, and *Gorgonaceae* (sea fans and sea whips) are by far the most widely distributed and studied. In *Alcyonaceae*, *Alcyoniidae* and *Nephtheidae* are the largest families. *Alcyonaria* possess a diverse array of chemical compounds and are particularly rich in isoprenoids, including terpenes, carotenoids, and steroids [7.25–27]; hence, they were one of the first marine groups to be systematically screened for secondary metabolites [7.28]. Each species of octocorals seems to have its own specific set of compounds [7.29].

Potential therapeutics identified in soft corals include anticancer agents, immunomodulators, and useful antifouling agents. The striking presence of cembranoid diterpenes in soft corals outnumbers the occasional occurrence in other taxa, which are also interesting in terms of pharmacological research. They show significant biological activity, including antimicrobial,

Ca-antagonistic and anti-inflammatory properties; the antitumoral effect of cembranes is, however, the most important activity. The ability of soft corals to produce a wide array of diversified cembranoid structures is remarkable (this is reviewed in [7.30]). Furthermore, a particular class of cembranes, namely furanocembranolides are exclusive to octocorals, which were found only in the families *Gorgoniidae* and *Alcyonidae* (see [7.31, 32] and earlier reviews referred to therein). The concentrations of various secondary metabolites vary in different genera of octocorals and hard coral species; therefore, this review first introduces the richest, most studied genera, for which potential pharmaceutical properties have been reported.

7.2.1 Family *Alcyoniidae* (Subclass *Octocorallia*)

Genus *Sinularia*

Extensive studies on the *Sinularia* revealed that about 60% of this genus contains toxins [7.35]; it is a rich source of cembranoid diterpenes and shows a large variation in chemical elaboration [7.11, 24, 36]. Secondary metabolites of *S. flexibilis* marked the presence of terpenoids associated with the following bioactivities: antifouling [7.37, 38], antimicrobial [7.39, 40], cytotoxicity [7.17, 41], and feeding deterrence [7.42]. Of these compounds, sinulariolide, flexibilide, and dihydroflexibilide are the most significant, whose concentrations vary depending upon the site of collection [7.37]. They ranged from 7.5–32.0 mg terpenoid/g tissue (0.75–3.2% dry mass, mean = 1.7%) in the field (see [7.37]). Sinulariolide and flexibilide showed marked antimicrobial activity and inhibited growth of Gram-positive bacteria at effective concentrations as low as 5 and 10 ppm, respectively; hence, they show potential as antibiotics [7.17]. The anti-inflammatory potency of flexibilide was stated to be similar to phenylbutazone [7.43]. One metabolite of low concentration in *S. flexibilis*, 7,8-deoxyflexibilide, was responsible for ichthyotoxicity against the Japanese medaka fish (*Oryzias latipes*: [7.44]). Diterpene cembranoids, sinularin and its dihydro congener, obtained from *S. flexibilis* were found to be effective (ED_{50} = 0.3–16 $\mu g\,mL^{-1}$) in the NCI's screens of potential anticancer agents [7.41]. 11–Episinulariolide from *S. flexibilis* exhibited strong algacidal properties [7.38]. *S. flexibilis* also yielded cembrane-type diterpenoids, sinulaflexiolides A–K, among which sinulaflexiolides D and E showed selective inhibitory activity against gastric gland carcinoma cell line BGC-823 at 8.5 and 0.12 μM, respectively [7.45].

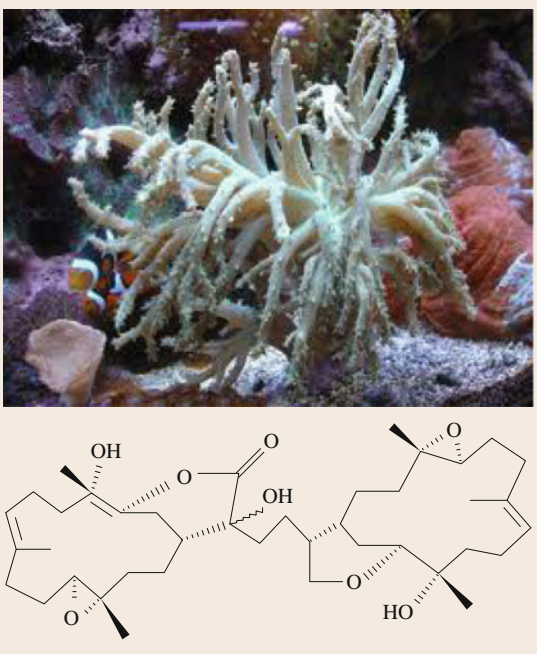
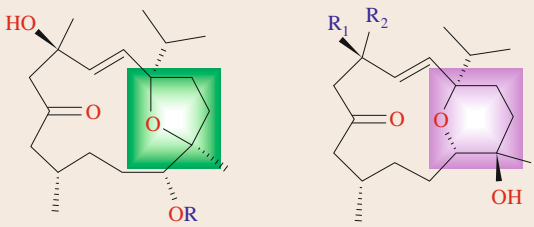


Fig. 7.1 *Sinularia flexibilis* (after [7.33]) and one of its terpenes, sinuflexlin (after [7.34])

The cembranoid sinulariol D obtained from *Sinularia* sp. [7.46] was mildly cytotoxic (reviewed by [7.47]). A sesquiterpene furanoic acid isolated from *Sinularia* sp. was found to inactivate bee venom, phospholipase AZ (bvPLA) in vitro [7.48]. *S. ovispiculata* from Andaman and Nicobar Islands (India) presented a cytotoxic sterol [7.49]. A study of *S. gardineri* from the Red Sea revealed a heptacyclic norcembranoid dimer singardin, which showed cytotoxicity to murine leukemia (P-388), human lung carcinoma (A-549), human colon carcinoma (HT-29), and human melanoma cells (MEL-28) [7.50]. A cembranolide, capillolide, from the Chinese *S. microclavata* exhibited potent cytotoxic activity against tumor cell lines (A-549) with an IC_{50} value of 0.5 $\mu g\,mL^{-1}$; *S. microclavata* also yielded microclavatin with cytotoxicity against tumor cell lines KB and MCF (Michigan Cancer Foundation-7) with IC_{50} values of 5.0 and 20.0 $\mu g\,mL^{-1}$ [7.51]. A cytotoxic lobane diterpene, ineleganene, was isolated from the Formosan *S. inelegans* [7.52]. Three cytotoxic acylspermidines were reported from a Japanese *Sinularia* sp. [7.53]. Extract of an Okinawan *Sinularia* sp., containing norcembrenolide and sinuleptolide, demonstrated potent inhibition of tumor necro-



2 R = Ac
3 R = CHO

4 R_1 = OH, R_2 = CH_3
5 R_1 = CH_3, R_2 = OH

Fig. 7.2 *Sinularia crassa* and one of its cembranoids (after [7.58])

sis factor-α (TNF-α) production dose-dependently (IC_{50} = 20 and 15 $\mu g\,mL^{-1}$, respectively); it showed a more potent effect than prednisolone at a concentration of 33 $\mu g\,mL^{-1}$ [7.54]. β-Caryophyllene-type terpenoids from the Taiwanese *S. nanolobata* presented cytotoxicity against the growth of a limited panel of cancer cell lines [7.55]. Scabrolides, as two epimeric norditerpenoids, isolated from the Taiwanese *S. scabra*, were found to show significant cytotoxicity against KB and Hepa59T/VGH cancer cell lines (ED_{50}s 2.3–2.6 $\mu g\,mL^{-1}$) [7.10]. The sphingosine derivative from *S. crassa* [7.56] produced maximum anti-inflammatory effect at a dose of 10 $mg\,kg^{-1}$, which was comparable to that of indomethacin (2 $mg\,kg^{-1}$). *S. crassa* also yielded crassarosterol A and four steroidal glycosides, crassarosterosides A–D, which significantly inhibited the expression of pro-inflammatory iNOS protein at 10 μM, and some also showed cytotoxicity toward selected human liver cancer cells [7.57].

Sinularolides from *S. gibberosa* exhibited cytotoxicity against HL-60, Hela, BGC-823, MDA-MB-435, Bel-7402, and PC-3MIE8 tumor cell lines [7.36]; *S. gibberosa* also produced sesquiterpene peroxygibberol, which showed mild cytotoxicity [7.59]. Gibberoketosterol from *S. gibberosa* of Taiwan was shown to significantly inhibit the up-regulation of the pro-inflammatory proteins of macrophage cells [7.60, 61]. Novel norditerpenoids and diterpenoids extracted from the Formosan *S. gibberosa* showed weak cytotoxicity in vitro toward a few cancer cell lines [7.62]. *S. firma* from coastal Indian Ocean waters yielded two glycosides named Firmacosides A and B, previously found to possess significant antibacterial activity [7.63]. Two polyhydroxylated steroids from a Formosan *Sinularia* sp. showed significant inhibition of the accumulation of the pro-inflammatory COX-2 protein of RAW264.7 macrophage cells at 10 μM [7.64]. Five novel cytotoxic steroids, and four new hydroxycembranes isolated from the Formosan *S. facile*; one of the hydroxycembranes exhibited cytotoxicity toward Hep G2 cancer cell line with IC_{50} value of 12.9 $\mu g\,mL^{-1}$ [7.65, 66]. Anti-inflammatory cembranoids querciformolides A–D and granosolides A and B were reported from *S. quercifformis* and *S. granosa* [7.67]. *S. gyrosa* yielded three diterpenoids, gyrosanols A–C, with antiviral activity against HCMV (human cytomegalovirus) at IC_{50}s of 2.6 and 3.7 μM; the compounds also showed significant anti-inflammatory activity by reducing the levels of the COX-2 protein in RAW 264.7 macrophages [7.68]. Reference [7.69] reviewed the latest progress in the chemistry and pharmacological activities of terpenoids from *Sinularia* soft corals providing a perspective on future areas of research interest.

Genus *Sarcophyton*

Two cembranoids isolated from the hexane extract of the *Sarcophyton trocheliophorum* (Pacific Ocean) were cytotoxic to Ehrlich ascites tumor cells [7.71]. A new macrocyclic diterpenoid lactone, sartrochine, was also reported from *S. trocheliophorum*, which showed cytotoxic activity against S180 cells and an antibiotic effect on *Streptococcus* [7.72]. A sarcophine-related cembranoid from an Australian *Sarcophyton* species showed convulsant activity [7.73]. A cembranoid diterpene possessing antifouling activity was reported from a Thai *Sarcophyton* sp. [7.74]. Two new cytotoxic tetraterpenes, methylsarcophytotate and methylchlorosarcophytoate, were isolated from a species in Japanese seawater [7.75]. Reference [7.76] deals with a discovery of tubulin stabilizing activity of sarcodictyin A derived from a Mediterranean stolonifer *Sarcodictyon roseum*; accordingly, this compound was re-

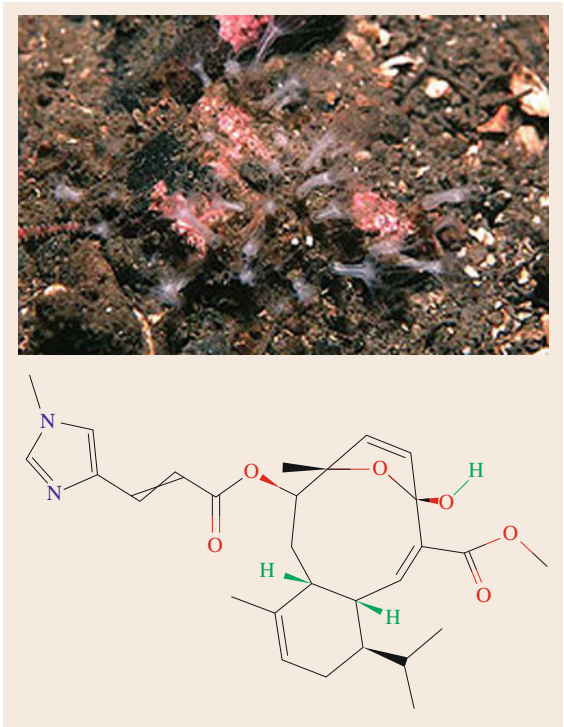


Fig. 7.3 Sarcodictyin A and *Sarcodictyon roseum* (after [7.70])

ported to be in preclinical development [7.77]. However, sarcodictyin showed relatively weak antiproliferative activity (e.g., the IC_{50} values for growth inhibition of the human ovarian carcinoma cell line 1A9 were 300 nM for both sarcodictyin A and B versus 4 nM for taxol; [7.78]. The Australian *S. crassocaule* contained cembranoids, sarcophine and sarcophytoxide, which were released into the seawater as allelochemicals [7.35]. Three cytotoxic cembrenolide diterpenes, sarcocrassolide, crassolide, and 13-acetoxysarcocrassolide, with two cytotoxic steroids were isolated from the Formosan *S. crassocaule* [7.79]. The cytotoxicity of crassocolides A–M from a Taiwanese *S. crassocaule* was determined against a limited panel of cancer cells [7.80, 81]. Sarcophytol A (from *S. glaucum*), due to its cancer preventive activity, was extensively studied by the National Cancer Institute at preclinical trials level [7.82]. Sarcophytol A and its analogs isolated from *S. glaucum* were shown to inhibit the development of large bowel cancer in female rats, to suppress carcinogenesis in liver, breast, thymus, and skin in mice, and to prevent skin cancer [7.83, 84]. Sarcophine, obtained from red *S. glaucum* has been investigated since 1998 for its potential as a chemopreventive, cytotoxic, and antimicrobial agent, as well as competitive cholinesterase inhibitor, noncompetitive phosphofructokinase inhibitor, and a Na^+, K^+-ATPase inhibitor [7.85]. A study on *S. glaucum* resulted in 7β-acetoxy-8α-hydroxydeepoxysarcophine showing cytotoxicity against HepG2 (Hepatoma Growth2), HCT-116 (human colorectal tumor), and HeLa (Henrietta Lacks) cells with IC_{50} values of 3.6, 2.3, and 6.7 $\mu g\,mL^{-1}$, respectively [7.86]. Sarcotriol is a derivative of sarcophine, which displayed a potent chemopreventive activity against skin cancer [7.87]. Three furano-cembranoids and sarcoglaucol from *S. cherbonnieri* collected from the Great Barrier Reef [7.88] were found to be cytotoxic towards several tumor cell lines (IC_{50} values ranged from 0.15 to 8.6 $\mu g\,mL^{-1}$). Moreover, two tetraterpenoids, methyltortuoates A and B from the soft coral *S. tortuosum* showed cytotoxicity on human nasophyringeal carcinoma (CNE-2) and murine lymphocytic leukemia (P-388) tumor cell lines [7.89]. 7β-hydroxy-8α-methoxydeepoxysarcophytoxide reported from the Vietnamese *S. mililatensis*, was found to stimulate the growth of preosteoblastic MC3T3-E1 cells, suggesting the compound to be a potential cure for osteoporosis [7.90]. Sarcostolide E extracted from *S. stolidotum* [7.91] exhibited weak to moderate cytotoxic activity against human WiDr and Daoy tumor cell lines.

Genus *Lobophytom*

Lobophytum depressum from the Red Sea is one of the earliest corals studied [7.92]. Denticulatolide, an ichthyotoxic cembranoid diterpene, was isolated from the *L. denticulatum* [7.93]. Later, [7.94] presented evidence that extract of the Indian *L. strictum* showed promising hypotensive activity in *in vitro* tests (25 $mg\,kg^{-1}$, 27–30 min). Subsequently, it was reported in [7.95] that one of the two amine chlorides from the same species exhibited hypotensive and vasodilator activities. A cembranolide diterpene from *L. cristagalli* was identified as a potent (IC_{50} 5.3 μM) inhibitor of farnesyl protein transferase [7.96]. A sterol that was active against human ovarian tumor and human leukemia cell lines was isolated from an Indonesian *Lobophytum* sp. [7.97]. A cembrenoid diterpene, lobohedleolide, derived from *Lobophytum* sp. [7.56] produced maximum anti-inflammatory effect at a dose of 10 $mg\,kg^{-1}$, which was comparable to that of indomethacin (2 $mg\,kg^{-1}$). Lobohedleolide was also reported to have cytotoxic [7.98] and anti-HIV activities (EC_{50} approximately 3–5 $\mu g\,mL^{-1}$) [7.99]. Ex-

tracts obtained from *L. crassum* indicated ceramideas, a moderately antibacterial component [7.100]. The Formosan *L. crassum* yielded three glycolipids, all of which exhibited cytotoxicity against the growth of cancer cells of HepG2, Hep3B (human liver carcinoma), MDA-MB-231 (human breast carcinoma), and Ca9-22 (human gingival carcinoma) with IC_{50}s from 9.2 to 15 $\mu g\,mL^{-1}$ [7.101]. In [7.102] crassumolides A and B and D–F, which were cytotoxic toward Ca9-22 cancer cells, and broadly cytotoxic toward all six test cancer cell lines used were also isolated. *L. crassum* also produced a cembrane-type diterpenoid, lobocrassin F, which displayed a significant inhibitory effect on the release of elastase by human neutrophils [7.103]. Investigations on the *L. durum* [7.104] led to the isolation of three hemiketal cembranolides, durumhemiketalolides A–C with anti-inflammatory activities. Moreover, *L. durum* succumbed anti-inflammatory durumolides F–L (10 μM), which significantly reduced the levels of the iNOS and COX-2 proteins, and also exhibited weak antibacterial activity against *Salmonella enteritidis* [7.105].

Recently, six new cytotoxic cembranolides from *L. michaelae* were isolated and their anti-HCMV activity as well as cytotoxicity against selected cell lines were evaluated [7.106].

Genus *Eleutherobia*

Anti-inflammatory activity was observed for three related xenicane diterpenes, 9-deacetoxy-14,15-deepoxyxeniculin isolated from specimens of the South African octocoral, *Eleutherobia aurea* [7.107]. In reference [7.108] initially isolated eleutherobin, a tricyclic diterpene from the Australian soft coral *Eleutherobia* sp. was reported. Later, in reference [7.109] it was shown that eleutherobin was a potent inducer of tubulin polymerization *in vitro*. Eleutherobin showed selective cytotoxicity toward breast, renal, ovarian, and lung cancer cell lines and was found to be generally more inhibitory than sarcodictyin A (reviewed in [7.110]). Treatment of cancer cells with eleutherobin produced all the characteristic effects associated with those observed with taxol (reviewed by [7.111]. The Carribbean octocoral *Erythropodium caribaeorum* (family Anthothelidae) and the soft coral *Bellonella albiflora* (family Alcyoniidae) provided alternative sources for eleutherobin [7.112, 113].

Genus *Alcyonium*

The valdivones, anti-inflammatory diterpene esters, from the South African *Alcyonium valdivae* were introduced by [7.114]. *A. fauri* from the southeast coast of southern Africa yielded a sesquiterpene, hydroquinone rietone, which exhibited moderate activity in the NCI's in vitro anti-HIV bioassays [7.115]. *A. patagonicum* from the South China Sea succumbed a sterol cytotoxic against the P-388 cell line with an IC_{50} value of 1 $\mu g\,mL^{-1}$ [7.116]. Identified steroids in the Korean *A. gracillimum* exhibited moderate cytotoxicity and antiviral activity [7.117]; its glycoside was found to be toxic to the larvae of *Balanus amphitrite* [7.118].

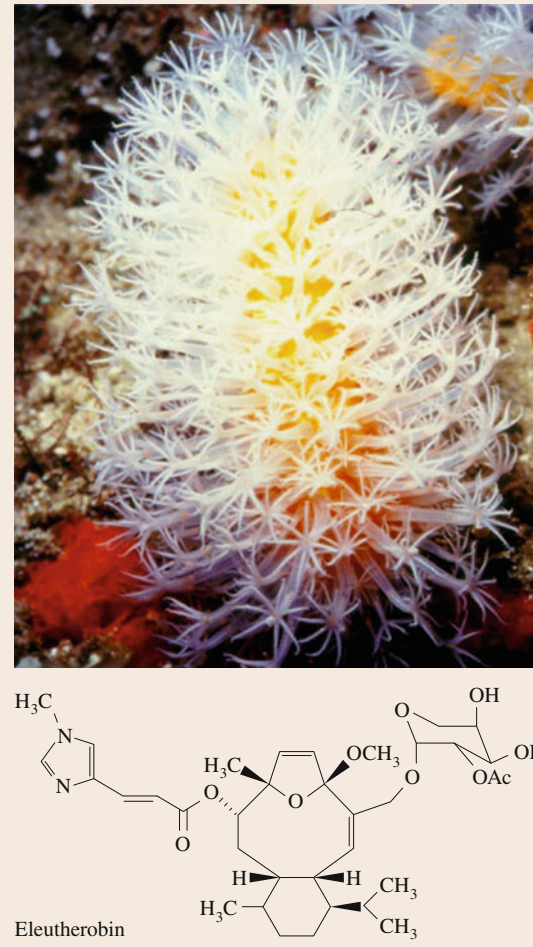


Fig. 7.4 *Eleutherobia* sp. and its tricyclic diterpene eleutherobin (after [7.70])

Genus *Cladiella* (= *Microspiculariа*)

Cladiella sp. surrendered a new dienone sterol, named cladiellin A, with antioxidant activity [7.119]. An aus-

tralins A–D isolated from Taiwanese *C. australis* was mildly cytotoxic [7.59]. Most diterpenoids, hirsutalins A–H, isolated from *C. hirsuta* exhibited cytotoxicity toward several cancer cell lines with two hirsutalins being significantly anti-inflammatory in vitro [7.120]. An Indonesian *Cladiella* sp. produced cladielloides A and B; the latter exhibited moderate cytotoxicity toward CCRF-CEM (Human Caucasian acute lymphoblastic leukaemia) tumor cells as well as significant inhibitory effects on superoxide anion generation and elastase release by human neutrophils [7.121]. From the same species, cladieunicellin H was purified with moderate inhibitory effects on the generation of superoxide anion and the release of elastase by human neutrophils [7.122].

Genus *Klyxum*

Nine diterpenoids, simplexins A–I, from *Klyxum simplex* were found to be cytotoxic toward a limited panel of cancer cell lines, or to significantly inhibit the accumulation of the pro-inflammatory iNOS and COX-2 proteins in RAW264.7 macrophage cells [7.123]. Three novel diterpenoids, namely klysimplexin sulfoxides A–C, were isolated from the cultured soft coral *K. simplex*, which significantly inhibited the accumulation of the pro-inflammatory iNOS protein in RAW264.7 macrophage cells [7.124]. Diterpenoids from Taiwanese *K. molle* named klymollins A–H displayed a similar activity together with significant in vitro anti-inflammatory activity by inhibiting the expression of the iNOS protein [7.125].

Genus *Paraminabea*

The Formosan *Paraminabea acronocephala* yielded withanolides, paraminabeolides A–F along with minabeolides, which were either cytotoxic toward Hep G2 cancer cells, or significantly inhibited the accumulation of the pro-inflammatory iNOS protein, or could effectively reduce the expression of COX-2 protein [7.126].

Genus *Parerythropodium*

Extracts of the Red Sea *Parerythropodium fulvum fulvum* exhibited antimicrobial activity against several co-occurring and potentially pathogenic marine bacteria [7.40].

Genus *Anthomastus*

3-oxo-sterols were reported as moderately cytotoxic metabolites of Antarctic *Anthomastus bathyproctus* [7.127].

Genus *Bellonella*

The cytotoxic diterpene sarcodictyin A, which was reported in the stoloniferan soft coral *Sarcodictyon roseum* [7.76], was also isolated from *Bellonella albiflora* [7.113]. Sarcodictyin A showed strong cytotoxicity against HeLa human cervix cells.

7.2.2 Family *Anthothelidae*

Genus *Erythropodium*

The Caribbean octocoral *Erythropodium caribaeorum* provided methyl-caribaeorane, which was active in a cell-based assay for antimitotic activity at 10 mM [7.112].

7.2.3 Family *Gorgoniidae*

Both soft corals and gorgonians are abundant in tropical reef habitats, with the Indo-Pacific being dominated by soft corals and the western Atlantic reefs by gorgonians. Both are prolific producers of terpenoids, especially diterpenes [7.128]. Gorgonians (horny corals) from the Caribbean were the first octocorals studied for antimicrobial activity of corals [7.129]. In the late 1960s and early 1970s, it was discovered that some gorgonians contain large quantities of prostaglandins rendering, in part, the rapid growth of the field of marine natural products. The corals that were found to possess antibacterial activity were *Antillogorgia turgida*, *A. americana*, *Rhipidogorgia flabellum*, *Briareum asbestinum*, *Plexaura homomalla*, *P. dichotoma*, and *Plexauropsis crassa*. In reference [7.27] the absolute configuration and species distribution of 28 terpenoid compounds isolated from 19 species of gorgonians were described. Over 200 novel secondary metabolites isolated from gorgonians were discussed in [7.25], for many of which the pharmacological effects were described in [7.130]. Extracts from eight coral species from San Blas Islands, Panama, [7.131] displayed intermediate levels of antimicrobial activity against five marine and nonmarine bacteria. It was found that nonpolar fractions were much more active than polar fractions. Extracts of several species of 39 Caribbean gorgonians were broadly studied against 15 strains of marine bacteria, including marine opportunistic pathogens, and strains isolated from healthy and decayed gorgonians inhibited the growth of bacteria [7.132], hence, as the source of a number of bioactive terpenoids. Nine of ten gorgonian extracts from the four families tested showed antimicrobial activity against Gram-positive bacteria [7.133]. These corals, therefore, maintain a wealth of novel

Fig. 7.5 *Pseudopterogorgia elizabethae* and its major diterpene pseudopterosin A (courtesy of NSF (Natural Products Science), Oregon State University)

structures with potentially useful biological activities, from which 602 new compounds have been reviewed by [7.134].

Genus *Pseudopterogorgia*

The genus *Pseudopterogorgia* was considered as one of the most chemically defended among gorgonians. This ability seems to derive from high concentrations of secosteroids present in these gorgonians [7.25]. The anti-inflammatory potential of pseudopterosins was found to be superior to that of standard drugs such as indomethacin [7.135]. The extracts from *P. elizabethae* (containing pseudopterosins) and *Eunicea fusca* (containing fucoside-A or fuscol) could possibly be used in cosmetic industries [7.136–138]. A semisynthetic pseudopterosin derivative, methopterosin, also displayed anti-inflammatory activity [7.139], hence it has successfully completed Phase II clinical trials as a topical anti-inflammatory agent [7.140]. Therefore, and because the pseudopterosins are potent anti-inflammatory and analgesic agents (ED_{50} ca. $3.0\,mg\,kg^{-1}$ [7.141]), they have been licensed to a small pharmaceutical firm for medical use as potential anti-inflammatory drugs [7.142]. A furanogermacrene isolated from the *P. americana* inhibited both diatoms *Nitzchia salinicola* and *Nitzchia* spp. at naturally occurring concentrations in in situ experiments [7.143]. Up until 1999, 12 different congeners, pseudopterosins A–L, were reported in *P. elisabethae* from various geographic locations around the West Indian region [7.141]; pseudopterosins A–D were reported as the primary metabolites in their collections of *P. elisabethae* from the Bahamas, being major metabolites in the gorgonian; pseudopterosins C comprised as much as 7.5% of the lipid extract. Furthermore, pseudopteroxazole, a benzoxazole diterpene alkaloid isolated from the West Indian *P. elisabethae*, induced 97% growth inhibition for *Mycobacterium tuberculosis* H37Rv at a concentration of $12.5\,g\,mL^{-1}$ [7.144]. In reference [7.145] the synthesis of elisabethatriene, a diterpene cyclase, from *P. elisabethae*, was reported. Similarly, ergorgiaene, a diterpene (also known as biflorane) was isolated from the hexane extract of the same West Indian gorgonian, which induced 96% growth inhibition for *M. tuberculosis* H37Rv at a concentration of $12.5\,g\,mL^{-1}$ [7.146]. A partially purified extract of *P. elisabethae*, containing pseudopterosin E, is being used as an additive in a cosmetic preparation, a face cream under the brand name Resilience (see the review in [7.30]). In reference [7.142] elisabethin H from the southwestern Caribbean *P. elisabethae* with anti-inflammatory properties was further isolatedİt was also demonstrated that the compounds isolated from *P. elisabethae*, collected in the southwestern Caribbean, were promising molecules with an anti-inflammatory activity both in vivo and in vitro [7.147].

An analog of lophotoxin (from the genus *Lophogorgia*), bipinnatin-B, isolated from the gorgonian *P. bipinnata* [7.148] was found to be a potent neurotoxin (at concentrations of $10\,\mu M$); *P. bipinnata* also produced caucanolides A–F, which exhibited mild activity towards *Plasmodium falciparum*, and cytotoxicity towards a panel of tumor cell lines [7.149]. Providencin and kallolide A were isolated from the West Indian gorgonian *P. kallos*; providencin showed moderate anticancer activity against human breast (MCF7), lung (NCI-H460), and CNS (SF-268) cancer cell lines [7.150]. This octocoral also yielded seven cembranolides, including bipinnatin Q, which displayed significant cytotoxic activity against the NCI (Na-

tional Cancer Institute) tumor cells MCF breast cancer, NCI-H460 nonsmall cell lung cancer, and SF-268 CNS cancer [7.151]. In reference [7.150] the isolation of the structurally intriguing diterpenes providencin, intricarene, and bielschowskysin from *P. kallos* was also reported. Bielschowskysin exhibited strong activity against certain human cancer cell lines and was also effective against *Plasmodium falciparum*, the cause of malaria [7.152].

Pseudopterolide, an unusual diterpene from the gorgonian *P. acerosa* showed unusual cytotoxic properties [7.155]. The genus *pseudopterogorgia* also yielded pseudopetrocin-E, a tricyclic diterpene pentoside with anti-inflammatory and analgesic activities equal in potency to industrial standard indomethicine [7.156].

Genus *Pterogorgia*

Pterogorgia guadalupensis was reported to contain a cristaline lactone that exhibited mild antibiotic activity against bacterial species [7.157].

Genus *Gorgonia*

Crude extracts from the sea fan coral *Gorgonia ventalina* showed antibacterial activity against the bacterium *Listonella anguillarum* [7.158]. In reference [7.159] exochitinase activity against the fungal pathogen, *Aspergillus sydowii* in *G. ventalina* from the Florida Keys was also explored.

Genus *Eunicella*

Spongouridine previously isolated from the sponge *Cryptotethia crypta* and found to possess antiviral activity, was also isolated from the gorgonian *Eunicella cavolini* (reviewed in [7.160]). *E. verrucosa* from Spain gave the cytotoxic verrucoside [7.161].

7.2.4 *Subergorgiidae*

Genus *Subergorgia*

The gorgonian coral *Subergorgia suberosa* occurs widely in Indo-Pacific waters. Subergorgic acid, a cardiotoxin obtained from the Pacific *S. suberosa* [7.162] inhibited neuromuscular transmission at $0.16\,\mu g\,mL^{-1}$ in isolated guinea-pig heart assay. A further study of *S. suberosa* yielded the sesquiterpene suberosols A–D as well as a subergane-based sesquiterpene, subergorgiol, which exhibited cytotoxicity towards the P-388 murine leukaemia and the A-549 and HT-29 tumor cell lines; subergorgiol was moderately cytotoxic against the growth of HeLa cancer cells [7.163, 164]. A sesquiterpene alkaloid, 6-(9′-purine-6′,8′-diolyl)-2β-suberosanone, was also reported from *S. suberosa* as a mildly cytotoxic metabolite ($IC_{50} = 8.87\,\mu g\,mL^{-1}$ [7.165]. Reference [7.166] reported that the most potent antifoulant produced by *S. suberosa* was subergorgic acid.

7.2.5 Family *Briareidae*

Genus *Briareum (= Solenopodium)*

Solenolides as anti-inflammatory and antiviral diterpenoids were introduced from the gorgonian *Briareum* (*Solenopodium*) sp. [7.167]. A study of *B. excavatum* resulted in briantheln A [7.168], which was shown to reverse multidrug resistance in human carcinomas, and in briaexcavatolides, one of which shown to exhibit significant cytotoxicity toward P-388 and HT-29 cancer cells [7.169]. A diterpenoid, excavatoid L, from the cultured *B. excavatum* moderately inhibited superoxide anion generation and elastase release by human neutrophils [7.170]. Violides O, briarlides I–R and briviolides derived from *Briareum* sp. showed mild cytotoxicity toward Vero and MDCK cells [7.171, 172]. In addition, diterpenes briarenol A and briaranolides A–J were isolated from Taiwanese and Okinawan collections of *Briareum* sp., respectively [7.173, 174]. Very recently, briarenolide F from *Briareum* sp. was reported to show significant in vitro anti-inflammatory effect on the generation of superoxide anions by human neutrophils [7.175].

7.2.6 Family *Plexauridae*

Genus *Pseudoplexaura*

Horny gorgonians *Pseudoplexaura porosa*, *P. wagenaari*, *P. flagellosa*, and *P. crucis* provided a lactonic cembrane diterpene, the antineoplastic crassin acetate, which is reported to be toxic to *Entamoeba histolytica* at $20\,\mu g\,mL^{-1}$ in vitro [7.176]. Crassin acetate may constitute as much as 1.5% of the dry weight of the cortex of *P. porosa* [7.177], which was cytotoxic ($1-10\,\mu g\,mL^{-1}$) to mouse fibroblasts and human leukemic and HeLa cells in vitro [7.178]. Both crassin acetate and eunicin also inhibited the growth of *Clostridium feseri* and *Staphylococcus* species [7.179]. Furthermore, two cytotoxic antitumor cembranes, named 14-deoxycrassin and pseudoplexaurol were isolated from the Caribbean *P. porosa* [7.180].

Genus *Plexaura*

Two prostaglandins, 15-epi-PGA2 and its diester and PGA2α were isolated from the air-dried cortex of the

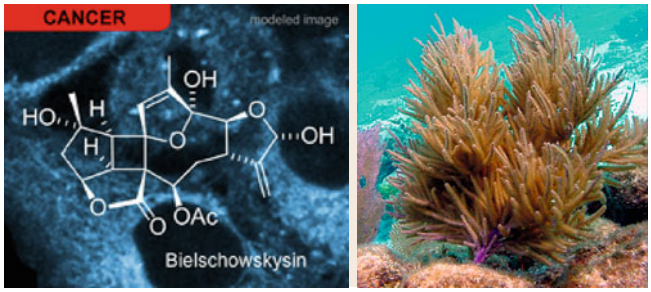


Fig. 7.6 Bielschowskysin, a diterpene isolated from the Caribbean gorgonian octocoral *Pseudopterogorgia kallos* (after [7.153, 154])

Caribbean gorgonian *Plexaura homomalla* (Esper) in high yields of 0.2 and 1.3%, respectively [7.16, 18]; hence, there is a great interest in *P. homomalla* as a potential commercial scale source of prostaglandins either by pruning in a natural way or by marine farming [7.19]. These findings stimulated a worldwide survey of prostaglandins in *Cnidaria* (formerly coelenterates), with an eye toward the commercial harvesting of these important metabolites from marine *Cnidaria*, resulting in extensive harvesting of wild corals. An antimalarial inhibitor of plasmepsin II (enzyme belonging to Plasmodium family) was detected in *P. homomalla*, which showed 5.9-fold specific inhibitory activity [7.181].

Genus *Eunicea*

A diterpene, eunicin exhibiting antibacterial activity has been isolated from the gorgonian *Eunicea mammosa* [7.182, 183]. Seven sesquiterpenoid metabolites including elemane, eudesmane, and germacrane types were reported as mildly antiplasmodial constituents of a Caribbean *Eunicea* species [7.184], which are among the most abundant octocorals of the region. Eupalmerin acetate, a novel anticancer agent from Caribbean gorgonians *E. succinea* and *E. mammosa* induced apoptosis in human malignant glioma cells ($IC_{50} = 5.1–6.9\,\mu mol\,L^{-1}$), hence it was suggested to be promising as a clinical anticancer agent [7.2]. A diterpene, fuscoside E, extracted from *E. fusca* showed strong anti-inflammatory in ear edema bioassay and also presented antifouling activity against bacterial strains [7.185]. A dilophol diterpene, eunicidiol, along with fuscol and eunicol were isolated from the crude extract of *E. fusca*, collected from Hillsboro Ledge, Florida. Topical application of a 100 µg/ear dose of these diterpenes significantly reduced ear edema by 44, 46, and 54%, respectively, which was superior to the effect of indomethacin, a known anti-inflammatory [7.186].

Genus *Euplexaura*

Guaiazulene from the gorgonian *Euplexaura erecta* [7.187] exhibited mild activity against *Pseudomonas aeruginosa*. New tetraprenylated purine alkaloids, nuttingins A–E, and new closely related malonganenones D–G were isolated from the gorgonian *E. nuttingi* collected on Pemba Island, Tanzania, displayed inhibitory activity against both K562 and UT7 tumor cell lines, and also induced apoptosis in transformed mammalian cells at a concentration of $1.25\,\mu g\,mL^{-1}$ [7.188]. *E. robusta* collected from Weizhou Island, China, yielded malonganenones I–K; the compounds showed moderate cytotoxicities against K562 and HeLa tumor cell lines with IC_{50} values ranging from 0.35–10.82 µM, and one with moderate inhibitory activity against c-Met kinase at a concentration of 10 µM [7.189].

Genus *Muricea*

Muricea species such as *M. californica* and *M. fruticosa* provided four new esterified aminogalactose saponins called muricins, which inhibited the growth of the diatom *Phaeodactylum tricornum* at 100 ppm concentrations, suggesting that it has a significant role in reducing fouling due to the diatoms [7.190]. From *M. austere* collected on the Pacific coast of Panama, eight compounds including three tyramine derivatives, two steroidal pregnane glycosides, and three sesquiterpenoids were isolated, and their antiprotozoal activities were evaluated in vitro against a drug-resistant *Plasmodium falciparum* and intracellular form of *Trypanosoma cruzi* [7.191].

Genus *Anthoplexaura*

The dimorphosides from the gorgonian *Anthoplexaura dimorpha* inhibited the development of fertilized eggs of sea urchins [7.192].

Genus *Paramuricea*

Linderazulene and two congeners were isolated from a deep-sea collection of the gorgonian *Paramuricea*

sp. in Curaçao and found to be mildly cytotoxic against the P388 murine leukemia cell line with IC_{50}s of 18.8, 2.7, and 15.6 $\mu g\,mL^{-1}$, respectively [7.193]. The Mediterranean gorgonian *P. clavata* resulted in two new alkaloids bufotenine and 1,3,7-trimethylisoguanine, which showed significant antiadhesion activity against one bacterial strain while being nontoxic [7.194].

Genus *Plexaurella*

Plexaurella grisea from the Caribbean (Dominican Republic) produced sterols and sesquiterpenes with selective activity against the HT 29 ($ED_{50} = 0.1\,\mu g\,mL^{-1}$) and P-388 tumor cell lines, respectively [7.195, 196].

Genus *Echinomuricea*

A xanthine derivative, named caffeine, isolated from the gorgonian *Echinomuricea spendens* displayed mild antifouling activity against marine fouling organisms [7.197]. A new halimane-type diterpenoid, echinohalimane A, was isolated from *Echinomuricea* sp.; the compound exhibited cytotoxicity toward various tumor cells and displayed an inhibitory effect on the release of elastase by human neutrophils [7.198]. Echinoclerodane A from *Echinomuricea* sp. exhibited moderate cytotoxicity toward MOLT-4 (molybdate uptake transporter), HL-60, DLD-1 (dihydrolipoamide dehydrogenase), and LoVo (human colon adenocarcinoma) tumor cells, and inhibitory effects on the generation of superoxide anions and the release of elastase by human neutrophils [7.199].

Genus *Calicogorgia*

Calciferols first reported from Japanese *Calicogorgia* sp. Calciferols A and B were found to be toxic to brine shrimp; calciferol D exhibited potent antiviral activity and calciferols F–I showed cytotoxicity and PLA2 inhibitory behavior [7.117, 200].

Genus *Menella*

A south China Sea gorgonian, *Menella* sp., resulted in menellins A–C, which exhibited modest anti-inflammatory inhibition effects against RAW264.7 macrophages with IC_{50} of 71.3, 33.9 μM [7.201]. A sesquiterpenoid, menelloide A, isolated from *Menella* sp. inhibited generation of superoxide anions by human neutrophils [7.202].

7.2.7 Family *Acanthogorgiidae*

Genus *Astrogorgia*

Astrogorgiadiol from *Astrogorgia* sp. inhibited cell division in starfish eggs [7.203]. Five compounds of astrogorgols A–N from Chinese *Astrogorgia* sp. showed significant inhibitory activities against human tumor related protein kinases, including ALK, AXL, FAK, IGF-1R, MET wt, SRC, and VEGF-R2 [7.204]. Twelve new eunicellin-based diterpenoids, astrogorgins B–M, were isolated from the Chinese gorgonian *Astrogorgia* sp., among which significant antifouling activity was observed for 14-deacetoxycalicophirin B against the larval settlement of the barnacle *Balanus amphitrite* at nontoxic concentrations with an $EC_{50} = 0.59\,\mu g\,mL^{-1}$, while the other analogs were effective within an EC_{50} range of 5.14–17.8 $\mu g\,mL^{-1}$ [7.205].

Genus *Acalycigorgia*

Xenicane diterpenoids including acalycixeniolide H and D–G, and five further xenicanes isolated from the gorgonian, *Acalycigorgia inermis* inhibited cell division of fertilized starfish eggs and significant cytotoxicity against a human leukemia cell line [7.192, 206, 207].

Genus *Muricella*

A sterol, calicoferol D, from the Korean gorgonian *Muricella* sp. showed antiviral activity and brine shrimp lethality [7.117, 200].

7.2.8 Family *Nephtheidae*

Genus *Nephthea*

Cytotoxic sterols were isolated from the soft coral *Nephthea erecta* [7.208]. Another Formosan *N. brassica* yielded two new cytotoxic cembranoid diterpenes, brassicolide and brassicolide acetate; a new cytotoxic sesquiterpene, (−)-4*R*-*O*-acetyl-selin-11-en; and six cytotoxic terpenoids, (−)-selin-11-en-4*R*-ol, 2-hydroxynephthenol, nephthenol, cembrene A, epoxycembrene A, and (−)-β-elemene, all of which showed significant cytotoxicity in A549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), KB (human epidermoid carcinoma), and P-388 (mouse lymphocytic leukemia) [7.209]. An isolate from *Nephthea* sp. (called decaryiol) collected from the Fiji Islands was found to be cytotoxic towards several tumor cell lines with IC_{50} values ranging from 0.15–8.6 $\mu g\,mL^{-1}$ [7.88]. The Taiwanese *N. armata* yielded two new cytotoxic 19-oxygenated ergosterols, armatinols A and B [7.210].

Chabrolonaphthoquinone B, chabrolobenzoquinones E–H and chabrolohydroxybenzoquinones E–G were isolated as mildly cytotoxic constituents of *N. chabrolii* [7.211]. Furthermore, three new steroids, chabrolosteroids A and B, and C, were isolated from the Taiwanese *N. chabrolii* [7.212], from which chabrolosteroid B exhibited weak cytotoxicity toward the Hep 3B ($IC_{50} = 19.9\,\mu g\,mL^{-1}$) cancer cell line. The diterpene pacificins, isolated from Taiwanese *N. pacifica* were mildly cytotoxic [7.213]. The extract of the Formosan *Stereonephthya crystalliana* showed significant cytotoxicity to A549, HT-29, and P-388 cell cultures [7.214]. A novel unusual pentacyclic hemiacetal sterol, nephthoacetal, was isolated from *Nephthea* sp.; it exhibited a significant inhibitory effect with an EC_{50} value of $2.5\,\mu g\,mL^{-1}$ and moderate cytotoxicity against HeLa cells with IC_{50} values of $12.3\,\mu g\,mL^{-1}$ [7.215].

Genus *Dendronephthya (= Spongodes)*

Isogosterones found in *Dendronephthya* sp. were reported to inhibit larvae settlement at low concentrations (ca. $2.2\,\mu g\,mL^{-1}$), which makes them promising as nontoxic antifoulants [7.118]. Dendronesterol B from the Japanese *D. gigantia* was shown to be mildly cytotoxic [7.216]. Tissue extracts of *Dendronephthya* sp. showed antibacterial activity against epibiont bacteria [7.217]. Two steroids, 3-oxocholest-1,22-dien-12β-ol and 3-oxocholest-1,4-dien-20β-ol from *D. gigantea* showed notable inhibitory activity against farnesoid X-activated receptors (FXR) with IC_{50}s of 14 and 15 µM [7.218]. The Chinese *Spongodes* (*Dendronephthya*) sp. yielded a steroid, methyl spongoate, found to have cytotoxicity against BEL-7402 at a moderately low concentration [7.219]. The Chinese *Dendronephthya* sp. resulted in the isolation of 18 new cembranoid diterpenes, namely, dendronpholides A–R, along with 11-episinulariolide and an enantiomer of sandensolide; the cytotoxicity of several compounds against human tumor cell lines was also evaluated [7.220]. *D. griffini* yielded griffinisterones F–I, which were found to significantly inhibit the accumulation of the pro-inflammatory iNOS protein of the LPS-stimulated RAW264.7 macrophage cells at 10 µM [7.221].

Genus *Gersemia*

Gersemia fruticosa, a cold water species of the White Sea (Arctic Ocean), presented polar sterols, among which 9,11-secosterol was found to be endowed with cytotoxicity and 5,6-epoxy sterol was highly antiproliferative [7.222, 223]. Antibacterial effects were observed in the extract of sub-Arctic *G. rubiformis* [7.224]. The Antarctic *G. antarctjca* released metabolites containing a 1 : 1 mixture of homarine and trigonein that served antibacterial roles to the surrounding seawater [7.225].

Genus *Capnella*

Capnella thyrsoidea yielded a series of xenicane diterpenes, e.g., the anti-inflammatory tsitsixenicin A [7.226]. *C. imbricata* is known for producing a range of sesquiterpene alcohols known as capnellenes [7.227]. All the capnellene compounds identified by [7.228] were cytotoxic in all cell lines, including renal leiomyoblastoma, ovarian, promyelogenous leukaemia, and HL-60 as well as K562 leukaemia cancer cell lines (IC_{50} values 0.7–4500) with the greatest activity against K562 leukaemia. A study on the Formosan *C. imbricata* revealed three anti-inflammatory capnellenes and a calamenene, which showed more specific inhibition ($IC_{50} = 10\,\mu M$) against the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of LPS-stimulated RAW264.7 macrophage cells [7.229]. Capnellenes, GB9 and GB10, were also found to have antineuroinflammatory and antinociceptive properties in IFN-gamma-stimulated microglial cells and in neuropathic rats, respectively [7.230].

Genus *Litophyton*

Litosterol, a C-19 hydroxysteroid, was isolated from an Okinawan octocoral *Litophyton viridis*. It inhibited 90% of the growth of *M. tuberculosis* with an MIC (minimum inhibitory concentration) of $3.13\,\mu g\,mL^{-1}$ [7.231]. Litophynols A, B, and litophynins E, H, and I monoacetate isolated from the mucus secreted by *Litophyton* sp. were reported to possess hemolytic activity [7.232].

Genus *Lemnalia*

The Kenyan *Lemnalia flava* yielded lemnaflavoside and three monoacetate derivatives [7.233]. Lemnalol isolated from the Formosan *L. cervicorni* produced anti-inflammatory and analgesic effects in carrageenan-injected rats. As a potential therapeutic agent for neuropathic pain, lemnalol, in a single intrathecal administration (0.05–10 µg) was demonstrated to significantly attenuate thermal hyperalgesia and mechanical allodynia induced by chronic constriction injury (CCI) in a well-established rat model, 14 days postsurgery. Lemnalol (10 µg) also significantly inhibited CCI-induced

up-regulation of microglial and astrocytic immunohistochemical activation markers in the dorsal horn of the lumbar spinal cord. Additionally, intrathecal injection of lemnalol (10 μg) markedly inhibited spinal pro-inflammatory mediator tumor necrosis factor-α expression in microglial cells and astrocytes in neuropathic rats [7.234]. It was recently reported in reference [7.235] that treatment with lemnalol (intramuscular: 30 mg kg^{-1}, for 6–168 h) significantly attenuated mechanical allodynia, paw edema, and knee swelling induced by monosodium urate; the elevated expression of c-Fos and pro-inflammatory proteins observed in synovial tissue were also significantly inhibited by lemnalol administration rendering the compound a promising candidate for the development of a new treatment for gouty arthritis and other acute neutrophil-driven inflammatory diseases [7.235].

Genus *Scleronephthya*

Scleronephthya pallida from Thailand produced a fucoside steroid that exhibited moderate antimalarial activity and cytotoxicity [7.236].

7.2.9 Family *Clavulariidae*

Genus *Clavularia*

Kericembrenolides from *Clavularia koellikeri* were shown to display growth inhibition against B-16 melanoma cells (IC_{50}s of 1.2–3.8) [7.237]. A cembrane diterpene isolated from the Okinawan *C. koellikeri* [7.238] showed cytotoxic activity against human colorectal adenocarcinoma cells (DLD-1, IC_{50} 4.2 μg mL^{-1}) and strong growth inhibition against human T lymphocytic leukemia cells (MOLT-4, IC_{50} 0.9 μg mL^{-1}). *C. koellikeri* also yielded a new diterpenoid with modest growth-inhibition effect in vitro toward tumor cells [7.239]. Six cytotoxic dolabellane diterpenes were isolated from the Formosan soft coral *C. inflata*, and their cytotoxicity against selected cancer cells was measured in vitro [7.240]. Three cytotoxic prostanoids, claviridenone E–G, and three cytotoxic steroids, stoloniferone E–G, were isolated from the Formosan *C. viridis* [7.241]. A cytotoxic cembranoid, claviolide, was also isolated from the Formosan *C. violacea*, as reported in reference [7.241]. Clavubicyclone from *C. viridis*, a common western Pacific coral, exhibited mild cytotoxicity towards MCF-7 and OVCAR-3 tumor cell lines [7.242]. Extract of *C. viridis* further afforded seven new prostanoids [7.243], the pharmacological study of which revealed that bromovulone III and chlorovulone II exhibited the most promising cytotoxicity (0.5 μg mL^{-1}) against human prostate (PC-3) and colon (HT29) cancer cells. Antitumor and anticarcinogenic compounds were isolated from the symbiont of the Okinawan soft coral *C. viridis* and showed significant growth-inhibitory activity in vitro toward cancer cells [7.244].

Corals obviously produce various prostaglandins as an antipredatory defense [7.245]. Prostaglandins including chlorovolones, clavulones, claviridenones (antitumor prostanoids), and C-20 acetoxy clavulones were also reported to have been isolated from the stoloniferan *C. viridis*, which exhibited unique antineoplastic activity and were potent growth inhibitors in a variety of cultured cells; thus, they may represent a new chemical class of cancer therapeutics [7.246]. A prostanoid and steroids of *C. viridis* were also found to inhibit the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of LPS-stimulated RAW264.7 macrophage cells [7.227].

Genus *Telesto (= Carijoa)*

Diverse collections of *Carijoa* (*Telesto*) *riisei* produce a variety of natural products geographically. *C.* (*Telesto*) sp. presented extracts with high cytotoxic activity [7.12]. The Brazilian *C. riisei* yielded the known 18-acetoxypregna-1,4,20-trien-3-one, which displayed cytotoxic activity against the cancer cell lines SF295, MDA-MB435, HCT8, and HL60, as well as antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter* spp. and *Proteus* spp., all of which are resistant to antibiotics [7.247]. Unusual prostanoids named punaglandins with antiviral and anticancer activities (e.g., an antitumor that inhibits leukemia cell proliferation) from the octocoral *T.* (*Carijoa*) *riisei* have been reported [7.248]. A steroid (18-acetoxipregna-1,4,20-trien-3-one) isolated from *C. riisei* displayed a strong antileishmanial activity, with an IC_{50} value of 5.5 μg mL^{-1} against promastigotes and 16.88 μg mL^{-1} against intracellular amastigotes [7.249]; the antitrypanosomal activity of the steroid resulted in an IC_{50} value of 50.5 μg mL^{-1}. The steroid also displayed mammalian cytotoxicity (IC_{50} of 10.6 μg mL^{-1}). Pregnane compounds from the Indopacific octocoral *Carijoa* sp. showed a strong activity in the preliminary biological assay for the inhibition of the integrated electron transfer chain (NADH oxidase activity) in beef heart submitochondrial particles [7.250]. Inhibitory concentration 50% (IC_{50}) ranged from 1.1–1.9 μM, with full inhibition at approximately 21 μM. Riiseins A and B from *C. riisei* showed *in vitro* cytotoxicity toward HCT-

Fig. 7.7 *Capnella imbricata* Quoy and Gaimard, 1833 and capnellene

116 human colon adenocarcinoma with IC_{50} values of $2.0\,\mu g\,mL^{-1}$ [7.251]. Cytotoxic amides found in the Micronesian *C. riisei*, were reported to be mildly toxic to murine leukemia cells (P-388) in culture [7.252].

7.2.10 Family *Tubiporidae*

Genus *Pachyclavularia*

Pachyclavulariolide F derived from *Pachyclavularia violacea* collected in Papua New Guinea showed in vitro cytotoxicity [7.253]. Organic extract from *P. violaceae* exhibited significant cytotoxicity toward P-388 tumor cells ($ED_{50} = 0.3\,mg\,mL^{-1}$) [7.254]. Pachyclavulariaenone G was shown to exhibit significant cytotoxicity toward P-388 and HT-29 cancer cells [7.163]. Five new briarane-type diterpenoids, pachyclavulides E–I, were isolated from the Okinawan *P. violacea* [7.255]; pachyclavulides B and E showed weak cytotoxicity in vitro toward the CNS (SNB-75; $IC_{50} = 5.2\,\mu M$) and the lung (A549; $IC_{50} = 5.1\,\mu M$) cancer lines.

Genus *Leptogorgia* (= *Lophogorgia*)

Lophotoxin from Pacific sea whips of the genus *Lophogorgia* was found to be an irreversible inhibitor of the nicotinic acetylcholine receptor [7.256]. Lophotoxin, a neurotoxin originally isolated from the Pacific gorgonian *L. rigida*, was the most potent feeding deterrent among the furanocembranolides isolated from the Brazilian gorgonian *L. violacea* [7.257]. The steroids found in the gorgonian *L. sarmentosa* of the Gibralter Strait, exhibited significant cytotoxicity against four tumor cell lines ($ED_{50} = 1\,\mu g\,mL^{-1}$) [7.258]. *Lophogorgia* sp. presented extracts with high cytotoxic activity [7.259]. The common sea whip *L. virgulata*, a temperate/subtropical coral of the western Atlantic Ocean, yielded antimicrobial extracts named homarine [7.260].

7.2.11 Family *Ellisellidae*

Genus *Ctenocella*

Ctenocella pectinata from New Caledonia produced pectinoacetals A–C [7.263], of which their two analogs from *Ctenocella* sp. of the same region were cancer cell antiproliferative [7.264].

Genus *Ellisella*

Two briarane diterpenes from the gorgonian *Ellisella* sp. inhibited cytokinesis, causing multinuclei formation on NBT-II cells [7.265]. *E. robusta* sucumed robustolides J and K, which displayed inhibitory effects on superoxide anion generation by human neutrophils [7.266].

Genus *Rumphella*

A fraction isolated from the gorgonian *Rumphella aggregata* was studied in vitro on asynchronous cells of a human non-small-cell-bronchopulmonary-carcinoma line, which appeared to irreversibly inhibit cell growth in the G1 phase of the cell cycle [7.267]. Two norsesquiterpenoid alcohols, designated as rumphellatins B and C, were obtained from the Formosan gorgonian coral *Rumphella antipathies*. Rumphellatin B showed antibacterial activity toward the Gram-positive bacterium *Staphylococcus aureus* [7.268].

Genus *Junceella*

Junceellonoids C and D from *Junceella fragilis* exhibited cytotoxicity toward human breast carcinoma MDA-MB-231 and MCF-7 cells at a concentration of $100\,\mu M$ [7.269]. The sea whip gorgonian *Junceella juncea* led to the isolation of five new 8-hydroxybriarane diterpenoids, junceols D–H [7.270]; junceols D and F–H exhibited cytotoxicity toward CCRF-CEM and DLD-1 tumor cells, and junceols E–H displayed weak inhibitory effects on superoxide anion

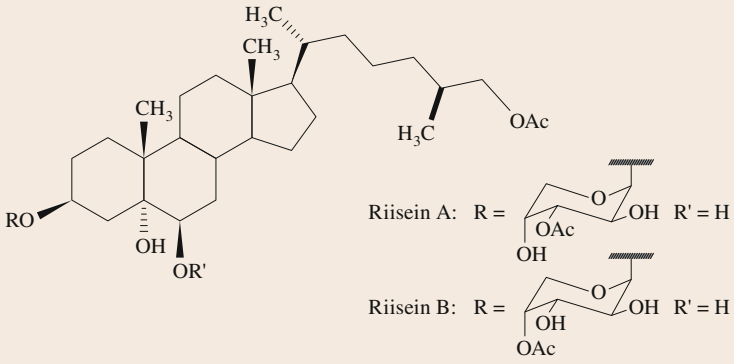


Fig. 7.8 *Carijoa riisei* and riiseins A and B (after [7.261, 262])

generation by human neutrophils. Structures, names, biological activities, and references of 137 briarane-type diterpenoids from various octocorals are summarized in [7.271].

7.2.12 Family *Xeniidae*

Genus *Xenia*

The soft coral *Xenia macroscopiculata* was shown to be the most active species among the Red Sea corals examined, with the highest and most potent antimicrobial activity. This characteristic, based upon bioassay-directed fractionation, was due to the presence of a range of compounds of different polarities. One of the isolated antibiotics was identified as desoxyhavannahine with an estimated volumetric concentration of ca. $590\,\mu g\,mL^{-1}$ (assuming 100% recovery) in tissues of *X. macroscopiculta*. The MIC of purified desoxyhavannahine was $48\,\mu g\,mL^{-1}$ against a marine bacterium; it is about tenfold lower than its estimated natural concentration, whereas the MIC of the crude extract of *X. macroscopiculta* was $25\,\mu g\,mL^{-1}$. This may suggest that the extract of this coral contains additional antimicrobial compounds [7.272]. Furthermore, eight diterpenoids were isolated from the Formosan *X. blumi*, and their cytotoxicity against selected cancer cells was measured in vitro [7.273]. Xenicane diterpenes and xeniolactones A–C were reported from Taiwanese *X. blumi* and *X. florida*, for which mild cytotoxicity was observed [7.273]. *X. florida* also afforded florxenilides A and B, which exhibited cytotoxicity against human colon cancer (WiDr) cells at 4.5 and 3.7 μM, respectively [7.274]. The cytotoxic sesquiterpenes, xenitorins A–F, were isolated from *X. puerto-galerae*; xentorins A and E exhibited cytotoxicity towards the A and P388 tumor cell lines [7.275]. Seven cytotoxic xenicane-type diterpenoids, 9-deoxyxeniloide-E, 9-deoxy-7,8-epoxyxeniloide-E, xeniolide-G, 9-deoxyxenialactol-C, xenibecin, xeniolide-H, and xenitacin, were isolated from the methylene chloride solubles of the Formosan *X. umbellata* [7.276]. An extract from Taiwanese *X. umbellata* led to the isolation of mildly cytotoxic diterpenoids, xenibellols A and B, and umbellactal [7.277]. The norxenicane metabolite xenibellal was reported as

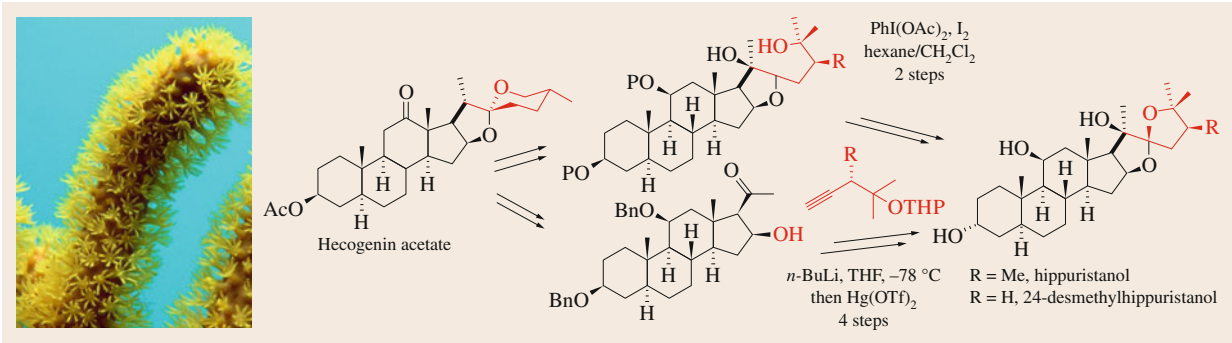


Fig. 7.9 *Isis hippuris* and its cytotoxic polyoxygenated steroid, hippuristanol

a mildly cytotoxic component of Taiwanese *X. umbellata* [7.278].

Genus *Heteroxenia*

The *Heteroxenia* sp. collected in the Philippines yielded biologically active sarcoaldosterol, which was active against the phytopathogenic fungus *Cladosporium cucumerinum*; it was also found to be cytotoxic [7.279]. The venom of *H. fuscescens* was found to be lethal (LD_{50}: 0.7 mg kg^{-1}) in dermonecrosis and vasopermeability potency assays in mouse skin, with one prominent 97 kDa protein fraction (LD_{50}: 0.55 mg kg^{-1}) [7.280]. The Egyptian Red Sea *H. ghardaqensis* led to the isolation of a gorgostane sterol that showed moderate activity as growth inhibitor of human colon tumor cell lines [7.281].

Genus *Cespitularia*

Cespitularia hypotentaculata yielded diterpene cespitularins A–H and I–Q, for which variable potency and selectivity was observed towards tumor cell lines A-549, HT-29, and P388 [7.282, 283]. Alcyonolide from an Okinawan *Cespitularia* sp. was cytotoxic against HCT 116 cells (IC_{50} 5.85 μM) [7.284]. Cespitulin G produced by *C. taeniata* displayed significant activities on superoxide-anion generation and elastase release by human neutrophils [7.285].

Genus *Asterospicularia*

An investigation of *Asterospicularia laurae*, collected on the Great Barrier Reef, Australia, afforded the cytotoxic diterpenes 13-epi-9-deacetoxyxenicin and 13-epi-9-deacetoxyxenicin [7.286]. The compounds showed cytotoxicity against a cultured suspension of P388D1 mouse lymphoma cells, with IC_{50}s of 0.1 and 1.0 μg mL^{-1}, respectively. Asterolaurins A–F, xenicane diterpenoids were extracted from Taiwanese *A. laurae*; asterolaurin A exhibited moderate cytotoxicity against HepG2 cells with an IC_{50} of 8.9 μM, while asterolaurin D showed potent inhibition of elastase release and superoxide anion generation in vitro [7.287].

7.2.13 Family *Melithaeidae*

Genus *Acabaria*

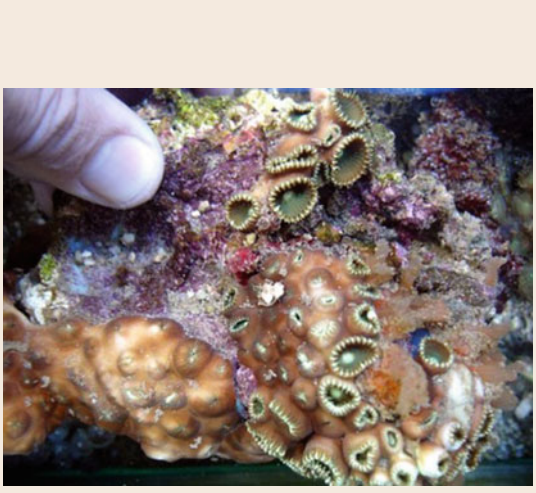
Extracts of *Melitodes ornata* exhibited spasmolytic activity [7.288]. Three sterols from the Korean *Acabaria undulata* exhibited moderate cytotoxicity and inhibitory activity against phospholipase A_2 [7.289].

7.2.14 Family *Isididae*

Genus *Isis*

Isis hippuris has proven to be a rich source of new cytotoxic polyoxygenated steroids. The authors of reference [7.291] worked on Okinawan the gorgonian *I. hippuris* and found that hippuristanol and 2R-hydroxy hippuristanol were potent anticancer agents. Epihippuristanol from the same species [7.292] was stated to be in the patenting process for its cytotoxic properties [7.293].

I. hippuris also yielded polyoxygenated gorgosterols showed moderate activity for reversal of multidrug resistance with cancer cells [7.294]. Several hippuristanol steroids were reported from the Taiwanese *I. hippuris*, including isishippuric acids A and B; isishippuric acid B was shown to exhibit potent cytotoxicity toward a limited panel of cancer cells [7.295]. Hippuristanol was further shown to be a selective and potent inhibitor of eIF4A RNA-binding activity that can be used to distinguish between eIF4A-dependent and eIF4A-independent modes of translation initiation in vitro and in vivo [7.296]. From the extracts of the South China Sea gorgonian *Isis minorbrachyblasta*,



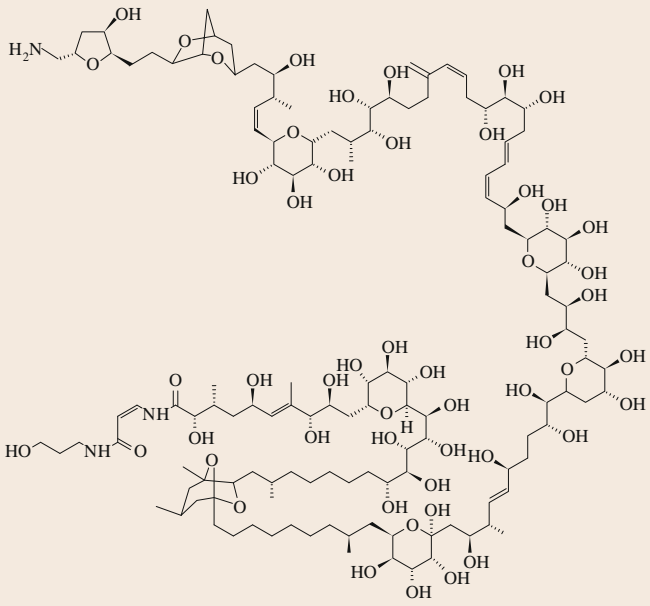


Fig. 7.10 Structure of palytoxin and a zoanthid *Palythoa* sp. (after [7.290])

two polyoxygenated steroids, gorgost-5-ene and 11-*O*-acetyl-22-epihippuristanol were isolated; they showed weak and moderate cytotoxicities against the examined cancer cell lines [7.297].

Genus *Dasystenella*

The first Antarctic gorgonian to be reported to contain polyoxygenated steroids was *Dasystenella acanthina* of the eastern Weddel Sea. All steroidal compounds from this species showed significant activities as growth inhibitors of several human tumor cell lines, as well as cytostatic and cytotoxic effects on selected tumor cell lines [7.225, 298].

Genus *Ainigmaptilon*

Ainigmaptilone A, extracted from the Antarctic gorgonian *Ainigmaptilon antarcticus* was one of two sesquiterpenes showing antibiotic activity [7.299].

7.2.15 Family *Zoanthidae* (Subclass *Hexacoralia = Zoantharia*)

Zoanthids containing palytoxins are reportedly among the most toxic marine organisms known [7.300, 301]. The extremely poisonous palytoxin, as one of the most potent known toxins, was originally found in the zoanthid *Palythoa toxica* (for a review, see [7.6]). Its intravenous lethality (LD_{50}), which ranges from $0.025\,\mu g\,kg^{-1}$ in rabbits to $0.45\,\mu g\,kg^{-1}$ in mice, is exceeded only by certain proteins and polypeptides [7.302]

Palythoa liseia yielded several punaglandins exhibiting antineoplastic properties [7.246]. The zoanthid, *Gerardia savaglia* was found to be an unexpected new rich source of the molting hormone, ecdysterone [7.303], which was found to be effective in inducing contractions in guinea pig uterus due to the active component oxytocin, reported as 2-deoxyecdysterone in *Zoanthus* sp. as well [7.304]. Prostaglandins such as PGA_2 isolated from the zoanthid *Palythoa kochii*, stabilize microtubules in a manner similar to paclitaxel (Taxol) [7.305]. The isolated zoanthamine and norzoanthamine alkaloids produced by the *Zoanthus* spp. [7.306] and *Z. nymphaeus* showed antitumor and antiplatelet activities, restrained the growth of murine leukemia cells, inhibited inflammation in mouse ears, and were suggested as promising candidates for antiosteoporotic drugs [7.307, 308]. The suppressive effect of norzoanthamine hydrochloride ($2\,mg\,kg^{-1}\,d^{-1}$) on osteoporosis was confirmed in vivo [7.309]. These findings suggest that norzoanthamine may act as both a suppressor of bone resorption and an enhancer of bone formation. In addition, a high concentration of norzoanthamine was detected to be present in the epidermal

tissue of *Zoanthus* sp. [7.310]; it was also found to be a collagen-strengthening function for norzoanthamine. Parazoanthoxanthin A, a related structure of zoanthoxanthin isolated from the zoanthid *Parazoanthus axinellae* [7.311] showed anticholinesterase activity [7.312]. *Zoanthus* sp. was also reported to produce a steroidal hormone, 2-deoxyecdysterone, which exhibited promising oxytocic activity compared to that of clinical standards, oxytocin and prostaglandin $F_{2\alpha}$ [7.304].

An alkaloid, antipathine A, together with three known zoanthoxanthin alkaloids extracted from the South China Sea black coral *Antipathes dichotoma* (zoanthid, family *Antipathidae*). Two alkaloids showed moderate cytotoxicity against human stomach carcinoma SGC-7901 cell line with IC_{50} of 67.38 and 86.40 $\mu g\,mL^{-1}$, respectively; the other two alkaloids also showed weak cytotoxicity toward human liver carcinoma Hep_G2 cell line [7.313].

7.3 Potential Pharmaceuticals from Hard Corals

Stony corals (hard coral/scleractinian) have rather been disregarded as a source of bioactive compounds; this may be because many secondary metabolites assumedly function in defense, and the calcareous skeleton of these animals is supposed to satisfy the defensive role [7.314]. Growth inhibition of smaller soft corals by some hard corals revealed the biological role of terpenes in hard corals [7.315].

7.3.1 Family *Poritidae*

A vasoactive and cardiotonic peptide (12 000 Da) from *Goniopora* sp. was reported to prolong action potential in amphibian and mammalian atria caused by delayed inactivation of Na^+ current, analogous to the mechanism of the action of ATX-II [7.316]. Extracts of *Porites lichen* and *P. lutea* exhibited antifertility at 80 and 100%, respectively ($LD_{50} > 1000\,mg\,kg^{-1}$ in mice) [7.288]. Aqueous extracts from the Hawaiian *Porites lobata* revealed antibacterial activity against nine bacterial strains [7.317].

7.3.2 Family *Dendrophylliidae*

A compound, tubastrene, with antiviral activity was found in the scleractinian *Tubasterea aurea* [7.318]. Sponge-derived aplysinopsins, with anticancer and antimicrobial properties that were first reported from *Astroides calycularis* collected in the Mediterranean Sea [7.319], were also isolated from the Indo-Pacific corals, *T. aurea* and *Tubastraea* sp., as inhibitors of the development of fertilized sea urchin eggs [7.320]. The isolation of two cytotoxic macrolides, mycalolides D and E, was reported from a collection of *T. faulkneri* [7.321]. The extract of *T. faulkneri* had previously been found to be cytotoxic in the antitumor screening of the NCI. Chemical extracts from *T. faulkneri* were also tested for deleterious effects on competent larvae of 11 other species of coral belonging to seven genera of four scleractinian families. Larvae exposed to extract concentrations from 10–500 $mg\,mL^{-1}$ consistently suffered higher mortality than larvae in solvent controls [7.322]. These compounds could, therefore, function as allelochemicals.

7.3.3 Family *Milleporidae*

A calcium-dependent smooth muscle excitatory effect was elicited by the venom of the hydrocoral *Millepora complanata* [7.183]. The nucleoside 1-methylisoguanosine isolated from some marine animals, including the coral *Madracis mirabilis*, showed potent muscle relaxant, blood pressure lowering, cardiovascular, and anti-inflammatory activity [7.323, 324].

7.3.4 Family *Acroporidae*

Extracts from *Acropora formosa* and *A. millepora* exhibited weak and strong hemolytic activity, respectively, and were also toxic to mice [7.325]. *Acropora corymbosa*'s extracts showed 100% antifertility ($LD_{50} > 1000\,mg\,kg^{-1}$, in mice). *Montipora divaricata* showed spasmolytic activity, LD_{50} more than 1000 ($mg\,kg^{-1}$, in mice) [7.288]. *Montipora* sp. and *M. mollis* succumbed polyacetylene compounds that exhibited ichthyotoxicity and inhibited the growth of some bacteria and fungi [7.326]. Six acetylenic compounds of *Montipora* sp. exhibited significant cytotoxicity against a small panel of human solid tumor cell lines, with Montiporyne A found to induce apoptosis in human colon tumor cells [7.314, 327]. The Korean *Montipora* spp. yielded three diacetylenes, one of which was the most potent cytotoxin towards a range of tumor cell lines [7.328]. Antimicrobial activity was found in the eggs of *Montipora digitata* [7.329], which had previ-

ously been reported to produce montiporic acids [7.330] and other cytotoxic diacetylenes [7.327]. Antibacterial activity against 9 bacterial strains was revealed in crude aqueous extracts from the Hawaiian *M. capitata* [7.317].

7.3.5 Family *Pocilloporidae*

Extracts from *Pocillopora demicornis* exhibited hypotensive activity (LD_{50} 681 mg kg^{-1} in mice) [7.288]. The Hawaiian *P. meandrina* displayed antibacterial activity in crude aqueous extracts against nine strains of bacteria including coral pathogens [7.317].

7.3.6 Family *Oculinidae*

From the coral *Galaxea fascicularis*, a crude mucus-like extract and subsequently its purified component were found to contain a DNase-like activity. It indiscriminately digested λDNA, as well as naked genomic DNAs isolated from a multiple-drug-resistant murine leukemia cell line, P388/VCR, and a nontransformed liver cell line, BL8L. Their apoptotic actions indicate their potential role in the development of an anticancer agent [7.331].

7.3.7 Family *Helioporidae*

Seven diterpenes, helioporins A–G, were isolated from the blue coral *Heliopora coerulea*; helioporins A and B showed antiviral activity against HSV1 and C-G cytotoxicity against P388 [7.332].

7.3.8 Family *Pectiniidae*

A polyacetylene from *Pectinia lactuca* exhibited ichthyotoxicity and inhibited the growth of some bacteria and fungi [7.326].

7.3.9 Family *Mussidae*

The extracts of *Lobophyllia corymbosa* showed 100% anti-implantation activity in female mice as the most promising activity observed [7.325].

7.4 Mycosporine-Like Amino Acids (MAAs)

Above all, because they are inhabitants of shallow waters exposed to ultraviolet radiation, symbiotic corals also produce mycosporine-like amino acids (MAA) that, as UV-absorbing compounds, possess photoprotective properties [7.333]; they also manufacture other mycosporines referred to as true *multipurpose* secondary metabolites against detrimental UV effects, in combination with other functionalities such as prevention of oxidation reactions [7.334]. *Palythoa tubereulosa* was the initial source of the mycosporine-like amino acids as natural sunscreens [7.335]. The unique physical and chemical properties of MAAs as natural sunscreens prompted an investigation of their use in healthcare applications and in the formulation of cosmetic products [7.336]. As an example, the commercially available synthetic fungicide, clotrimazole (mycosporin) is a chemical analog of naturally occurring mycosporine-like amino acids [7.337]. MAAs were also separated from the *Acropora formosa*, *Stylophora pistillata* [7.338–341], *Pocillopora damicornis*, and *Montipora verrucosa* [7.342].

Moreover, the microscopic algal symbionts of corals, referred to as zooxanthellae, and not coral cells are in some cases the likely source of the secondary metabolites of interest [7.343]. For example, anti-inflammatory pseudopterosins were shown to be produced by the endosymbiotic algae [7.344]. Ultimately, it is worth mentioning that antitumor and anticarcinogenic activities have also been reported for the unique pigment of dinoflagellate symbionts of corals, namely peridinin and related compounds [7.345]. The genus *Symbiodinium*, for instance, a symbiont of the Okinawan soft coral *Clavularia viridis* containing abundant antitumor marine prostanoids (e.g., clavulones), yielded compounds with significant growth-inhibitory activity in vitro toward cancer cells (IC_{50} of 2.0−2.8 g mL^{-1}) [7.244].

7.5 Conclusion

Table 7.1 summarizes various coral-derived compounds with diverse therapeutic properties, most of which necessitate further preclinical examinations to be commercialized. The substances in Table 7.1 account

Table 7.1 Componds with various properties extracted from soft and hard coral species

Compounds	Coral species	Properties	References
Alcyoniidae			
Flexibilide and dihydroflexibilide	*Sinularia flexibilis*	Antibacterial, cytotoxic, cardiac vasorelaxant, anti-inflammatory, anti-arthritic	[7.17, 37, 39, 41, 43]
Sinulariolide and 11–episinulariolide	*S. flexibilis*	Cytotoxic, algicide	[7.17, 28, 38]
Sinuflexlin	*S. flexibilis*	Cytotoxic	[7.11]
2-Phenylethylamides	*S. flexibilis*	Atrial stimulants	[7.43]
Sinulaflexiolides A−K	*S. flexibilis*	Anticancer	[7.45]
Scabrolides	*S. scabra*	Cytotoxic	[7.10]
Sinularolides, gibberosins, norhumulene, xeniaphyllane and gibberoketosterol	*S. gibberosa*	Cytotoxic, anti-inflammatory	[7.36, 60–62]
β-Caryophyllene terpenes	*S. nanolobata*	Cytotoxic	[7.55]
Sphingosine derivative, crassarosterol A and crassarosterosides A–D	*S. crassa*	Anti-inflammatory, cytotoxic	[7.56, 57]
Querciformolides A–D, granosolides A and B	*S. querciformis*, *S. granosa*	Anti-inflammatory	[7.67]
Gyrosanols A–C	*S. gyrosa*	Anti-inflammatory and antiviral	[7.68]
24-Methylenecholest-4-ene-3β,6β-diol, singardin	*S. ovispiculata*, *S. gardineri*	Cytotoxic	[7.49, 50]
Microclavatin and capillolide	*S. microclavata*	Cytotoxic	[7.51]
Sinuleptolide, sinulariol D and acylspermidines	*Sinularia* spp.	Anticancer, cytotoxic	[7.46, 53, 54]
Furanoic acid	*Sinularia* sp.	Bee venom inactivator	[7.48]
Firmacosides A and B	*S. firma*	Antibacterial	[7.63]
Polyhydroxylated steroids	*Sinularia* sp.	Anti-inflammatory	[7.64]
Polyoxygenated steroids and hydroxycembranes	*S. facile*	Cytotoxic	[7.65, 66]
Methylsarcophytotate and methylchlorosarcophytoate	*S. glaucum*	Cytotoxic	[7.75]
Sarcodictyins	*S. roseum*	Cytotoxic	[7.76]
Sarcophine, sarcophytol A, sarcotriol and hydroxydeepoxysarcophine	*Sarcophyton glaucum*	Antitumor, Anticancer, cytotoxic	[7.83, 84, 86, 87]
Sarcoglaucol, methyl tortuoates A and B, sarcostolides A–G	*S. cherbonnieri*, *S. tortuosum*, *S. stolidotum*	Cytotoxic	[7.88, 89, 91]
Sarcophine, sarcophytoxide, sarcocrassolide, crassolide, 13-acetoxysarcocrassolide and crassocolides A−M	*S. crassocaule*	Allelochemicals, cytotoxic	[7.35, 79–81]
7β-Hydroxy-8α-methoxydeepoxy sarcophytoxide	*S. mililatensis*	Antiosteoporosis	[7.90]
Neocembrenes and sartrochine	*S. trocheliophorum*	Cytotoxic, antibiotic	[7.71, 72]
Denticulatolide	*Lobophytum denticulatum*	Ichthyotoxic	[7.93]
Amine chlorides	*L. strictum*	Hypotensive and vasodilator	[7.94, 95]
Cembranolide deterpenes	*L. cristagalli*	Farnesyl inhibitor	[7.96]
Secosterol and lobohedleolide	*Lobophytum* spp.	Antitumor, anti-inflammatory, anti-HIV, cytotoxic	[7.56, 97, 99]
Ceramideas, lobocrassin F, glycolipids, crassumolides	*L. crassum*	Anti-inflammatory, antibacterial, anti-cancer	[7.100, 101, 103]

Table 7.1 (continued)

Compounds	Coral species	Properties	References
Alcyoniidae			
Durumhemiketalolides A−C and durumolides	*L. durum*	Antibacterial, anti-inflammatory	[7.104, 105]
Cembranolides	*L. michaelae*	Antiviral	[7.106]
Eleutherobin	*Eleutherobia sp.*	Cytotoxic	[7.109]
9-Deacetoxy-14,15- deepoxyxeniculin	*Eleutherobia aurea*	Anti-inflammatory	[7.107]
Sarcodictyin A	*Bellonella albiflora*	Cytotoxic	[7.113]
Cladiellin A, Cladielloides A and B, cladieunicellin H	*Cladiella* sp.	Antioxidant, cytotoxic and anti-inflammatory	[7.119, 120, 122]
Australins A–D	*C. australis*	Cytotoxic	[7.59]
Hirsutalins A–H	*C. hirsuta*	Cytotoxic	[7.120]
Valdivones	*Alcyonium valdivae*	Anti-inflammatory	[7.114]
Rietone	*A. fauri*	Anti-HIV	[7.115]
Sterol	*A. patagonicum*	Cytotoxic	[7.116]
Steroids	*A. gracillimum*	Cytotoxic and antiviral	[7.117]
Klysimplexin sulfoxides A–C, klymollins A–H, simplexins A–I	*Klyxum simplex*, *K. molle*	Cytotoxic, anti-inflammatory	[7.123–125]
Paraminabeolides A–F	*Paraminabea acronocephala*	Cytotoxic	[7.126]
Extracts	*Parerythropodium fulvum fulvum*	Antimicrobial	[7.40]
Anthothelidae			
Caribaeorane	*Erythropodium caribaeorum*	Anticancer	[7.112]
Plexauridae			
Crassin acetate	*Pseudoplexaura porosa* and *P. wagenaari*	Antibacterial and antineoplastic	[7.176–178]
14-Deoxycrassin and pseudoplexaurol	*P. porosa*	Antitumor	[7.180]
Guaiazulene	*Euplexaura erecta*	Antibacterial	[7.187]
Nuttingins A–E and malonganenones D–G	*E. nuttingi*	Antitumor, cytotoxic	[7.188]
Malonganenones	*E. robusta*	Cytotoxic	[7.189]
Muricins	*Muricea californica* and *M. fruticosa*	Antifouling	[7.190]
Steroids and sesquiterpenoids	*M. austere*	Antiprotozoal	[7.191]
Dimorphosides	*Anthoplexaura dimorpha*	Cytotoxic	[7.192]
Linderazulene	*Paramuricea* sp.	Cytotoxic	[7.193]
Sterols and sesquiterpenes	*Plexaurella grisea*	Anticancer	[7.195]
Caffeine	*Echinomuricea spendens*	Antifouling	[7.197]
Echinohalimane A and echinoclerodane A	*Echinomuricea* spp.	Antitumor, cytotoxic	[7.198, 199]
Calicoferols A–H, menelloide A	*Muricella* sp., *Calicogorgia* sp.	Antiviral, cytotoxic	[7.117, 200, 202]
Menellins A–C	*Menella* sp.	Anti-inflammatory	[7.201]
Eunicin	*Eunicea mammosa*	Antibacterial	[7.182, 183]
Fucoside-A (fuscol), fuscoside E, and eunicidiol	*E. fusca*	Skin care, anti-inflammatory, antifouling	[7.137, 185, 186]
Sesquiterpenoids	*Eunicea* sp.	Antimalarial	[7.184]
Eupalmerin acetate	*E. succinea* and *E. mammosa*	Anticancer	[7.2]

Table 7.1 (continued)

Compounds	Coral species	Properties	References
Plexauridae			
Spongouridine	*Eunicella cavolini*	Antiviral	[7.160]
Verrucoside	*E. verrucosa*	Cytotoxic	[7.161]
Prostaglandins	*Plexaura homomalla*	Hormon-like	[7.16]
Ellisellidae			
Briarane diterpenes	*Ellisella* sp.	Antitumor	[7.265]
Robustolides J and K	*E. robusta*	Anti-inflammatory	[7.266]
Rumphellatin B	*Rumphella antipathies*	Antibacterial	[7.268]
Pectinoacetal analogs	*Ctenocella* sp.	Anticancer	[7.264]
Junceols D and F–H	*Junceella juncea*	Cytotoxic	[7.270]
Gorgoniidae			
Pseudopterolide	*Pseudopterogorgia acerosa*	Cytotoxic	[7.155]
Bipinnatin-B and caucanolides A–F	*P. bipinnata*	Neurotoxin, cytotoxic, antimalarial	[7.148, 149]
Pseudopterosins, elisabethin H and methopterosin	*P. elisabethae*	Anti-inflammatory	[7.135, 139, 142]
Pseudopteroxazole and ergorgiaene (biflorane)	*P. elisabethae*	Antimycobacterial	[7.144, 146]
Providencin, kallolide A, Bielschowskysin and bipinnatin Q	*P. kallos*	Anticancer and antimalarial	[7.150–152]
Pseudopetrocin-E	*Pseudopterogorgia* sp.	Anti-inflammatory	[7.156]
Ancepcenolide, furanogermacrene	*Pterogorgia guadalupensis*, *P. americana*	Antibacterial	[7.143, 157]
Antikinases, crude extracts	*Gorgonia ventalina*	Antibacterial, antifungal	[7.158, 159]
Briareidae			
Solenolides	*Briareum* (*Solenopodium*) sp.	Anti-inflammatory and antiviral	[7.167]
Briantheín A, briaexcavatolides and excavatoid L	*B. excavatum*	Cytotoxic, anti-inflammatory	[7.168–170]
Violides Q–V, briarlides I–R, briviolides, biarenol A, briaranolides A–J, and briarenolide F	*Briareum* spp.	Cytotoxic, anti-inflammatory	[7.171–175]
Nephtheidae			
Naphthoquinones, chabrolidiones, chabrolosteroid B and armatinols	*Nephthea chabrolii* and *N. armata*	Cytotoxic	[7.210–212]
Elongatols	*N. elongate*	Cytotoxic	[7.106]
Brassicolide and brassicolide acetate	*N. brassica*	Cytotoxic	[7.209]
Erectasteroids and various sterols	*N. erecta*	Cytotoxic	[7.208, 346]
Pacificins, decaryiol	*N. pacifica*, *Nephthea* sp.	Cytotoxic	[7.88, 213]
Dendronesterone A, dendronesterol B, 3-oxocholest-1,22-dien-12β-ol and 3-oxocholest-1,4-dien-20β-ol	*Dendronephthya gigantea*	Cytotoxic	[7.216, 218, 347]
Dendronpholides A−R, isogosterones and tissue extracts	*Dendronephthya* sp.	Cytotoxic, antibacterial, antifoulant	[7.118, 217, 220]
Methyl spongoate	*Spongodes* (*Dendronephthya*) sp.	Cytotoxic	[7.219]
Griffinisterones F–I	*D. griffin*	Anti-inflamatory	[7.221]
9,11-Secosterol and 5,6-epoxy sterol	*Gersemia fruticosa*	Cytotoxic, antiproliferative	[7.223]
Extract, homarine, and trigonein	*G. rubiformis*, *G. antarctjca*	Antibacterial	[7.224, 225]

Table 7.1 (continued)

Compounds	Coral species	Properties	References
Nephtheidae			
Lemnalol, cervicol and isolemnalol	*Lemnalia cervicorni*	Cytotoxic	[7.347]
Lemnalol	*L. cervicorni*	Antinociceptive	[7.234, 235]
Laevinols	*L. laevis*	Cytotoxic	[7.348]
Lemnaflavoside	*L. flava*		[7.233]
Tsitsixenicin	*Capnella thyrsoidea*	Anti-inflammatory	[7.226]
Capnellenes, calamenene and cytotoxic, capnellenes GB9 and GB10	*C. imbricata*	antineuroinflammatory and antinociceptive	[7.228–230]
Fucoside steroid	*Scleronephthya pallida*	Antimalarial and cytotoxic	[7.236]
Litosterol	*Litophyton viridis*	Antimycobacterial	[7.231]
Stereonsteroids	*Stereonephthya crystalliana*	Cytotoxic	[7.214]
Clavulariidae			
Krecembrenolides and sesquiterpenoids	*Clavularia koellikeri*	Cytotoxic, antitumor	[7.237–239]
Prostadienoate, prostanoids, stoloniferones A–D and E–G, claviridenones, clavubicyclone, bromovulone III, clavulones, and chlorovulone II	*C. viridis*	Anticancer, antitumor, cytotoxic	[7.238, 241–243, 246, 296, 349]
Claviolide, dolabellane diterpenes	*C. violacea*, *C. inflata*	Cytotoxic	[7.240, 241]
Amides, riiseins and 18-acetoxypregna. . .-one	*Carijoa riisei*	Antiprotozoan, cytotoxic, antimicrobial	[7.247, 249, 251, 252]
Punaglandins	*Telesto* (*Carijoa*) *riisei*	Antitumor	[7.248]
Pregnane compounds	*Carijoa* sp.	Cytotoxic	[7.250]
Subergorgiidae			
Suberosols, subergorgic acid, and suberosanone	*Subergorgia suberosa*	Cardiotoxin, antifoulant, cytotoxic	[7.162, 163, 165, 166]
Acanthogorgiidae			
Astrogorgiadiol, astrogorgols, and astrogorgins B–M	*Astrogorgia* sp.	Anticancer, antitumor, antifouling	[7.203–205]
Acalycixeniolides	*Acalycigorgia inermis*	Cytotoxic	[7.206, 207]
Xeniidae			
Desoxyhavannahine	*X. macroscopiculta*	Antimicrobial	[7.272]
Xenitorins A and E	*X. puerto-galerae*	Cytotoxic	[7.275]
Xenicanes, xeniolactones, and florxenilides A and B	*X. blumi*, *X. florida*	Cytotoxic	[7.273, 274]
Xenibellols A and B, umbellactal and xenibellal	*X. umbellata*	Cytotoxic	[7.276–278]
Sarcoaldosterol	*Heteroxenia* sp.	Cytotoxic and antifungal	[7.279]
Gorgostane	*H. ghardaqensis*	Antitumor	[7.281]
Cespitularins A–Q	*Cespitularia hypotentaculata*	Antitumor, cytotoxic	[7.282, 283]
Alcyonolide, cespitulin G	*Cespitularia* sp., *C. taeniata*	Cytotoxic	[7.284, 285]
13-Epi-9-deacetoxyxenicin, 3-epi-9-deacetylxenicin and asterolaurins A–F	*Asterospicularia laurae*	Cytotoxic	[7.286, 287]
Isididae			
Polyoxygenated gorgosterols, epihippuristanol, isishippuric acid B and hippuristanols	*I. hippuris*	Anticancer, cytotoxic	[7.291, 293–296]

Table 7.1 (continued)

Compounds	Coral species	Properties	References
Isididae			
Polyoxygenated gorgosterols, epihippuristanol, isishippuric acid B and hippuristanols	*I. hippuris*	Anticancer, cytotoxic	[7.291, 293–296]
Gorgost-5-ene- and 11-*O*-acetyl-22-epihippuristanol	*I. minorbrachyblasta*	Cytotoxic	[7.297]
Steroids	*Dasystenella acanthina*	Cytotoxic	[7.225, 298]
Ainigmaptilone A	*Ainigmaptilon antarcticus*	Antibacterial	[7.299]
Tubiporidae			
Pachyclavulariolide F and pachyclavulariaenone G	*Pachyclavularia violacea*	Cytotoxic	[7.253, 254]
Lophotoxin	*Lophogorgia* sp.	Paralytic (neuro)toxin	[7.256]
Steroids	*L. sarmentosa*	Cytotoxic	[7.258]
Homarine	*L. virgulata*	Antimicrobial	[7.260]
Melithaeidae			
Polyhydroxysteroids	*Acabaria undulata*	Cytotoxic	[7.289]
Zoanthidae			
Palytoxin	*Palythoa toxica* and *P. tuberculosa*	Cytotoxic	[7.6]
Punaglandins	*Palythoa liseia*	Antineoplastic	[7.246]
2-Deoxyecdysterone	*Zoanthus* sp.	Oxytocic	[7.304]
PGA2 (Prostaglandin)	*P. kochii*	Microtubules stabilizer	[7.305]
Zoanthamine alkaloids	*Zoanthus* spp.	Anti-inflammatory and antiosteoporotic	[7.306]
Norzoanthamine	*Zoanthus* sp. and *Z. nymphaeus*	Antiplatelet and antiosteoporotic	[7.307, 308, 350]
Parazoanthoxanthin	*Parazoanthus axinellae*	Anticholinesterase	[7.312]
Antipathine A	*Antipathes dichotoma*	Cytotoxic	[7.313]
Acroporidae			
Polyacetylenes	*Montipora* sp., *M. mollis*	Ichthyotoxic, antibacterial	[7.326]
Montiporyne A	*Montipora* sp.	Anticancer	[7.327]
Diacetylene	*Montipora* spp.	Antitumor	[7.328]
Montiporic acids	*M. digitata*	Antimicrobial	[7.329, 330]
Poritidae			
Peptide	*Goniopora* sp.	Vasoactive and cardiotonic	[7.316]
Helioporidae			
Helioporins A–G	*Heliopora coerulea*	Antiviral and cytotoxic	[7.332]
Dendrophylliidae			
Tubastrene	*Tubasterea aurea*	Antiviral	[7.318]
Mycalolides	*T. faulkneri*	Cytotoxic	[7.321]
Aplysinopsins	*Tubasterea* spp. and *Astroides calycularis*	Anticancer, antimicrobial	[7.319]
Milleporidae			
1-Methylisoguanosine	*Madracis mirabilis*	Cardiovascular and anti-inflammatory	[7.323, 324]
3-Oxo sterols	*Anthomastus bathyproctus*	Cytotoxic	[7.127]
Venom	*Millepora complanata*	Muscle excitatory	[7.183]

Table 7.1 (continued)

Compounds	Coral species	Properties	References
Mussidae			
Extracts	*Lobophyllia corymbosa*	Antiimplantation activity	[7.325]
Pocilloporidae			
Extract	*Pocillopora demicornis*	Hypotensive	[7.288]
Pectiniidae			
Polyacetylene	*Pectinia lactuca*	Antibacterial and antifungul	[7.326]
Oculinidae			
Crude extract	*Galaxea fascicularis*	Anticancer	[7.331]
Mixed hard corals			
Crude extracts	*M. capitata*, *Porites lobata* and *Pocillopora meandrina*, *M. divaricata*, *Acropora formosa*, *A. millepora*, *A. corymbosa*, *Porites lichen*, and *P. lutea*	Antibacterial, spasmolytic, hemolytic, anti-implantation activities	[7.288, 317]
Mycosporine-like amino acids (MAAs)	Various soft and hard corals	Photoprotective (sunscreens)	[7.338]

Table 7.2 Distribution of bioactive compounds among members of soft and hard corals

Soft corals				***Hard corals***			
Family	Genera	Species	Compounds	Family	Genera	Species	Compounds
Alcyoniidae	14	43	75	Acroporidae	2	6	4
Plexauridae	11	23	27	Dendrophylliidae	2	4	3
Nephtheidae	7	21	36	Milleporidae	3	3	3
Zoanthidae	5	8	8	Poritidae	1	1	1
Gorgoniidae	4	8	16	Helioporidae	1	1	1
Xeniidae	4	10	16	Pectiniidae	1	1	1
Ellisellidae	4	5	5	Total	10	16	13
Acanthogorgiidae	3	2	4				
Isididae	3	3	7				
Tubiporidae	2	4	5				
Clavulariidae	2	6	17				
Briareidae	1	3	10				
Subergorgiidae	1	1	3				
Melithaeidae	1	1	1				
Anthothelidae	1	1	1				
Total	63	139	231				

for ≈ 32% cytotoxic, ≈ 11% anticancer and antitumor, ≈ 11% anti-inflammatory, > 13% antibacterial, antiviral, antifungual, antiprotozoan and antimicrobial, and 33% other bioactivities including antifouling and anti-HIV properties. Accordingly, the majority of the tabulated compounds are promising as potential cancer therapeutics.

Table 7.2 shows that a total of 244 compounds (excluding crude extracts) have been introduced from 15 and 6 families each with 139 and 16 species of soft and hard corals, respectively. These compounds have mostly been identified in *Alcyoniidae* (soft corals) and *Acroporidae* (hard corals), in which the genera *Sinularia* and *Montipora* are prominent, respectively, with regard to bioactive compound production. This survey scores the *Alcyoniidae* family as the first followed by the families, *Nephtheidae*, *Plexauridae*, *Clavulariidae*, *Xeniidae*, and *Gorgoniida* as the most prolific among the octocorals studied (Fig. 7.11). Corals and in particular soft corals are, therefore, outstanding among marine

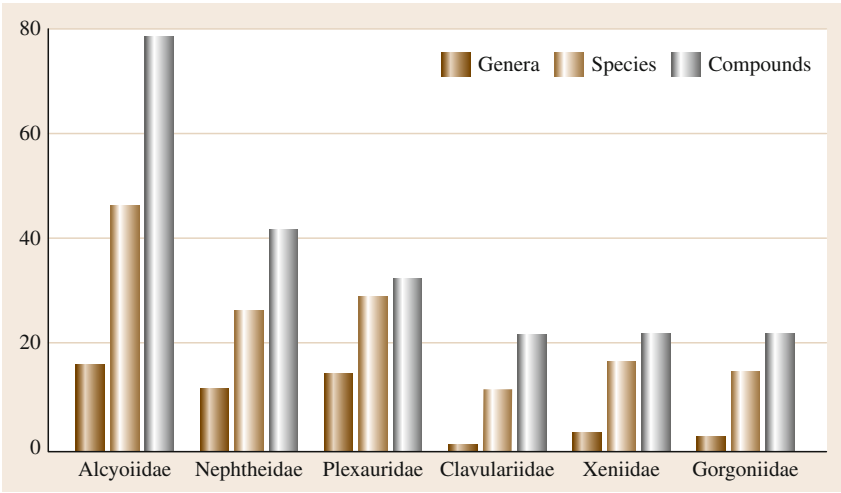


Fig. 7.11 Prolific families of soft coral species

organisms in the quantitative investment they make in terpenoid-based defence and competition. This wealth of bioactive substances renders these invertebrates potential with a variety of therapeutic agents.

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8. Marine Sponges – Molecular Biology and Biotechnology

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Marine sponges are an ancient and diverse animal phylum that host well-established symbiotic microbial communities. The vast majority of the microbial genetic diversity in sponges is, however, currently inaccessible by traditional methods. This large genetic resource may be of use for biotechnological applications, particularly as the physicochemical parameters under which the genes within these microbes function are likely to dictate that they may be significantly different from similar genes or gene products currently in use in industry, offering in some instances improved performance. Emerging tools and technologies in the field of metagenomics offer enormous potential for the discovery and exploitation of new biosynthetic entities. Both sequence-based and function-based technologies have to date been employed to identify genes with novel or improved functions. Marine sponges, as well-recognized sources of novel marine natural products with varied applications, are the ideal target for the implementation of these new technologies. We detail here some successes in the discovery and development of marine natural products for industrial or pharmaceutical applications and we also highlight some technical impediments to gene and gene product exploitation which as yet still need to be overcome.

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8.1 Marine Sponges

Marine sponges (phylum *Porifera*) are the oldest extant metazoan animals (Fig. 8.1), with the oldest fossils dating back almost 630 million years [8.1]. Sponges are globally distributed (Fig. 8.2) and are impor-

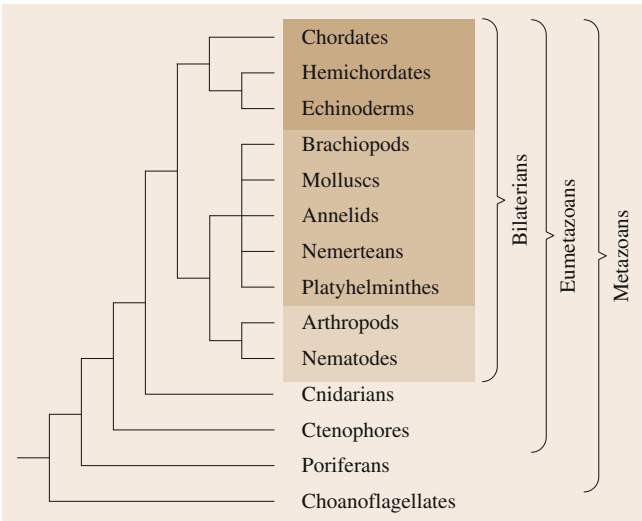


Fig. 8.1 Phylogeny of metazoa

tant members of all benthic communities. Sponges have been reported to be more abundant (area coverage/biomass/volume) than other benthic organisms [8.2], with sponge species diversity often outnumbering all other benthic species combined [8.2]. Sponges play vital roles in marine nutrient cycling as important sources of dissolved inorganic nitrogen (DIN), mediated by nitrifying endosymbiotic microbes resulting in high concentrations (40 μM) of nitrate near the ocean floor. They are also important sinks and sources of particulate organic carbon (POC), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON) [8.3].

The World Porifera Database [8.5] currently lists > 8370 valid sponge species, which are distributed amongst 680 genera in four distinct classes: *Calcarea*, *Hexactinellida*, *Demospongiae*, and the recently recognized *Homoscleromorpha* [8.6]. *Demospongiae* is by far the largest class, comprising ≈ 83% of valid species. Almost all sponges are found in seawater, however, one suborder of *Demospongiae* (*Spongillina*) comprising ≈ 250 species is freshwater in origin [8.4].

8.1.1 Sponge Anatomy and Physiology

Although marine sponges exhibit a wide range of morphologies, the basic physiological features are common to all filtering sponges. Anatomical features, particularly different sponge skeleton types have traditionally been used to aid taxonomic classification of sponges.

8.1.2 Sponge Skeletons

Porifera exhibit a wide range of morphologies, from encrusting, through branching to barrel types. Sponge skeletal systems are comprised of spicules which may be calcareous, composed of calcium carbonate ($CaCO_3$); siliceous, composed of silicon dioxide (SiO_2); or spongin – a collagenous protein. The class *Calcarea* have calcareous spicules, *Hexactinellida* have siliceous spicules, while *Demospongia* and *Homoscleromorpha* can be spiculate, with a combination of siliceous spicules and spongin, or aspiculate, which contain spongin skeletons.

8.1.3 Sponge Cell Types

The sponge body is composed of very few differentiated cell types. The sponge epidermis (pinacoderm) is composed of pinacocyte cells interspersed with porocyte cells, which form a porous aquiferous system throughout the sponge body. Choanocyte cells line choanosome *chambers*, where these flagellated cells, through a whipping action, create a water current which flows from outside the sponge body, through ostia – pores in the pinacoderm, through the sponge aquiferous system and is expelled through the osculum (Fig. 8.3). The sponge body is composed of a mesohyl – collagenous material through which archaeocytes travel. These archaeocyte cells play a role in the phagocytosis of food and can also differentiate into oocytes for sexual reproduction or gemmules for asexual reproduction. Pinacocyte cells are also capable of digesting food particles while sclerocyte cells produce and excrete spicules.

8.1.4 Sponge Physiology

Sponges do not possess distinct systems or organs; with the aquiferous system serving a role that is analogous to the circulatory, digestive and excretory systems found in higher metazoans. Most adult sponges are sessile filter-feeding animals that filter bacteria, micro-eukaryotes and particulate matter from ambient seawater, which they then pump through the canal systems in their bodies. Oxygen is delivered to cells by diffusion, food is engulfed and digested by phagocytosis in the mesohyl, and metabolic waste is removed in the constant water current generated throughout the body of the sponge. Sponges can pump remarkable volumes of seawater through their bodies with reports of 24 000 $L\,kg^{-1}\,d^{-1}$ in some sponge

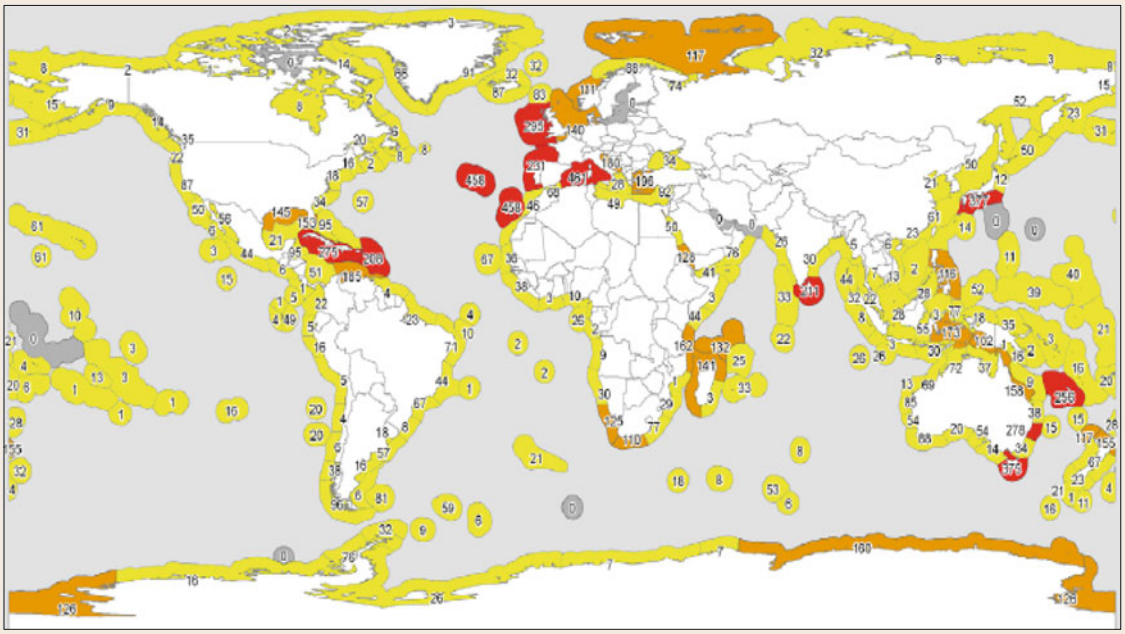


Fig. 8.2 Global distribution of marine sponges [8.4]

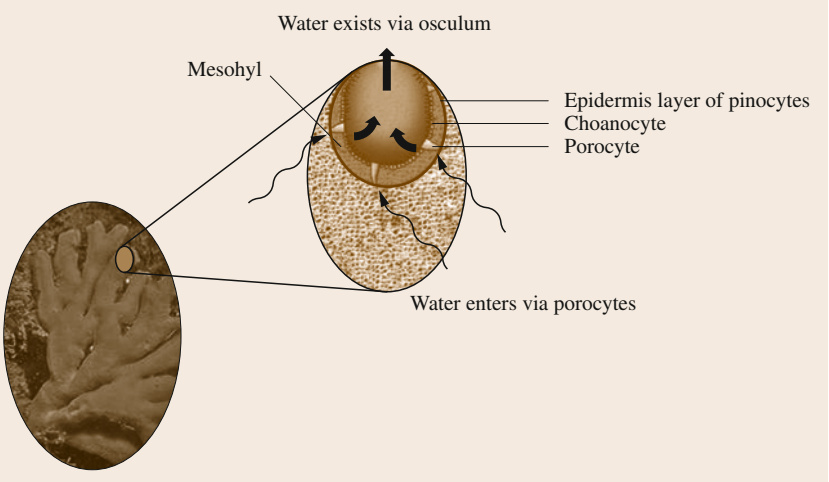


Fig. 8.3 Anatomy of a marine sponge

species [8.7]. Some sponges (≈ 120 species) do not possess aquiferous canal systems and thus are not filter feeders. Instead, they are carnivorous, capturing prey on *hooks* on the outer surface of the body where specialized cells migrate to the captured prey and phagocytose and digest the food prey. Carnivorous sponges have to date only been found in the deep sea [8.4].

Sponges do not possess adaptive immunity although innate immunity featuring an interferon-like 2′,5′-adenylate-synthetase system, a variable immunoglobulin-like system, and lipopolysaccharide LPS activated

kinase cascades are all present [8.8], and compounds with antimicrobial and anti-inflammatory properties have been extracted from sponge tissues. The primary producer of sponge-derived secondary metabolites is, however, still quite unclear although many of the sponge-derived compounds identified to date strongly resemble compounds that are known to be produced by microbes [8.9].

8.2 Sponge-Associated Microorganisms

Marine sponges play host to microbes from all domains of life; *Eukarya* [8.10, 11], *Archaea* [8.12, 13], and *Bacteria* [8.7]. Viruses and bacteriophages have also been detected in sponge tissues [8.14, 15]. These close and consistent associations are thought to be based on various symbiotic relationships including commensalist and mutualist [8.16], as well as parasitic. Microbes are also a significant food source for marine sponges [8.17] which, as sessile animals, must derive their nutrition by active filter-feeding from ambient seawater. This water filtering activity results in a remarkable enrichment of microbes in sponge tissues where 10^8–10^{10} bacteria/g wet weight have been recorded [8.18], with microorganisms constituting up to 35% of the total sponge biomass and densities exceeding 10^9 microbial cells/cm of sponge tissue; which is three to four orders of magnitude greater than the density of bacteria in the surrounding seawater (10^6 mL^{-1}).

8.2.1 Sponge-Associated Bacteria

Bacterial associates of sponges have been investigated through the use of both culture-dependent and culture-independent methods. Culture isolation from sponges is, like all other environmental sources, hampered by *the great plate anomaly* where less than 1% of taxa observed through other methods have proven amenable to culture under laboratory conditions through traditional or, indeed, more novel innovative approaches [8.9].

Researchers have used a wide range of culture conditions (including various growth media and different incubation temperatures) in attempts to access as wide a variety of bacterial diversity as possible [8.19–25]. Others have targeted the isolation of particular taxa of interest [8.26–40]. In addition, a number of innovative culture isolation methods have been employed, including the manipulation of bacterial communities through antibiotic administration prior to isolation [8.41], or other quite imaginative approaches such as liquid culturing and floating-filter culturing methodologies [8.42].

Despite these efforts the same bacterial phyla repeatedly appear following culture isolations, with members of only seven bacterial phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Cyanobacteria*, and *Bacteroidetes*) [8.7] to date being isolated in culture from sponge tissues; despite the observation that > 30 phyla or candidate phyla can be found in close association with sponges through molecular methods [8.9]. Notwithstanding this, diverse novel bacterial taxa are regularly isolated from sponge species worldwide (Table 8.1).

Culture-Independent Analyses

Microscopy. The presence of bacteria in the mesohyl of sponges was first confirmed in the early 1960s [8.69] by use of electron microscopy (EM). Subsequently, EM studies reported various cell types, including *Cyanobacteria*, in sponge tissues [8.70], and later still dense bacterial cell populations in sponge mesohyl tissues [8.71] were estimated to comprise 30% of the sponge biomass. Scanning electron microscopy (SEM) was subsequently employed to report the presence of unicellular cyanobacteria and non-photosynthetic filamentous cyanobacteria in the tissues of *Theonella swinhoei* [8.72]. The development of fluorescence in situ hybridization (FISH) allowed subsequent investigators to identify particular bacterial taxa and their spatial distribution within sponge tissues by designing probes to target particular 16S ribosomal ribonucleic acid (rRNA) genes. This allowed for the identification of *Cyanobacteria* [8.73, 74], *Actinobacteria*, *Bacteroidetes*, *Planctomycetes* as well as γ- and β-*Proteobacteria* [8.38] in sponges, and also demonstrated the vertical transmission of eubacteria and archaea in sponge larvae [8.75].

16S rRNA Clone Libraries. Polymerase chain reaction (PCR)-based approaches involving the amplification of 16S ribosomal deoxyribonucleic acid (rDNA) molecules and their subsequent cloning and analysis have been successfully employed to allow the species' composition of unculturable sponge-associated bacte-

Table 8.1 Novel bacteria isolated from marine sponges

Genus/species	Phylum	Host	References
Desulfoluna spongiiphila	*Proteobacteria* (δ)	*Aplysina aerophoba*	[8.43]
Kangiella spongicola	*Proteobacteria* (γ)	*Chondrilla nucula*	[8.44]
Fulvitalea axinellae	*Bacteroidetes*	*Axinella verrucosa*	[8.45]
Spongiibacter marinus	*Proteobacteria* (γ)	*Haliclona* sp.	[8.46]
Spongiispira norvegica	*Proteobacteria* (γ)	*Isops phlegraei*	[8.47]
Rubritalea squalenifaciens	*Verrucomicrobia*	*Halichondria okadai*	[8.48]
Planococcus plakortidis	*Firmicutes*	*Plakortis simplex*	[8.49]
Streptomyces tateyamensis	*Actinobacteria*	*Haliclona* sp.	[8.50]
Winogradskyella poriferorum	*Bacteroidetes*	*Lissodendoryx isodictyalis*	[8.51]
Fabibacter halotolerans	*Bacteroidetes*	*Tedania ignis*	[8.52]
Roseivirga spongicola	*Bacteroidetes*	*Tedania ignis*	[8.53]
Stenothermobacter spongiae	*Bacteroidetes*	*Lissodendoryx isodictyalis*	[8.53]
Shewanella irciniae	*Proteobacteria* (γ)	*Ircinia dendroides*	[8.54]
Thalassococcus halodurans	*Proteobacteria* (α)	*Halichondria panicea*	[8.55]
Marinobacter xestospongiae	*Proteobacteria* (γ)	*Xestospongia testudinaria*	[8.56]
Leptobacterium flavescens	*Bacteroidetes*	*Clathria eurypa*	[8.57]
Salegentibacter agarivorans	*Bacteroidetes*	*Artemisina* sp.	[8.58]
Endozoicomonas numazuensis	*Proteobacteria* (γ)	*Haliclona* sp.	[8.59]
Pseudovibrio axinellae	*Proteobacteria* (γ)	*Axinella dissimilis*	[8.29]
Mycobacterium poriferae	*Actinobacteria*	*Halichondria bowerbanki*	[8.60]
Saccharopolyspora cebuensis	*Actinobacteria*	*Haliclona* sp.	[8.61]
Streptomyces axinellae	*Actinobacteria*	*Axinella polypoides*	[8.62]
Pseudomonas pachastrellae	*Proteobacteria* (γ)	*Pachastrella* sp.	[8.63]
Lysobacter spongiicola	*Proteobacteria* (γ)	*Pachastrella* sp.	[8.64]
Rubritalea marina	*Verrucomicrobia*	*Axinella polypoides*	[8.65]
Spongiibacterium flavum	*Bacteroidetes*	*Halichondria oshoro*	[8.66]
Aquimarina spongiae	*Bacteroidetes*	*Halichondria oshoro*	[8.67]
Formosa spongicola	*Bacteroidetes*	*Hymeniaciden flavia*	[8.68]

rial communities to be conducted, as well as studies on other aspects of sponge microbial ecology to be undertaken. In this way, the bacterial community structures in many sponges have to date been elucidated [8.13, 31, 37, 40, 76–81]. In addition both inter and intra-sponge species microbial community comparisons have been performed [8.18, 82, 83]. The structures of communities within taxa of particular interest have been examined including: *Actinobacteria* [8.34], *Chloroflexi* [8.84], and *Cyanobacteria* [8.73, 85–87]. Differences in community profiles between inner and outer sponge tissues have also been explored [8.88–90]. Cloning of 16S rRNA genes led to the discovery of a novel candidate bacterial phylum, *Poribacteria* [8.91], which is common to many sponge species [8.92] but almost exclusively known from sponges.

These investigations have spanned a large range of sponge species from all of the worlds' oceans (Table 8.2). The sequencing of 16S rRNA clone libraries initially led to the identification of 16 bacterial phyla or candidate phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Lentisphaerae*, *Nitrospira*, *Planctomycetes*, *Poribacteria,* *Proteobacteria* [8.α-, β-, δ- and γ-], *Spirochaetes*, TM6, and *Verrucomicrobia*), which have been found in close association with sponges [8.7]. Subsequently, sequencing of sponge-derived denaturing gradient gel electrophoresis (DGGE) bands [8.78] added the phyla *Aquificae*, *Dictyoglomi*, and *Deferribacteres*, and the candidate phylum TM7 to the list of taxa found in association with sponges.

Table 8.2 Sponge species from which bacterial 16S rRNA gene clone libraries have been reported

Sponge species	References	Sponge species	References	Sponge species	References
Agelas oroides	[8.90]	*Halichondria panicea*	[8.18]	*Raspailia topsenti*	[8.93]
Amphimedon sp.	[8.31]	*Haliclona (? gellius)*	[8.79]	*Rhabdastrella globostellata*	[8.92]
Ancorina alata	[8.93, 94]	*Haliclona foraminosa*	[8.18]	*Rhopaloides odorabile*	[8.38, 95]
		Haliclona rufescens	[8.18]	*Smenospongia aurea*	[8.91]
Aplysina aerophoba	[8.82, 86, 87, 91, 95]	*Haliclona* sp.	[8.86, 87]	*Sphaerotylus antarcticus*	[8.13]
Aplysina archeri	[8.87]	*Homaxinella balfourensis*	[8.13]	*Spheciospongia floridae*	[8.87]
Aplysina fistularis	[8.91, 92]	*Hymeniacidon perleve*	[8.34]	*Stelleta kallitetilla*	[8.87]
Aplysina fulva	[8.78]	*Hyrtios erectus*	[8.31]	*Stelleta maori*	[8.93]
Aplysina insularis	[8.91]	*Ircinia fasciculata*	[8.76, 96]	*Stelleta pudica*	[8.87]
Aplysina lacunose	[8.91]	*Ircinia felix*	[8.87]	*Stylinos* sp.	[8.97]
Axinella polypoides	[8.90]	*Ircinia oros*	[8.76, 96]	*Stylissa carteri*	[8.98]
Callyspongia sp.	[8.97]	*Ircinia variabilis*	[8.76, 87, 96]	*Suberites zeteki*	[8.37]
Callyspongia vaginalis	[8.98]	*Kirkpatrickia variolosa*	[8.13]	*Svenzea zeai*	[8.87]
Candidaspongia flabellata	[8.87]	*Lamellodysidea chlorea*	[8.73]	*Terpios hoshinota*	[8.99]
Carteriospongia foliascens	[8.87]	*Lamellodysidea herbacea*	[8.73]	*Tethya calaforniana*	[8.89]
Chondrilla australiensis	[8.86, 87]	*Latrunculia apicalis*	[8.13]	*Tethya* sp.	[8.90]
Chondrilla nucula	[8.87, 88]	*Lendenfeldia chondrodes*	[8.73]	*Tethya stolonifera*	[8.93]
Chondrilla sp.	[8.86]	*Mycale acerata*	[8.13]	*Theonella conica*	[8.87]
Chondrilla sp.	[8.87]	*Mycale adhaerens*	[8.18]	*Theonella swinhoei*	[8.82, 87, 95]
Chondrosia reniformis	[8.90]	*Mycale armata*	[8.86]	*Theonella swinhoei*	[8.92]
Cinachyra sp.	[8.100]	*Mycale hentscheli*	[8.85]	*Tsitsikamma favus*	[8.101]
Clathria pennata	[8.18]	*Mycale loveni*	[8.18]	*Ulosa* sp.	[8.100]
Craniella austrialiensis	[8.102]	*Myxilla intruscans*	[8.18]	*Verongula gigantean*	[8.91]
Crella cyathophora	[8.98]	*Niphates digitalis*	[8.98]	*Vetulina* sp.	[8.40]
Cribochalena vasculum	[8.87]	*Oscarella lobularis*	[8.90]	*Xestospongia hispida*	[8.18]
Cymbastela concentrica	[8.97, 103]	*Petrosia ficiformis*	[8.86, 87, 90]	*Xestospongia muta*	[8.83, 87]
Cymbastela marshae	[8.86]	*Petrosia* sp.	[8.87, 100]	*Xestospongia proxima*	[8.87]
Discodermia dissoluta	[8.104]	*Phakella fusca*	[8.105]	*Xestospongia testudinaria*	[8.83]
Dysidea avara	[8.90]	*Phyllospongia papyracea*	[8.73]		
Dysidea granulosa	[8.22]	*Polymastia* sp.	[8.94]		
Gelliodes carnosa	[8.106]	*Polymastia* sp.	[8.93]	*Pseudoceratina fistularis*	[8.92]
Geodia sp.	[8.90]	*Pseudoaxinella tubulosa*	[8.87]		

Pyrosequencing. Next generation sequencing allows for the generation of hundreds of thousands of sequencing reads from metagenomic deoxyribonucleic acid (DNA) samples. Barcoding of samples allows for the pooling and parallel processing of samples, and very robust and comprehensive descriptions of bacterial community structures from diverse sources can thus be generated. The large datasets generated by pyrosequencing analyses have allowed for the identification of members of the *rare-biosphere* [8.107]. Also, more accurate descriptions of community structures and rank-abundance profiles of bacterial communities from a huge diversity of biomes have been made.

Various aspects of the aquatic bacterial consortia from lakes [8.108, 109], seawater [8.110], and hydrothermal vents [8.111], for example, have been reported; together with the bacterial communities associated with marine animals (fish [8.112], squid [8.113], corals [8.114, 115], and a marine polychaete [8.116]). The same is true for marine sponges. A recent re-

Table 8.3 Sponge species from which pyrosequencing of bacterial 16S rRNA genes has been reported

Sponge species	Reference	Sponge species	Reference
Ianthella basta	[8.119]	*Aplysina aerophoba*	[8.93]
Ircinia ramosa	[8.119]	*Aplysina cavernicola*	[8.93]
Rhopaloides odorabile	[8.119]	*Ircinia variabilis*	[8.93]
Hyrtios erectus	[8.118]	*Petrosia ficiformis*	[8.93]
Stylissa carteri	[8.118]	*Pseudocorticium jarrei*	[8.93]
Xestospongia testudinaria	[8.118]	*Axinella corrugata*	[8.122]
Raspailia ramosa	[8.120]	*Arenosclera brasiliensis*	[8.121]
Stelligera stuposa	[8.120]		

view of publicly available sponge-associated 16S rRNA sequences [8.117] analyzed a dataset of ≈ 7500 sequences. However, pyrosequencing analyses have generated > 700 000 sponge-derived bacterial 16S rRNA gene sequences which were not included in that study. These datasets have investigated various aspects of sponge-bacterial associations, including bacterial community structures [8.118–121], seasonal variations in community composition [8.122], bacterial-archaeal relative abundances [8.118], vertical symbiont transmission [8.119], and core variable and species-specific bacterial communities from a range of sponge species [8.84]. These pyrosequencing studies have thus far investigated 15 sponge species (Table 8.3) leading to the identification 35 bacterial phyla or candidate phyla which have been found in close association with sponges. Taxa identified in sponges for the first time by pyrosequencing include BRC1, *Chlamydiae*, *Fibrobacteres*, *Fusobacteria*, *Tenericutes* and WS3 [8.119], *Chlorobi*, *Chrysiogenetes*, OD1, *ε-Proteobacteria* and *Thermodesulfobacteria* [8.118], OP10, OS-K [8.84] and *Thermotogae*, *Elusimicrobia*, and *Synergistetes* [8.121]. Many of these extra taxa are amongst the rarest members of the sponge-associated communities. Highly diverse communities described at genus, family, order, and class levels have been described with ≈ 3000 OTUs (95% sequence identity) reported from the marine sponge *Rhopaloides odorabile* [8.119].

8.2.2 Sponge-Associated Archaea

Archaea were first reported in association with marine sponges in 1996 when *Cenarchaeum symbiosum* was found in the tissues of *Axinella mexicana* [8.123], and it has subsequently been reported to be consistently found in sponges of the family *Axinellidae* [8.12]. Many reports of sponge-associated archaea followed [8.13, 38, 124–131] and included studies which demonstrated the vertical transmission of archaea in sponge larvae, suggesting a very close co-evolutionary relationship [8.75, 132]. In a recent study using the aforementioned pyrosequencing-based approaches the relative abundance of bacteria and archaea in sponges from the Red Sea was assessed with values of between 4–28% in different sponges being reported, but significantly all were comprised almost exclusively of *Crenarchaeota* [8.118].

8.2.3 Sponge-Associated Eukaryota

Sponge-Associated Fungi

Although an as yet quite underexplored group of microorganisms, marine fungi are known in some cases to form symbiotic relationships with sponges, although the function of these associations remains largely unknown. While the majority of fungi recovered from marine sponges to date appear to be halotolerant, few, if any, appear to have a growth requirement for dissolved sea salts. Many of these halotolerant fungi are believed to have originated from the terrestrial environment and may have been washed from the terrestrial environment into the sea and subsequently adapted to the marine environment. Indeed, many of the fungi reported to have been isolated from sponges are closely related to terrestrial species with *Penicillium* sp. and *Aspergillus* sp. in particular being commonly found in marine sponges.

Despite the discovery that marine fungi are often derived from terrestrial environments, a coherent case for fungal involvement in a symbiotic relationship with marine sponges has also been advanced. A yeast has been shown to be maternally transmitted in a *Chondrilla* sponge, while putative fungal genes have been detected in sponge mitochondria, and yeasts have been proposed as a potential source of vitamins for marine sponges. In addition, transmission electron microscopy (TEM) has been employed to observe the close association of a filamentous fungus with sponge oocytes, while six fungal species have been reported to be cultured from in vitro cultures of sponge primmorphs and

single cells, further suggesting that fungi may, in fact, be true sponge symbionts and might, therefore, play an important role in host physiology.

Sponge-associated fungi have been shown to display a range of quite diverse biological activities through the production of a wide range of chemically diverse compounds with novel antibacterial, anti-inflammatory, antiviral, and anticancer activity. Some examples include *Fusarium oxysporum* DLFP2008005 isolated from *Hymeniacidon perlevis*, which has been reported to exhibit antibacterial and antifungal activities against *Staphylococcus epidermidis*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and the yeast *Candida albicans*. *Penicillium* cf., *montanense* isolates obtained from *Xestospongia exigua* from the Bali Sea in Indonesia have been reported to produce xestodecalactone B, a novel decalactone metabolite which displays antifungal activity against *Candida albicans*. *Aspergillus versicolor*, isolated from *Petrosia* sp. in Korea is known to produce the cytotoxic lipopeptide fellutamide C, while *Emericella variecolor* isolated from a Venezuelan sponge, has been shown to produce varitriol, which displays activity against breast cancer cell lines and varixanthone, which has antimicrobial activity. In addition, the fungus *Microascus longirostris* SF-73 isolated from a New Zealand sponge has been shown to produce the protease inhibitors cathestatin A, B, and C, which may potentially be used in inactivating target proteases in the pathogenic processes of various human diseases such as emphysema, arthritis, pancreatitis, thrombosis, and many others. Finally, extracts from the fungus *Engyodontium album* isolated from the Mediterranean sponge *Suberites domuncula* have been shown to display cytotoxicity against murine lymphoma cells.

As a result there has been an increased recent interest in molecular-based approaches to assess the fungal biodiversity of sponges with a view to assessing their overall chemical potential. These studies have primarily involved the use of 18S rRNA to phylogenetically analyze the fungal ecology of these sponges, resulting in fungi from 32 orders, from three phyla (*Ascomycota* [8.22] orders, *Basidiomycota* [8.8] orders, *Zygomycota* [8.2] orders), representing > 120 genera having to date been found in or on sponges. In addition, at least 18 orders of fungi have been isolated in culture. Examples of phylogenetic studies include the analysis of 80 fungi isolated from *Haliclona simulans* which were assessed by using both culture-dependent and metagenomic approaches. With the use of different fungal media containing either agar or gellan gum, a total of 19 different genotypes were detected, which were subsequently classified as members of *Agaricomycotina*, *Mucoromycotina*, *Saccharomycotina*, and *Pezizomycotina*; with the majority of the isolates being associated with the latter class. Some of these fungal isolates showed antimicrobial activity against *Escherichia coli*, *Bacillus* sp., *Staphylococcus aureus*, and *Candida glabrata*. Fungal 18S rRNA gene sequences belonging to *Eurotiales*, *Calosphaeriales*, and *Chaetothyriales* were amplified from deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) extracted from this marine sponge. Further studies have reported on the isolation of fungi from three Hawaiian sponges, from the Mediterranean sponges *Psammocinia* sp. and *Tethya aurantium* and from China Sea sponges. The secondary metabolic potential of these fungal isolates is reflected in the fact that 15 polyketide synthase (PKS) genes and 4 NRPS (non-ribosomal peptide synthase) genes were identified in the fungi isolated from the Chinese sponges.

It is fair to say that, in general, fungal associations with marine sponges are less well understood than their microbial counterparts. This may be due in part to the fact that metagenomic 18S rRNA-based studies typically encounter problems with respect to contamination with sponge rRNA coupled with difficulties in extracting fungal DNA. Even though sponge specific clusters have recently been detected in the genus *Penicillium* and 530 possibly obligate marine fungi have to date been described, many if not most sponge-derived fungal isolates or 18S rRNA clones which have been classified as genera typically found in terrestrial environments, and many compounds derived from those isolates, have also been shown to be present in their terrestrial counterparts. Nonetheless, the tremendous biochemical potential of fungi and the lack of understanding of sponge-fungi ecology should ensure that an ongoing and active research interest is maintained in this area.

Other Sponge-Associated Eukaryotes

Other eukaryotes have been reported to be present in close association with sponges. Polychaetes (annelid worms) and shrimp have been reported from Caribbean sponges [8.133]. *Ophiuroidea* (brittle stars), *Cnidaria* (sessile *Anthozoa*), *Turbellaria* (flatworms), *Nemertinia* (ribbon worms), *Sipuncula* (sipunculid worms), *Polychaeta*, *Mollusca*, *Crustacea*, *Pycnogondia* (sea spiders), *Echinodermata* (sea cucumbers), *Ascidiacea* (sea squirts), and *Pisces* (fish) have all been observed in association with the Brazilian sponge *Zygomycale*

parishii over a 5 year study period [8.134]. *Ophiuroidea* were also found to be consistently associated with sponges, but the authors suggest that this relationship is species-specific between *Callyspongia vaginalis* and *Ophiothrix lineata* [8.135]. Although many of these phyla are known parasites, their precise roles within their sponge hosts are as yet not known. A mutualist relationship between a sponge (*Halichondria panicea*) and a scallop (*Chlamys varia*) has, however, been reported, where the sponge obtains increased suspended nutrients while the scallop gains protection from predation [8.136].

8.2.4 Sponge-Specific Microorganisms

In a meta-analysis performed in 2002 of all the then publicly available ($n = 190$) sponge-derived 16S rRNA gene sequences, including 5 sponge species from different geographical regions such as the Mediterranean Sea (France, Israel and Croatia), the Red Sea, the North Pacific (Japan and USA), Australian waters (Davies Reef), and from the Philippine Sea (Palau), researchers revealed monophyletic clusters of sponge-derived sequences more closely related to each other than to sequences of the same taxa derived from non-sponge sources; leading to the hypothesis of sponge-specific microbes [8.82]. That study established that 14 monophyletic sequence clusters from 7 bacterial phyla, representing 70% of all sponge-derived sequences, were termed as *sponge-specific*. This term was defined to apply to groups of at least three sequences, which were (i) recovered from different sponge species and/or from individuals of the same species from different geographic locations, (ii) more closely related to each other than to sequences from non-sponge sources, and (iii) clustered together independently of the tree-building algorithm used.

Further interrogation of this hypothesis followed with the subsequent analysis in 2007 of $\approx$ 1700 sponge derived publicly available 16S rRNA sequences, with reports that 32% of all sponge-derived sequences from at least 10 bacterial phyla and also from a major archaeal lineage (*Crenarchaeota*) recruited to sponge-specific clusters [8.7]. These sponge-specific clusters included 100% ($n = 21$) of all sequences, then available, from the putatively sponge-specific candidate phylum *Poribacteria*. High proportions of sponge-derived sequences from *Chloroflexi* (62%), *Cyanobacteria* (79%), *Nitrospira* (57%), and *Spirochaetes* (67%) were classified as being sponge-specific. Notable proportions of sequences from *Actinobacteria* (38%), *Gemmatimonadetes* (25%) and *β-/γ-Proteobacteria* (34%) were assigned to sponge-specific clusters. Conversely, only 5% of *Acidobacteria* sequences, 9% of *Firmicutes* sequences, and 0% of *Bacteroidetes* sequences were determined to be sponge-specific.

In a subsequent study in 2011, by which time the number of publicly available (non-pyrosequencing-derived) sponge-derived 16S rRNA sequences had risen to $\approx$ 7500, *Simister* and colleagues found that 27% of sponge-derived sequences could be assigned to sponge-specific clusters from 14 bacterial phyla and one major archaeal lineage (*Thaumarchaeota*) [8.117]. Similar to previous findings, large proportions of sponge-derived *Chloroflexi* (61%), *Cyanobacteria* (53%), *Nitrospirae* (39%), and *Spirochaetes* (92%) were classified as being sponge-specific. While this study analyzed a dataset of $\approx$ 7500 sponge-derived sequences, it like the previous study, only considered relatively long sequencing reads. However, with the emergence of pyrosequencing $\approx$ 700 000 sponge-derived 16S rRNA sequences are currently available in public databases, with reads varying in length from 50–60 bp [8.119] up to an average 430 bp [8.120]. Recent reports which have analyzed $>$ 110 000 sponge-derived sequences derived by pyrosequencing, assigned between 36–65% of sequences from sponge individuals to previously described sponge-specific clusters [8.118].

8.3 Symbiotic Functions of Sponge-Associated Microorganisms

While the diversity and abundance of microbes found associated with sponges is well established, little experimental evidence exists to elucidate the symbiotic roles of those microbes in the sponge hosts. Instead, indirect evidence is used to support the hypotheses of commensal, mutualistic, or parasitic activities in the sponge holobiont.

8.3.1 Molecular Methods to Elucidate Sponge Symbiont Functions

The detection of microbial biomarker gene sequences from sponge metagenomes has led to speculation about possible symbiotic functional roles for those taxa. While known physiological functions of microbes may

be used to predict possible functions, empirical conclusions cannot be conclusively drawn from phylogenetic biomarker data analyses. In addition, such predictions can only be made for microbes which have been cultured and from which physiological characterizations have been elucidated. Other methods which can be employed to determine sponge symbiont functions include genome reconstruction [8.137], single-cell genomics [8.138, 139], metatranscriptomics [8.94, 140], shotgun cloning and sequencing of sponge metagenomic DNA [8.141], shotgun pyrosequencing [8.121], and the targeted PCR amplification of functional genes from sponge metagenomes [8.20, 105, 142–148].

A good example of the successful application of single-cell genomics to predict sponge symbiont functionality from uncultured microbes is the recent work on the genome of *Cenarchaeum symbiosum* derived from the marine sponge *Axinella mexicana* [8.138] and on Poribacteria from the sponge *Aplysina aerophoba* [8.139]. While a shotgun sequencing-based approach has also been used in the reconstruction of an unidentified δ-proteobacterium from shotgun sequence data from the sponge *Cymbastela concentrica* [8.137], pyrosequencing of cDNA has also recently been used to elucidate the diversity and abundance of actively transcribed genes from the sponge *Geodia barretti* ([8.140]; while shotgun approaches (cloning – [8.141]; pyrosequencing – [8.121] have identified functional genes in the sponges *Cymbastela concentrica* and *Arenosclera brasiliensis*, respectively.

Researchers have also targeted functional genes of particular interest for PCR amplification and sequencing, with genes involved in ammonia-oxidation, nitrification, and putative host defence in particular being targeted. Ammonia-oxidation (amoA) genes have been identified in the metagenomes of *Aplysina aerophoba* [8.145], *Ircinia strobilina*, *Mycale laxissima* [8.147], and *Phakiella fusca* [8.105]; and nitrification genes (nirS) have been amplified from the sponge *Astrosclera willeyana* [8.148]. Genes involved in the production of bioactive secondary metabolites which may contribute to sponge defence have also been targeted, with PKS genes being identified from the sponges *Pseudoceratina clavata* [8.143], *Discodermia dissoluta* [8.142], *Theonella swinhoei*, *Aplysina aerophoba* [8.1–6], and *Haliclona simulans* [8.77].

8.3.2 Discrimination Between Food Microbes and Symbiotic Microbes

A long-standing question in the sponge microbiology area centers on how sponges discriminate between food and symbionts when both occur in the sponge mesohyl. Some recent progress has been made in this regard, with genomic, metagenomic, and metatranscriptomic analyses being employed to identify factors which may play crucial roles in the symbiosis between sponges and microbes. These include factors associated with cell recognition, adhesion, and signalling. Gene transcripts for cell recognition factors such as polycystic kidney domain-like (PKD) factor have been identified in the *Geodia barretti* metatranscriptome [8.140], while Ig-like domain protein encoding gene sequences have been found in the genome of *Candidatus Poribacteria* [8.139]. Adhesion related genes (ankyrin repeat, tetratrico peptide repeat, fibronectin type III, and laminin-G domain proteins) were also noted in the genomes of sponge-derived *Poribacteria* [8.139] and δ-proteobacteria [8.137], and adhesion related gene transcripts (ankyrin repeat domain proteins, tetratrico repeat domain proteins, TonB-dependent receptors, and collagen binding surface proteins) were observed from the metatranscriptome of *Cymbastela concentrica* [8.141] and *Geodia barretti* with cell signalling related protein transcripts also being noted [8.140].

Transposable insertion elements have been identified in the metagenome of *Cymbastela concentrica* [8.141] with transposase gene transcripts being reported from the metatranscriptome of *Geodia barretti* [8.140]. These elements are thought to play roles in microbial genomic rearrangements and streamlining to help with adaptation to a symbiotic lifestyle [8.141]. Factors with possible roles in the maintenance of a symbiotic relationship including tetracycline resistance genes and gene encoding for multidrug resistance proteins were found in the genome of a sponge-associated unidentified δ-proteobacterium [8.137], while clustered regularly interspaced short palindromic repeat (CRISPR) gene sequences with possible roles in resistance to viral infection have been found in the metagenome *C. concentrica* [8.141]. Genes and gene transcripts involved in the biosynthesis of essential vitamins (B_2 or B_{12}) have been noted in the genomes of *Cenarchaeum symbiosum* [8.138], *candidatus Poribacteria* [8.139], and a sponge-associated δ-proteobacterium [8.137],

in the metagenome of *C. concentrica* [8.141] and in the metatranscriptome of *G. barretti* [8.140], suggesting that symbiotic microbes may be an important source of these essential vitamins for their hosts. Sponge-associated microbes have also been noted to be a remarkably rich source of various classes of chemicals with a wide range of bioactive properties and are thought to potentially play important roles in sponge host defence ranging from infection to predation [8.7].

8.4 Biotechnological Potential of Marine Sponges – Pharmacological Potential

Much of the recent research interest in the area of marine sponges and marine sponge-associated microbes has been primarily driven due to the pharmacological potential of many of the diverse chemical entities, with wide ranging biological activities which have been and continue to be discovered from this quite unique marine ecosystem [8.149]. The physicochemical properties of the marine environment (pH, pressure, temperature, osmolarity) in which many marine sponges reside means that any bioactive substances produced in that environment may have sufficiently different properties to terrestrially produced products to make them of interest for novel drug discovery [8.150]. While the search for novel drugs has involved many phyla of marine invertebrates, the phylum *Porifera* has proved to be the most promising (Fig. 8.4) [8.151]. As previously mentioned, sponges are sessile filter feeders, with no adaptive immunity, and thus rely on the production of chemical entities to defend themselves against infection, parasitism, and disease, and also to gain a competitive advantage [8.150].

Quite a diverse range of chemical classes with bioactive properties have to date been obtained from sponges and sponge-derived microbes, and include alcohols [8.152], alkaloids (Table 8.5), amino acid derivatives [8.153–155], aromatic compounds [8.156], fatty acids [8.157–159], lactones [8.160–162], peptides (Table 8.6), polyacetylenes [8.163, 164], polyketides (Table 8.7), quinones, and quinolones [8.165–168], sphingolipids [8.169, 170], and sterols [8.171–173], together with terpenes and terpenoids (Table 8.8). These bioactivities have been identified from bacterial or fungal isolates from sponges or from aqueous or organic extracts from the sponge tissues. In many cases, the bioactive compounds have been identified, purified, and characterized.

Numerous compounds and small molecules with activities against important human infections and diseases have been reported. Important bioactive compounds which have been reported include antibacterial compounds (including anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) and antituberculosis) (Table 8.4), antifungal compounds (Table 8.5), antiparasitic compounds (including antimalarial) (Tables 8.5–8.8), antiviral compounds (including anti-HIV) (Tables 8.5, 8.6 and 8.8), anticoagulant compounds [8.174, 175], antihelminthic compounds [8.176], antibiofouling compounds [8.177–179], anti-inflammatory compounds (Tables 8.7 and 8.8), neuromodulatory compounds [8.162, 180, 181], a UV-A protectant compound [8.173], and a large array of cytotoxic compounds with potential uses as anticancer drugs (Tables 8.5–8.8).

8.5 Exploiting the Pharmacological Potential of Marine Sponges

Although many novel bioactive compounds have been, and continue to be, isolated from sponges and/or their symbiotic microbes, these compounds are typically produced in minute quantities and the potential utility of these compounds to the pharmaceutical industry is, therefore, often somewhat limited [8.231]. For example, when halichondrins were isolated from the marine sponge *Halichondria okadai* [8.232], they were identified as very potent antitumor compounds with enormous clinical potential. However, it was estimated that 1 t of sponge biomass would need to be harvested to obtain 300 mg of a mixture of the halichondrin analogs [8.233]. With 1–5 kg of the drug being potentially required annually for the treatment of cancer patients, natural harvest was obviously unrealistic. To help overcome supply problems such as this, much recent effort has focused on marine products of potential bacterial origin. Indeed, evidence continues to emerge

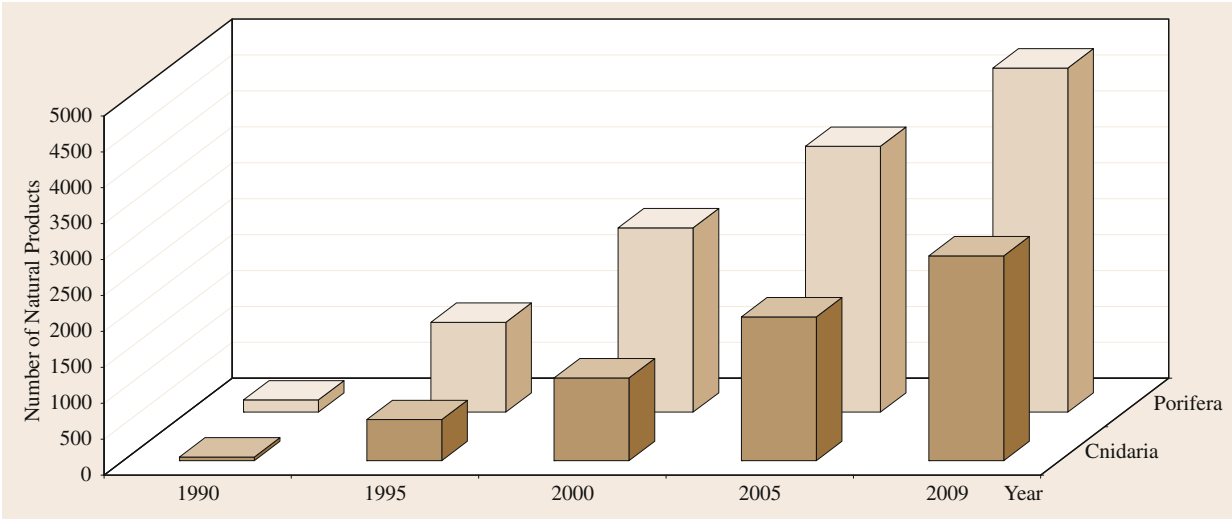


Fig. 8.4 Marine natural product discovery from marine phyla from 1990–2009

to suggest that many of the bioactive compounds isolated from sponges may, in fact, be secondary metabolite products of symbiotic bacteria [8.9, 234]. Given that bacteria have long been used for industrial products and that systems and tools for the manipulation of bacteria for industrial purposes are well established, then where marine natural products are of bacterial origin, industrial, and biotechnological manipulations are likely to be readily available for the production of sufficient quantities for clinical trials.

The extensive search for pharmaceutical products from marine sponges has led to some success stories. The nucleosides Ara-A (Acyclovir) and Ara-C (Cytarabine) from the sponge *Cryptotethya crypta* are commercially available as antiviral and antitumor drugs, respectively [8.235]. The chemical synthesis of Halichondrin B (Eribulin) has been achieved and was recently approved for breast cancer treatment [8.236, 237]. The synthetic tripeptide Hemiasterlin first identified in the marine sponge *Cymbastela* sp. has entered phase I clinical trials for cancer treatment [8.234], while a derivative of the hydroxamic acid, psammaplin [8.Panobinostat (LBH-589)], from the sponge *Psammaplysina* sp. is also currently in phase II clinical trials.

8.6 Metagenomic Strategies for Natural Product Discovery

It is now well established that in most environments, including marine environments, only $< 1\%$ of microbes present within these ecosystems can currently be isolated using traditional culturing techniques, thereby leaving the vast majority of these potentially biotechnologically important microorganisms and their biochemical pathways inaccessible and, therefore, unexploitable. The emergence of culture-independent and metagenomic techniques has, however, provided us with additional tools to allow us to determine the full extent of the uncultured microbial diversity within these ecosystems, thereby allowing access to the biochemical pathways within these as yet uncultured microorganisms. The term *metagenome* was first coined by Handelsman and colleagues [8.238] in the context of a description of the collective genomes of soil microbes. Metagenomic analyses involve describing either the sequence-based or function-based characteristics of a particular metagenome. Where the sequence of particular gene of interest is known, PCR primers or hybridization probes can be designed to interrogate a metagenome for the presence of the desired genes [8.239]. When focusing on genes and/or gene products for which sequence data is not known, a functional metagenomics approach can be employed [8.240]. This involves the extraction of total DNA from the metagenome of choice, fractionating the DNA to provide DNA fragments large enough to

Table 8.4 Examples of antibacterial activities from marine sponge aqueous or organic extracts, bacterial or fungal isolates from sponges, or from compounds purified from sponges, bacterial or fungal extracts

Reference	Sponge species	Source of activity	Target of activity
[8.182]	*Haliclona* aff *tubifera*	Organic extract	*E. coli*, *S. aureus*, *S. epidermis*
[8.183]	*Aplysina aerophoba*	*Bacillus* sp.	*E. coli, S. aureus*
	Melophlus sarassinorum	Melophlins (tetramic acids)	*S. aureus, B. subtilis*
[8.184]	*Brachiaster* sp.	Heteronemin (sesterterpene)	*M. tuberculosis*
[8.160]	*Luffariella* sp.	Manoalides	*S. aureus*
[8.150]	*Suberites domuncula*	α-*Proteobacteria*	*S. aureus, S. epidermis*
[8.10]	*Haliclona simulans*	*Penicillium* sp. *Pezizomycotina* sp. *Hypocreales* spp. *Phaeosphaeriaceae* sp.	*B. subtilis; S. aureus*
[8.20]	*Haliclona simulans*	*Pseudoalteromonas* sp., *Halomonas* sp., *Psychrobacter* sp.	*B. cereus, B. subtilis, E. coli,* MRSA
[8.159]	*Siliquariaspongia* sp.	Motualevic acid	MRSA
[8.33]	*Halichondria panicea*	Myamycin (polyketide) *Microbacterium* sp. *Rhodococcus* sp. *Streptomyces* sp. *Micromonospora* sp.	MRSA *S. aureus; E. faecalis* *S. aureus* *S. aureus* *S. aureus; E. faecalis*
[8.185]	*Plakortis halichondrioides*	Plaktoride J (lactone)	*M. tuberculosis*
[8.186]	*Halichondria* sp.	*Bacillus licheniformis*	*P. aeruginosa, S. aureus, V. cholerae,* MRSA
[8.187]	*Cliona viridis*	Ethanol extracts	*E. coli; B. subtilis; P. flourescens; S. aureus*
[8.188]	*Haplosclerida spp.* *Cliona celata* *Ircinia dendroides* *Haliclona mediterranea* *Haliclona viscosa* *Axinella dissimilis, Polymastia boletiformis, Haliclona simulans*	*Pseudovibrio* spp.	MRSA
[8.21]	*Suberites carnosus*	*Arthrobacter* sp., *Pseudovibrio* spp., *Spongiobacter* spp.	*E. coli; B. subtilis; S. aureus*
[8.168]	*Hippospongia* sp.	Epi-ilimaquinone	MRSA
[8.163]	*Xestospongia* sp.	Methanol extracts	*P. aeruginosa, M. intracellulare*
[8.22]	*Dysidea granulosa*	*Acinetobacter calcoaceticus*	*A. hydrophila, V. alginolyticus, V. parahaemolyticus*
[8.189]	*Clathria compressa*	Organic extract	*Gram positive bacteria*
[8.190]	*Petromica citrina*	Aqueous extract	*S. aureus, S. epidermidis, E. faecalis*
[8.191]	*Agelas mauritiana*	Ageloxime B (alkaloid)	MRSA

include complete gene clusters and/or operons, and cloning the large fragments via bacterial artificial chromosomes (BACs) or fosmids into a heterologous host such as *E. coli* (Fig. 8.5). The generation of large metagenomic libraries in this way allows for the high-throughput functional screening of the libraries for desired functions, by culturing the clones on media incorporating appropriate substrates to reveal phenotypic functions [8.238]. Large-insert BAC and fosmid clone libraries have to date been constructed from a wide variety of different marine environmental niches, including: marine plankton [8.241], seawater [8.242–

Table 8.5 Examples of sponge-derived alkaloids with bioactive properties

Reference	Sponge species	Compound	Target of activity
[8.192]	*Haliclona* sp.	Manzamine A	*Plasmodium berghei*
[8.193]	*Monanchora* sp.	Crambescidin 826	HIV
[8.194]	*Agelas* sp.	Nagelamides A–H	Gram positive bacteria
[8.195]	*Leucetta chagosensis*	Naamine G	*Cladosporium herbarum*
[8.36]	*Halichondria panicea*	Circumdatin I	UV-A protectant
[8.196]	*Agelas* sp.	Nagelamide O	Gram positive bacteria
[8.181]	*Ianthella flabelliformis*	Bastadin 25	δ-opoid receptor
[8.197]	*Pandaros acanthifolium*	Pandaroside G	*Trypanosoma brucei rhodesiense*
[8.198]	*Hyattella* sp.	Psammaplysin G	*Plasmodium falciparum*
[8.199]	*Aaptos aaptos*	Aaptamine	NT2 (embryonal carcinoma) cells
[8.200]	*Aaptos suberitoides*	Suberitines B & D	P388 (lymphoblastic) cells
[8.201]	*Haliclona* sp.	Papuamine & Haliclonadiamine	MCF-7 (breast), LNCap (prostate), Caco-2 (colon) and HCT-15 (colon) cells
[8.191]	Agelas mauritiana	Ageloxime B	MRSA

Table 8.6 Examples of sponge-derived peptides with bioactive properties

Reference	Sponge species	Compound	Target of activity
[8.202]	*Haliclona nigra*	Haligramides A & B	cytotoxic
[8.178]	*Haliclona* sp.	Haliclonamides C, D & E	*Mytilus edulis galloprovincialis*
[8.183]	*Aplysina aerophoba*	lipopeptides	*S. aureus, E. coli, Vibrio* sp.,*C. albicans*
[8.203]	*Neamphius huxleyi*	Neamphamide A	HIV
[8.204]	*Siliquariaspongia mirabilis*	Mirabamides A–D	HIV
[8.205]	*Siliquariaspongia mirabilis*	Celebesides A–C & Theopapuamides B–D	HIV
[8.206]	*Eurypon laughlini*	Rolloamides A & B	cytotoxic
[8.207]	*Tedania* sp.	Valinomycin	*Leishmania major*
[8.208]	*Phakellia fusca*	Phakellistatins 15−18	P388 (lymphoblastic) cells
[8.209]	*Holoxea* sp.	*L*-Trp-*L*-Phe	cytotoxic
[8.210]	*Discodermia calyx*	Calyxamides A & B	P388 (lymphoblastic) cells
[8.211]	*Cinachyrella apion*	Lectin	HeLa cells
[8.212]	*Pipestela candelabra*	Pipestelides A–C	cytotoxic

Table 8.7 Examples of sponge-derived polyketides with bioactive properties

Reference	Sponge species	Compound	Target of activity
[8.213]	*Theonella swinhoei*	Theopederin	Antitumor
[8.214]	*Cacospongia mycofijiensis*	Fijianolide	Antitumor
[8.215]	*Siliquariaspongia mirabilis*	Mirabilin	Antitumor
[8.216]	*Plakortis halichondrioides*	aromatic compounds	Anti-inflammatory
[8.217]	*Plakortis* cf., *simplex*	Manadoperoxides A–D	*Plasmodium falciparum*
[8.218]	*Plakortis* sp.	Plakortide Q	*Trypanosoma brucei brucei*
[8.185]	*Plakortis halichondrioides*	Plakortide O	*Plasmodium falciparum*
[8.33]	*Halichondria panicea*	Mayamycin	Anticancer, antibacterial

246], from sediment [8.247–249], and also from the metagenome of marine sponges [8.142, 143, 250–254]. Screening of these marine sponge-derived metagenomic libraries has led to the identification of a number of novel polyketide synthase (PKS) genes from the sponges *Discodermia dissoluta* [8.142] and *Pseudocer-*

Table 8.8 Examples of terpene/terpenoids compounds from marine sponges with bioactive properties

Reference	Sponge species	Compound	Target of activity
[8.166]	*Dysidea* sp.	Bolinaquinone	Anti-inflammatory
[8.219]	*Fasciospongia cavernosa*	Cacospongionolide B	Anti-inflammatory
[8.184]	*Brachiaster* sp.	12-deacetoxyscalarin 19-acetate	*M. tuberculosis*
[8.220]	*Stelletta* sp.	sesquiterpenoids	Anti-inflammatory
[8.221]	*Negombata corticata*	Negombatoperoxides	cytotoxic
[8.222]	*Rhabdastrella globostellata*	Isomalabaricane	cytotoxic
[8.223]	*Ircinia* sp.	Dorisenone D	*Trypanosoma* sp.
[8.224]	*Phorbas gukulensis*	Gukulenins A & B	cytotoxic
[8.225]	*Hippospongia* sp.	Hippospongide A	cytotoxic
[8.226]	*Stylissa* cf., *massa*	8-isocyano-15-formamidoamphilect-11	*Plasmodium falciparum*
[8.227]	*Carteriospongia flabellifera*	Flabelliferans A & B	cytotoxic
[8.228]	*Xestospongia testudinaria*	Aspergiterpenoid A	Bacteria
[8.189]	*Clathria compressa*	Clathric acid	Gram positive bacteria
[8.229]	?	Manoalide	Hepatitis C
[8.230]	*Phorbas* sp.	Phorbasone A	Anti-inflammatory

atina clavata [8.143], together with novel NRPS genes from the sponges *Haliclona okadai* [8.252] and *A. aerophoba* [8.253]. With respect to novel biocatalysts, quite a diverse range of enzymes has been discovered from a wide variety of marine metagenomic libraries, including esterases, lipases, and chitinases, amongst others.

8.6.1 Functional Screening of Large-Insert Metagenomic Clone Libraries

Such strategies involve screening the library for functional activities which result from the expression of genes within the bacterial metagenomic DNA. In many instances, this involves phenotypic-based detection methods which employ chemical dyes and substrates of the enzyme, which are often linked to chromophores. When the products of the individual metagenomic clones act upon these dyes or chromophores the product can be detected either visually or spectrophotometrically. The clone(s) displaying the relevant activities can then be genetically characterized, and following identification of the gene encoding the putative activity, cloned in *E. coli*, allowing subsequent purification and biochemical characterization studies on the enzyme to be undertaken. A number of routinely used functional assays will be described later in this chapter. The major advantage of functional screening-based approaches is given that sequence-based information is not required, no bias is introduced into the screening process, thereby increasing the likelihood of identifying entirely new classes of genes encoding either known or more likely novel functions.

One of the most commonly used functional screens currently employed to screen terrestrial metagenomic libraries and which is now finding increased utility in marine metagenomic studies has been for the identification of lipase/esterase activity. These are an important group of biocatalysts which are frequently used in organic synthesis strategies primarily due to their enantio/stereoselectivity and high level of activity in the presence of organic solvents. Metagenomic clones exhibiting lipolytic activity can be readily identified by the formation of clear halos surrounding the positive clones following growth on agar plates containing tributyrin as the indicator substrate (Fig. 8.6). Tributyrin-positive clones can then subsequently be tested on trioleinrhodamine B agar, which is used to detect lipolytic activity against long-chain fatty acids (C18).

With the use of this screen quite a large number of lipases have been reported from marine environments. Examples include novel lipolytic enzymes from *Aplysina aerophoba* and *Hyrtios erecta* marine sponge metagenomic libraries, as well as 15 different lipolytic genes from a metagenomic library constructed from South China Sea marine sediment. Esterases that appear to be particularly adapted to high hydrostatic pressure and salinity have also been isolated using this screen from metagenomic libraries constructed from the brine interface of a deep-sea hypersaline anoxic basin. More recently, this screen was successfully used by the authors' group to identify a novel halo tolerant li-

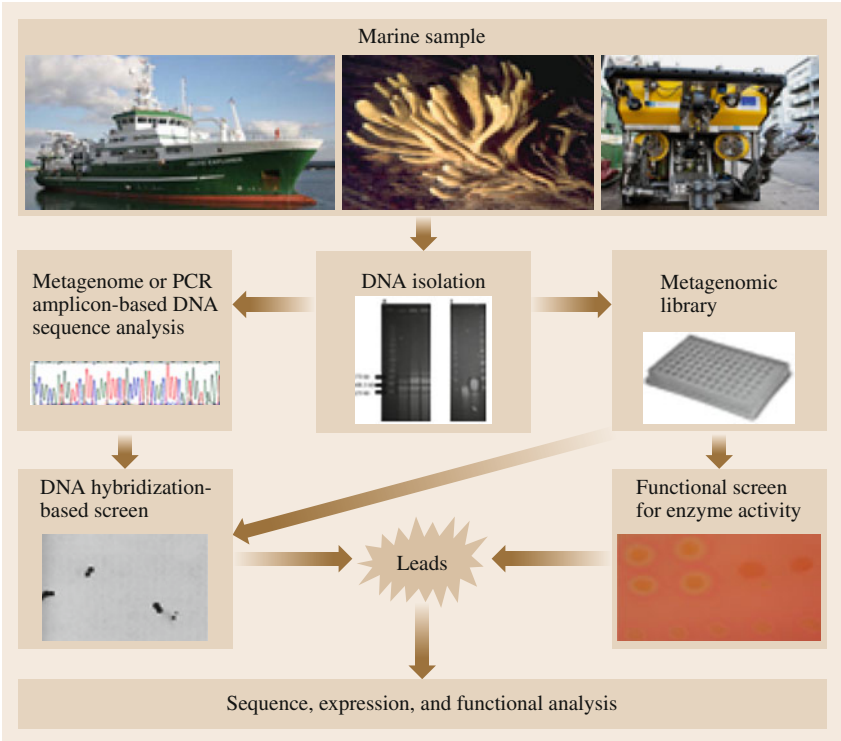


Fig. 8.5 Metagenomic strategies

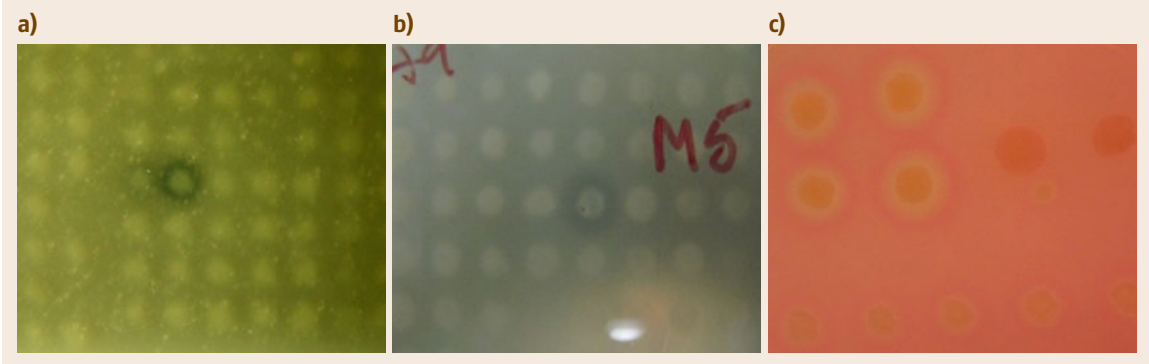


Fig. 8.6a–c Identification of a metagenomic clone exhibiting **(a)** lipase activity on 1% tributyrin agar, **(b)** protease activity on 1% skimmed milk agar and **(c)** cellulase activity on 0.1% Ostazin Brilliant Red hydroxyethyl cellulose

pase from a *Haliclona simulans* metagenomic library. Heterologous expression of the recombinant lipase in *E. coli* and subsequent biochemical characterization of the recombinant protein, showed an enzyme with the highest substrate specificity for long-chain fatty acyl esters, with optimal activity with p-nitrophenyl palmitate (C16) at 40 °C, in the presence of 5 M NaCl at pH 7. The lipase was also active over a broad temperature (4–60 °C) and pH (3–12) range and displayed high levels of stability at NaCl concentrations as high as 5 M and at temperatures ranging from 10–80 °C.

Another functional screen which is also now being increasingly used in marine metagenomics is the use of Luria Bertani agar supplemented with 1% skimmed milk, with a clear halo around the metagenomic clone being indicative of protease activity (Fig. 8.6). Using

this approach two proteases have been identified from the aforementioned *Haliclona simulans* library [8.255], while a zinc-dependent metalloprotease has also been isolated from a deep-sea sediment metagenomic library using this screen [8.256]. Interestingly, this enzyme also displayed an ability to hydrolyze both fibrin and azocasein, thereby raising the possibility of its utility as a potential therapeutic agent in the treatment of thrombosis. A novel subtilisin-like serine protease has also recently been cloned from an Antarctic coastal sediment metagenomic library [8.257]. This protease displayed good thermostability with an optimum temperature of 60 °C with approximately 73% of its activity being retained after incubation at 50 °C for 2 h.

The fluorogenic analog of chitin 4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside (MUF-diNAG) can also been successfully employed in function-based screens to identify chitinases in marine metagenomic libraries; it has been successfully employed to identify two cloned chitinase genes from coastal seawater metagenomic samples [8.242].

Metagenomic clones displaying cellulase activity can be detected by using colorimetric assays involving a cellulosic substrate. An example is the dye Congo red, which interacts with intact β-D-glucans and can thus be used to screen for β-D-glucan-hydrolase activity. Cellulolytic metagenomic clones can be identified on carboxymethylcellulose containing Luria Bertani (LB) and subsequently staining with Congo red. Metagenomic clones exhibiting activity can be visualized due to the formation of a yellow halo, which is due to the formation of the dye-glucan complex (Fig. 8.6). While a large number of cellulases have to date been isolated from metagenomic libraries constructed from different terrestrial and in particular mammalian gut microbiomes [8.258], to the authors' knowledge there are no reports to date of cellulases being isolated from marine metagenomic libraries. This is surprising, given the recent increased interest in this family of enzymes from a bioenergy perspective, particularly with respect to production strategies involving lignocellulose containing plant material/biomass, coupled with the fact that a number of cellulases have been isolated from marine bacteria, including a *Marinobacter* strain associated with the marine sponge *Dendrilla nigra*. Therefore, it appears highly likely that marine sponge metagenomic libraries do contain large numbers of novel cellulase genes; a fact reinforced by the recent cloning of a novel cellulase from a metagenomic library constructed from the gut microflora of abalone [8.259].

Laccases are a family of blue multicopper oxidases, which are mostly known from fungi but which have been found in all domains of life. Bound copper atoms catalyze the oxidation of aromatic compounds while reducing molecular oxygen to water. Fungal laccases are known to play a key role in lignin degradation, while bacterial laccases are involved in the production of spore coat pigment production for UV production and in copper homeostasis. Laccases possess significant biotechnological potential, including degradation and detoxification of industrial dyes, particularly azo dyes which are used extensively in the textile industry and are thus present in many wastewater discharges. Laccases are known to degrade azo dyes and thus constitute a promising, environmentally friendly method for the treatment of azo dye containing waste streams. These discharges are typically alkaline in nature and contain high concentrations of salts such as NaCl, which limits the use of fungal laccases. Thus, there is an increased interest in new laccases from marine sources, particularly of bacterial origin, given that bacterial laccases have been shown to possess excellent activity under alkaline conditions and to be very salt tolerant [8.260].

A number of functional screens are thus potentially available for the detection of laccase activity in marine metagenomic libraries. These include the polyphenol dye Remazol Brilliant Blue R (RBBR), guiacol and 2′,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). RBBR is typically incorporated into standard growth media such as LB at a final concentration of 0.04 w/v %, together with 250 µM copper chloride ($CuCl_2$). The copper chloride is added as laccases require copper ions to work and activity is indicated by a clear or brown halo around the colony displaying the activity. Guaicol $C_6H_4(OH)(OCH_3)$ is a colorless natural organic compound which when oxidized by laccases produces an amber color. The inclusion of guaicol at levels of around (0.01 w/v %) in standard bacterial growth media coupled with the addition of copper chloride (250 µM) ensures the ready detection of laccase activity. ABTS can also be employed to functionally screen for laccase activity. ABTS is an aromatic compound, which can be used to determine the antioxidant capacities of foodstuffs. When ABTS is incorporated to standard bacterial growth at a concentration of 1 mM, together with copper chloride (250 µM), laccase activity can be detected by the appearance of a green-blue color. Finally, syringaldazine [8.*N*,*N*′-bis (3,5-dimethoxy-4-hydroxybenzylidene) hydrazine] is also a good a good substrate that can be used to detect laccase activity. The incorporation of syringal-

dazine (50 μM) to standard bacterial growth allows the detection of laccase activity due to the appearance of a purple halo resulting from the oxidation of the substrate.

Syringaldazine has been successfully employed in the functional screening of a bovine rumen metagenomic library to clone a novel laccase gene [8.261], while guaicol has similarly been used in a functional screen to clone a novel laccase from a mangrove soil metagenomic library [8.262]; detailed protocols have been developed for the functional screening of metagenomic libraries from numerous environmental sources by the Golyshin group [8.263]. Thus, with the availability of these functional screens coupled with the fact that sequence-based screens have already resulted in the cloning of novel sponge-derived laccases, which will be discussed later in this chapter, it is likely that metagenomic libraries constructed from marine sponges will contain large numbers of novel laccases, many with potentially biotechnologically important biochemical characteristics.

Function-based screens continue to be developed as evidenced by the recent functional screen involving the use of chrome azurol S (CAS) as an indicator. This screen involves CAS changing color from orange to blue in the presence of iron and has recently been used in the cloning of gene clusters for two known siderophores, namely vibrioferrin and bisucaberin from marine metagenomic libraries [8.264].

8.6.2 Problems Associated with Functional Screening of Metagenomic Libraries

A number of problems continue to hamper the discovery of novel genes and gene products from metagenomic clone libraries. These include the choice of an appropriate heterologous host system and appropriate screens for the detection of activities. *E. coli* is the heterologous host of choice in most cases [8.265]. Uchiyama and colleagues reported that ≈ 40% of foreign genes can successfully be expressed in *E. coli* [8.224]. However, the expression of foreign genes can be impeded by host codon usage preferences, problems with gene promoter recognition, transcription initiation factors, improper protein folding, and the inability to export gene products from the host cell [8.265]. In addition, the expression of foreign gene products can sometimes be toxic to the heterologous host [8.266]. The abundance of genes of interest in the source environment and the cloned insert size and library size can also have an effect on the probability of cloning particular genes [8.266].

Efforts to increase the rate of gene and product discovery can be improved by the use of alternative heterologous host expression systems other than *E. coli*. In this respect, an increasing number of host systems are now becoming available, including *Agrobacterium tumefaciens*, *Bacillus subtilis, Burkholderia graminis, Caulobacter vibrioides, Pseudoalteromonas haloplanktis, Pseudomonas putida*, *Ralstonia metallidurans, Rhizobium leguminosarum*, *Streptomyces* spp., *Sulfolobus solfataricus*, *Thermus thermophilus*, and *Thiocapsa roseopersicina* [8.267, 268]. In addition, increases in the rate of novel gene discovery are likely to be improved through the use of multiple heterologous host expression systems, such as through the use of shuttle vectors that can be transformed from *E. coli* into different hosts, thereby increasing the likelihood of expression [8.265]. In this respect, the authors' group has developed a novel cloning vector pCCERI (Fig. 8.7), which incorporates elements from *Streptomyces* phage φC31 for chromosomal integration into alternative hosts and an inducible replicon for ease of handling. pCCERI incorporates the φC31 integrase gene and attachment site for site specific integration into hosts containing the cognate *att*B site. The vector also incorporates the *ori*V from the inducible copy number system developed by *Wild* et al. [8.269], allowing induction from single to multicopy in a modified *E. coli* host. In addition, the vector has apramycin and chloramphenicol resistance genes for selection in different hosts, and *ori*T for conjugal transfer. A helper plasmid pERI3-50-1 was also constructed to allow efficient high-throughput conjugation of entire metagenomic libraries to the alternative host strains *Pseudomonas putida* and *Streptomyces lividans*, thereby increasing the likelihood of obtaining the expression of metagenomic inserts.

While, as previously mentioned, functional screening of marine sponge metagenomic libraries has been successful in the isolation of numerous industrially relevant biocatalysts, primarily involving the use of screens that employ colony color or colony appearance changes. However, many enzymes are not amenable to identification using these types of approaches. In an attempt to overcome this problem approaches involving the use of *gene-expression* reporter-based assays have been developed. This involves the use of a transcriptional regulator that responds to the product of a reaction, which is catalyzed by the enzyme being targeted, resulting in the transcription factor-dependent

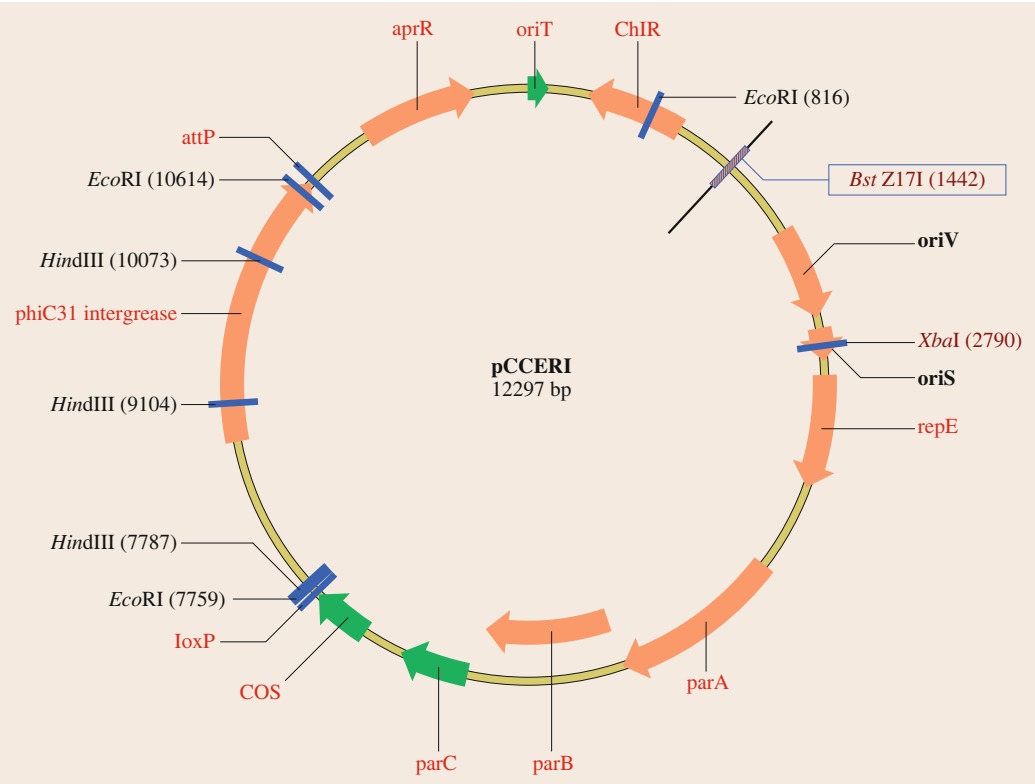


Fig. 8.7 Cloning vector pCCERI

activation of a promoter which is itself fused to a reporter gene, with a readily available phenotype such as β-galactosidase activity. The idea is to employ bacteria containing reporter systems such as this as *hosts* for a sponge metagenomic library. Hereby clones encoding enzymes, which catalyzes a reaction, which produces the desired reaction product, would activate the reporter gene. Other similar approaches that have been successfully employed for screening terrestrial-based metagenomic libraries which may also prove useful in the context of marine metagenomic libraries include the substrate-induced gene expression (SIGEX) assay, which was originally designed for the identification of quorum sensing attenuation in *Pseudomonas* and specific catabolic enzymes [8.264]. Another novel approach that may also prove useful is the use of complementation to identify novel enzymes, where the ability of the product of a metagenomic clone to complement a mutation in a reporter is used in the detection of the expression of the enzyme being targeted. This was previously used to clone novel DNA polymerases from a glacial ice metagenomic library. Another major problem with heterologous expression of genes, irrespective of the particular host system employed, centers on the decreased sensitivity due to the likelihood of intracellular accumulation of the enzymatic activity. This can be overcome by facilitating enzyme release from the cell by ensuring cell lysis using detergents such as Tween or Triton X, allowing release while still ensuring that the heterologously expressed protein retains its native configuration. This approach is particularly suited to high-throughput screens, where metagenomic libraries can be functionally screened in a microtiter plate format in the presence of substrate, allowing the use of liquid handling and colony-picking robots, together with microtiter readers.

8.6.3 Sequence-Based Screening of Metagenomic Libraries to Identify Novel Biocatalysts

Sequence-based approaches involves targeting the presence of specific gene encoding proteins of interest by

Fig. 8.8 Partial amino acid alignment of bacterial laccase genes including gene sequences derived from metagenomic studies (ADV52199 – drained peat soil metagenome, ADM87301: South China Sea marine microbial metagenome, SNB11 and SNB12: marine sponge metagenome). Histidine-rich copper binding domains are indicated by vertical boxes. ZP 01036720: multicopper oxidase domain protein *Roseovarius* sp. 217, ZP 00964414: multicopper oxidase domain protein *Sulfitobacter* sp. NAS14.1, ZP00961617: multicopper oxidase domain protein *Roseovarius nubinhibens*, YP 002289160: CumA *Oligotropha carboxidovorans* OM5, ADV52199: laccase uncultured bacterium, YP 004619745 cueO *Ramlibacter tatouinensis* TTB310, ZP 02186341: alpha proteobacterium BAL 199, ADM87301: laccase uncultured bacterium, SNB11 and SNB12: sponge metagenome-derived sequences

using either hybridization probes or PCR primers for these genes. This involves the design of DNA probes or PCR primers typically derived from conserved regions of already well-characterized genes or protein families. Using the homology-based approach, PCR amplification is performed, following which the putative gene is sequenced and then subsequently cloned into an appropriate expression vector where the heterologously expressed protein is then purified and biochemically characterized to ascertain its function. While such ap-

proaches are often successful, they at best lead to the identification of new variants of already well-known classes of proteins, with very few really novel genes being detected.

Notwithstanding this, such homology-based strategies have led to the identification of genes encoding novel enzymes from marine metagenomic libraries. Examples include the use of degenerate PCR primers designed based on the conserved regions of alkane hydroxylase gene sequences, to clone two alkane hydroxylase (*alkB*) genes from a Pacific deep-sea sediment metagenomic library [8.270]. Moreover, the recent cloning of four unique $FADH_2$-dependent halogenases from the metagenome of the Mediterranean sponge *Aplysina aerophoba* using PCR primers designed based on conserved sequences from previously cloned halogenases indicates that the microbial consortia of sponges are likely to be a valuable source for novel halogenases [8.271].

Other examples include the cloning of a novel peptidase encoding gene using PCR primers designed to amplify cellulase genes, from the metagenome of the western Arctic Ocean [8.272]. In addition, a new laccase gene was cloned from a marine microbial metagenomic library from the South China Sea, using PCR primers based on the relatively highly conserved sequence coding for copper binding sites in bacterial laccases (Cu1F ACMWCKGTTCAYTGGCACGG; Cu4R TGNTCNAGNAWGTGRCARTG) [8.260]. This novel laccase shared less than 40% sequence identity with previously characterized multicopper oxidases and when heterologously expressed in *E. coli* resulted in the production of a recombinant protein that exhibited alkalescence-dependent, chloride tolerant laccase activity. Similar primers were employed by the authors' group to clone two candidate laccase encoding genes (SNB11 and SNB12) from the metagenomic library of the sponge *Stelletta normani*, which was collected from a depth of 1348 m from the Atlantic Ocean in Irish waters. Basic Local Alingment Search Tool (BLAST) analyses and sequence alignments of the cloned sequences show that although the copper binding domains in these novel genes are conserved, the overall gene sequences are quite divergent from known gene sequences (Fig. 8.8). The quite marked difference between these newly cloned SNB11 and SNB12 genes from *S. normani* and already known genes suggests that marine sponges and those from deep sea ecosystems in particular may prove a valuable source for laccases with potentially new and improved functionality.

8.6.4 Sequence-Based Screening of Metagenomic Libraries to Identify Genes Involved in the Biosynthesis of Natural Products

As previously mentioned, sponges are a rich source of natural products, many of which are now widely believed to be metabolites, which are, in fact, synthesized by symbiotic bacteria. There are a number of examples of marine sponge-associated bacterial isolates which produce compounds that are similar and in many cases identical to sponge-derived compounds. These include jasplakinolide (jaspamide) from the sponge *Jaspis* sp. and cyclodepsipeptide chondramide D isolated from the myxobacterium *Chrondromyces crocatus* and salicylihalamide A produced by *Haliclona* sp., which is almost identical to the myxobacterial metabolite apicularen A. In addition, there are a number of sponge-associated bacteria that synthesize important natural products, including the *Salinispora* spp., which produces rifamycins and a *Micromonospora* sp., from *Acanthostrongylophora* sp., which produces the antimalarial compound manzamine A.

Many of the structurally diverse classes of natural products most commonly associated with microorganisms include polyketides, non-ribosomal peptides, or hybrid peptide-polyketides, which are synthesized by polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS), or hybrid PKS-NRPS synthases which are encoded in gene clusters in the producing bacteria. These multifunctional biosynthetic enzymes typically consist of a series of modules which are usually distributed among several polypeptides. These modules consist of a series of enzymatic domains each of which is involved in the catalysis of individual biosynthetic steps. Biosynthesis occurs in an *assembly line*-like process with the nascent polyketide or peptide chain passing from module to module, with each module adding extender units until synthesis of the compound is complete. Polyketide-derived natural products which are believed to be produced by symbiotic sponges microbes include halichondrin B, discodermolide, peloruside, and laulimalide; while polyketide/non-ribosomal peptides include hemiasterlins, the aforementioned jaspamide, and the salicylihalimides. Thus, perhaps it is not surprising that numerous sequence-based screens, many of which have been PCR-based, have been undertaken on sponge metagenomic libraries; specifically targeting PKS and NRPS gene clusters as a means of assessing overall diversity and the secondary metabolite/chemical

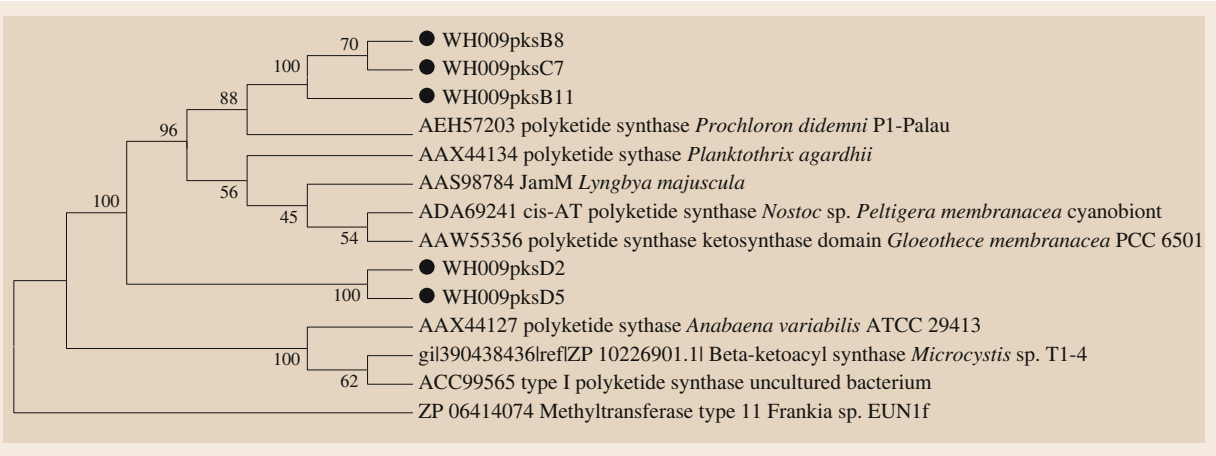


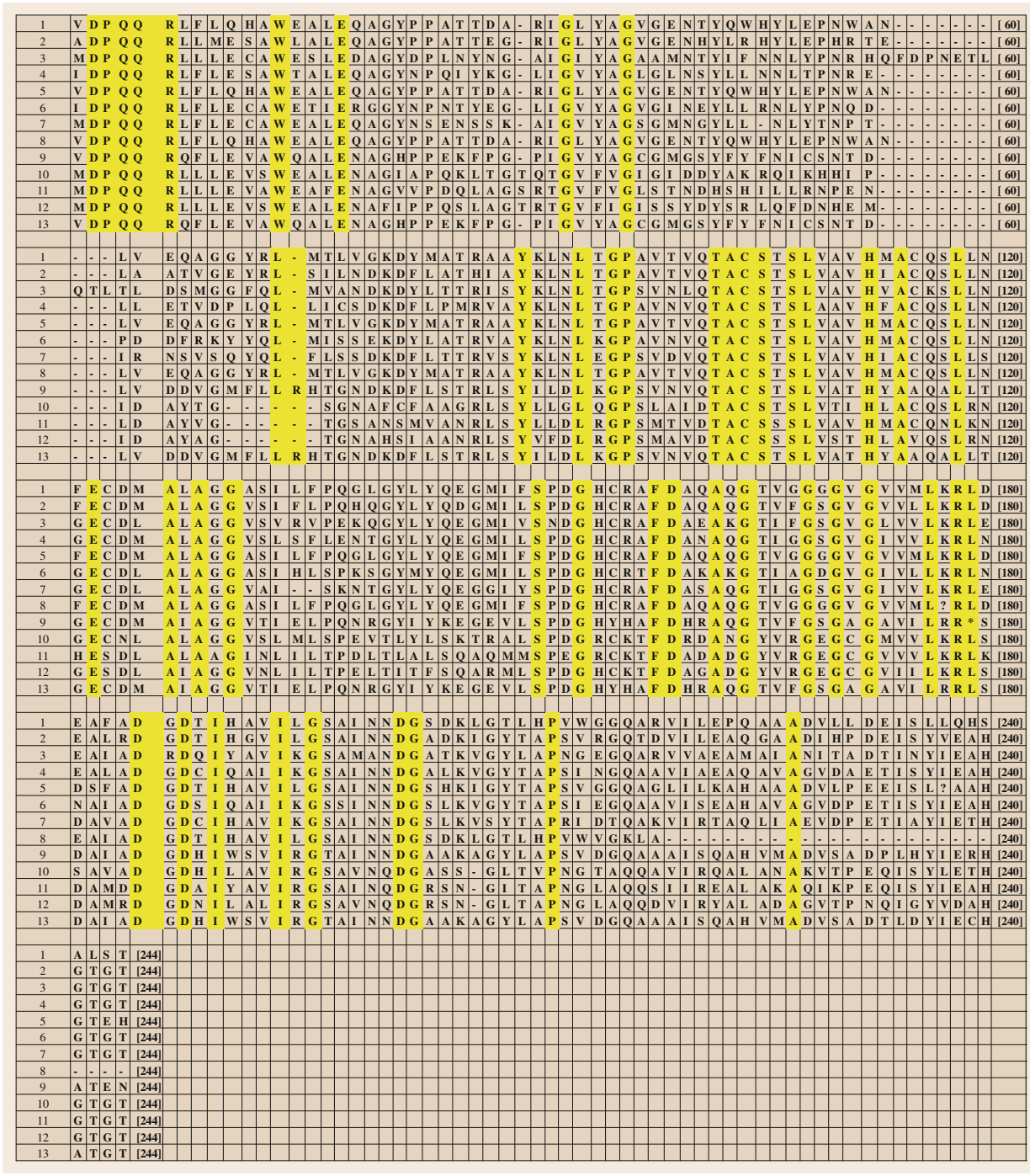
Fig. 8.9 UPGMA bootstrap-consensus tree showing the evolutionary relationship of inferred amino acid sequences from translated putative PKS gene fragments cloned from the metagenome of the marine sponge *R. ramosa*. Included are the closest BLAST relatives and a methyltransferase outgroup. • – denotes sponge-derived sequences

potential of the microbiota associated with these sponges. With this approach, PKS genes have been identified in a number of sponges including *Pseudoceratina clavata*, *Discodermia dissoluta*, *Theonella swinhoei*, *Aplysina aerophoba*, *Haliclona simulans*, *Arenosclera brasiliensis*, amongst others, together with novel NRPS genes from the sponges *Haliclona okadai* and *A. aerophoba*.

We also employed such an approach to assess the metabolic potential of the marine sponge *Raspailia ramosa*, which we isolated from the Lough Hyne Marine Nature Reserve in Cork, Ireland. Many bioactive secondary metabolites and bioactivities have previously been reported from sponges of this genus [8.273]. The authors' group also previously identified antimicrobial activities from cultured isolates from *R. ramosa* [8.267]. These data combined suggest that there is a high likelihood that the metagenome of this sponge might be a good source of potentially exploitable bioactivity. Thus using degenerate primers targeting the ketosynthase domains of Type I PKS genes namely (MDPQQRf 5′-RTRGAYCCNCAGCAICG-3′ and HGTGTr 5′-VGTNCCNGTGCCRTG-3′ [8.143]), we analyzed metagenomic DNA from the sponge. This resulted in the cloning of five partial putative polyketide synthase genes, which, following blast analyses of the deduced amino acid sequences of these putative PKS and subsequent sequence alignment and phylogenetic tree construction, indicated the likely presence of two distinct types of PKS (Fig. 8.9). One tree branch included two highly similar ketosynthase gene fragments which were found to be only distantly related (40–45% amino acid sequence identity) to previously known genes, with the closest known related sequences being derived from cyanobacterial (*Anabaena* sp., *Microcystis* sp.) PKS genes. The other clade included 3 sequences more related to each other than to any other known gene sequence, with significant amino acid sequence homology (58–73% similar) to known ketosynthase genes, including genes involved in the biosynthesis of Jamaicamide, a neurotoxic polyketide from *Lyngbya majuscula*. In all cases a highly conserved cysteine residue (residue 104, Fig. 8.10) at the enzyme active site was present.

A similar approach was subsequently employed to identify putative non-ribosomal peptide encoding gene fragments from the *R. ramosa* metagenome by

Fig. 8.10 Alignment of inferred amino acid sequences of cloned putative PKS gene fragments from the metagenome of the marine sponge *R. ramosa* with the closest known BLAST relatives. Conserved residues are highlighted in yellow. (1) WH009pksB8, (2) *Prochloron didemni* P1-Palau polyketide synthase, (3) *Planktothorix agardhii* polyketide synthase, (4) *Nostoc* sp. cis-AT polyketide synthase, (5) WH009pksB11, (6) JamM *Lyngbya majuscula*, (7) *Gloeothece memranacea* polyketide synthase ketosynthase domain, (8) WH009pksC7, (9) WH009pksD2, (10) *Anabaena variabilis* polyketide synthase, (11) *Microcystis* sp. β-ketoacyl synthase, (12) type I polyketide synthase, uncultured bacterium, (13) WH009pksD5. Sponge-derived sequences are denoted WH009 ►



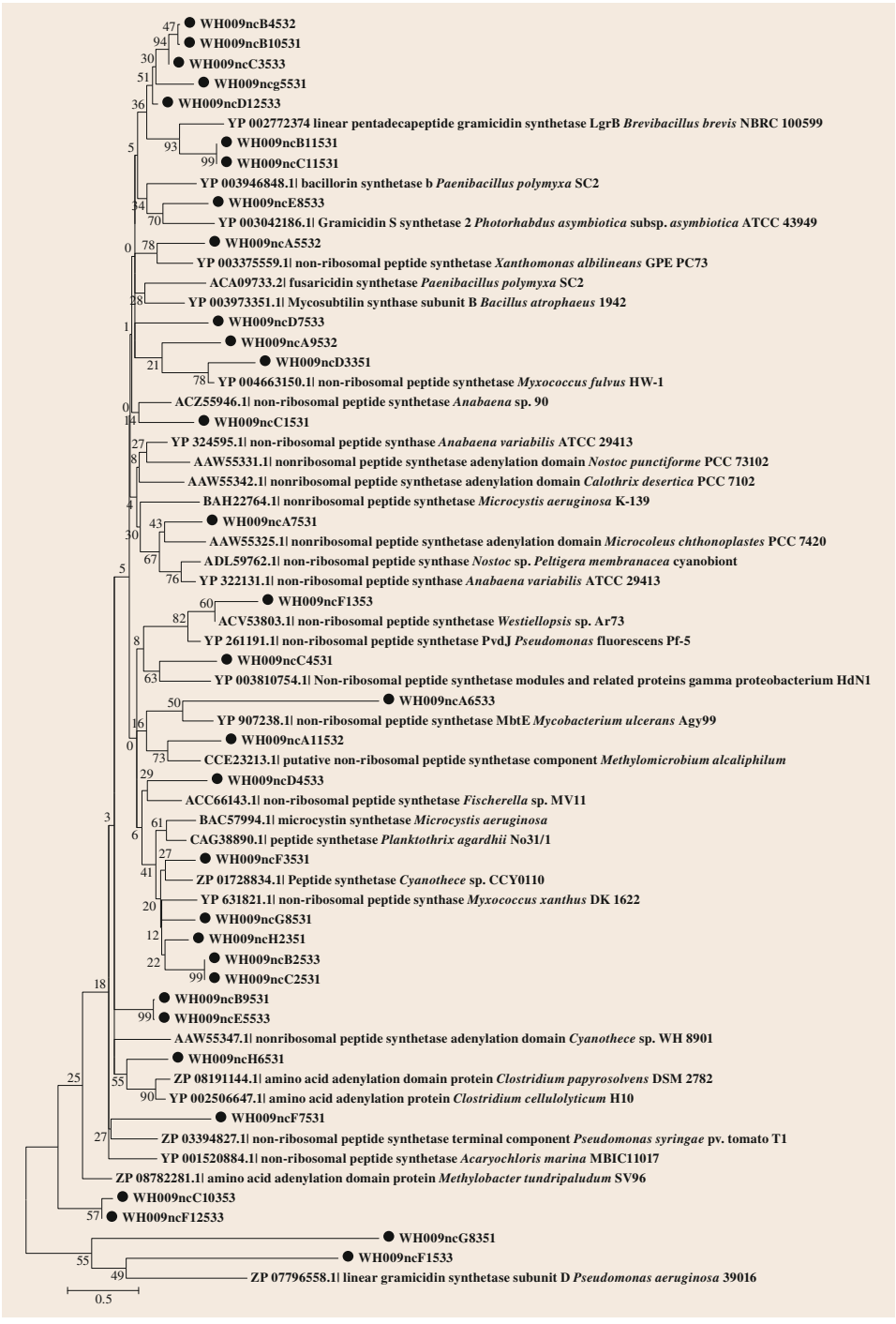


Fig. 8.11 Maximum likelihood bootstrap-consensus tree showing the evolutionary relationships between inferred amino acid sequences of partial putative NRPS gene sequences cloned from the metagenome of the marine sponge *R. ramosa* and their closest BLAST relatives. • – denotes sponge-derived sequences

employing degenerate primers to target the adenylation domain of potential NRPS genes (MTF2 5′-GCNGGYGGYGCNTAYGTNCC-3′ and MTR 5′-CCNCGDATYTTNACYTG-3′ [8.274]). This resulted in the cloning of 32 putative partial NRPS genes which, following BLAST analyses and phylogenetic tree construction, revealed a high level of diversity with many sharing homology (amino acid sequence identities ranging from 40–99%) with genes known to be involved in antimicrobial compound biosynthesis, and the cloned PCR products being related to varying degrees to gene products from at least 21 bacterial genera from 4 bacterial phyla. Examples of partial genes cloned here, which were similar known antimicrobial biosynthetic genes, include a sponge-derived gene sequence with similarity to the biosynthetic genes responsible for the production of the cytotoxic cyanobacterial product, microcystin and putative NRPS genes, which shared significant homology with genes involved in the biosynthesis of antibacterial (fusaricidin) and antifungal (bacillomycin L, mycosubtilin) compounds.

The high levels of bacterial diversity associated with *R. ramosa* together with the identification of diverse biosynthetic gene fragments of interest and the previous isolation of bacteria showing antimicrobial activities from *R. ramosa* make this species of particular research interest. Moreover, the wide diversity of potential non-ribosomal peptides that may be produced by bacteria associated with the sponge is also especially interesting. A PCR-based approach has recently also been employed to demonstrate a ribosomal origin for the bioactive metabolites polytheonamides A and B isolated from the marine sponge *Theonella swinhoei*. The biosynthetic gene cluster was cloned from the sponge metagenome, revealing a bacterial gene architecture involving six candidate enzymes which appear capable of carrying out up to 48 posttranslational modifications. This work, together with the work reported here, further highlights the biosynthetic scope of ribosomal systems within sponge metagenomes, and may ultimately provide further opportunities for peptide bioengineering strategies [8.275].

8.7 Conclusions

The results presented here clearly identify the considerable promise of metagenomic technologies and techniques to discover and exploit novel genes and gene products with potential commercial value. However, much work remains to be done to determine if the enzymes and compounds discovered here can realize that potential.

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Tools and Methods Part B

Part B Tools and Methods in Marine Biotechnology

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9. Bioprocess Engineering of Phototrophic Marine Organisms

Gregory L. Rorrer

Phototrophic marine organisms, particularly photosynthetic marine algae, are a diverse source of natural products ranging from lipid-based biofuels to pharmacologically-active compounds. The engineered production of both biomass and natural products is accomplished within enclosed, illuminated bioreactor systems called photobioreactors. This chapter describes the basic principles of photobioreactor analysis and design, and the biological factors that limit photobioreactor performance for the controlled cultivation of phototrophic marine organisms. There are three parts to this chapter. First, the quantitative growth characteristics of photosynthetic marine organisms in liquid cell suspension culture are described, including the use of photosynthetic biomass stoichiometry to describe nutrient incorporation into cellular biomass, and the combined effects of dissolved nutrient concentration and light intensity on cell growth rate. Second, the basic elements of photobioreactor design and operation are presented, focusing on models for predicting biomass production in batch or continuous cultivation modes within common photobioreactor configurations. Third, the limiting factors in photobioreactor design and operation are critically assessed from a quantitative point of view, focusing on light and carbon dioxide transfer limitations, and their effects on biomass production. Finally, future directions for the design and applications of enclosed photobioreactors are overviewed.

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9.1 Introduction to Marine Process Engineering

Marine bioprocess engineering focuses on the design and analysis of processes that manufacture valuable compounds from marine organisms within an enclosed, controlled environment. Marine bioprocesses use liv-

ing marine organisms as *cell factories* for biological synthesis of these compounds. Marine organisms can be broadly classified into two categories, those requiring an organic carbon source for growth (heterotrophs) and those that rely on photosynthesis for growth (phototrophs). This chapter focuses solely on phototrophic marine organisms. Phototrophic marine organisms comprise a vast group that includes photosynthetic bacteria, cyanobacteria, microscopic algae (microalgae), and seaweeds (macroalgae).

9.1.1 Phototrophic Marine Organisms: A Diverse Source of Valuable Natural Products

A marine natural product is defined as any valuable material that is biologically produced by a marine organism. This section overviews natural products of current or future value commercial value from phototropic marine organisms, with a particular focus on marine algae.

Phototrophic marine algae are particularly diverse sources of natural products, as detailed in several reviews [9.1–8]. The biosynthesis of natural compounds by phototrophic marine organisms falls into two categories. The first category comprises organic compounds biologically synthesized through primary metabolism. Primary metabolism represents a system of biochemical pathways that are essential for cell growth and reproduction. For example, the biosynthesis of sugars, amino acids, nucleic acids, proteins, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA), are usually associated with primary metabolism in phototrophic marine organisms. The second category comprises organic compounds derived from secondary metabolism. Biochemical pathways within secondary metabolism are not essential for cell growth or reproduction but serve some specialized function in time of need, for example, lipids for chemical energy storage, or bioactive chemical defense compounds to avoid being eaten by other organisms. In general, secondary metabolites are the source of pharmacologically-active compounds. Natural products derived from marine organisms do not have to be in a pure form. Often, the cell biomass itself or extracts of the cell biomass are valuable. These extracts, which can include both primary and secondary metabolites of human nutritional value, are called *nutraceuticals*.

Microalgae are a promising future platform for the production of advanced biofuels, as discussed in several recent reviews and papers [9.9–15]. Microalgae are single-celled photosynthetic organisms that grow, reproduce themselves, and biosynthesize energy-dense molecules through metabolic processes that utilize only sunlight, atmospheric CO_2, water, and macronutrients – principally nitrogen and phosphorous – as inputs. The variety of lipids biosynthesized within the algae, which can constitute up to 50 wt% of the algal biomass, can be chemically converted to liquid transportation fuels such as biodiesel ($\approx C_{14}--C_{22}$ fatty acid methyl esters) or green diesel by catalytic processes [9.16]. Relative to oil-bearing land plants for biodiesel production, algae can thrive on saline waters not suitable for food production, and have much higher production rates. Furthermore, algal biofuel production systems can be sited on non-arable land because the organisms are grown in water within engineered systems (e.g., open ponds or enclosed photobioreactors), not in soil.

Other than algal biofuels, commercially important natural products from phototrophic marine organisms fall into five categories:

1. Nutritional supplements or nutraceuticals
2. Specialty pigments
3. Industrial polysaccharides
4. Products for aquaculture
5. Pharmaceutical compounds.

Examples of commercial natural products from phototrophic marine organisms are provided in Table 9.1. Commercial natural products include beta-carotene, the polyunsaturated fatty acids (PUFAs) EPA and DHA, *Spirulina* dried biomass, astaxanthin (an aquaculture feed supplement), and the hydro-colloids. Most of the products listed in Table 9.1 are primary metabolites, although some polyunsaturated fatty acids and polysaccharides can also be produced as a consequence of secondary metabolism. Discussions of the metabolic pathways associated with the biological synthesis of each of these compounds within the cell are beyond the scope of this chapter.

Phototrophic marine organisms will play a major role in emerging marine biotechnology revolution because they are a rich source of novel drugs for the future. Below, a few exciting new pharmaceutical candidates from cyanobacteria, microalgae, and macroalgae are overviewed.

Marine cyanobacteria and microalgae offer a diverse array of pharmaceutical candidates. For example, a sulfolipid from the filamentous marine cyanobacteria *Lyngbya* sp. is in preclinical trials for treatment against the human immunodeficiency virus (HIV). The thiazoline-containing lipid Curacin A from *Lyngbya*

Table 9.1 Commercial high value products from phototrophic marine organisms

Product area	Product class	Examples	Representative organisms Species	Classification
Nutritional supplements	Carotenoids	Beta-carotene	*Dunaliella salina*	Green microalga
	PUFAs	Eicosapentaenoic acid (EPA) Docosahexanaeoic acid (DHA)	*Nannochloropsis* sp. *Phaeodactylum tricornutum* *Isochrysis galbana*	Green microalga diatom Green microalga
	Foodstuffs	Dried biomass rich in vitamins & proteins	*Spirulina platensis*	Cyanobacteria, green micoalga
Specialty pigments	Phycobiliproteins	Red phycoerythrin, blue phycocyanin	*Porphyridium cruentum* *Spirulina platensis*	Red microalga Cyanobacteria
Industrial polysaccharides	Hydro-colloids	Sulfated polysaccharides	*Porphyridium cruentum*	Red microalga
		Agars, alginates, carrageenans	*Gracilaria* sp., *Laminaria* sp., *Eucheuma* sp.	Red, brown macroalgae
Aquaculture	Carotenoids	Astaxanthin for fish aquaculture	*Haematococcus* sp.	Green microalga
	Feedstock	Fresh biomass for bivalve and shrimp larvae	*Isochrysis, Dunaliella* sp.	Green microalgae

is also under research and development as an anticancer drug. Other cyanobacteria contain linear peptides that act as immunosuppressive agents. Brevetoxins from various dianoflagellates possess potent antiinfective properties. These are just a few examples of the broad spectrum of pharmacologically active compounds found in marine cyanobacteria and microalgae.

Many red and brown marine macroalgae (seaweeds) biochemically oxidize simple complex polyunsaturated fatty acids to complex compounds called eicosanoids. In eicosanoid biosynthesis, the stereochemically specific oxidation of arachidonic acid is accomplished through lipoxygenase, epoxygenase, and cyclooxygenase pathways to produce a variety of pharmacologically important compounds, including hydroxy fatty acids, prostaglandins, hepoxlins, and leukotrienes. Eicosanoids are important in maintaining normal physiological conditions in mammals, and aberrant production of these metabolites underlies several diseases, including inflammation, asthma, heart disease, and cancer. Unique eicosanoids produced by marine macroalgae possess potent pharmacological properties as well, and may ultimately lead to the development of new pharmaceutical agents to treat these diseases.

Halogenation of secondary metabolites, while rare in land plants and microorganisms, occurs frequently in marine organisms due to the abundance of chlorine and bromine in seawater. Red macroalgae in particular have evolved several unique biosynthetic pathways for the production of organohalogen compounds, primarily as a means for chemical defense against predation by herbivores. A novel pathway is the stereoselective halogenation of terpenoids. Monoterpenes and their biohalogenated derivatives have emerged as a class of chemopreventive agents for alleviating the proliferation and progression of cancer-related tumors. However, bioprocess technologies are needed for securing future supplies of these drug candidates in the quantities required for product development, testing, and eventual commercial production. Toward this end, research in marine biotechnology has focused on the development of cell and tissue suspension cultures of macroalgae that biologically synthesize pharmacologically active compounds in vitro [9.7].

9.1.2 Marine Bioprocess Engineering and the *Cell Factory*

Many of the natural products derived from phototrophic marine organisms are complex organic compounds that possess unique structures and stereochemistry. Often, these compounds are so complex that they cannot be duplicated by chemical synthesis in the laboratory. In this case, the phototrophic marine organism itself must serve as the living *cell factory* for the biological synthesis of these compounds. The living cell factory can assume the form of an intact organism, a tissue culture derived from the organism, or a liquid cell suspension culture derived from the organism. The liquid cell suspension culture is the most desired form for process

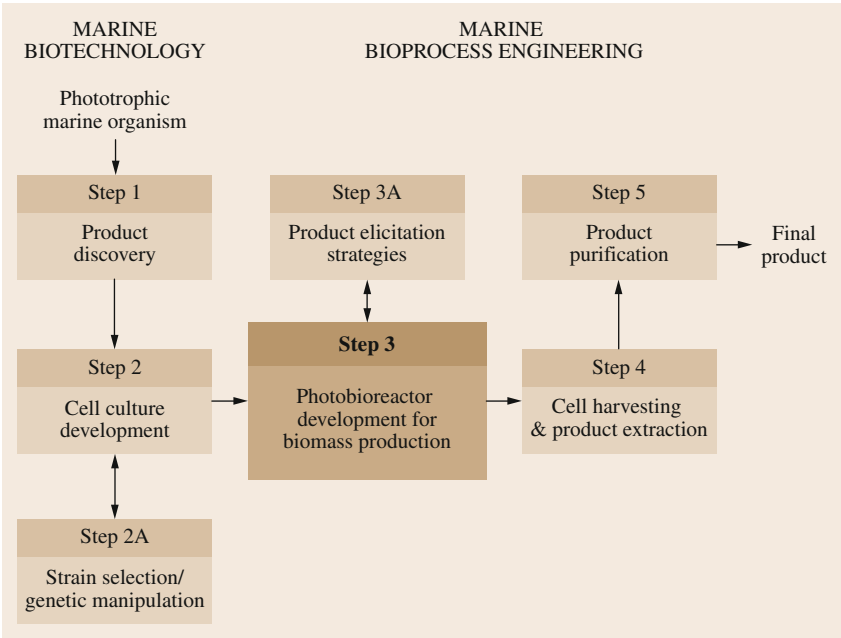

Fig. 9.1 The development of bioprocess technology for phototrophic marine organisms requires five steps, spanning both marine biotechnology and marine bioprocess engineering

biotechnology applications, as it resembles a fermentation broth. The cell factory can produce compounds resulting from primary metabolism, which are essential to cell growth, or compounds from secondary metabolism, which are not essential for cell growth but may serve some special function in time of need. Primary and secondary metabolites are often produced by a complex series of reactions within the cell – pathways that cannot be easily duplicated by other means. So how can the microscopic *cell factory* serve as a macroscopic *real factory* on a large scale? The answer lies in understanding how marine bioprocesses are developed, which is described below.

All bioprocesses include biomass production and product purification stages. The development of marine bioprocesses requires five steps, as illustrated in Fig. 9.1. In step 1, a valued natural product within a given marine organism is identified and characterized. In step 2, the specific marine organism that naturally produces the target compound is isolated and cultured. From the culture isolates, a cell suspension culture that grows on a nutrient medium is established. Often, several cell lines within a pure culture are established in order to find the one that biosynthesizes the most desired compound in the highest amount, a process called strain selection.

In step 3, a biological reactor is developed for cultivation of the marine organism under tightly controlled conditions on a process scale. In essence, the biological reactor, or bioreactor, houses the cell factories that produce cellular biomass containing the valued natural compounds. Usually, if cell biomass is produced, the target compounds are produced as well. However, often, elicitation strategies specific to the marine organism or its class of compounds must be developed. Elicitation strategies seek to coax metabolic pathways within the living *cell factory* to produce more of a desired compound or compounds. For example, the concentration of polyunsaturated fatty acids within an algal cell can often be improved by switching the culture medium from a nutrient-rich growth medium to a nitrogen-limited medium. This forces cell metabolism to produce compounds that do not contain nitrogen, such as fatty acids.

In step 4, the cell biomass in the liquid suspension culture is harvested by various techniques including any combination of flocculation, de-watering, sedimentation, centrifugation, or filtration. This step is often very costly, because often 100–5001 of water must be processed for each gram of dry cell mass harvested! Sometimes, the product is excreted from the cells to the surrounding liquid medium, but more often the product is retained within the cellular biomass. After harvesting, the desired natural product is then extracted from the cellular biomass with the appropriate aqueous or organic solvent. In step 5, established chemical process technologies such as liquid–liquid ex-

traction or chromatography are used to fractionate the cell extract into the final purified compounds. Topics related to *downstream bioseparation processes* such as biomass extraction and chromatography are beyond the scope of this chapter because they are generic to all forms of bioprocess technology, not just marine bioprocess technology. Excellent references on bioprocess engineering fundamentals and bioseparations are available [9.17–19].

This chapter focuses on the cultivation of phototrophic marine organisms in enclosed photobioreactor systems. However, it is also possible to cultivate phototrophic marine organisms outdoors in tanks or ponds. In open pond or tank cultures, the liquid surface is exposed to ambient air and direct sunlight. The open pond or tank culture is industrially practiced species of photosynthetic microalgae that can grow under conditions of salinity or pH where potential airborne contaminants cannot survive. A prominent example is the cultivation of the green microalga *Dunaliella* at elevated salinity in raceway ponds for production of beta-carotene. The two major limitations on open pond culture are sterility and process control. The manufacture of bioproducts requires a *GMP* or *good manufacturing practices* environment. Therefore, bioreactors must be enclosed and sterilizable to control contamination. Furthermore, bioreactor cultivation must be carried out with pure cultures under precisely controlled environmental conditions to ensure that the same product is reliably produced in the cell culture from batch to batch. Simultaneous control of contamination, temperature, pH, nutrient composition, aeration, and light is impossible to achieve in open pond or tank cultures.

9.2 Growth Characteristics of Phototrophic Suspension Cultures

Phototrophic marine organisms in liquid suspension culture share common growth characteristics. Below, concepts underlying photosynthetic biomass stoichiometry and specific growth rate are used to estimate the amounts and rates of photosynthetic biomass production.

9.2.1 Nutrient Requirements and Photosynthetic Biomass Stoichiometry

Photosynthetic Biomass Production

Phototrophic liquid suspension cultures utilize dissolved carbon dioxide as the carbon source and light as the energy source for photosynthesis. A detailed discussion of the complex biochemistry behind photosynthesis is beyond the scope of this chapter, and so the reader is encouraged to consult any of the excellent textbooks that cover photosynthesis, particularly in algal systems [9.20–22]. However, the basic idea of photosynthesis is to convert carbon dioxide to carbohydrates and ultimately to cellular biomass by an array of reduction reactions. Within the cell, photosynthesis is localized within organelles called chloroplasts. Within the chloroplasts are thylakoid membranes that contain complex molecules called chlorophylls that absorb light in the photosynthetically active radiation (PAR) range of 400–700 nm wavelength. The absorption of light by chlorophyll induces electron flow and transport that is used to drive reactions within two photosystems (I and II). Photosystem I carries out the reduction of the chemical energy carrier $NADP^+$ to NADPH (nicotinamide adenine dinucleotide phosphate) whereas Photosystem II splits water into O_2 and hydrogen ions (H^+). The proton gradient generated by Photosystem II produces the chemical energy carrier adenosine triphosphate (ATP) through the process of photophosphorylation. The NADPH and ATP generated by these processes subsequently reduce CO_2 to carbohydrates by a series of non-light requiring (dark) reactions collectively called the Calvin cycle. The overall stoichiometric equation for photosynthesis is

$$6CO_2 + 6H_2O \xrightarrow{h\nu} C_6H_{12}O_6 + 6O_2 \,, \qquad (9.1)$$

where $h\nu$ is the light energy. Note that CO_2 is consumed and O_2 is generated in a 1 : 1 stoichiometric ratio. However, this stoichiometric equation ignores the incorporation of other elements such as nitrogen and phosphorous into cellular material.

Medium Components

In phototrophic liquid suspension cultures, carbon dioxide is supplied to the culture by placing the liquid medium into contact with an aeration gas containing CO_2. The CO_2 dissolves into the liquid phase from the gas phase. In addition to dissolved CO_2 and light, phototrophic liquid suspension cultures require both

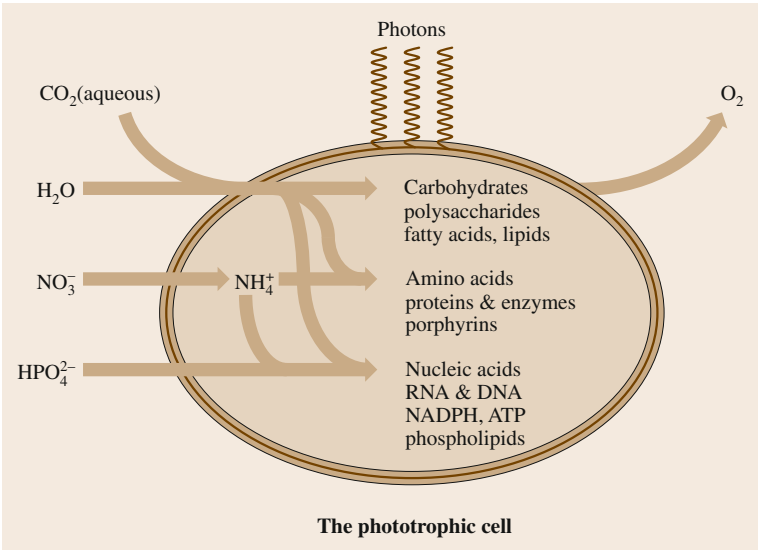

Fig. 9.2 Simplified schematic of macronutrient inputs to a phototrophic cell

macronutrients and micronutrients for growth. A very simplified schematic of the assimilation of macronutrients and CO_2 into the phototrophic cell is presented in Fig. 9.2. Both macronutrients and micronutrients are dissolved in a liquid base medium. The liquid base medium mimics the composition of seawater, which contains salts of sodium (Na), magnesium (Mg), potassium (K), and calcium (Ca) in order of decreasing abundance.

Macronutrients include nitrogen and phosphorous, usually supplied as inorganic salts. Nitrogen is typically supplied to the liquid medium as salt containing the nitrate anion NO_3^- or the ammonium cation NH_4^+. Nitrogen is assimilated into amino acids and ultimately protein. Phosphorous is typically supplied to the liquid medium as sodium or potassium salt containing the phosphate anions HPO_4^{2-} or $H_2PO_4^-$. Phosphorous is assimilated into nucleic acids, genetic material (RNA, DNA), chemical energy carriers (ATP, NADPH), and phospholipids.

Inorganic micronutrients include elements such as boron (B), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn), supplied as salts. Organic micronutrients, if used, are most commonly vitamins. Micronutrients usually serve as cofactors for enzymatic processes within the cell. The generic roles of macronutrients and micronutrients in the primary metabolism of phototrophic marine organisms and their typical concentrations in the liquid medium are summarized in Table 9.2. Liquid medium formulations also typically contain metal ion chelators such as ethylenediaminetetracetic acid (EDTA) to maintain iron solubility and alkaline buffers such as sodium bicarbonate to adjust the medium pH to the typical ambient seawater pH of 8.0.

Biomass Stoichiometry

The overall biomass stoichiometry approximates the incorporation of externally supplied carbon, nitrogen, and phosphorous (the macronutrients) into cellular material. The elemental composition of phototrophic marine organisms in a nutrient-rich environment typically follows the well-known Redfield ratio of 106C : 16N : 1P. Assuming that the elemental composition of phototrophic biomass follows the Redfield ratio, the photosynthetic biomass stoichiometry for incorporation of CO_2, nitrate (NO_3^-), and phosphate (PO_4^{3-}) into cellular material is

$$106\,CO_2 + 122\,H_2O + 16\,NO_3 + PO_4 \xrightarrow{h\nu} |\,(CH_2O)_{106}(NH_2)_{16}(PO_4)\,|_{\text{biomass}} + 138\,O_2\,. \tag{9.2}$$

Note that the CO_2 consumed and O_2 generated are not in a 1 : 1 stoichiometric ratio. The biomass stoichiometry for a specific organism can be proposed if the elemental composition ($C_x\,H_y\,O_z\,N_r\,P_s$) of the cellular biomass is known and the type of nutrients dissolved in the liquid medium is also specified.

The biomass yield coefficient relates biomass production to nutrient consumption. Biomass yield coefficients can be estimated from photosynthetic biomass

Table 9.2 Typical growth medium composition for phototrophic marine organisms

Typical medium component & concentration				Major cellular constituents/role or function
Macronutrients				
Nitrogen (nitrate)	N	$NaNO_3$	1–10 mM NO_3^-	Amino acids and proteins (enzymes), purines, porphyrins, amino sugars, amines
Phosphorous (phosphate)	P	Na_2HPO_4	0.05–0.5 mM HPO_4^{2-}	Nucleic acids and genetic material, chemical energy carriers (ATP, NADPH), phospholipids
Sulfur (sulfate)	S	$MgSO_4$	40 mM SO_4^{2-}	Amino acids (methionine, cysteine), sulfated polysaccharides, sulpholipids
Macrosalts				
Sodium	Na	NaCl	450 mM Na^+	Major component of seawater/water balance
Magnesium	Mg	$MgSO_4$	40 mM Mg^{2+}	Major component of seawater, component of chlorophyll
Potassium	K	KCl	10 mM K^+	Osmotic regulation, pH balance, protein conformation
Calcium	Ca	$CaCl_2$	10 mM Ca^{2+}	Major component of seawater/enzyme activation, ion transport
Micronutrients – trace elements a				
Boron	B	H_3BO_3	0.4 mM BO_4^{3-}	Diverse roles in primary metabolism and reproduction
Cobalt	Co	$CoCl_2$	0.2 µM Co^{2+}	Constituent of vitamin B_{12}
Copper	Cu	$CuCl_2$	0.2 µM Cu^{2+}	Constituent of plastocyanin/enzyme co-factor, electron transport (photosynthesis)
Iron	Fe	$FeCl_3$	0.02 mM Fe^{3+}	Constituent of ferredoxin and cytochrome/enzyme co-factor for redox reactions including nitrate reductase
Molybdenum	Mo	Na_2MoO_4	5 µM MoO_4^{2-}	Enzyme co-factor: nitrate reductase
Manganese	Mn	$MnCl_2$	5 µM Mn^{2+}	Constituent of photosystem II, maintenance of chloroplast membranes
Silicon	Si	Na_2SiO_3	0.5 mM SiO_3^{2-}	Cell wall component (diatoms)
Zinc	Zn	$ZnCl_2$	5 µM Zn^{2+}	Enzyme co-factor: carbonic anhydrase
Micronutrients – vitamins				
Cyanocobalamin		B_{12}	0.001 µM	Enzyme co-factor/growth factor
Thiamin		B_1	1 µM	Enzyme co-factor/growth factor
Biotin			0.001 µM	Enzyme co-factor/growth factor

stoichiometry. For example, the biomass yield coefficients based on CO_2 consumption and for nitrate consumption for the biomass stoichiometry given in (9.2) are

$$\begin{aligned} Y_{X/CO_2} &= \frac{\text{g cellular biomass produced}}{\text{mol } CO_2 \text{consumed}} \\ &= \frac{1\text{ mol biomass produced}}{106\text{ mol CO }_2\text{consumed}} \frac{3531\text{ g biomass}}{1\text{ mol biomass}} \\ &= \frac{33.3\text{ g cells}}{1\text{ mol } CO_2}\,, \end{aligned} \tag{9.3}$$

$$\begin{aligned} Y_{X/N} &= \frac{\text{g cellular biomass produced}}{\text{mol nitrate consumed}} \\ &= \frac{1\text{ mol biomass produced}}{16\text{ mol } NO_3^- \text{consumed}} \frac{3531\text{ g biomass}}{1\text{ mol biomass}} \\ &= \frac{221\text{ g cells}}{1\text{ mol } NO_3^-}\,. \end{aligned} \tag{9.4}$$

It cannot be inferred from (9.3) and (9.4) that the molecular weight of biomass is 3531 $gmol^{-1}$, as this value simply represents the biomass composition referenced to the stated biomass stoichiometry. Published values of biomass yield coefficients must be interpreted with caution, as they often change at high N:P ratios in the liquid medium or under nitrogen limitation. Nevertheless, biomass yield coefficients are very useful for material balance calculations. For example, biomass yield coefficients can be used to predict the maximum cell density for a given dissolved nutrient composition, as detailed in Sect. 9.3.2.

CO_2 Speciation

Dissolved CO_2 speciates to bicarbonate and carbonate ions if the medium is alkaline. Phototrophic marine organisms grow best in a seawater-based medium at pH 8 to 9, where CO_2 speciates to bicarbonate (HCO_3^-) and

carbonate (CO_3^{2-})

$$CO_2(g) \leftrightarrow CO_2(aq)\,,$$
$$CO_2(aq) + H_2O \leftrightarrow HCO_3^- + H^+\,,$$
$$HCO_3^- \leftrightarrow CO_3^{-2} + H^+\,,$$
or
$$CO_2(aq) + OH^- \leftrightarrow HCO_3^-\,. \qquad (9.5a)$$

It must be emphasized that the speciation of CO_2 is a gas–liquid equilibrium process. The equilibrium absorption of CO_2 from the gas phase to the liquid phase is described by Henry's law

$$P_A = H[CO_2]_{aq} \qquad (9.5b)$$

where P_A is the partial pressure of CO_2 in the gas in contact with the liquid, $[CO_2]$ is the concentration of CO_2 dissolved in the liquid medium that is in equilibrium with the CO_2 partial pressure in the aeration gas, and H is the Henry's law constant. The subsequent mass action expressions for dissolved CO_2 speciation in a liquid medium are

$$K_1 = \frac{[H^+][HCO_3^-]}{[CO_2]_{aq}}\,,$$
$$K_2 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]}\,. \qquad (9.6)$$

From the mass action expressions and Henry's law for absorption of CO_2, it can be shown that the predicted equilibrium concentrations of bicarbonate $[HCO_3^-]$ and carbonate $[CO_3^{2-}]$ ions are

$$[HCO_3^-] = 10^{-(+pK_{a,1}-pH)}\frac{P_A}{H}[CO_3^{2-}]$$
$$= 10^{-(+pK_{a,2}-pH)}[HCO_3^-]\,. \qquad (9.7)$$

The total dissolved carbon concentration ($C_{A,T}$) is the sum of all specie concentrations

$$C_{A,T} = [CO_2]_{aq} + [HCO_3^-] + [CO_3^{2-}]\,. \qquad (9.8)$$

The pK_a values for dissociation of HCO_3^- and CO_3^{2-} are 6.05 and 9.23, respectively, in seawater of 35 ppt salinity at 15 °C. The Henry's law constant (H) for dissolution of CO_2 is 0.026 L atm mmol^{-1} at the same conditions. Selected equilibrium properties of CO_2 and O_2 in fresh water and seawater are presented in Table 9.3.

Representative plots of equilibrium species concentration versus pH are shown in Fig. 9.3. The dissolved concentration of CO_2 in seawater is a function of temperature and CO_2 partial pressure but is not a function of pH, whereas the ionic bicarbonate and carbonate species concentrations are also a function of pH. For example, if ambient air containing 350 ppm CO_2 (CO_2 partial pressure P_A of 0.00035 atm CO_2) is bubbled into a seawater medium at 15 °C, then at pH 8.0 the equilibrium dissolved CO_2, HCO_3^- and CO_3^{-2} concentrations are 0.014 mM, 1.2 mM, and 0.071 mM respectively. However, at pH 7.0 the equilibrium concentrations of CO_2, HCO_3^- and CO_3^{2-} are 0.014 mM, 0.12 mM and 0.00071 mM, respectively. At high pH, this bicarbonate reservoir can serve as ballast for dissolved CO_2.

9.2.2 Specific Growth Rate

The specific growth rate is a convenient tool for characterizing the intrinsic biomass production rate in the exponential phase of growth. It is defined as the derivative of the cell density (C_x, g cells/L^{-1} culture) versus cultivation time (t) curve divided by the cell density

$$\mu = \frac{1}{C_x}\frac{dC_x}{dt}\,. \qquad (9.9)$$

The specific growth rate (μ) is a normalized parameter with units of reciprocal time, e.g., h^{-1}, similar to a first-order rate constant associated with a first-order chemical reaction.

The specific growth rate of a particular phototrophic marine organism in a liquid suspension culture is affected by both intrinsic environmental conditions (pH, temperature, salinity, light intensity) and the available nutrient composition. At optimal pH, temperature, and salinity, the specific growth rate is principally affected by three variables: 1) the dissolved carbon dioxide concentration in the liquid medium; 2) the dissolved limiting nutrient concentration in the liquid medium; and 3) the incident light intensity to the cell surface for photosynthesis. Like most living organisms, the specific growth rate of phototrophic cells exhibits saturation with respect to each of these variables. The Monod model is most commonly used to describe saturation growth kinetics. By the Monod approach, the combined effect of these three variables on the specific growth rate is given by

$$\mu = \frac{C_A}{K_A + C_A}\frac{C_N}{K_N + C_N}\frac{I}{I_k + I}\mu_{max}\,, \qquad (9.10)$$

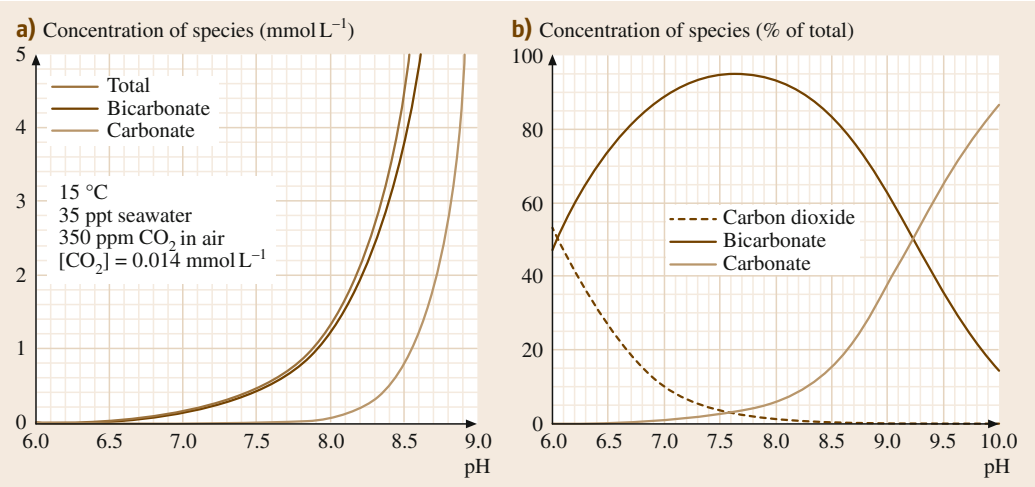

Fig. 9.3a,b Calculated speciation of dissolved carbon dioxide to carbonate and bicarbonate ions as a function of pH in a seawater-based liquid medium using air as the aeration medium. Conditions: 15 °C, 35 ppt seawater, CO_2 partial pressure of 0.00035 atm (350 ppm CO_2 at 1 atm total pressure), Henry's law constant for CO_2 of 0.026 L atm mmol^{-1}, equilibrium dissolved CO_2 concentration of 0.014 mM. **(a)** Molar concentrations of bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) versus pH; **(b)** molar percentage of dissolved CO_2, bicarbonate, and carbonate versus pH

Table 9.3 Equilibrium properties for the speciation of CO_2 in fresh water and seawater. All values are obtained from *Raven* [9.21]

Equilibrium property	Variable name	T (°C)	Value		Units
			fresh water	35 ppt seawater	
Dissolved CO_2 concentration in equilibrium with 35 Pa gas phase CO_2	(CO_2) or C_A*	5.0	22.43	18.75	μmol L^{-1}
		15.0	15.95	13.46	
		25.0	11.90	10.18	
		35.0	9.27	8.06	
Henry's law constant for CO_2	$H(CO_2)$	5.0	0.0154	0.0184	L atm mmol^{-1}
		15.0	0.0217	0.0257	
		25.0	0.0290	0.0339	
		35.0	0.0373	0.0428	
Dissolved O_2 concentration in equilibrium with 20.95 kPa gas phase O_2	(O_2)	5.0	391.5	310.8	μmol L^{-1}
		15.0	298.3	240.8	
		25.0	236.0		
Henry's law constant for O_2	$H(O_2)$	5.0	0.5280	0.6651	L atm mmol^{-1}
		15.0	0.6930	0.8584	
		25.0	0.8759	1.0683	
Dissociation constant for bicarbonate (HCO_3^-)	$pK_{a,1}$	5.0	6.52	6.11	
		15.0	6.42	6.05	
		25.0	6.35	6.00	
		35.0	6.31	5.97	
Dissociation constant for carbonate (CO_3^{2-})	$pK_{a,2}$	5.0	10.55	9.34	
		15.0	10.43	9.23	
		25.0	10.33	9.10	
		35.0	10.25	8.95	

where C_A is the dissolved carbon dioxide concentration ($\mathrm{mol\,L^{-1}}$), C_N is the dissolved limiting nutrient concentration ($\mathrm{mol\,L^{-1}}$), I is the light intensity incident to the cell surface ($\mu\mathrm{mol\ photons\,m^{-2}\,s^{-1}}$), I_k is the half-saturation constant for light intensity ($\mu\mathrm{mol\ photons\,m^{-2}\,s^{-1}}$), K_A is the half-saturation constant for dissolved carbon dioxide ($\mathrm{mol\,L^{-1}}$), K_N is the half-saturation constant for the dissolved nutrient concentration, and μ_{max} is the maximum possible specific growth rate of the phototrophic organism ($\mathrm{h^{-1}}$). It is important to note here that the dissolved nutrients are expressed as concentration in the liquid surrounding the cell whereas light is expressed as the photon flux incident to the cell. If the culture is not illuminated ($I = 0$), then the specific growth rate is zero. Furthermore, if the dissolved limiting nutrient concentration C_N or the dissolved CO_2 concentration C_A are zero, the specific growth rate is zero.

For many phototrophic marine organisms, values for K_A and K_N are typically very small, often in the range of only 10–100 $\mu\mathrm{mol\,L^{-1}}$. The specific growth rate is assumed to follow zero-order kinetics with respect to the dissolved carbon dioxide concentration C_A. A zero-order process means that as long as C_A is finite, the specific growth rate μ is not affected by C_A. However, if C_A is zero, then μ is zero even if sufficient dissolved nutrients and light are present. For zero-order kinetics with respect to CO_2, (9.10) reduces to

$$\mu = \frac{C_A}{K_A + C_A}\frac{C_N}{K_N + C_N}\frac{I}{I_k + I}\mu_{max} \cong \frac{C_N}{K_N + C_N}\frac{I}{I_k + I}\mu_{max}\,. \tag{9.11}$$

A short-hand form of (9.11) is

$$\mu \cong \frac{C_N}{K_N + C_N}\mu'\,, \tag{9.12}$$

where μ' represents the effect of light intensity alone on the specific growth rate. Often, in liquid suspension culture the dissolved carbon dioxide concentration C_A is much higher than K_A, *and* the limiting nutrient concentration C_N is much higher than K_N. Under these conditions, the growth kinetics are zero-order with respect to both of these variables and (9.12) becomes

$$\mu \cong \mu_{max}\frac{I}{I_k + I}\,. \tag{9.13}$$

The effect of light intensity on the specific growth rate deserves special attention, as this variable has the most profound effect on the specific growth rate. Light intensity is formally referred to as photosynthetically active radiation (PAR or irradiance) in the range of 400–700 nm wavelength. Irradiance is best expressed as quantum photon flux with units of $\mu\mathrm{mol\ photons\,m^{-2}\,s^{-1}}$. Further details on units of irradiance are provided in Sect. 9.3.3. The specific growth rate exhibits saturation growth kinetics with respect to light intensity. A representative plot of specific growth rate versus light intensity is shown in Fig. 9.4. At low light intensities, the specific growth rate linearly increases with increasing light intensity. At high light intensities, the specific growth remains constant at its maximum value with increasing light intensity, as all photoreaction centers within the cell are saturated by incident photons. At light saturation, the growth is CO_2 fixation rate limited. At very high light intensities above light saturation, oxidation processes resulting in a reduction in growth rate can occur, a process known as photoinhibition.

Four models that quantify the effect of I on μ are compared in Table 9.4. The parameters I_k and μ_{max} and photoinhibition constant K_i are unique for a given phototrophic marine organism and are determined experimentally by fitting I versus μ data to the desired model. Values of I_k range widely but typically vary from about 10–200 $\mu\mathrm{mol\ photons/m^{-2}\,s^{-1}}$. Published values of I_k and μ_{max} for a given phototrophic marine organism must be used with extreme caution since they

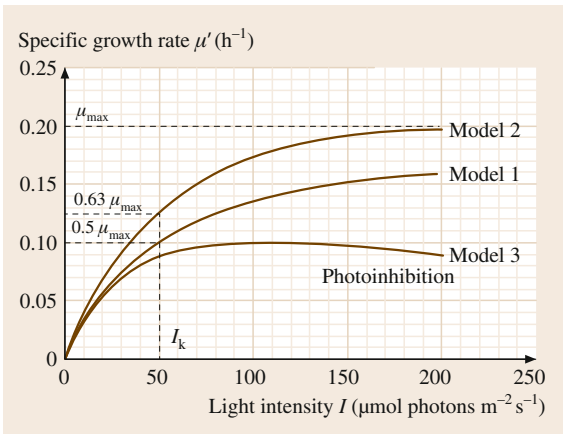

Fig. 9.4 Specific growth rate (μ') versus light intensity (I): comparison of Monod model (Model 1) with the exponential model (Model 2) and the photoinhibition model (Model 3). Model input parameters: $I_k = 50\,\mu$ mol photons $\mathrm{m^{-2}\,s^{-1}}$, $\mu_{max} = 0.2\,\mathrm{h^{-1}}$, $K_i = 0.005\,\mathrm{m^2\,s\,\mu}$ mol photons^{-1}

Table 9.4 Common light-saturation growth models for phototrophic marine organisms

Light-saturation growth model	Equation	Adjustable parameters	Limiting cases
Monod model (Model 1)	$\mu' = \frac{\mu_{max} I}{I_k + I}$	μ_{max}, I_k	$I \ll I_k$, $\mu' = \mu_{max}\frac{I}{I_k}$; $I = I_k$, $\mu' = 0.5\,\mu_{max}$; $I \to \infty$, $\mu' \to \mu_{max}$
Exponential model (Model 2)	$\mu' = \mu_{max}\left(1 - e^{-\frac{I}{I_k}}\right)$	μ_{max}, I_k	$I \ll I_k$, $\mu' = \mu_{max}\frac{I}{I_k}$; $I = I_k$; $\mu' = 0.632\mu_{max}$; $I \to \infty$, $\mu' \to \mu_{max}$
Monod model with photoinhibition (Model 3)	$\mu' = \frac{\mu_{max} I}{I_k + I + K_i I^2}$	μ_{max}, I_k, K_i	$I \ll I_k$, $\mu' = \mu_{max}\frac{I}{I_k}$; $I \to \infty$, $\mu' \to 0$
Bannister–Monod model (Model 4)	$\mu' = \frac{\mu_{max} I}{(I_k^m + I^m)^{1/m}}$	μ_{max}, I_k, m	$m = 1$, Monod model; $I \to \infty$, $\mu' \to \mu_{max}$

are usually dependent on the conditions under which the organism is maintained. Specific factors include the cell line of the organism, maintenance conditions for pH, temperature, salinity, and light intensity, and finally the particular constituents of the liquid medium formulation.

Phototrophic marine organisms are often subjected to an illumination cycle, commonly known as a photoperiod. Usually, the photoperiod is referenced with respect to a 24 h day and abbreviated as, for example, 16 : 8 LD, which refers to 16 h of light to 8 h of dark for one cycle on a 24 h photoperiod. During the dark phase of the photoperiod, it is assumed that no photosynthetic biomass production occurs. Therefore, the net specific growth rate over the entire photoperiod can be estimated by

$$\mu_{photoperiod} = \mu_{continuous\ light} f\,, \tag{9.14}$$

where f is the fractional photoperiod, defined as the time of culture illumination divided by the total time of the photoperiod, e.g., for a 16 : 8 LD photoperiod, f is equal to 0.67.

9.3 Basic Elements of Photobioreactor Design and Operation

Photobioreactors can assume many configurations. The common requirements and process material balances for well-mixed and tubular photobioreactors in batch and continuous operation are described below.

9.3.1 Common Requirements

A photobioreactor is an enclosed, illuminated culture vessel designed for the controlled biomass production of phototrophic liquid cell suspension cultures. All photobioreactor systems, regardless of configuration, must provide illumination, gas exchange, and mixing. Phototrophic cultures need light as the energy source to drive photosynthesis, and so the photobioreactor must deliver sufficient light to the culture vessel. Phototrophic cultures also use dissolved CO_2 as the inorganic carbon source for photosynthetic biomass production and evolve O_2.

Gas exchange is the process of adding CO_2 to the culture and removing evolved O_2 from the culture. Usually, gas exchange is accomplished by sparging an aeration gas into the liquid suspension culture. The aeration gas contains CO_2 that dissolves into the liquid. For example, ambient air contains about 350 parts per million (ppm) CO_2. The aeration gas also removes evolved O_2 by stripping the dissolved O_2 from the culture liquid to the aeration gas stream. The aeration gas stream exits the culture and carries out the evolved O_2 along with it.

Mixing of the liquid culture is required to suspend the biomass and to promote contact between the liquid nutrient medium and the cells. Mixing also promotes the process of gas exchange. A uniform biomass suspension allows light to uniformly penetrate the culture.

9.3.2 Biomass Production in Well-Mixed Batch or Continuous Operation

Photobioreactors can be operated in either batch or continuous modes, as illustrated in Fig. 9.5. The concepts underlying biomass production in batch and continuous bioreactors are described below.

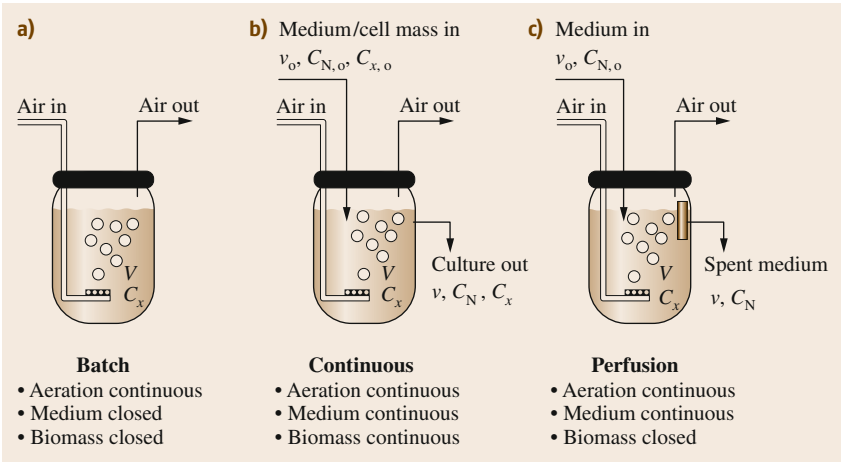

Fig. 9.5a–c Comparison of (**a**) batch, (**b**) continuous, and (**c**) perfusion bioreactors

Well-Mixed Batch Cultures: Unsteady-State Material Balances

In a batch bioreactor, the biomass suspension is retained within the cultivation vessel. Initially, a small amount of suspension culture is added to the vessel containing a liquid medium, which is a process called inoculation. As long as adequate amounts of carbon dioxide and light are continuously provided to the liquid suspension culture, the cell mass grows and consumes the macro and micronutrients dissolved in the liquid medium. The cell density within the culture vessel increases with time until a limiting nutrient within the liquid medium is completely consumed, as illustrated in Fig. 9.6. After the limiting nutrient is completely consumed, cell growth moves to a stationary phase. Other processes can also lead to the stationary phase of growth. For example, the buildup of toxic components in the liquid medium produced by the cells can halt further growth.

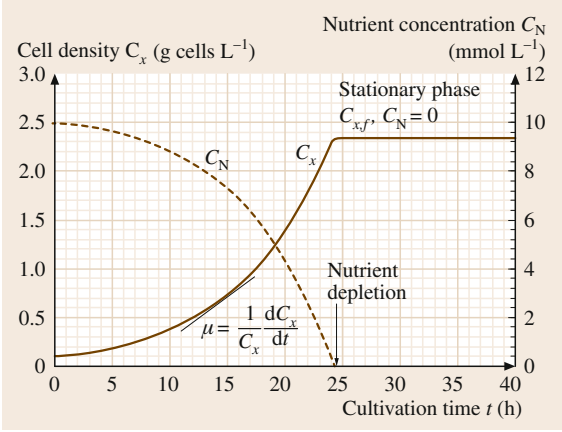

Fig. 9.6 Computed cell density (C_x) and limiting nutrient concentration (C_N) versus cultivation time in a well-mixed batch culture with light-saturated growth showing the final cell density at nutrient depletion. Model input parameters: $K_N = 0.1\,\text{mmol}\,L^{-1}$, $Y_{X/N} = 224\,\text{g cell mol}^{-1}\,\text{N}$, $\mu_{max} = 0.2\,h^{-1}$, $I_k = 50\,\mu\,\text{mol photons}\,m^{-2}\,s^{-1}$, $I = 100\,\mu\,\text{mol photons}\,m^{-2}\,s^{-1}$, $C_{N,i} = 10\,\text{mmol N}\,L^{-1}$, $C_{x,i} = 0.1\,\text{g cell}\,L^{-1}$

Material balances on cell biomass and the dissolved limiting nutrient describe the rates and amounts of biomass production within the batch bioreactor. Here, the cell suspension within the culture vessel is well mixed and the total culture volume is constant. The rate of biomass production is determined from a differential material balance on the cell mass, described in words as

$$\begin{pmatrix}\text{rate of cell mass}\\ \text{added to vessel}\end{pmatrix} - \begin{pmatrix}\text{rate of } \textit{cell} \text{ mass}\\ \text{removed from vessel}\end{pmatrix} + \begin{pmatrix}\text{rate of generation of}\\ \text{cell mass within vessel}\end{pmatrix} = \begin{pmatrix}\text{rate of accumulation of}\\ \text{cell mass within vessel}\end{pmatrix}. \tag{9.15}$$

In a batch reactor, the rates of cell mass addition and removal from the vessel are zero. Mathematically, the differential material balance on cell mass in the batch bioreactor is given by

$$0 - 0 + \mu C_x V = \frac{d\,(C_x V)}{dt}. \tag{9.16}$$

Simplification of (9.16) and combination with (9.12) yields

$$\frac{\mathrm{d}C_x}{\mathrm{d}t} = \mu C_x = \mu' \frac{C_\mathrm{N}}{K_\mathrm{N} + C_\mathrm{N}} C_x \,, \tag{9.17}$$

where C_x is the cell density in the liquid suspension culture (g cells L^{-1} culture), V is the total culture volume, and μ/describes the dependence of specific growth rate on light intensity as presented earlier in (9.11). Equation (9.17) is subject to an initial condition $C_x = C_{x,\mathrm{i}}$ at $t = 0$, the beginning of the cultivation cycle. For now, (9.17) is left in differential form because μ also depends on the delivery of light and CO_2 to the photobioreactor. Detailed methods on how to estimate growth rate in light-limited or CO_2-limited growth are described in Sects. 9.4.2 and 9.4.3.

In a batch bioreactor, the cell mass will not grow forever because at some point the limiting nutrient will be exhausted ($C_\mathrm{N} = 0$). The cell density at nutrient depletion is determined by an integral material balance on the cell mass, which is described in words as

$$\begin{pmatrix} \text{final cell mass} \\ \text{at end of cultivation} \end{pmatrix} - \begin{pmatrix} \text{initial cell mass} \\ \text{at beginning of cultivation} \end{pmatrix} - \begin{pmatrix} \text{amount of cell mass produced} \\ \text{by limiting nutrient consumption} \end{pmatrix} = 0 \,. \tag{9.18}$$

Mathematically, (9.18) is expressed as

$$C_x V - C_{x,\mathrm{i}} V - (C_{\mathrm{N,i}} - C_\mathrm{N}) V Y_{X/\mathrm{N}} = 0 \,, \tag{9.19}$$

where $C_{\mathrm{N,i}}$ is the initial concentration of the limiting nutrient (mol $\mathrm{N\,L}^{-1}$), and $C_{x,\mathrm{i}}$ is the initial biomass concentration (g cells L^{-1}), and $Y_{X/\mathrm{N}}$ is the biomass yield coefficient for the limiting nutrient (g cell produced mol^{-1} N consumed). At nutrient depletion ($C_\mathrm{N} = 0$), (9.19) reduces to

$$C_{x,\mathrm{f}} = C_{x,\mathrm{i}} + C_{\mathrm{N,i}} Y_{X/\mathrm{N}} \,, \tag{9.20}$$

where $C_{x,\mathrm{f}}$ is the final cell density at $C_\mathrm{N} = 0$.

Well-Mixed Continuous Cultures: Steady-State Material Balances

In a continuous bioreactor, the biomass suspension is continuously removed from the culture vessel. Fresh liquid medium enters the culture vessel at a constant volumetric flow rate equal to the volumetric flow rate of the suspension culture exiting the vessel so that the total liquid volume within the vessel is constant. The aeration gas and light are also continuously delivered to the culture vessel. Continuous bioreactors are designed to operate at steady state, meaning that the rate of biomass production within the vessel equals the rate of biomass removal from the vessel. In steady-state operation, the cell density in the vessel does not change with time, and there is no accumulation of cell mass within the culture vessel. If the liquid suspension culture is well mixed within the vessel, then the cell density inside the vessel equals the cell density of liquid suspension culture exiting the vessel.

Material balances on cell biomass and the dissolved limiting nutrient describe the rates and amounts of biomass production within the continuous bioreactor. Again, the cell suspension within the culture vessel is well mixed and the total culture volume is constant. Using (9.15) as a template, the mathematically stated material balance on the cell mass in the continuous bioreactor is given by

$$C_{x,\mathrm{o}} v_\mathrm{o} - C_x v + \frac{\mu' C_x C_\mathrm{N} V}{K_\mathrm{N} + C_\mathrm{N}} = 0 \,. \tag{9.21}$$

In a similar manner, the material balance on the limiting nutrient is

$$C_{\mathrm{N,o}} v_\mathrm{o} - C_\mathrm{N} v + \frac{\mu' C_x C_\mathrm{N} V}{(K_\mathrm{N} + C_\mathrm{N})\, Y_{X/\mathrm{N}}} = 0 \,. \tag{9.22}$$

In continuous culture, D is the dilution rate (h^{-1}), defined as

$$D = \frac{v_\mathrm{o}}{V} \,. \tag{9.23}$$

Usually, there are no cells in the liquid inlet ($C_{x,\mathrm{o}} = 0$) and the volumetric flow rates of the inlet and outlet are equal ($v_\mathrm{o} = v$). In this case, (9.21) and (9.22) are rearranged to yield the following:

$$D = \mu = \frac{K_\mathrm{N}}{K_\mathrm{N} + C_\mathrm{N}} \mu' \,, \tag{9.24}$$

$$C_x = Y_{X/\mathrm{N}} \left(C_{\mathrm{N,o}} - \frac{D K_\mathrm{N}}{\mu' - D} \right) \,, \tag{9.25}$$

$$C_\mathrm{N} = \frac{D K_\mathrm{N}}{\mu' - D} \,. \tag{9.26}$$

Note that (9.25) and (9.26) depend on the dilution rate D. However, D cannot exceed μ'. If D exceeds μ' then

Table 9.5 General comparisons of enclosed photobioreactor configurations

Photobioreactor configuration	Mixing and biomass suspension	Aration and gas exchange	Light transfer	Shear damage to cells	Scale-up difficulty	Comments
Bubble or airlift aerated – planar vessel	Poor–adequate	Excellent	Good	Low	Moderate	Simple design
Bubble or airlift aerated – cylindrical vessel	Good	Excellent	Adequate	Low	Moderate	Simple design
Stirred tank – externally illuminated	Excellent	Excellent	Poor	High	Difficult	Suitable only for bench or small pilot scale
Stirred tank – internally illuminated	Excellent	Excellent	Adequate	High	Unproven	Design derived from established fermenter technology
Tubular – horizontal array	Poor–adequate	Poor–adequate	Excellent	Low–High	Easy	Airlift pumping can be used
Tubular – vertical array	Poor– adequate	Poor–adequate	Excellent	Low–high	Easy	Compact tube bank layout
Tubular – helical array	Poor–adequate	Adequate	Excellent	Low–high	Easy	Efficient light transfer & Compact tube layout
Airlift tubular	Adequate–good	Good	Good	Low	Easy	Simple design but large footprint for tube layout

a phenomenon known as *washout* occurs, where the residence time of the cells within the vessel is not sufficient to sustain culture growth at constant cell density. In this instance, the cells leave the vessel faster than new cells can be formed, and the cell density C_x goes to zero. Therefore, continuous flow bioreactors have a limit on the range of dilution rate D. Furthermore, as detailed later in Sect. 9.4.2, within photobioreactor vessels the cell density C_x reduces μ' by a process known as light attenuation. Finally, with C_x known by (9.25), the volumetric biomass production rate of continuous bioreactors is given by

$$r_x = DC_x \,. \tag{9.27}$$

The design equations above all assume that the CO_2 transfer to the culture is always provided at a sufficient rate to avoid CO_2-limited growth, and that the culture growth within the photobioreactor is not subject to light attenuation. It will be shown in Sects. 9.4.2 and 9.4.3 how these equations are used under conditions of light or CO_2-limited growth.

The perfusion bioreactor is the intermediate case between batch and continuous operation (Fig. 9.5). In a perfusion operation, the fresh medium inflow and waste medium outflow are continuous, but the cell mass is retained within the vessel. Therefore, cell density still increases with time. There are many methods to achieve perfusion operation, but the simplest is to put a retaining screen on the culture output line to retain the biomass. Since nutrients are continuously added to the bioreactor, perfusion operation is commonly used to avoid nutrient depletion until some other variable becomes limiting. Thus perfusion operation can increase final cell density.

9.3.3 Enclosed Photobioreactor Configurations

A myriad of elaborate and elegant photobioreactor configurations have been developed. Each configuration seeks to provide the best venue for illumination, aeration, and mixing of the photosynthetic cell suspension culture to promote optimal growth. In general, photobioreactors fall into three configurations, each with several variations on a given theme. These three configurations are the bubble-column/airlift photobioreactor, the stirred-tank photobioreactor, and the tubular photobioreactor. The general characteristics of each configuration are compared in Table 9.5. The photobioreactor vessel can be externally or internally illuminated. Specific examples of externally illuminated photobioreactors are provided in references [9.23–28], and examples of novel internally illuminated photobioreactor configurations are described in references [9.29–33].

Table 9.6 Approximate conversion factors for flux irradiance in the photosynthetically active range (PAR). For example, to convert PAR flux irradiance from energy units of $W\,m^{-2}$ to quantum units of $\mu mol\ photons\,m^{-2}\,s^{-1}$, multiply the numerical value for $W\,m^{-2}$ by 4.6, e.g. $10\,W\,m^{-2}$ multiplied by $4.6\,\mu mol\,m^{-2}\,s^{-1}$ per $1.0\,W\,m^{-2}$ gives $46\,\mu mol\ photons\,m^{-2}\,s^{-1}$

Required units conversion	Light source conversion factor		
	Natural sunlight	Metal halide lamp	White fluorescent lamp
$W\,m^{-2}$ to $\mu mol\ photons\,m^{-2}\,s^{-1}$	4.6	4.6	4.6
klx to $\mu mol\ photons\,m^{-2}\,s^{-1}$	18	14	12
klx to $W\,m^{-2}$	4.0	3.1	3.1

Illumination System and Vessel Material Requirements

Illumination of photobioreactors can be either external or internal. The light source for the externally illuminated photobioreactors is positioned outside the culture vessel. Therefore, the culture vessel wall is constructed of a transparent material to allow the transfer of light to the photosynthetic liquid suspension culture. The light source for internally illuminated photobioreactors is placed within the culture vessel.

Light sources for illumination of photobioreactors can be either artificial or natural. Common artificial light sources include fluorescent lamps and metal halide lamps, which provide irradiance in the photosynthetically active range of 400–700 nm. Irradiance is best expressed in terms of quantum photon flux, with common units of $\mu mol\ photons\,m^{-2}\,s^{-1}$. Common conversion factors that relate quantum photon flux to energy flux or other units of light measurement for common light sources are provided in Table 9.6. Fluorescent lamps are relatively inexpensive and can easily provide incident light intensities up to $150\,\mu mol\ photons\,m^{-2}\,s^{-1}$, whereas halogen lamps are more expensive but provide higher incident light intensities up to about $500\,\mu mol\ photons\,m^{-2}\,s^{-1}$. The lamps are arrayed on a grid to uniformly illuminate the vessel surface. More exotic and expensive artificial light sources include light emitting diodes or Xenon lamp sources with fiber optic light delivery systems. These light sources are used for internally illuminated photobioreactors. References on fiber optic illumination systems are provided in the Suggested Reading section at the end of this chapter.

Natural sunlight obviously provides the best quality PAR at high irradiance near $1000–2000\,\mu mol\ photons\,m^{-2}\,s^{-1}$ and is free. However, natural sunlight also has many drawbacks for bioprocess technology applications. First, the photobioreactor must be placed outdoors, which places severe constraints on sterility and temperature control. Second, it is very difficult to achieve reproducible light delivery as the light intensity of natural sunlight is dependent on several variables, including the weather, the time of day, the time of year, and geographic location.

Wasted light energy input to photobioreactors is dissipated as heat, and so the vessel must be temperature controlled. Usually, a heat exchanger is installed into the culture vessel to control the temperature of the liquid suspension culture. The heat exchanger usually consists of coiled metal tube bank that contacts the liquid culture within the vessel. Cold water pumped through the heat exchanger tubes cools the culture. Alternatively, hot water or steam pumped through the heat exchanger tubes heats the culture. Principles of heat exchanger design are beyond the scope of this presentation.

In general, externally illuminated photobioreactors are easier to design, fabricate, and operate than internally illuminated photobioreactors. This presentation of photobioreactor configurations focuses on externally illuminated photobioreactors with a uniform light source. A parameter common to all externally illuminated photobioreactors is the illumination surface area to volume ratio

$$S/V = \frac{\text{illuminated surface area of vessel exposed to culture}}{\text{culture volume inside vessel}} . \qquad (9.28)$$

The externally illuminated photobioreactor configurations described below seek to maximize S/V while providing adequate mixing and gas exchange.

Suitable materials for externally illuminated photobioreactor culture vessels depend on the vessel configuration. Furthermore, phototrophic marine organisms are cultivated in a salt water medium which is highly corrosive. Therefore, wetted parts are constructed of stainless steel or inert plastics such as polyethylene, polypropylene, Plexiglass, or clear polyvinylchloride (PVC). The transparent portions of column and planar photobioreactor vessels are constructed of glass or plexiglass sheets. Tubular photobioreactors are constructed of transparent rigid plexiglass tubing, translucent rigid

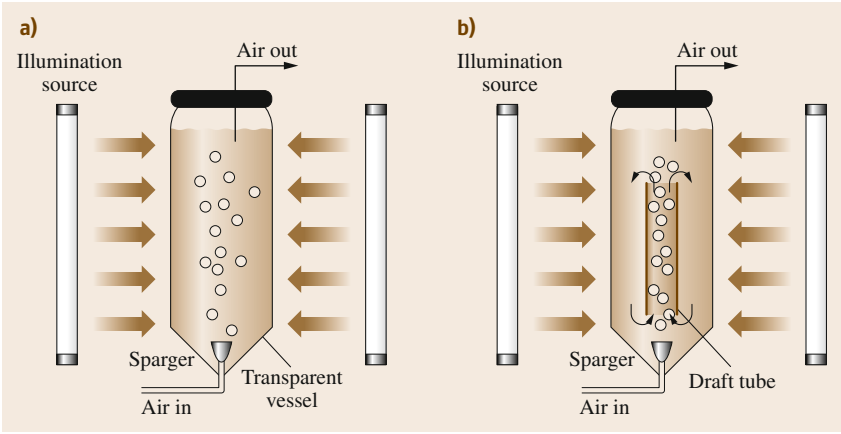

Fig. 9.7a,b Comparison of **(a)** bubble column aeration with **(b)** airlift aeration. In bubble column aeration, the bubbles fill the entire culture volume whereas in the airlift aeration with a draft tube, the bubbles are localized inside the draft tube

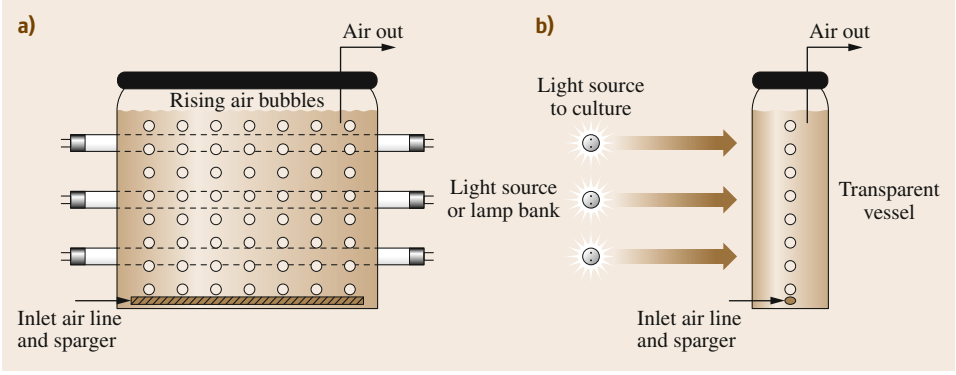

Fig. 9.8a,b The externally illuminated, aerated planar photobioreactor, showing modes of aeration gas and light delivery. **(a)** Front view, **(b)** side view

or flexible PVC tubing, or translucent flexible silicone tubing. The plastic materials also must be resistant to UV radiation from the illumination source.

Bubble-Column and Airlift Photobioreactors

The basic features of bubble-column and airlift photobioreactors in both cylindrical and planar vessel configurations are provided in Figs. 9.7 and 9.8 respectively. Aeration provides both mixing and gas exchange. The aeration gas stream is continuously injected to the bottom of the vessel through a sparger. The sparger forms small bubbles in the liquid medium. The bubbles rise through the culture and generate liquid convection currents, which mix and suspend the cells in the liquid medium. In the bubble column configuration, air is uniformly injected across the bottom of the vessel, and the rising bubbles fill the liquid volume. In the airlift configuration, air is injected only into the riser section. The air bubbles break up at the liquid surface, and the upward liquid flow generated by the rising air bubbles now moves downward through the downcomer section. Although the airlift configuration only aerates a portion of liquid in the vessel, the overall liquid circulation pattern within the vessel is uniform and promotes mixing and suspension of biomass. Airlift or bubble column aeration is a gentle means of mixing and biomass suspension and thus is appropriate for marine organisms that are fragile and easily damaged by fluid shear forces. Bubble-column and airlift photobioreactors can be operated in either batch or continuous biomass production modes.

Light delivery to bubble-column or airlift photobioreactors determines the vessel configuration. The two most basic vessel configurations are planar and cylindrical. The planar photobioreactor configuration (Fig. 9.8) is designed to minimize the path length for light penetration into the suspension culture and maximize the illumination surface area to culture volume ratio. The flat surface of the vessel is illuminated, either from one or both sides. The aeration gas is injected uniformly across the bottom of the vessel. Although light transfer is generally good, mixing is often

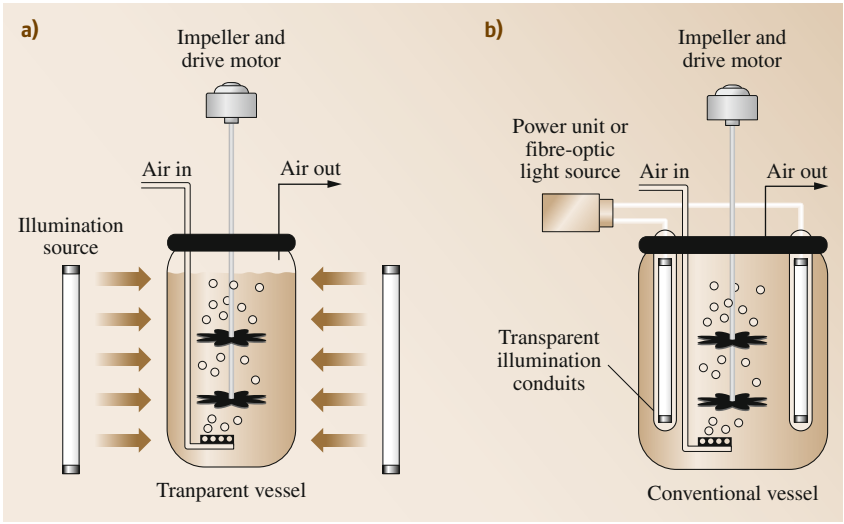

Fig. 9.9a,b Comparison of externally and internally illuminated stirred-tank photobioreactors. **(a)** External illumination, **(b)** internal illumination

non-uniform and dead zones occur where cell mass settles to the bottom corners of the vessel. Non-uniform mixing becomes more problematic as the culture volume increases upon process scale-up, particularly above 2000 L. The cylindrical photobioreactor configuration (Fig. 9.7) is designed to promote mixing and biomass suspension while maintaining an acceptable illumination surface area to culture volume ratio. The illumination source is aligned around the outside surface of cylindrical vessel or placed within an annular space in the center of the vessel. There are many variations to these basic planar and column photobioreactor configurations.

Stirred-Tank Photobioreactors

Externally and internally illuminated stirred-tank photobioreactor configurations are illustrated in Fig. 9.9. Stirred-tank photobioreactors provide the best venue for mixing and gas exchange because the impeller mechanically agitates the culture to suspend the biomass and to break up and disperse the aeration gas bubbles into the liquid phase. Stirred tank bioreactors of cylindrical vessel geometry typically have a height to diameter ratio of less than 3. Consequently, externally illuminated stirred tank photobioreactors are not practical at culture volumes exceeding 100 L because the path length for light penetration to the suspension culture is too large. Internal illumination can circumvent this light transfer constraint but complicates mixing and vessel design. Transparent glass or plastic tubes sealed at one end are mounted through the head plate of the vessel to accommodate fluorescent lamps. The lamps can be removed or replaced without opening the vessel. More advanced internally illuminated photobioreactors use fiber optics to deliver light to the culture [9.29–33].

Tubular Photobioreactors

The tubular photobioreactor is designed to minimize the path length for light penetration into the suspension culture and concurrently maximize the illumination sur-

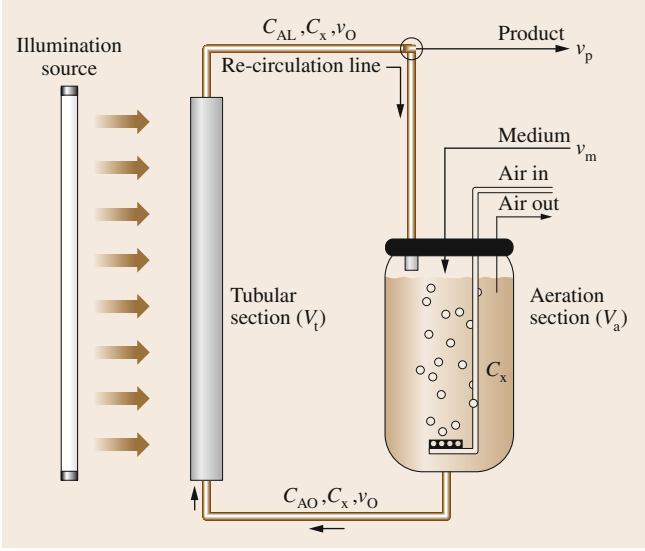

Fig. 9.10 Basic configuration of the externally illuminated tubular photobioreactor, featuring tubular section, aeration tank, and re-circulation line for batch or continuous operation

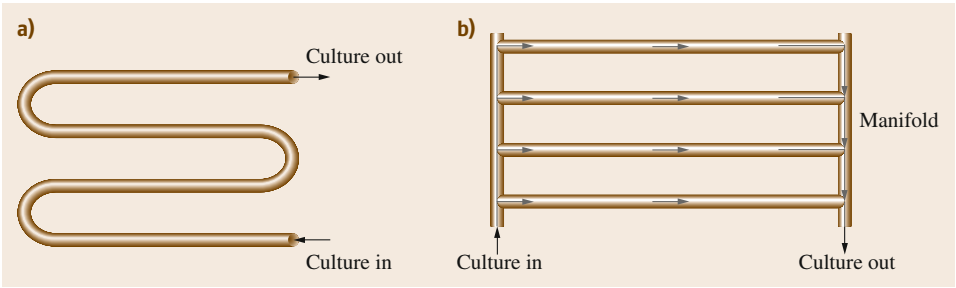

Fig. 9.11a,b Tubular photobioreactors: comparison of **(a)** single-tube bank and **(b)** parallel tube bank with manifold

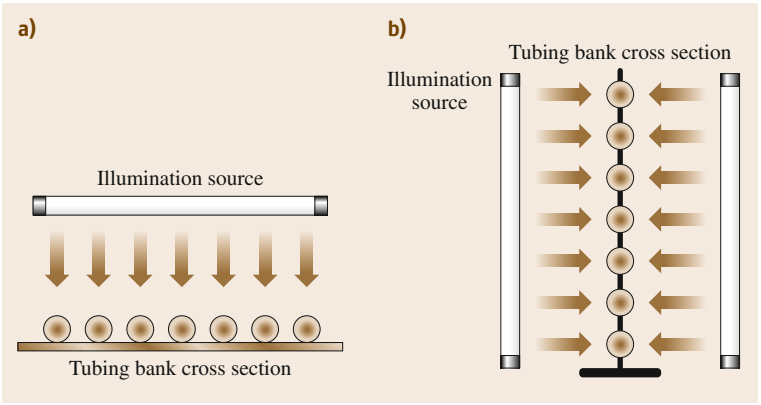

Fig. 9.12a,b Tubular photobioreactors: illumination of **(a)** horizontal tube bank array versus **(b)** vertically stacked tube bank array

face area to culture volume ratio. Tubular photobioreactors are much easier to scale up to large culture volumes exceeding 2000 L. Tubular photobioreactors can also be operated in batch or continuous biomass production modes, as described at the end of this section.

Most tubular photobioreactors have an aeration section connected to a tubular section, as shown in Fig. 9.10. The aeration section carries out the gas exchange processes for the entire photobioreactor. The aeration section is usually not illuminated. The tubular section is illuminated to promote photosynthetic biomass production. The tubular section is not usually aerated, as it is often difficult for air bubbles to travel down the length of tube. Therefore, the aeration section delivers CO_2 to the liquid suspension culture, whereas the culture within the tubular section consumes CO_2 by photosynthesis. Since the tubular section is not aerated, the CO_2 concentration in the liquid medium decreases and the dissolved O_2 concentration increases as the suspension culture moves down the length of the tube. Consequently, the phototrophic suspension culture is continuously re-circulated between the aeration section and the tubular section to replenish the suspension culture with CO_2 consumed by photosynthesis and to remove the O_2 buildup created by photosynthesis. Carbon dioxide-limited growth occurs when the dissolved CO_2 concentration inside the tube goes to zero. In order to avoid CO_2-limited growth, the residence time of the culture within the tube is short and so the biomass cell density is essentially constant for a single pass through the tubes. Cumulative biomass production occurs over many passes between the aeration tank and tubular section.

Light delivery to tubular photobioreactors is usually excellent because the maximum path length for light transfer is the diameter of the tubing. Typically, even for large-scale systems exceeding 1000 L, tubing diameters are below 6 cm. The light path length for the tubular photobioreactor is the shortest of all photobioreactor systems. The configuration of a particular tubular photobioreactor is determined by the arrangement of the tubing bank relative to the external illumination system. Three common tubular photobioreactor configurations are the horizontal tube bank, the vertically stacked tube bank, and the helical tube bank. Most tubing banks use a manifold to distribute the culture flow between several tubes running in parallel (Fig. 9.11). Manifold tube banks also reduce the pressure drop associated with frictional losses as the culture moves through the tubing, since the tubing length is reduced proportional to

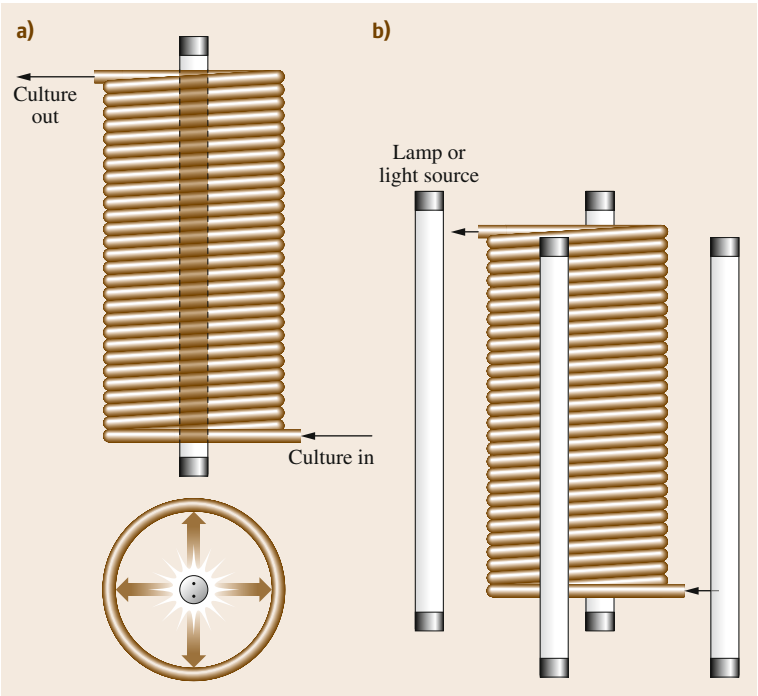

Fig. 9.13a,b Tubular photobioreactors: **(a)** internal versus **(b)** external illumination of helically wrapped tube array

the number of manifolds. The horizontal tube bank is illuminated from only one side (Fig. 9.12a), whereas the vertically stacked tube bank can be illuminated from two sides (Fig. 9.12b), similar to the planar photobioreactor. The helical tube bank is wrapped around a cylindrical support, and the tubing is essentially vertically stacked (Fig. 9.13). The lamps are placed within the tubing coil (Fig. 9.13a) or outside of the tubing coil (Fig. 9.13b).

There are two important parameters that characterize the flow of the liquid suspension culture inside the tube. The culture flow rate inside the tubing should be turbulent to adequately suspend the cell suspension as it travels down the length of the tube. The Reynolds number is a dimensionless parameter that defines the conditions for turbulent flow. The Reynolds number for flow of the liquid suspension culture through a tube is given by

$$\mathrm{Re} = \frac{v\rho_c d}{\mu_c} , \tag{9.29}$$

where d is the inner diameter of the tubing, v is the bulk linear velocity of the liquid culture (volumetric flow rate divided by cross-sectional area, $\mathrm{m\,s^{-1}}$), μ_c is the apparent viscosity of the liquid suspension culture ($\mathrm{kg\,m^{-1}\,s^{-1}}$), and ρ_c is the overall density of the liquid suspension culture ($\mathrm{kg\,m^{-3}}$). For simplicity, μ_c and ρ_c approximate the properties of the liquid medium, which in turn approximate the properties of water. Turbulent flow occurs at $\mathrm{Re} \geq 2000$. If the tube diameter is 2 cm, then the linear velocity for turbulent flow begins at about $10\,\mathrm{cm\,s^{-1}}$. Common linear velocities in tubular photobioreactors range from $30–50\,\mathrm{cm\,s^{-1}}$. Another important flow parameter for tubular photobioreactors is the culture residence time inside the tube

$$\tau_{\mathrm{tube}} = \frac{V_t}{v_o} , \tag{9.30}$$

where V_t is the volume of the culture in the tube.

There are two basic methods for circulating the liquid suspension culture between the aeration tank and the tubular section, as illustrated in Fig. 9.14. In Fig. 9.14a, the culture is mechanically pumped from the exit of the aeration tank to the entrance of the tubular section. The culture flow exiting the tubular section is returned to the aeration tank. In Fig. 9.14b, the culture exiting the tubular section is sent to an airlift riser. Air injected into the bottom of the riser section moves the culture up to a holding tank and simultaneously promotes gas exchange. The liquid suspension culture exits the holding tank and flows by gravity through the

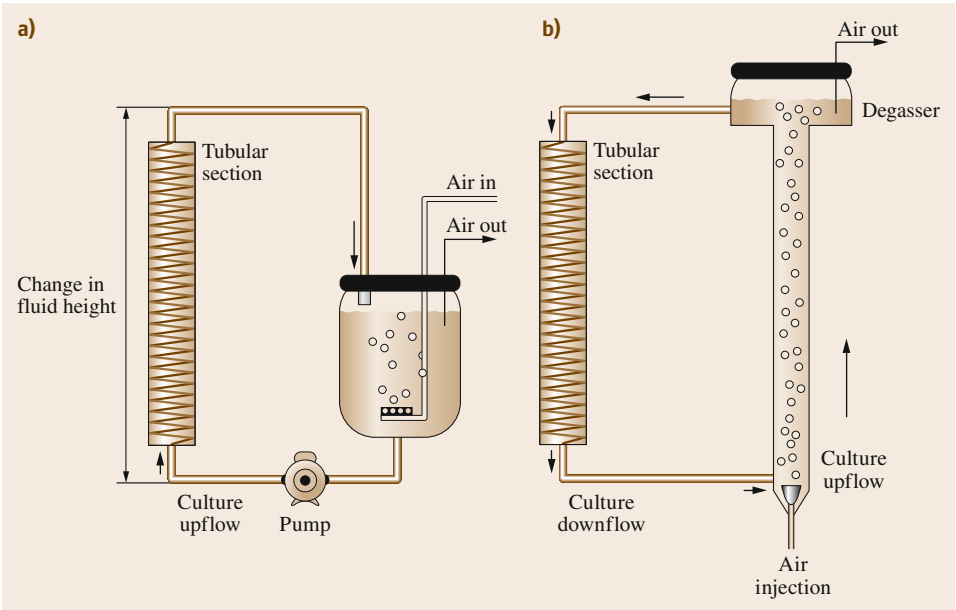

Fig. 9.14a,b Tubular photobioreactors: **(a)** mechanical versus **(b)** airlift pumping

tubular section. In both configurations, the culture recirculation system must compensate for pressure drop due to friction of the culture with the tubing walls and the difference in elevation between the tubular section entrance and exit points. The airlift system is relatively gentle on the cells and has no moving parts, but cannot accommodate high flow rates or pump against high fluid heads. The mechanically pumped system can provide high flow rates against high pressure drops. However, the pump can potentially damage the culture if the cells are fragile towards hydrodynamic or mechanical shear forces. In this context, volumetric pumps, where the pump rotor speed is proportional to flow rate, are preferred over centrifugal pumps, where the rotor speed is fixed independently of culture flow rate. Diaphragm pumps also provide relatively low shear.

A variant of the airlift pumping system for the tubular photobioreactor is the airlift tubular photobioreactor. It combines the elements of an airlift and tubular bioreactors into one system, as illustrated in Fig. 9.15. In the airlift tubular photobioreactor, the tube is inclined upward at an angle. Air is injected into the bottom of the tube to provide gas exchange and to move the suspension culture up the tube. Since the aeration gas is inside the tube, the tubing diameter is larger than that for conventional tubular reactors. Furthermore, the reactor volume must be increased to compensate for the space occupied by the bubbles. At the top of the tube is a degassing vessel where the aeration gas exits the system, and the culture flows back down to the bottom of the tube.

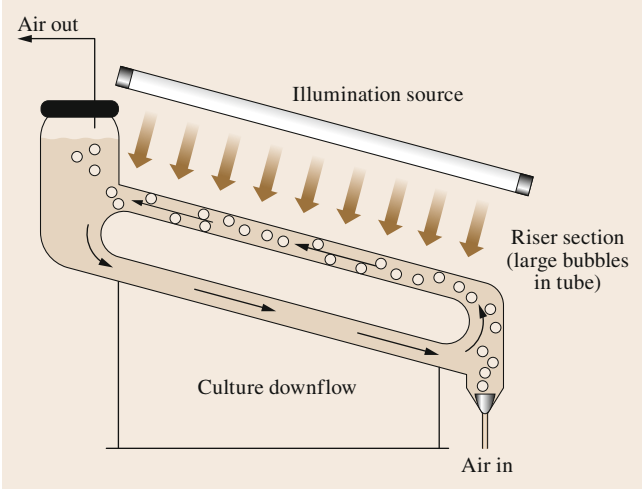

Fig. 9.15 The inclined tubular airlift photobioreactor

Tubular Recycle Photobioreactors

The tubular photobioreactors described above are designed for batch operation. Even though the culture is continuously flowing in and out of the tubing bank, it is still recirculating between the tubular section and

the aeration section and so there is no net flow of the culture out of the system. However, the tubular photobioreactor can be converted from batch operation to continuous operation by adding a product line to the re-circulation loop, as shown in Fig. 9.10. In the continuous recycle mode of operation, the liquid suspension culture exiting the tubular section is divided into two streams. The first stream, called the recycle line, is returned to the aeration section. The second stream, called the product line, is withdrawn from the system at a constant volume flow rate. The recycle ratio is defined as

$$R_p = \frac{v_o}{v_p} , \tag{9.31}$$

where v_o is the culture volumetric flow rate in the recycle line and v_p is the volumetric flow rate in the product line. As described earlier in Sect. 9.3.2, to maintain steady-state, constant cell density operation, fresh liquid medium is added to the aeration section at a volumetric flow rate equal to the volumetric flow rate of culture removal (i. e., $v_p = v_m$).

9.4 Limiting Factors in Photobioreactor Design and Operation

There are three major processes that limit photosynthetic biomass production in a photobioreactor. The first process, macronutrient limitation, was discussed in Sect. 9.3.2. The second process is the delivery of light for photosynthesis. The third process is the delivery of CO_2 for photosynthesis. Light delivery is traditionally seen as the limiting process to biomass production. However, CO_2 delivery limitations can be significant and are often overlooked in photobioreactor design and operation. In this section, methods are presented to estimate the combined effects of light delivery and CO_2 delivery on biomass production in photobioreactors. The modeling of light and CO_2 limitations in enclosed photobioreactors is described in several excellent references [9.34–40]. Process scale-up, measurement, and control issues in photobioreactor design and operation are also described, as they follow from the presentation of light and CO_2 delivery limitations.

9.4.1 Five Steps for Photobioreactor Design

Successful photobioreactor design depends on understanding light and CO_2 limitations. In this context, photobioreactor design has five major steps:

1. Use biomass stoichiometry to calculate the limiting nutrient concentration in the liquid medium necessary to achieve the desired final cell density in the vessel (Sect. 9.2.1).
2. Select a batch or continuous biomass production process. For relatively low rates of biomass production (e.g., $10–100\,kg\,d^{-1}$), consider a batch process. For higher biomass production rates, consider a continuous process (Sect. 9.3.2).
3. Select the photobioreactor configuration and incident light intensity which provides sufficient light delivery to the culture, and then compute the path length for light transfer so that the mean light intensity $I_m \geq I_k$ (Sect. 9.4.2).
4. Based on *step 3* above, design the aeration system to provide a CO_2 transfer rate (CO_2-TR) that is sufficient to avoid CO_2-limited growth at the final cell density in the culture vessel (Sect. 9.4.3).
5. Based on *step 3*, above, determine the cultivation time (batch process) or the culture residence time (continuous process) and the vessel volume required to satisfy the biomass production schedule.

Engineering analysis falls into two primary categories: design and performance analysis. Design starts with a set of process performance targets, e.g., required biomass production rate or cell density, and determines the photobioreactor design parameters necessary to achieve the production target. Specific design parameters include nutrient concentration in the liquid medium, vessel size and configuration, illumination system settings, and aeration system settings. In contrast, performance analysis usually begins with a photobioreactor system already in place that is operating at a given set of medium, illumination, and aeration conditions. Performance analysis estimates the biomass production at this set of process conditions. Below, the quantitative relationships needed for both process design and performance analysis are presented.

9.4.2 Light-Limited Growth

Light Attenuation

The specific growth rate of phototrophic organisms exhibits saturation growth kinetics with respect to incident light intensity. Therefore, the delivery of light to the photobioreactor in part determines the biomass pro-

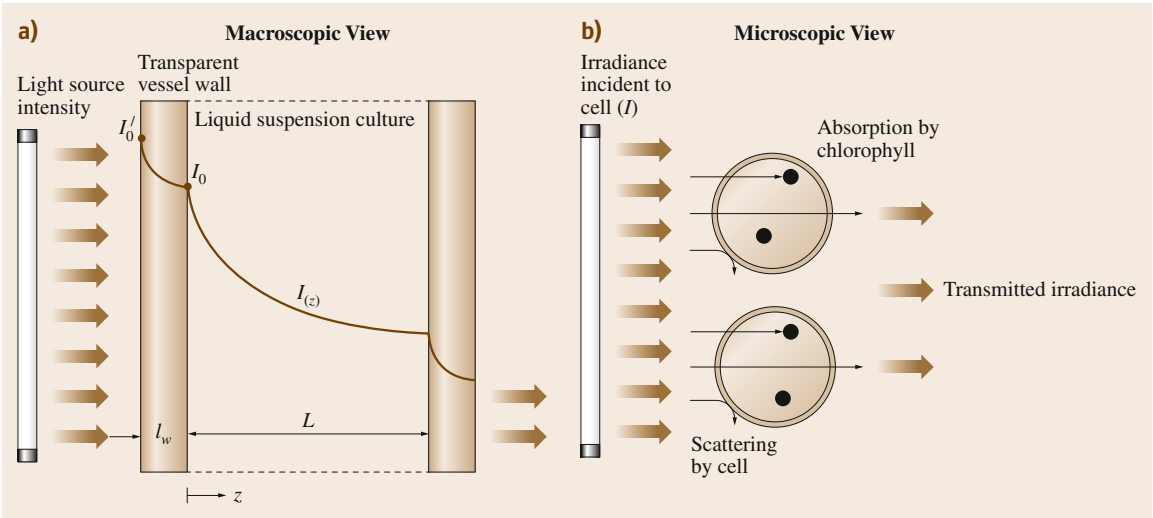

Fig. 9.16a,b Light attenuation for one-dimensional light transfer through the vessel wall and liquid suspension culture. **(a)** Macroscopic view, **(b)** microscopic view

ductivity in the photobioreactor. Consider a simple case illustrated in Fig. 9.16, where a transparent vessel of planar configuration is uniformly illuminated from one side. Recall that light intensity is expressed in units of flux. Light transfers through the transparent vessel wall and then penetrates the suspension culture (Fig. 9.16a). As the light penetrates into the suspension culture, photons are absorbed by the chlorophyll in the cells for photosynthesis or scattered by the cells in the suspension (Fig. 9.16b). The superimposing effects of absorption and scattering reduce the light flux. The light intensity decreases with increasing path length for the light flux and increasing biomass concentration in the liquid suspension. The reduction in light intensity is called light attenuation. The Beer–Lambert law for one-dimensional light transmission approximates the light intensity $I(z)$ as a function of path length z and biomass concentration C_x

$$I(z) = I_o e^{-k_c C_x z}\,, \tag{9.32}$$

where k_c is the specific attenuation constant of the phototrophic cell suspension culture (L(g cells cm)$^{-1}$). Values for k_c can range from 0.1–2.0 L(g cells cm)$^{-1}$ and are linear function of the specific chlorophyll concentration (Y_c) in the cell. Values for k_c are unique for a given phototrophic organism and must be experimentally determined, since the concentration of light absorbing pigments and the size of the cell aggregates can vary considerably even within a given species.

As seen in Fig. 9.17, light attenuation follows an exponential decay function. At long path lengths and high cell densities, the local light intensity $I(z)$ can approach zero. In this situation, the specific growth rate will also go to zero and no growth will occur. This behavior suggests three basic regimes for light attenuation within the vessel. Each regime can be referenced with respect to the I_k value for a given organism. In the first regime,

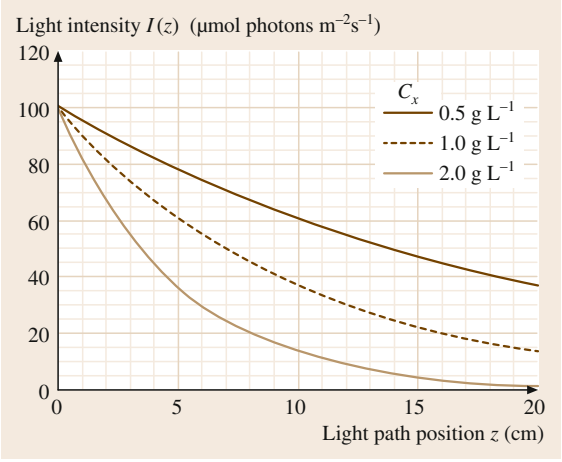

Fig. 9.17 Calculated light intensity (I) versus light path position (z) and cell density (C_x) for one-dimensional light transfer through well-mixed cell suspension culture of total light path length $L = 20$ cm. Model input parameters: $I_o = 100\,\mu$mol photons m^{-2} s^{-1}, $k_c = 0.1$ L (g cell cm)$^{-1}$

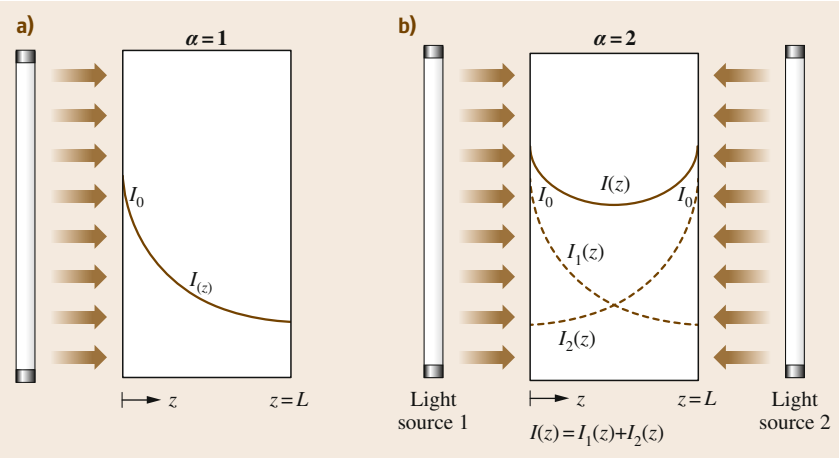

Fig. 9.18a,b Superposition of light intensity distribution for one-dimensional light transfer: comparison of **(a)** single-plane ($\alpha = 1$) versus **(b)** dual-plane ($\alpha = 2$) external illumination

$I(z) \gg I_k$, and the specific growth rate is not significantly affected by light intensity as the light intensity is near saturation. In the second regime, $I(z)$ is on the same order of magnitude as I_k, and the specific growth rate is now affected by the reduction in light intensity. In the third regime, $I(z) \ll I_k$, and the specific growth rate is now close to zero, the so-called dark zone.

Mean Light Intensity

It follows from the above discussion that the light intensity distribution sets the specific growth rate distribution of the phototrophic suspension culture within the photobioreactor. If the suspension culture is well mixed, then the cells circulate between areas of relatively high light intensity near the surface of the vessel and relatively low light intensities deeper within the vessel. In essence, all the cells within the suspension culture experience a mean light intensity that, in turn, sets the average specific growth rate of the culture. The mean light intensity is best determined by integrating the light distribution function over path length for light transfer. For the one-dimensional transfer of light across the width of a planar vessel shown in Fig. 9.18, the mean light intensity I_m is

$$I_m = \frac{1}{L}\int_0^L \alpha\, I(z)\,\mathrm{d}z = \frac{1}{L}\int_0^L \alpha\, I_o \mathrm{e}^{-k_c C_x z}\,\mathrm{d}z$$
$$= \frac{\alpha\, I_o}{k_c C_x L}\left(1 - \mathrm{e}^{-k_c C_x L}\right) , \qquad (9.33)$$

where α is the view factor for one-dimensional light transfer, representing the number of planes of light delivery: $\alpha = 1$ for one-sided illumination, $\alpha = 2$ for two-sided illumination.

Basic concepts for light attenuation in the one-dimensional system described by Fig. 9.18 and (9.33) can be extended to photobioreactors with more sophisticated vessel geometry and view factors for light delivery to the vessel surface. The mathematical aspects of these analyses can become quite complicated and are beyond the scope of this chapter. However, three cases are considered here. First, consider the photobioreactor vessel of planar geometry with two planes of light delivery shown in Fig. 9.18b. In this configuration, light is symmetrically delivered to the vessel surface from opposite sides. The light flux incident to the cells superimposes, so that the light flux from the left side and the light flux from the right side are simply added together at a given position within the path length for light transfer. In this illumination arrangement, α is equal to 2. Second, consider a photobioreactor vessel of cylindrical geometry of radius R uniformly illuminated around the vessel circumference with incident light intensity I_o (Fig. 9.19). In this more complicated case, the cross-sectional area for light transfer is a function of radial position within the vessel. The mean integral for I_m is given by

$$I_m = \frac{4 I_o \mathrm{e}^{-k_c C_x R} \sinh(k_c C_x R)}{k_c C_x R} . \qquad (9.34)$$

Finally, consider a parallel array of narrow cylindrical tubes, where the entire bank of tubes is uniformly illuminated from one side (e.g., Fig. 9.12). The diameter of the tube represents the longest light path for light. If the bank of tubes is approximated by a flat plate with the

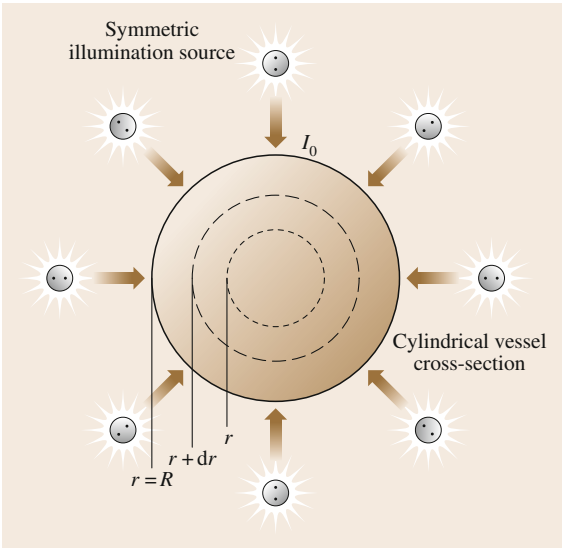

Fig. 9.19 Symmetrical, external light delivery to a cylindrical photobioreactor vessel

tubing diameter as path length for light transfer, then (9.33) can still be used to provide the most conservative approximation for the mean light intensity I_m.

Often the vessel wall material itself is not perfectly transparent and attenuates light, as illustrated in Fig. 9.16. Therefore, the transfer of light from the outer surface of the vessel to the culture encounters two resistances in series: the vessel wall and the liquid suspension culture itself. From the Beer–Lambert law, the light intensity incident to the outer vessel surface is related to the light intensity incident to the culture on the inner vessel surface by

$$I'_o = I_o e^{-k_w l_w} , \tag{9.35}$$

where k_w is the specific light attenuation constant for the vessel wall material (cm $^{-1}$), I'_o is the light intensity incident to the outer vessel wall surface, and l_w is the thickness of the vessel wall. Typical values of k_w for a transparent Plexiglass sheet and translucent silicon rubber tubing are 0.05 and 0.5 cm^{-1}, respectively. Values for k_w are best determined experimentally. The mean light intensity in terms of I'_o can be estimated by simply inserting (9.35) into (9.33).

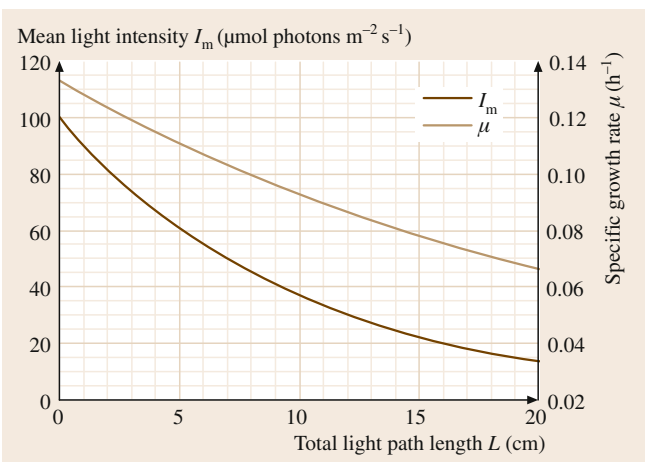

Fig. 9.20 Calculated effect of total light path length (L) on mean light intensity (I_m) and specific growth rate (μ) for one-dimensional light transfer. Model input parameters: $I_o = 100\,\mu mol\ photons\ m^{-2}\ s^{-1}$, $k_c = 0.1\,L\,(g-cell-cm)^{-1}$, $C_x = 2\,g\,cell\,L^{-1}$, $\alpha = 1$

Effects of Mean Light Intensity on Growth Rate and Biomass Production

Light attenuation reduces the mean light intensity (I_m) experienced by the phototrophic liquid suspension culture within a photobioreactor vessel. If the culture is uniformly mixed, the mean light intensity sets the specific growth rate. Therefore, in (9.11) the light intensity (I) is replaced with I_m, so that

$$\mu = \frac{C_N}{K_N + C_N} \frac{I_m(C_x)}{I_k + I_m(C_x)} \mu_{max} . \tag{9.36}$$

Recall from (9.33) that I_m is a function of the incident light intensity I_o, the path length for light transfer into the photobioreactor, and the cell density C_x. During phototrophic cultivation, the incident light intensity and path length for light transfer are fixed. However, as cell density C_x increases, I_m decreases and hence the specific growth rate μ decreases. The process of light attenuation therefore reduces biomass production. In particular, if the culture is not CO_2-limited, then biomass production for the well-mixed batch bioreactor described by (9.17) now becomes

$$\frac{dC_x}{dt} = \mu C_x = \frac{C_N}{K_N + C_N} \frac{I_m(C_x)}{I_k + I_m(C_x)} \mu_{max} C_x , \tag{9.37a}$$

with

$$\frac{dC_N}{dt} = -\frac{\mu}{Y_{X/N}} C_x . \tag{9.37b}$$

Equations (9.37a) and (9.37b) are subject to the initial conditions $C_x = C_{x,i}$, $C_N = C_{N,i}$ at $t = 0$.

The output cell density and limiting nutrient concentration for the well-mixed continuous-flow bioreactor described by (9.25) and (9.26) now become

$$C_x = Y_{X/N}\left(C_{N,o} - \frac{DK_N}{\frac{I_m(C_x)}{I_k + I_m(C_x)}\mu_{max} - D}\right) \tag{9.38}$$

and

$$C_N = \frac{DK_N}{\frac{I_m(C_x)}{I_k + I_m(C_x)}\mu_{max} - D}\,. \tag{9.39}$$

If the path length L is increased, then I_m and μ also decrease. To illustrate, the effect of light path length L on I_m and μ for a planar vessel with one-sided illumination ($\alpha = 1$) is presented in Fig. 9.20.

Biomass production for light-limited growth of phototrophic liquid suspension cultures in tubular photobioreactors follows from the equations described above. In the tubular photobioreactor, the tubular section improves the access of the culture to light. However, in order to avoid CO_2-limited growth, the residence time of the culture within the tube is short and so the biomass cell density is essentially constant for a single pass through the tubes. Cumulative biomass production occurs over many passes between the aeration tank and tubular section. Consequently, for batch operation of the tubular photobioreactor, the material balance development parallels that of (9.37a), but with one important modification. In the tubular photobioreactor, the aeration tank is not illuminated, and so only the tubular section culture volume V_t can promote photosynthetic growth. In this case, the biomass production rate for batch growth of the photosynthetic tubular photobioreactor under light-limited conditions is given by

$$\frac{dC_x}{dt} = \frac{V_t}{V}\mu C_x = \frac{V_t}{V}\frac{C_N}{K_N + C_N}\frac{I_m(C_x)}{I_k + I_m(C_x)}\mu_{max}C_x\,. \tag{9.40}$$

Model predictions reveal how light transfer affects biomass productivity in photobioreactors. The effect of light attenuation on cell growth kinetics is shown in Fig. 9.21. The batch cultivation is carried out in a well-mixed planar photobioreactor illuminated from one side. The growth curve (C_x versus t) and substrate consumption curve (C_N versus t) are generated by numerically integrating (9.37a) and (9.37b) over time using the model input parameters given in the caption of Fig. 9.21 and (9.20) as the limiting nutrient balance for estimation of C_N. As the cell density (C_x) increases with time, the mean light intensity I_m decreases, and the biomass production rate slows down. When the limiting nutrient is completely consumed the cell density becomes constant. As the path length for light transfer in the planar photobioreactor is increased from 5 to 20 cm, the biomass production rate decreases markedly. The effect of light attenuation on cell growth kinetics in a well-mixed continuous photobioreactor is shown in Fig. 9.22. Increasing dilution rate D (9.23) by increasing the volumetric flow rate of feed medium increases biomass productivity until washout is observed. Increasing the light path length L from 5 to 10 cm markedly decreases the optimum biomass production rate. Therefore, increasing light attenuation by increasing the path length for light transfer significantly lowers the performance of both batch and continuous photobioreactors in light-limited growth.

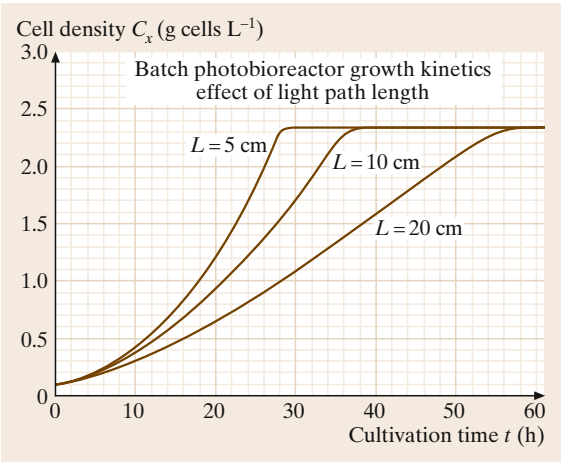

Fig. 9.21 Calculated effect of total light path length (L) on the cell density versus time curve within a well-mixed planar photobioreactor under *batch*, light-limited growth. Model input parameters: $\mu_{max} = 0.2\,h^{-1}$, $I_k = 50\,\mu mol\ photons\ m^{-2}\ s^{-1}$, $K_N = 0.1\,mmol\,L^{-1}$, $Y_{X/N} = 224\,g\ cell\ mol^{-1}\ N$, $k_c = 0.5\,L(g\,cell\,cm)^{-1}$, $C_{x,i} = 0.1\,g\,cells\,L^{-1}$, $C_{N,i} = 10\,mmol\,N\,L^{-1}$, $I_o = 150\,\mu mol\ photons\ m^{-2}\ s^{-1}$, $\alpha = 1$ (one-sided illumination)

9.4.3 Carbon Dioxide–Limited Growth

Phototrophic liquid suspension cultures utilize dissolved CO_2 as the sole inorganic carbon source for photosynthetic biomass production. Carbon dioxide is supplied to the culture by contacting the liquid medium with an aeration gas containing CO_2, for example by

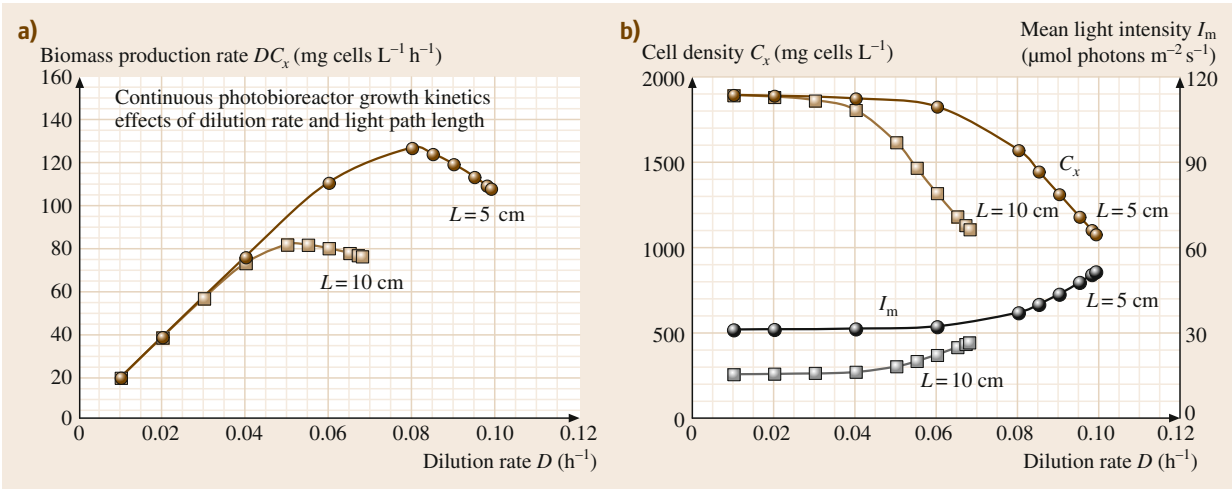

Fig. 9.22a,b Calculated effect of total light path length (L) and dilution rate on biomass productivity in well-mixed planar photobioreactor under *continuous*, light-limited growth. Model input parameters: $\mu_{max} = 0.2\,h^{-1}$, $I_k = 50\,\mu mol\ photons\ m^{-2}\ s^{-1}$, $K_N = 0.1\,mmol\,L^{-1}$, $Y_{X/N} = 224\,g\,cell\,mol^{-1}\,N$, $k_c = 0.5\,L\,(g\,cell\,cm)^{-1}$, $C_{x,o} = 0\,g\,cells\,L^{-1}$, $C_{N,o} = 10\,mmol\,N\,L^{-1}$, $I_o = 150\,\mu mol\ photons\ m^{-2}\ s^{-1}$, $\alpha = 1$ (one-sided illumination). **(a)** Biomass productivity, DC_x; **(b)** cell density C_x, and mean light intensity I_m at C_x

bubbling air containing CO_2 directly into the liquid suspension culture. The CO_2 entering the photobioreactor with the aeration gas can transfer from the gas phase to the liquid phase and become consumed by the photosynthetic cells, or simply exit with the aeration gas, as shown in Fig. 9.23.

The CO_2 is transferred from the gas phase to the liquid phase by a process known as interphase mass transfer. The CO_2 dissolved in the liquid phase is then consumed by the photosynthetic cells and incorporated into cellular biomass. If the CO_2 transfer rate provided by aeration and interphase mass transfer (i. e., the CO_2-TR) is not sufficient to meet the CO_2 consumption demand by the photosynthetically active culture, then the biomass production rate is CO_2 limited. Therefore, the rate at which CO_2 is delivered to the culture can have a significant impact on biomass productivity. The purpose of this section is to quantitatively define conditions that result in CO_2-limited growth and to identify strategies that avoid CO_2 limitations in photobioreactors.

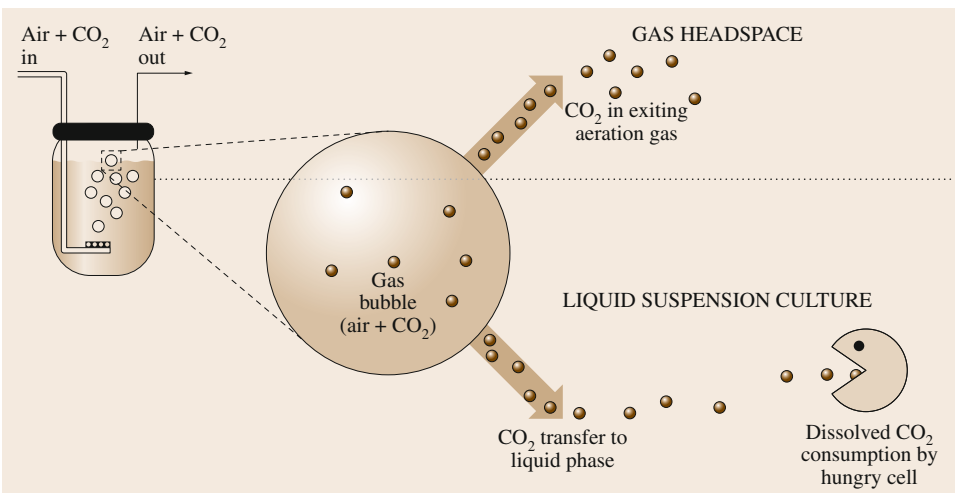

Fig. 9.23 Modes of CO_2 delivery and consumption in phototrophic culture

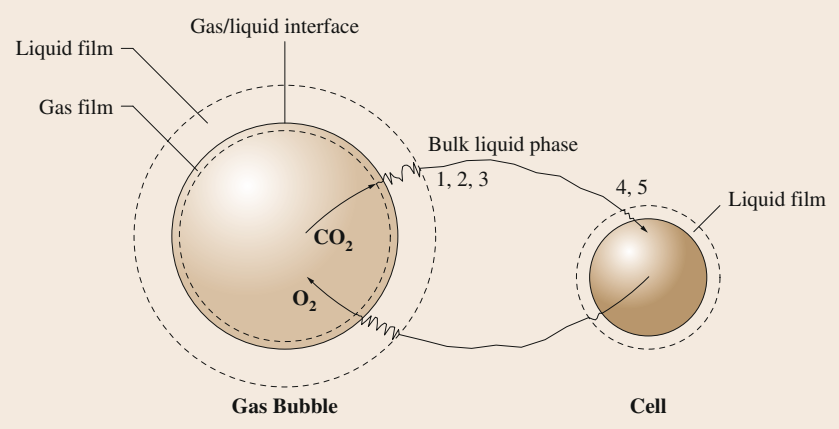

Fig. 9.24 Interphase mass transfer of CO_2 from the aeration gas bubble to the photosynthetic cell, and the concurrent transfer of O_2 evolved by the cell to the aeration gas bubble. The interphase mass transfer and consumption of CO_2 occurs in five steps: 1) mass transfer of gas-phase CO_2 through gas film inside gas bubble; 2) dissolution of CO_2 at the gas bubble/liquid interface; 3) mass transfer of dissolved CO_2 through the liquid film surrounding the gas bubble; 4) mass transfer of dissolved CO_2 through the liquid film surrounding the cell; 5) consumption of CO_2 by the cell

CO_2 Transfer Processes

The transfer of CO_2 from the aeration gas stream to the photosynthetically active cell occurs in five steps, as illustrated in Fig. 9.24:

1. Flux of CO_2 through the stagnant gas film inside the gas bubble to the gas/liquid interface (gas film mass transfer).
2. Equilibrium partitioning of CO_2 between the gas and liquid at the interface (absorption).
3. Flux of dissolved CO_2 through the stagnant liquid film surrounding the bubble (liquid film mass transfer).
4. Flux of dissolved CO_2 through the stagnant liquid film surrounding the cell.
5. Uptake and consumption of CO_2 by the cell for photosynthesis.

Thus the aeration gas serves as the source for CO_2 mass transfer and the photosynthetically active cells serve as the sink for CO_2 mass transfer.

Four assumptions and conditions on the CO_2 transfer processes described above are invoked to make the system more tractable for bioprocess engineering calculations. First, the equilibrium absorption of CO_2 from the gas to the liquid is described by Henry's law

$$P_A = H\, C_A^{*}\,, \tag{9.41}$$

where P_A is the partial pressure of CO_2 in the gas, C_A^{*} is the concentration of CO_2 dissolved in the liquid in equilibrium with the CO_2 partial pressure in the gas, and H is the Henry's law constant in units of pressure/concentration. Henry's law is valid for dilute solutions where the gas is sparingly soluble in the liquid. Such is the case for CO_2 dissolved in seawater. Second, for a sparingly soluble solute, it is commonly accepted that the solute flux is limited by the transport of the dissolved solute across the stagnant liquid film surrounding the bubble, i. e., the interphase mass transfer process is liquid phase controlled. Third, the partial pressure of CO_2 remains relatively constant inside the bubble. Fourth, the dissolved CO_2 consumption rate is assumed to follow zero-order kinetics with respect to dissolved CO_2 concentration, and so the volumetric consumption rate of CO_2 is given by

$$Q_A = \frac{\mu C_x}{Y_{X/CO_2}}\,. \tag{9.42}$$

Photosynthetic O_2 evolution and transfer follows the same processes as CO_2 transfer and consumption, only in reverse order, as illustrated in Fig. 9.24. The photosynthetically active cell is the source for O_2, whereas the aeration gas is the sink for O_2 mass transfer. If air is used as the aeration gas, then typically the dissolved O_2 concentration is near its saturation value for the partial pressure of O_2 in air.

Material Balances for CO_2 Delivery and Consumption

Carbon dioxide limitation during growth of phototrophic cell suspension cultures in a given photobiore-

actor configuration is assessed by performing a material balance on CO_2 dissolved in the liquid culture. The CO_2 material balance is

$$\begin{pmatrix}\text{rate of } CO_2\\ \text{added to culture}\end{pmatrix} - \begin{pmatrix}\text{rate of } CO_2\\ \text{removed from culture}\end{pmatrix} + \begin{pmatrix}\text{rate of generation of}\\ CO_2 \text{ by culture}\end{pmatrix} = \begin{pmatrix}\text{rate of accumulation of}\\ CO_2 \text{ within culture}\end{pmatrix} . \quad (9.43)$$

Liquid-phase CO_2 material balances are developed for batch and continuous photobioreactors. The batch and continuous photobioreactors are well mixed and continuously aerated. Although the cell density (C_x) increases with time in batch culture, CO_2 consumption and mass transfer rates are at a nominal steady state for a given value of C_x. At these conditions, the CO_2 material balance for the culture in the batch photobioreactor is

$$k_L A_i (C_A{}^* - C_A) - \frac{\mu C_x V}{Y_{X/CO_2}} = 0 , \quad (9.44)$$

where A_i is the gas/liquid interfacial surface area of all the bubbles, and k_L is the liquid phase mass transfer coefficient for dissolved CO_2 flux across the liquid film surrounding the bubble ($m\,s^{-1}$). The first term in (9.44) represents the interphase mass transfer flux of CO_2 from the gas to the liquid phase. Note that $C_A{}^*$ is dependent on the gas phase CO_2 partial pressure P_A by (9.41). The second term in (9.44) represents the consumption rate of CO_2 by the culture. Note the sign is negative to denote consumption rather than generation. Similarly, the CO_2 material balance on the continuous photobioreactor is

$$v_o C_{A,o} + k_L A_i (C_A{}^* - C_A) - v_o C_A - \frac{\mu C_x V}{Y_{X/CO_2}} = 0 . \quad (9.45)$$

Usually, the $v_o C_{A,o}$ term is small in magnitude with respect to the other terms and is neglected.

At the point of CO_2 limitation, the CO_2 that is delivered to the liquid culture is immediately consumed by the cells, and so C_A will be near zero, but not at zero. In this case, equations (9.44) and (9.45) both reduce to

$$CO_2 - TR = k_L a\, C_A{}^* \geq \frac{\mu C_x}{Y_{X/CO_2}} , \quad (9.46)$$

where a is the gas–liquid interfacial area per unit volume of culture A_i/V ($m^2\,m^{-3}$). Therefore, to avoid CO_2-limited growth, the CO_2-TR must always be higher than the CO_2 demand. If the growth is CO_2-limited, then the biomass productivity μC_x is set by the CO_2-TR.

The CO_2 demand given by (9.42) is a function of both specific growth rate μ and cell density C_x. Recall from (9.36) that light attenuation lowers μ as C_x increases and so μ' is also implicitly a function of C_x. At a fixed aeration rate and CO_2 partial pressure in the aeration gas, as the cell density increases, the process moves closer to CO_2 limitation. Therefore, the critical cell density ($C_{x,c}$) at which CO_2 limitation occurs in a well-mixed, continuously aerated photobioreactor is found by combination and rearrangement of equations (9.46) and (9.36) to yield

$$C_{x,c} = \frac{k_L a C_A{}^* Y_{X/CO_2}}{\mu_{max} I_m(C_{x,c})/(I_k + I_m(C_{x,c}))} , \quad (9.47)$$

where $C_N \gg K_N$. Since I_m is a function of C_x, (9.47) must be solved implicitly for $C_{x,c}$ using the appropriate relationship for I_m, e.g., (9.33).

Effects of Aeration on CO_2 Transfer Rate

Equation (9.46) contains a new parameter, a, which is defined as

$$a = \frac{A_i}{V} = \frac{\text{gas–liquid interfacial area } (m^2)}{\text{volume of culture}(m^3)} . \quad (9.48)$$

The parameter a is difficult to determine independently. Therefore, a and k_L are usually lumped together into a single parameter called the volumetric mass transfer coefficient $k_L a$, which has units of reciprocal time (e.g., h^{-1}). Generally, $k_L a$ increases with increasing aeration rate and decreasing bubble size. Many correlations exist for estimation of $k_L a$ in aerated systems, including enclosed algal photobioreactors [9.41–44]. The $k_L a$ for CO_2 transfer can be readily scaled from $k_L a$ for O_2 transfer. Specifically, $k_L a$ for CO_2 is related to the $k_L a$ for O_2 using penetration theory for mass transfer, for example,

$$(k_L a)_{CO_2} = (k_L a)_{O_2} \left(\frac{D_{CO_2}}{D_{O_2}}\right)^{\frac{1}{2}} , \quad (9.49)$$

where D_{CO_2} and D_{O_2} are the liquid-phase diffusion coefficients for dissolved CO_2 and O_2 dissolved in seawater, respectively. The diffusivity ratio D_{CO_2}/D_{O_2} is

0.8 for seawater. It should be noted here that (9.49) should be used at pH 7.5 and higher, as speciation of CO_2 provides a chemical reaction within the liquid film that is described by penetration theory.

Values of k_La for O_2 transfer in various bioreactor configurations are determined by well-established correlations or by direct measurements within the bioreactor. For example, a simple but very approximate k_La correlation for O_2 transfer in a bubble-column bioreactor presented by *Chisti* and *Moo-Young* [9.41] is

$$k_La = 0.76\, u_{gs}{}^{0.8}\,, \tag{9.50}$$

where k_La has units of s^{-1} and u_{gs} is the superficial gas velocity in units of $m\,s^{-1}$, defined as the volumetric flow rate of the entering aeration gas divided by the cross-sectional area of the bioreactor vessel. Well-established experimental methods also exist for estimating k_La directly in the photobioreactor culture by measurement of the dissolved oxygen versus time profile induced by a step change in aeration gas composition. A detailed presentation of the many correlations available to measure or estimate k_La for a particular bioreactor configuration and aeration system is beyond the scope of this chapter, but representative approaches are provided in [9.41–44].

Models for CO_2-Limited Growth

Carbon dioxide-limited growth occurs if C_x is greater than $C_{x,c}$ ((9.47)). Under CO_2-limited growth, the volumetric biomass production rate μC_x is set by (9.46). Therefore, *if* $C_x > C_{x,c}$ then biomass production for CO_2-limited growth in a well-mixed batch photobioreactor is

$$\frac{dC_x}{dt} = \mu C_x = \frac{C_N}{K_N + C_N} k_La C_A{}^* Y_{X/CO_2} \approx k_La C_A{}^* Y_{X/CO_2} (\text{if } C_N \gg K_N)\,. \tag{9.51}$$

If the simplification $C_N \gg K_N$ is made, the right-hand side of (9.51) is a collection of constant terms. In this case, (9.51) is readily integrated from $t = t_c$ at $C_x = C_{x,c}$ to yield

$$C_x(t) = C_{x,c} + k_La\, C_A{}^* Y_{X/CO_2}(t - t_c)\,, \tag{9.52}$$

where C_x increases with time until nutrient limitation ($C_N = 0$) is reached. Note that (9.52) is linear. A hallmark of CO_2-limited growth in batch photobioreactors is that the cell density versus time profile assumes a straight line. Furthermore, in CO_2-limited growth, the biomass production rate is directly proportional to CO_2–TR. However, even in CO_2-limited growth biomass production eventually becomes limited by some other variable, such as complete consumption of the limiting nutrient, as described by (9.20).

Similarly, with some algebra it can be shown that the material balances in the well-mixed continuous photobioreactor under CO_2-limited growth conditions where $C_x > C_{x,c}$ are

$$C_x = \frac{C_N}{K_N + C_N} \frac{k_La C_A{}^* Y_{X/CO_2}}{D} \approx \frac{k_La C_A{}^* Y_{X/CO_2}}{D}\,, \tag{9.53}$$

for the cell biomass, and

$$C_N = C_{N,o} - \frac{C_N}{K_N + C_N} \frac{k_La C_A{}^* Y_{X/CO_2}}{DY_{X/N}} \approx C_{N,o} - \frac{k_La C_A{}^* Y_{X/CO_2}}{DY_{X/N}}\,, \tag{9.54}$$

for the limiting nutrient. Simultaneous solution of equations (9.53) and (9.54) for outlet cell density C_x and outlet nutrient concentration C_N is required if C_N is comparable in magnitude to K_N. However, if $C_N \gg K_N$, then cell density C_x and biomass productivity DC_x increase linearly with increasing CO_2-TR. Equations (9.53) and (9.54) are only valid if C_x is greater than $C_{x,c}$ and if the dilution rate D is below the washout condition.

The CO_2 mass transfer analysis assumes that the aeration gas flow rate is high and CO_2 is slightly soluble in the liquid, so that only a small fraction of the CO_2 in the gas phase is actually transferred to the liquid phase. However, if the liquid medium is alkaline (pH > 8.5), then CO_2 in the aeration gas can be absorbed more efficiently since dissolved CO_2 speciates to bicarbonate and increases the total dissolved inorganic carbon concentration. Furthermore, if the aeration rate is very low and the CO_2 demand is high, it is possible that the liquid medium can capture all of the gas phase CO_2 delivered to the culture so that no CO_2 exits with aeration gas. Under these limiting conditions, CO_2-TR is

$$CO_2 - TR = \frac{F_A}{V} = \frac{v_a P_A}{RTV}\,, \tag{9.55}$$

where F_A is the molar flow rate of CO_2 in the aeration gas ($mol\,CO_2\,min^{-1}$), R is the gas constant (e.g., $0.08206\,L\,atm\,(mol\,K)^{-1}$), T is the temperature (K) of the aeration gas, v_a is the volumetric flow rate of the aeration gas at T ($L\,min^{-1}$), and V is the culture volume.

9.4.4 Process Scale-Up and Other Limiting Factors

Process *scale-up* seeks to preserve the performance of a given photobioreactor design as it is moved from a laboratory scale to a process scale. Conservatively, process scale-up is carried out in increments of ten. For example, a 100 L laboratory photobioreactor is first scaled up to a 1000 L pilot-plant photobioreactor, which is then scaled up to a 10 000 L process-scale photobioreactor. The scale-up design of the photobioreactor follows a few simple guidelines if the five-step process for rational photobioreactor design described at the beginning of this section is followed. Scale-up guidelines for bubble-column airlift photobioreactors and tubular photobioreactors are suggested below.

Process scale-up for bubble-column and airlift photobioreactors requires that the mean light intensity (I_m) and volumetric CO_2 transfer rate (CO_2-TR) are kept constant as the vessel size is increased. Therefore, the width of the planar vessel or the diameter of the cylindrical vessel is usually kept constant as the vessel size is increased from the pilot scale to the process scale to preserve I_m. The incident light intensity to the vessel surface is also kept constant. The other vessel dimensions (height or length of the planar vessel, height of the cylindrical vessel) are increased until the vessel size reaches 2000 L. If culture volumes in excess of 2000 L are required, then it is best to construct multiple units operating in parallel. Once the final vessel size is determined, the aeration rate is increased to keep the aeration rate per unit volume constant from the pilot scale to the process scale. For example, if the required aeration rate is 20 L air min^{-1} for a 100 L vessel, then the required aeration rate for a 1000 L vessel is 200 L min^{-1}, so that the aeration rate per unit volume of culture is kept constant at 0.2 L air (L culture $min)^{-1}$. All other aeration parameters are kept constant, including the sparger design and the CO_2 partial pressure in the aeration gas, so that the volumetric CO_2-TR is preserved upon scale-up.

Process scale-up for tubular photobioreactors requires that six process parameters be kept constant, including:

1. The mean light intensity inside the tube
2. The culture residence time within the tube
3. The Reynolds number for culture flow within the tube
4. The pressure drop in the tube
5. The CO_2-TR in the aeration tank
6. The ratio of the aeration tank culture volume to the tubular section culture volume.

The simplest way to keep parameters 1–4 constant is to keep the diameter and length of the tube constant and then manifold the tubes in parallel until the desired culture volume is achieved. The culture flow rate to a given tube is equal to the total flow rate divided by the number of parallel tubes.

Following scale-up, there are several other potential limiting factors and problems associated with biomass production in photobioreactors that deserve a brief mention. These include cell damage due to agitation, particularly pumping operations, cell adhesion to vessel surfaces, and cell clumping. Cell damage due to pumping was already discussed in Sect. 9.3.3.

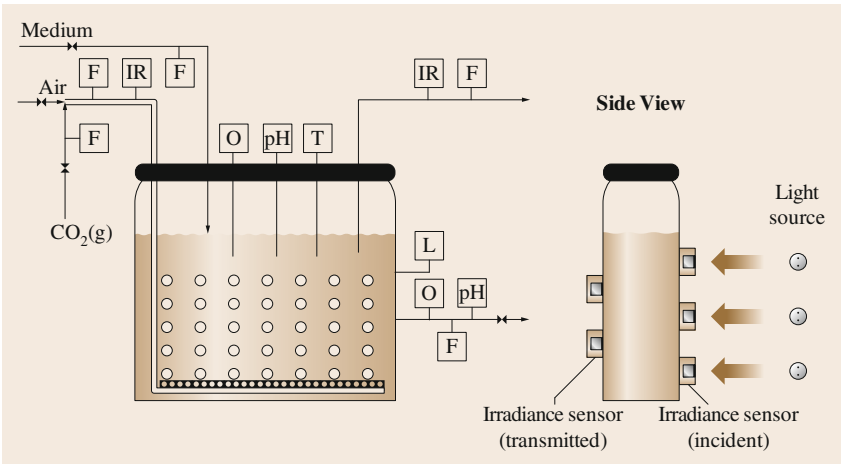

Fig. 9.25 Arrangement of bioprocess sensors for photobioreactor operation. F = flow meter, IR = gas phase CO_2 infrared sensor, L = liquid level, O = dissolved oxygen probe, pH = pH probe, T = temperature

9.4.5 Process Monitoring and Control

Photobioreactors require online process measurement sensors common to most bioprocess equipment for cell cultivation, including dissolved oxygen electrodes, pH electrodes, temperature probes, gas and liquid flowmeters, and liquid level indicators. Photobioreactors also require sensors for gas phase CO_2 concentration and light intensity. The suggested arrangement of process sensors is provided in Fig. 9.25.

Photosynthetic liquid suspension cultures produce oxygen and so the dissolved O_2 concentration in the liquid medium is usually near 100% of its saturation value with respect to the oxygen partial pressure in the aeration gas. However, if the culture is not adequately aerated, oxygen can build up in the liquid medium to higher concentrations which may be toxic to the cells, a phenomenon called oxygen toxicity. Therefore, the dissolved oxygen concentration should be monitored in all aerated vessels as well as the entrance and exit points to tubular sections of tubular photobioreactors. The consumption of dissolved CO_2 by photosynthesis can shift the equilibrium of dissolved organic carbon species by dissociating bicarbonate to dissolved CO_2 and OH- ions, which raises the pH. Therefore, the pH should be monitored at all points common to dissolved oxygen concentration measurement. Several commercially available dissolved oxygen (DO) and pH electrodes are suitable for these bioprocess measurements. The CO_2 ultimately consumed by photosynthesis is delivered to the photobioreactor by the aeration gas. Therefore, the total aeration gas flow rate and the gas-phase concentration entering and exiting the photobioreactor should be monitored. Online infra-red (IR) sensors are suitable for measurement of gas-phase CO_2 concentration in the inlet and exhaust aeration gas streams.

Online culture pH and gas-phase CO_2 concentration measurements can also be used to control photobioreactor operation during culture growth. For example, if CO_2 demand by actively growing culture is high, the pH will rise. The pH can be lowered by increasing the CO_2 concentration in the aeration gas or by direct injection of CO_2 to the culture. Both methods improve the CO_2 transfer rate and lower the culture pH by shifting the dissolved inorganic carbon equilibrium back to bicarbonate. For process controller operation, the gas phase CO_2 concentration serves as the manipulated variable and the pH serves as the response variable.

Photobioreactors should also be equipped with online irradiance sensors that measure the PAR (400–700 nm) light intensity in units of μmol photons $\cdot m^{-2} s^{-1}$. Irradiance sensors should be placed on the illuminated vessel surface with the face of the sensor pointed toward the light source. Other irradiance sensors should have the face of the sensor pointed to the culture to measure the light intensity transmitted through the culture vessel. If possible, several irradiance sensors should be laid out in a grid pattern along the illuminated vessel surface to monitor temporal distributions of both incident and transmitted light intensity. Irradiance sensors can also be used to control semi-continuous processes. For example, if the transmitted light intensity through the culture falls below a target value indicative of a certain cell density, then a portion of the suspension culture is pumped out and fresh medium is added to dilute the suspension culture and raise the transmitted light intensity back up to the setpoint value.

9.4.6 Illustration of a Photobioreactor Design Problem

This section illustrates the design of an enclosed photobioreactor system using concepts given in Sects. 9.2–9.4.

9.4.7 Design Needs Statement

It is necessary to design and scale up an aerated planar photobioreactor for cultivation of a phototrophic algal cell suspension culture. The cell mass contains an expensive antibiotic compound in the concentration of 25 mg per 1.0 g cell mass. The cultivation will be carried out as a batch process with an inoculum cell density of 0.10 g cells (L culture)$^{-1}$. To achieve the needed antibiotic production rate of 0.5 kg day^{-1}, the target biomass productivity is 20 kg cells d^{-1}, and a final cell density of 2.0 g cells (L culture)$^{-1}$ is desired to facilitate downstream processing. The intrinsic growth parameters for the organism, including biomass yield coefficients, are Monod parameters for light and limiting nutrient at 25 °C and pH 8.0, and a specific light attenuation coefficient for the cell suspension. These characteristic growth parameters are summarized in Table 9.7. The cell suspension culture becomes photo-inhibited at light intensities exceeding 300 μmol photons m^{-2} s^{-1}. To complete the photobioreactor design, specify the required nutrient loading in the culture medium, the illumination system delivery, the vessel dimensions, and the required CO_2 transfer rate necessary to avoid CO_2-limited growth.

Photobioreactor Process Design

The photobioreactor design process has five steps.

Step 1. Determine the initial concentration of limiting nutrient ($C_{N,i}$) necessary to achieve the target cell density ($C_{x,f}$) of 2 g cell L^{-1}. Using (9.20) and the $Y_{X/N}$ value provided in Table 9.7, $C_{N,i}$ is

$$\begin{aligned} C_{N,i} &= \frac{(C_{x,f} - C_{x,i})}{Y_{X/N}} \\ &= \frac{(2.0 - 0.1)\ \text{g cell l}^{-1}}{224\ \text{g cell mol}^{-1}\,\text{N}^{-1}} \frac{1000\,\text{mmol}}{1\,\text{mol}} \\ &= 8.48\,\text{mmol N L}^{-1}\,. \end{aligned}$$

Steps 2 and 3. Determine the light path length L. An aerated planar photobioreactor in the batch cultivation mode of operation must be used. Also, the vessel must be artificially illuminated since it will be located indoors. The light path width for the planar vessel configuration, the incident light intensity to the vessel surface, and the mode of light delivery (one or two-sided illumination) must be specified. With respect to the mode of light delivery, the two-sided illumination system improves light delivery and biomass productivity and hence reduces vessel size, but has a higher operating cost than the one-sided illumination system since the electricity to run the illumination system is a utility cost. First, the mean light intensity (I_m) at the final cell density in the process of 2 g cell L^{-1} is set to I_k for the culture of 50 μmol photon $m^{-2}\,s^{-1}$ so that $I_m \geq I_k$. Second, to avoid photoinhibition of cells residing near the wall of the vessel, the incident light intensity I_o is set to 150 μmol photon $m^{-2}\,s^{-1}$, which is one half the observed photoinhibition threshold of 300 μmol photon $m^{-2}\,s^{-1}$. Finally, the path length L is backed out from (9.33) for both $\alpha = 1$ (one-sided illumination) and for $\alpha = 2$ (two-sided illumination)

$$\begin{aligned} I_m &= \frac{\alpha I_o}{k_c C_x L}\left(1 - e^{-k_c C_{x,f} L}\right) \\ &= \frac{\alpha\ 150\,\mu\text{mol m}^{-2}\,\text{s}^{-1}}{0.21\,(\text{g cm})^{-1} 2.0\,\text{g l}^{-1} \cdot L} \\ &\quad \times \left[1 - \exp\left(-0.21\,\text{cm g}^{-1} 2.0\,\text{g l}^{-1} \cdot L\right)\right] \\ &\geq 50\,\mu\text{mol m}^{-2}\,\text{s}^{-1}\,. \end{aligned}$$

The required path length L is calculated using the root-solving function on any scientific calculator or spreadsheet program (e.g., Solver on Microsoft Excel). For $\alpha = 1$, L is 7.05 cm, and for $\alpha = 2$, L is 15 cm. Therefore, a path length of $L = 15$ cm with two-sided illumination is selected to make the final vessel size practical.

Step 4. Determine the volumetric CO_2 transfer rate (CO_2-TR) necessary to avoid CO_2-limited growth. According to (9.42), the CO_2 demand increases as cell density C_x increases and μ' increases. The maximum CO_2-TR required for the aeration process is determined at peak CO_2 demand. As the cultivation proceeds, C_x increases but μ' decreases because I_m, which determines μ', decreases as C_x increases. The magnitude of C_x increase is higher than the magnitude of μ decrease, and so the design is based on $C_{x,f}$. At final cell density $C_{x,f}$ of 2.0 g cell L^{-1}, I_m is 50 μmol photon $m^{-2}\,s^{-1}$, and μ' is 0.1 h^{-1} based on Model 1 in Table 9.4. Therefore, the maximum CO_2-TR at $C_{x,f}$ is determined by (9.46) in the form of

$$\begin{aligned} \text{Peak CO}_2 - \text{TR} = k_L a C_A{}^* &= \frac{\mu' C_{x,f}}{Y_{X/CO_2}} \\ &= \frac{0.1\,\text{h}^{-1} 2.0\,\text{g l}^{-1} 1000\,\text{mmol mol}^{-1}}{33.8\,\text{g mmol}^{-1}\text{CO}_2} \\ &= 5.92\,\text{mmol CO}_2\,(\text{L} - \text{h})^{-1}\,. \end{aligned}$$

Now, the aeration system itself can be further specified. First, consider that ambient air serves as the aeration gas, which contains 350 ppm CO_2 (0.00035 atm CO_2). Using a Henry's law coefficient of $H = 0.0339$ L atm $mmol^{-1}CO_2$ (Table 9.3), the required volumetric mass transfer coefficient $k_L a$ to achieve this CO_2-TR is

$$\begin{aligned} k_L a &= \text{CO}_2 - \text{TR}\frac{H}{P_A} \\ &= 5.92\,\text{mmol CO}_2\,\text{l} - \text{h}^{-1} \\ &\quad \times \frac{0.0339\,\text{l} - \text{atm mmol}^{-1}}{0.00035\,\text{atm}} \\ &= 573\,\text{h}^{-1}\,. \end{aligned}$$

A $k_L a$ value of 573.1 hr^1 is too large to accomplish within a bubble-column bioreactor. However, if the partial pressure of CO_2 in the aeration gas is increased to 3500 ppm (0.0035 atm CO_2), i. e.,

$$\begin{aligned} k_L a = \text{CO}_2 - \text{TR}\frac{H}{P_A} &= 5.92\,\text{mmol CO}_2\,\text{l} - \text{h}^{-1} \\ &\quad \times \frac{0.0339\,\text{l} - \text{atm mmol}^{-1}}{0.0035\,\text{atm}} \\ &= 57.3\,\text{h}^{-1}\,, \end{aligned}$$

Table 9.7 Example values of intrinsic growth characteristics of a phototrophic suspension culture

Input parameter	Value and units
μ_{max}	$0.2\,h^{-1}$
I_k	$50\,\mu mol\ photons\ m^{-2}\,s^{-1}$
K_N	$0.1\ mmol\,N\,L^{-1}$
$Y_{X/N}$	$224\ g\ cell\ mol^{-1}\ N$
Y_{X/CO_2}	$33.8\ g\ cell\ mol^{-1}\ CO_2$
k_c	$0.2\,L(g\ cell\ cm)^{-1}$

then the required k_La to avoid CO_2-limited growth is reduced to $57.3\,h^{-1}$, which is readily achievable. There are many ways the aeration rate and bubble size can be set to achieve this k_La value.

Finally, the sodium bicarbonate loading in the liquid medium necessary to maintain a pH of 8.0 at a CO_2 partial pressure of 3500 ppm must be calculated. From (9.7) and Table 9.3, at 25 °C the bicarbonate concentration needed in the liquid medium is

$$\begin{aligned}[HCO_3^-] &= 10^{-(pK_{a,1}-pH)}\frac{P_A}{H}\\ &= 10^{-(6.0-8.0)}\frac{0.0035\ atm}{0.03391-atm\,mmol^{-1}}\\ &= 10.3\ mmol\,l^{-1}\,.\end{aligned}$$

Step 5. Determine the cultivation time and photobioreactor vessel dimensions necessary to achieve the production schedule of 20 kg of biomass per day with a final cell density of $2.0\ g\ cell\,L^{-1}$ and initial cell density of $0.1\ g\ cell\,L^{-1}$. The cultivation time to achieve $C_{x,f}$ is determined from the computed growth curve of C_x versus cultivation time. The prediction of C_x versus t in the photobioreactor is determined by integrating equations (9.37a) and (9.37b) with respect to t using the input parameters given in Table 9.7 and $L = 15$ cm, $\alpha = 2$, and $C_{N,i} = 8.5\ mmol\,N\,L^{-1}$.

$$\frac{dC_x}{dt} = \mu C_x = \frac{C_N}{K_N + C_N}\frac{I_m(C_x)}{I_k + I_m(C_x)}\mu_{max}C_x\,,$$

with the initial condition $t = 0$, $C_x = C_{x,i} = 0.1\ g\ cell\,L^{-1}$. At at a given value for C_x, I_m and C_N are estimated by

$$I_m(C_x) = \frac{\alpha I_o}{k_c C_x L}\left(1 - e^{-k_c C_x L}\right),$$

$$C_N = \frac{(C_x - C_{x,i})}{Y_{X/N}}\,.$$

The differential equation can be numerically integrated without much trouble using differential equation solver utilities found in software packages such as MathCad or MatLab. Plots of C_x, C_N, I_m, and CO_2 demand versus cultivation time are presented in Figs. 9.26a,b. From Fig. 9.26a, the final cultivation time (t_f) is 22.0 h at the point where the limiting nutrient is consumed and the cell density becomes constant. Based on this cultivation time, the total culture volume to achieve the biomass

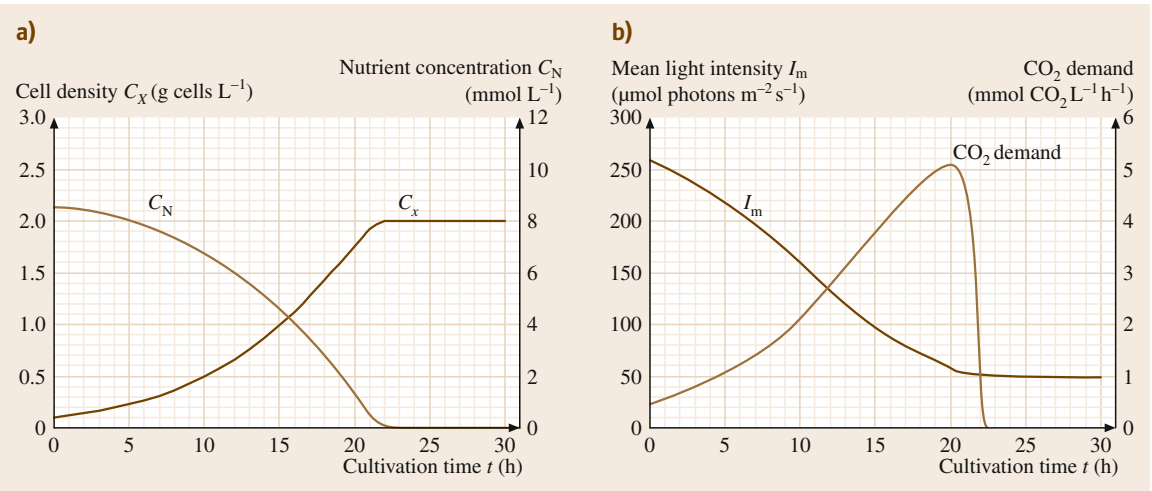

Fig. 9.26a,b Planar photobioreactor design illustration: model simulation results. **(a)** Cell density (C_x) and limiting nutrient concentration (C_N) versus cultivation time; **(b)** mean light intensity (I_m) and volumetric CO_2 demand versus cultivation time

Table 9.8 Summary of planar photobioreactor design parameters necessary to achieve 20 kg per day biomass production schedule

Parameter	Value and units
Initial cell density $C_{x,I}$	0.1 g cell L^{-1}
Final cell density $C_{x,f}$	2.0 g cell L^{-1}
Cultivation time in batch culture t_f	22 h
Initial nutrient concentration $C_{N,i}$	8.5 mmol N L^{-1}
Incident light intensity I_o	150 μmol photons m^{-2} s^{-1}
Mean light intensity at $C_{x,f}$ I_m	50 μmol photons m^{-2} s^{-1}
Planes of illumination α	2
Light path length L	15 cm
Total cultivation volume V	9648 L (five 2000 L units)
Single vessel width W	6.67 m
Single vessel height H	2.0 m
Peak CO_2-TR	5.69 mmol CO_2 $L^{-1}h^{-1}$
CO_2 partial pressure P_A	0.0035 atm (3500 ppm)
Bicarbonate concentration at P_A for pH 8.0, $[HCO_3^-]$	10.3 mmol L^{-1}
Aeration gas flow rate at P_A for peak CO_2-*TR* v_a/V	0.69 min^{-1}
Mass transfer coefficient for peak CO_2-*TR* k_La	57.3 h^{-1} (with 3500 ppm CO_2)

production rate (r_p) of 20 kg d^{-1} is

$$V = \frac{r_p t_f}{C_{x,f} - C_{x,i}} = \frac{20\,\text{kg}\,\text{d}^{-1}\,22\,\text{h}\,1\,\text{d}(24\,\text{h})^{-1}}{(2.0-0.1)\,\text{g}\,\text{l}^{-1}\,1\,\text{kg}(1000\,\text{g})^{-1}} = 9649\,\text{l}.$$

A culture volume of 9649 L may be too large for a single planar vessel, and so it is decided to build five 2000 L photobioreactors operating in parallel for a total cultivation volume of 10 000 L. For a single planar vessel, the light path length L or *thickness* of the vessel is fixed at 15 cm. Furthermore, the height of each vessel (H) is set at 2 m for the culture, 2.4 m for the entire vessel to accommodate the gas headspace. The width of each vessel is, therefore, 6.67 m. The final recommended design parameters are summarized in Table 9.8.

Based on these design parameters, chemical and energy inputs can be calculated. For example, the inlet mass flow rate of CO_2 in the aeration gas (F_A) based on the peak CO_2-TR and the required culture volume is

$$\begin{aligned} F_A &= \text{CO}_2 - \text{TR} \cdot V \\ &= 5.92\,\text{mmol}\,\text{CO}_2\,\text{l}-\text{h}^{-1}\,\frac{44\,\text{mg}\,\text{CO}_2}{1\,\text{mmol}^{-1}}\,\frac{1\,\text{kg}}{10^6\,\text{mg}}\,9649\,\text{l} \\ &= 2.51\,\text{kg}\,\text{CO}_2\,\text{h}^{-1}, \end{aligned}$$

which includes both CO_2 supplied by ambient air and the CO_2 addition to the aeration gas. The total aeration gas flow rate at this CO_2 molar flow rate of 2.51 kg CO_2 h^{-1} (0.95 mol CO_2 min^{-1}) at 25 °C and 3500 ppm CO_2 partial pressure is

$$\begin{aligned} v_{a,min} &= \frac{F_A RT}{P_A} \\ &= \left(0.95\,\text{mol}\,\text{CO}_2\,\text{min}^{-1}0.082061-\text{atm}\,\text{mol}^{-1}\,\text{K}^{-1}\right. \\ &\quad \left.\times(273+25)\,\text{K}\right)/\,0.0035\,\text{atm} \\ &= 6652\,\frac{1}{\text{min}}, \end{aligned}$$

and the minimum aeration rate per unit volume (*vvm*) at this peak CO_2-TR is

$$vvm = \frac{v_a}{V} = \frac{6652\,\text{l}\,\text{min}^{-1}}{9649\,\text{l}} = 0.68\,\text{min}^{-1}.$$

To achieve a k_La of 57.3 h^{-1} at a minimum aeration rate of 0.68 *vvm*, the bubble size of the aeration system is adjusted to achieve this value.

9.5 Future Directions for Process Scale Enclosed Photobioreactors

There are two pathways that will drive the future development of process scale photobioreactors.

First, high-value compounds from phototrophic marine organisms will drive the development of artificially illuminated photobioreactors. New process biotechnologies for the production of high-value compounds by phototrophic marine organisms in a GMP environment will require artificially illuminated photobioreactors located indoors within climate-controlled manufacturing facilities, similar to those used by fermentation processes. Furthermore, photobioreactors operating in a GMP environment must be easily sterilized and fully

controllable. Under controlled conditions for light and CO_2 delivery, cell densities exceeding 5 g dry cells L^{-1} of culture are possible. If 1) the achievable cell density is high, 2) the product is extremely valuable (> \$1000 kg^{-1}), and 3) required production slate of the target compound in the biomass is small (e.g., less than 1 kg d^{-1}), as is the case with many high-value specialty chemicals or pharmaceutical compounds, then photobioreactor culture volumes for process-scale units will most likely be under 20 000 L. Consequently, for process biotechnology applications, photobioreactor development is likely to move toward internally illuminated, stirred-tank photobioreactors, or batteries of externally illuminated bubble-column/airlift photobioreactors.

Second, environmental and renewable energy applications will drive large-scale photobioreactor development using natural illumination. The future development of photobioreactors will also focus on environmental or renewable energy applications, including CO_2 mitigation, waste water cleanup, and hydrogen gas production, and the production of biofuels. In the future, CO_2 emissions from industrial processes may be capped in the attempt to reduce the release of greenhouse gases to the atmosphere. One way to reduce CO_2 emissions is to capture the CO_2 and convert it to something else. Phototrophic marine organisms are ideal for this purpose because they convert CO_2 into biomass and lipids which can be processed into biofuels. However, there are several challenges to photobioreactor design for biological CO_2 mitigation. Stack gases from combustion processes typically contain at least 10% CO_2 by volume. In the simplest process, the stack gas is bubbled directly into an open pond or tank culture. However, this process is not efficient because only a fraction of the CO_2 in the stack gas is transferred to the liquid culture. In contrast, tubular photobioreactors may be more effective for CO_2 capture, but the residence time of the culture in the tubular section must be designed to completely consume the CO_2.

Whether the process application for cultivation of phototrophic organisms in enclosed photobioreactors is for high-value compounds at a small scale or CO_2 mitigation at large scale, this chapter has shown simple but effective approaches for the successful design of the cultivation system that incorporates nutrient consumption, light transfer, and CO_2 delivery into the process analysis.

9.6 Notation

a	Interfacial area of aeration gas bubbles in liquid suspension per unit culture volume, $m^2\ m^{-3}$
A_i	Total interfacial area of aeration gas bubbles in liquid suspension, m^2
C_A	Concentration of dissolved CO_2 in culture medium, mol L^{-1}
$C_A{}^*$	Concentration of dissolved CO_2 in equilibrium with the CO_2 partial pressure in the aeration gas, mol L^{-1}
C_{AL}	Concentration of dissolved CO_2 exiting tubular section or entering aeration tank (tubular photobioreactor), mol L^{-1}
C_{AO}	Concentration of dissolved CO_2 exiting aeration tank or entering tubular section (tubular photobioreactor), mol L^{-1}
$C_{A,T}$	Total dissolved carbon concentration, mol L^{-1}
C_N	Concentration of dissolved nutrient in liquid medium, mol L^{-1}
$C_{N,i}$	Initial concentration of dissolved nutrient in batch bioreactor liquid medium, mol L^{-1}
$C_{N,o}$	Concentration of dissolved nutrient in inlet flow stream of well-mixed continuous flow bioreactor, mol L^{-1}
C_x	Cell density in liquid suspension culture, g cells L^{-1} culture
$C_{x,c}$	Cell density where growth kinetics become CO_2 transfer rate limited at a given set of process conditions, g cells L^{-1} culture
$C_{x,f}$	Final cell density at nutrient depletion, g cells L^{-1} culture
$C_{x,i}$	Initial cell density in batch bioreactor, g cells L^{-1} culture
$C_{x,o}$	Cell density of inlet flow stream of well-mixed continuous flow bioreactor, mol L^{-1}
d	Inner diameter of tubing, cm or m
D	Dilution rate for well mixed continuous flow bioreactor, s^{-1} or h^{-1}
D_{CO_2}	Diffusion coefficient for CO_2 dissolved in seawater, $m^2\ s^{-1}$
D_{O_2}	Diffusion coefficient for O_2 dissolved in seawater, $m^2\ s^{-1}$

f Fractional photoperiod for illumination, h light on (24 h)$^{-1}$

F_A Molar flow rate of CO_2 in the inlet aeration gas, mol CO_2 h^{-1}

H Henry's law constant for dissolved CO_2 in seawater medium, atm L mol^{-1}

k_c Light attenuation constant for liquid cell suspension culture, L (g cells cm)$^{-1}$

$k_L a$ Volumetric mass transfer coefficient for CO_2, h^{-1}

k_w Light attenuation constant for transparent or translucent vessel wall material, cm^{-1}

K_A Dissolved CO_2 concentration at half saturation, mol L^{-1}

K_i Photoinhibition constant, m^{2}s μmol^{-1} photons

K_N Dissolved limiting nutrient concentration at half saturation, mol L^{-1}

I Light intensity incident to photosynthetic cell, μmol photons m^{-2} s^{-1}

I_k Light intensity at half saturation, μmol photons m^{-2} s^{-1}

I_m Mean light intensity delivered to liquid suspension culture, μmol photons m^{-2} s^{-1}

I_o Light intensity incident to liquid suspension culture inside vessel, μmol photons m^{-2} s^{-1}

I'_o Light intensity incident to outer vessel wall surface, μmol photons m^{-2} s^{-1}

$I(z)$ Light intensity distribution in response to light attenuation, μmol photons m^{-2} s^{-1}

L Light penetration path length for planar photobioreactor, or length of tubing in the tubular section, cm or m

l_w Wall thickness of transparent or translucent vessel material, cm or m

P_A Partial pressure of CO_2 in the aeration gas, Pa or atm

Q_A Volumetric consumption rate of dissolved CO_2 by cell suspension culture, mol L^{-1}h^{-1}

r Radial position within cylindrical vessel, cm or m

R Outer radius of cylindrical vessel, cm or m

R_p Recycle ratio in continuous flow tubular recycle photobioreactor, v_o/v_p

r_X Biomass production rate, g cells L^{-1}h^{-1}

t Cultivation time, h or day

u_{gs} Superficial gas velocity, volumetric aeration gas flow rate divided by bioreactor cross-sectional area, m s^{-1}

v Bulk linear velocity of the liquid culture flowing through the tube (volumetric flow rate divided by cross-sectional area), m s^{-1}

v_a Volumetric flow rate of the aeration gas, L h^{-1}

v_m Fresh medium addition to continuous flow tubular recycle photobioreactor, L h^{-1}

v_o Volumetric flow rate of liquid suspension culture through tubular section (tubular photobioreactor), L s^{-1}

v_o Volumetric flow rate of liquid medium added to continuous photobioreactor, L h^{-1}

v_p Volumetric flow rate of product suspension withdrawn from continuous flow tubular recycle photobioreactor, L h^{-1}

V Total liquid volume of culture, L

V_a Liquid volume of culture in aeration tank of tubular photobioreactor, L

V_t Liquid volume of culture in tubular section of tubular photobioreactor, L

Y_c Specific chlorophyll content of biomass, mg chl a g^{-1} cells

Y_{X/CO_2} Biomass yield coefficient based on CO_2 consumption, g cells mol^{-1} CO_2

$Y_{X/N}$ Biomass yield coefficient based on limiting nutrient consumption, g cells mol^{-1} nutrient

z Position along light path in planar photobioreactor or axial position down length of tubing for tubular photobioreactor, m

Greek Letters.

α Number of illumination planes

μ Specific growth rate of liquid suspension culture, h^{-1}

μ' Specific growth rate defined in (9.12), h^{-1}

μ_c Apparent viscosity of liquid suspension culture, g cm^{-1} sec^{-1}

μ_{max} Specific growth rate of liquid suspension culture at saturation light intensity, h^{-1}

ρ_c Apparent density of liquid suspension culture, g cm^{-3}

τ_{tube} Residence time of culture inside tubular section, h

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10. Bioinformatic Techniques on Marine Genomics

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Marine biotechnology is the industrial, medical or environmental application of biological resources from the sea. Since the marine environment is the most biologically and chemically diverse habitat on the planet, marine biotechnology has in recent years delivered a growing number of major therapeutic products, industrial and environmental applications, and analytical tools. These range from the use of a snail toxin to develop a pain control drug to metabolites from a sea squirt to develop anticancer therapeutic and marine enzymes to remove bacterial biofilms. In addition, well-known and broadly used analytical techniques are derived from marine molecules or enzymes, including green fluorescence protein gene tagging methods and heat resistant polymerases used in the polymerase chain reaction. Advances in bacterial identification, metabolic profiling, and physical handling of cells are being revolutionized by techniques such as mass spectrometric analysis of bacterial proteins. Advances in instrumentation and a combination of these physical advances with progress in proteomics and bioinformatics are accelerating our ability to harness biology for commercial gain. Single cell Raman spectroscopy and microfluidics are two emerging techniques that we try to touch a bit in this chapter. In this chapter, we provide a brief survey and update of the most powerful and rapidly growing analytical techniques as used in marine biotechnology, together with some promising examples of less well-known earlier stage methods which may make a bigger impact in the future.

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10.1 Background

Genomics is the study of the structure, function, and diversity of genomes; genome is the collective term for all the genetic information contained in a particular organism. Genomics has brought about a revolution in all fields of biology. Genomic analysis, from originally limited to one or a small number of genes to large numbers of genes or even to the complete set that make up a genome, differentiates the genomic approach from the classical biological approach. High throughput methodologies adapted for the analysis of multiple gene sets has led to substantial technical advances and to the development of new ways of thinking about bi-

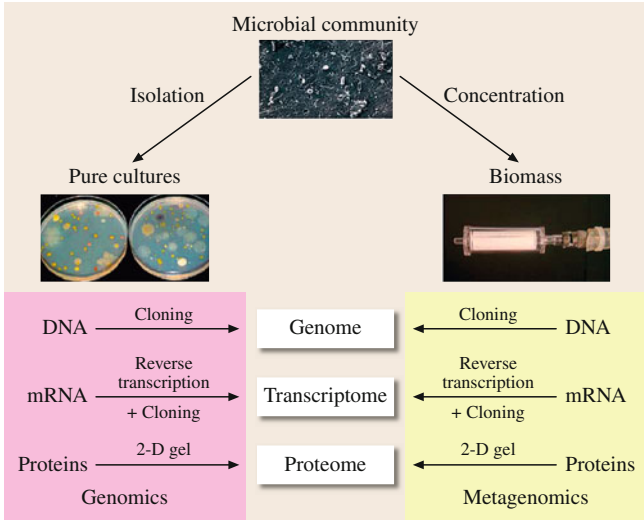

Fig. 10.1 Molecular and genomic approaches to the study of natural communities of microorganisms

ology. Genomics as a discipline began with the first attempts to obtain large-scale sequence data for individual organisms, either by sequencing the genomic DNA (deoxyribonucleic acid) or, where this was not practical, by sequencing large numbers of cDNA (complementary deoxyribonucleic acid) [10.1]. The availability of extensive sequence data for a large number of organisms has facilitated the development of additional genomic tools such as microarray systems for the analysis of gene expression and collections of sequence-tagged mutants.

Marine organisms were very poorly represented amongst these early genomic models and were to some extent left behind as the application of genomic approaches to several terrestrial models allowed an acceleration of our understanding of the biology, ecology, and evolutionary history of these species. Now the situation is different and, to a certain extent, advancements have been made in recent years, essentially due to the reduced cost of DNA sequencing and associated enhanced capacity for analysis of very large datasets. This cost reduction has not only allowed the application of genomic approaches to a much broader range of species but has also opened up new fields of genomics such as metagenomics and metatranscriptomics, in which sequencing methodologies are used in novel and groundbreaking ways. The rapid development of platforms for high-throughput experiments at lower costs can be observed in the fields of transcriptomics, proteomics, and metabolomics as well, providing scientists with a more holistic view of microbes in their natural, environmental context through multiomic studies. Multiomic studies not only significantly increase the size and complexity of genomic data; they demand the integration of diverse data to maximize scientific insights. Marine biology has been at the pole position of many of these new applications.

Novel genomic approaches are currently being applied to exploit the enormous phylogenetic diversity of marine organisms in order to explore the evolution of developmental processes, characterizing the marine ecosystems that play key roles in global geochemical cycles, searching for novel biomolecules, and understanding ecological interactions within important marine ecosystems. Genomics including ecological genomics is being transformed into a data-intensive science with an exponential increase of data [10.2]. The rate of sequence data generation is far outpacing the rate of increase in central processing units (CPU), and the cost of analyzing large datasets produced by, for example, Solexa, already exceeds the cost of generating them [10.3–6]. This situation is often characterized by fear-inducing metaphors such as *data tsunami*, *data avalanche*, and *data deluge*. Rather than being a threat to humankind, however, the technological improvements open challenging, but excellent opportunities for marine biology and biotechnology [10.7].

The burgeoning development of platforms for high-throughput experiments at lower costs can be observed in the fields of transcriptomics, proteomics, and metabolomics, providing scientists with a more all-inclusive view of microbes in their natural, environmental context through multiomic studies. Furthermore, these multiomic studies are extended to metatranscriptomics and metaproteomics, involving analysis of entire microbial communities. Modern marine microbial ecology can be considered to have started in the 1970s, when it was shown that most respiration in the oceans was in the bacterial size fractions [10.8], and that bacteria were very abundant [10.9, 10] (Fig. 10.1).

Nowadays marine microorganisms are known to be responsible for half of the total primary production on the planet [10.11], and the 1030 microbial cells present in the oceans [10.12] account for more than 95% of the total respiration [10.13]. The application of genomic approaches to marine microbial ecology in the past few years has caused a kind of Copernican revolution. Thanks to such techniques, novel functions have been discovered, a large diversity of microorganisms has been discovered, and the meaning of concepts such as species, genome, and niche has been challenged [10.14].

10.2 Marine and Bacterial Fluorescence Shining Light on Biological Questions

One of the most widespread and remarkable analytical techniques to be used in biology in recent years is the use of a green fluorescent protein (GFP) from a jellyfish (Fig. 10.2) to tag and study biological molecules under the microscope in vivo. The natural function of GFP in the jellyfish is unknown, although it has been suggested that the protein may act as a light driven electron transfer. Different GFP-like proteins have been discovered in a variety of marine organisms, including the marine chordate Amphioxus, underlying marine biodiversity as a rich source of biologically useful molecules which can have a major impact on the development of new analytical methods. It will be interesting to monitor the discovery of new fluorescent proteins from bacteria which may provide better markers or markers that allow the visualization of different processes (e.g., redox state) to be visualized in addition to the localization of specific proteins. One example is the development of fluorescent proteins as markers which emit light in the infrared region of the electromagnetic spectrum, allowing visualization of processes that occur deeper in tissues, which are impenetrable to visible light. One of these is an engineered phytochrome, named IPF1.4 from the bacterium *Deinococcus radiodurans*, a bacterium which is highly resistant to ionizing radiation. More recently, the photosynthetic bacterium *Rhodopseudomonas palustris* has provided a phytochrome, which has been engineered to produce the protein near-infrared fluorescent protein (iRFP for improved infrared imaging.

Fig. 10.2 The jellyfish *Aequoria victoria* source of green fluorescent protein which sparked an increase in the availability of fluorescent proteins as biological markers

10.3 Recent Advances in Imaging Techniques for Marine Biotechnology

The invention of the microscope in 1595 and its development and use by Van Leeuwenhoek, a Dutch draper, in his scientific observations of the first bacterial cells is a classic example of the need for new analytical tools as a prerequisite to the opening up of vast new areas of science that were hitherto invisible to us. Whilst this special issue covers a number of key metabolic and genomic breakthroughs which are also promising to reveal new worlds, there have also been some advances in imaging techniques that are proving useful for our ability to analyze biological systems in ever greater detail. One of these is super resolution microscopy. The resolution of images using light microscopy is limited by the physics of light diffraction and is $\approx 250\,\text{nm}$; however, super resolution microscopy has been developed to enable imaging at wavelengths below this limit. In addition to increasing the resolution of images obtained, three-dimensional imaging or subcellular structure is now becoming widely applied.

10.4 Chemical Analysis of Volatile Microbial Metabolites

Quorum sensing as a means by which bacterial cells can carry out the ultimate and original approach to crowdsourcing and collective decision-making for their ecological benefit was a major breakthrough in our understanding of the complexity of bacterial life, and provides the lead idea for the development of novel antibacterial compounds. Quorum sensing was discovered in bioluminescent marine bacteria and later found to regulate a wide variety of physiological responses, including virulence. There is no doubt that other cell–cell communication mechanisms exist and remain to be discovered. One thoroughly studied communica-

tion system occurring in higher animals and plants is through airborne volatile odorous molecules (olfaction). The evolutionary origins of olfaction are interesting, although unclear. Yeast cells, and more recently also bacteria, have been showed to respond physiologically to airborne odorous molecules produced by other bacteria, representing a new category of cell–cell communication in prokaryotes. The ability to quickly detect nearby competing life forms and/or nutrient sources by sensing of odorous molecules (unlike, for example, oxygen or carbon dioxide, which are not directly indicative of other life forms, which is probably why they do not have an odor) would have been an essential ability for survival even in the first bacterial cells to have evolved. In addition, a molecule of ammonia gas is one of the simplest molecules to be indicative of other living cells and would, therefore, have been a good candidate molecule for the first olfactory systems to sense and respond to. Indeed, sensing of ammonia by bacteria also appears to be connected not just to nitrogen metabolism but as a general signal which regulates a number of physiological responses such as biofilm formation and antibiotic resistance and chemotaxis. This, therefore, raises the question as to the use of new and powerful analytical techniques for detecting and analyzing volatile molecule production and function in interbacterial systems. Methods such as targeted metabolomics with mass spectrometry are increasingly being used not only to understand these volatile compounds but to develop artificial olfactory systems of the future.

10.5 Bioinformatics Resources

The sequencing value is realized only through its annotation, which expresses a scientific understanding of the raw data. Currently, a range of annotation systems exists for single-genome analysis. These systems support the management and integration of data from computational analyses using diverse sets of bioinformatics algorithms and software tools [10.15]. This is reflected by examples including sequence assembly [10.16], gene finding, protein domain prediction [10.17], protein function assignment [10.18, 19], prediction of gene expression, and gene regulation accompanied by data from laboratory studies. Until few years ago, most sequenced genomes belonged to bacteria of medical interest. However, thanks to the initiative of the Gordon and Betty Moore Foundation, over hundreds of genomes of marine bacteria have been sequenced in the last 2 years.

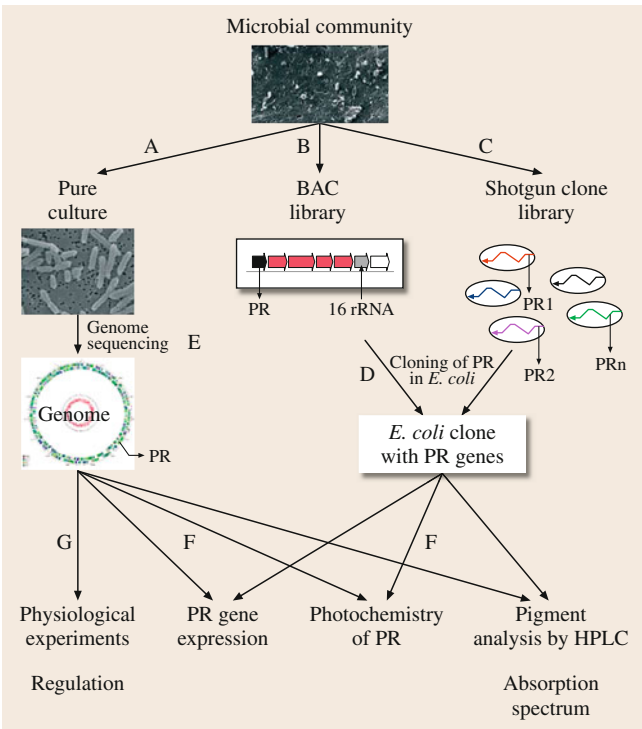

Fig. 10.3 Different approaches to environmental genomics

Of course, the ultimate genome project has surely been the 15-year effort to sequence the 3 billion base pair human genomes [10.20] The large investment in resources and people ($2.7 billion 1991) was paired with a period of rapid development in the technology of sequencing. The chemistry involved was basically the same as that originally developed by Sanger and colleagues (1977) [10.21], but the technology became hugely automated, largely due to the parallel non-governmental effort led by the Celera Corporation [10.22]. Similarly, corresponding advances in computational infrastructure and bioinformatics making sense of sequence data made it feasible to pair together millions of individual sequence reads by massively parallel pair-wise comparison techniques. Applications of these new methodologies helped spur on an explosion in the study of microbes and microbial communities. To date, sequence-based metagenomic analyses of ma-

rine microbiota have attempted to describe: *who is there?*; *what are they doing?*; *who is doing what?*, and *what evolutionary processes determine these parameters?* Recent conceptual and technological advances have allowed the increased use of sequence-guided metagenomic investigations of the marine environment. Recent advances in high-throughput sequencing technology and lower cost sequencing technologies have made random shotgun sequencing of environmental DNA economically feasible. The majority of metagenomic investigations to date have employed whole metagenome shotgun sequencing approaches for the cloning and sequencing of microbial DNA from marine environments. This involves the generation of small insert DNA clone libraries, and their subsequent analysis using Sanger dideoxy sequencing, generating sequences that can then be used to query databases, whereby phylogeny can be inferred or putative functional genes identified. Where putative protein encoding sequences are detected proximal to phylogenetic marker genes on a single cloned fragment, function can be linked to taxonomy. This approach can give read lengths ranging from 600–900 bp in length, which can be extended through the entire fosmid clone. The contigs generated using this approach give a fuller context for any given gene detected, thus giving a greater probability of reconstructing the metabolic pathways of individual members within a particular marine microbial consortium. While the benefits of the technology both from a sequence data generation and cost standpoint (25–40 Mb of DNA sequence per run at an accuracy of ≈ 98%) are obvious, the limitations of lower read lengths of between 200–300 bp can be problematic [10.23]. Read lengths achievable using pyrosequencing are currently around 450 bp, and this is likely to continue to improve, which should improve the amount of useful data generated from pyrosequencing of metagenomics samples (Fig. 10.3).

10.6 Large-Scale Sequence Analysis

With the advent of the *omics* era, more and more metagenomic and metatranscriptomic datasets are being generated from marine environments. While the computational needs for single-genome analysis are solved and can be performed on today's commodity hardware, the new large-scale metagenomic sequencing projects which generate 2000–3000 genome equivalents of sequence information per project bring new challenges. On the one hand, the challenge is the sheer amount of sequence data per metagenome. On the other hand, all metagenome sequences are mere fragments of unknown organismal origin. Taken together, these challenges demand further development of software for assembly, gene calling, and annotation. Recently, several new and dedicated data processing and database resources have emerged to address the current need for large-scale metagenomic data analysis and management, for example, the community cyber infrastructure for advanced marine microbial ecology research and analysis (CAMERA) and the meta genomics-rapid annotation using subsystems technology (MG-RAST) platform [10.24]. However, just a *simple* automatic annotation based on the basic local alignment search tool (BLAST) sequence similarity searches poses a severe computational bottleneck. Consider the following example: in November 2009, the MG-RAST server processed 278 metagenomes with an average of 33 Mbp of sequence data per project. On a single high-end server (Xeon E5540, 8 cpu's, 2.53 GHz, 16 GB ram) a complete BLAST analysis of a single project against

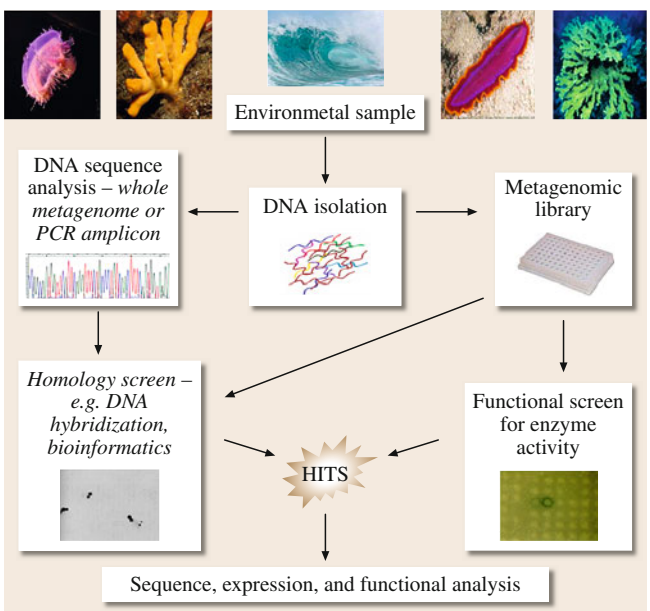

Fig. 10.4 Enzyme discovery from metagenomes: functional and sequence-based approaches

a 2 Gbp nonredundant reference database would take 3 days. Because BLAST computing time grows linearly with the reference database size, and the reference databases double every year, a comparable growth in the number of servers is needed. In other words, any institution that aims to keep pace with the growth of sequence data needs to double its budget each year. Even when accounting for the 15% yearly increase in CPU speed, a 1.7 factor budget growth is needed. This simplified example does not yet incorporate concomitant costs incurred as a result of increases in memory consumption, storage capacities, and network bandwidth for data exchange, as well as power consumption and cooling. The issue becomes even more severe when one considers that, unlike BLAST, several analysis tools have nonlinear computational time consumption and the growth of metagenomic datasets continues steadily.

The rapidly developing field of sequence-based metagenomic analysis of marine microbiota promises much for the future of marine biotechnology. While sequence-based metagenomic approaches rely on comparing sequence data, which is obtained with sequences deposited in databases, functional metagenomics focuses on screening DNA library clones directly for a phenotype, i. e., the genes are recognizable by their function rather than by their sequence. The power of such an approach is that it does not require the genes of interest to be recognizable by sequence analysis, ensuring that this approach has the potential to directly identify entirely new classes of genes for both known and, indeed, novel functions [10.25, 26]. Another advantage of such an approach is that while sequence-based approaches can result in the incorrect annotation of sequences with weak similarities to biochemically characterized gene products or of sequences similar to gene products with multiple functions, the results from a functional metagenomics approach are unambiguous. Data from the Global Ocean Sampling (GOS) expedition indicates that despite current large-scale sequencing efforts the rate of discovery of new protein families from the marine environment is linear, implying that marine microorganisms will continue to be a source of novel enzymes in the foreseeable future [10.27]. As many of these gene products are entirely novel, their activity cannot be inferred from comparison to known protein databases; thus a functional metagenomics approach has the ability to identify novel genes on the basis of phenotypes which lend themselves to high throughput screens Fig. 10.4.

10.7 Integrating Sequence and Contextual Data

From the time of field sampling to final step of sequence analysis, a range of diverse data is produced among different scientific communities, where individual researchers process the samples with different protocols in different time frames. Often, the data is not deposited in public resources at all, or the submitters have the choice of up to a dozen repositories. Therefore, current molecular, environmental and diversity data is fragmented, imprecise, or lost in lab books or proprietary private archives. Moreover, environmental data, such as temperature, cannot be stored consistently in the current records of sequences in the INSDC databases [10.28], nor can a genome sequence be stored in databases dedicated to environmental data. Unfortunately, even conceptually simple contextual data-driven requests, such as *Give me the temperature at the sampling site of the microbial isolate of interest* or *Give me all unknown genes sampled at temperatures greater than* 80 °C, are far from trivial. The reason is as simple as it is profound.

An important source of contextual data is the data taken in the field (on site), such as the geographic and environmental origin of the sample, as well as information about subsequent processing to obtain the DNA, and the sequencing itself. Given that each DNA sequence from the marine environment is properly georeferenced, with geographic location, depth in the water column or sediment, and time of sampling, the environmental context can be significantly complemented with data from environmental databases. Only recently have bioinformatic resources begun to invest in better management and integration of contextual data. CAMERA hosts the fully georeferenced GOS data set. IMG/M integrates rich details about the hosted genomes and metagenomes. Megx.net is the first resource to provide a comprehensive annotation of the environment of microbial genomes Kottmann et al., The Barcode of Life initiative [10.29] successfully collaborated with INSDC to store latitude and longitude in the public sequence repositories. Classical ecological and conservation marine studies focused on species and communities; the emphasis has now shifted to enhancing our understanding of the relationships among the various components

of biodiversity, especially their role in ecosystem services, such as global nutrient recycling and climate. Here, we highlight the significance of genomics and genetic principles in elucidating the interactions among different biological levels of diversity, from genetic and cellular, to community and ecosystem-level processes. Genomic methods are especially powerful in disclosing previously undetected taxonomic (e.g., DNA barcoding), genetic (e.g., 454 sequencing), and functional (e.g., gene expression, analysis of metabolites) diversity, including the identification of new species and metabolic pathways.

10.8 Proteomics as Potential Tool for Survey in Marine Biotechnology

Currently, newer regulations have been introduced to ensure that marine resources are exploited in a rational way, thus preventing depletion of the available resources. Accordingly, proteomic tools are aimed at the investigation of the proteome, which includes all changes in the quantities and post-translational modifications of all the proteins in cells. The changes may be brought about by growth, differentiation, senescence, changes in the environment, genetic manipulation, or other events. To date, the application of proteomic tools for the investigation of seafood and other marine products has been scarce. An idea of the current limited impact of proteomics in this research field can also be gained if one considers that SWISSPROT, EXPASSY, NCBI, and other databases include fewer than 23 000 amino acid sequences from teleost fish proteins, most of which correspond to the Euteleostei group. By contrast, there are nearly 610 000 nucleotide sequences reported for this group of aquatic vertebrates. Thus, the relative amount of information about protein sequences in this field is below 4% with respect to the number of DNA sequences available. Nevertheless, it should be stressed that proteomics has been useful in some biomedical applications of marine proteins. Additionally, proteomics has afforded valuable tools for the investigation of fish physiology and pathology. Among the research areas in which proteomics may have applied interest for the investigation of teleost fish, the following should be noted: antifreeze proteins, immunoglobulins, and enzymes. Note should also be taken of the application of proteomics to the sequencing of parvalbumins and to the characterization of their allergenic potential or calcium transport properties.

10.9 Proteomics and Seafood

From the nutritional and functional points of view, proteins are the basic components of seafood. The wide variability of proteins present in seafood and their highly variable composition offers a strong potential to originate a large variety of different products. In addition, the denaturation levels of seafood proteins are closely related to the sensory and technological properties of seafood products. Proteomic tools have seldom been applied to food nutritional analysis and, in the particular case of seafood technology, the shortage is even more relevant. Thus, MS methods have been considered in nutritional studies on milk and egg products. The application of proteomics to marine biotechnology began in the past decade. Important fields such as investigation of the expression of selected exogenous features introducing rough DNA manipulation techniques in transgenic fish have only been recently initiated. Although fish transgenesis involves gene integration and transmission steps achieved by gene mapping and other genomic tools, follow-up of the expression of such genes and the subsequent biosynthesis of recombinant proteins involves the use of proteomic tools. It should be noted that although the genomes of both wild-type and transgenic marine organisms are well-defined and not subject to variation; the proteome of these organisms may depend on several intracellular and/or extracellular factors and is hence subject to variation, thus being in a dynamic state. Accordingly, the follow-up of the advanced steps of gene expression by proteomic tools allows research scientists to elucidate the effect of such factors on the proteome, thereby providing valuable information aimed at obtaining the optimum levels of a target protein involved in prototrophy and growth, or in other features of technological interest. From the biotechnological point of view, traits of interest in aquaculture have focused on, amongst

other things, the improvement of the growth rate or the increase in thermotolerance. Growth enhancement strategies have been successfully applied to several fish species, such as carp, catfish, tilapia, and salmon, through the incorporation of homologous or heterologous growth hormone (GH) genes or cDNAs. The monitoring of gene expression in genetically modified marine organisms is thus a crucial step in the optimization of the use of such organisms in aquaculture and seafood technology. It should also be noted that the cross-effect of transgene expression on the biosynthesis of host proteins in genetically improved fish species is another relevant issue in which proteomics will surely play a significant role soon. In both issues, proteomics represents a valuable tool of remarkable applied interest.

10.10 Present Status and Future Trends of Proteomics in Marine Biotechnology

Proteomic tools, mainly 2-D PAGE (polyacrylamide gel electrophoresis) and mass spectrometry, represent a robust analytical strategy for the phenotypical investigation of marine organisms of industrial interest. These tools afford specific information about expression patterns, post-translational modifications, or protein interaction mechanisms. This information is extremely useful for the optimization of specific biotechnological processes. It should be stressed that high-throughput proteomics techniques have recently been coupled with newer bioinformatic tools, thus affording more robust and powerful strategies for both protein identification and characterization purposes. Recently, proteomic tools were successfully applied for the isolation and characterization of a calponin-like protein from the mussel *Mytilus galloprovincialis* 68 kDa polypeptide, a polypeptide involved in muscle contraction processes. The protein, isolated from the retractor muscle of this mollusc, was successfully separated from actin by 2-D PAGE and was identified by N-terminal amino acid sequence analysis. Likewise, the investigation of mussel adhesive proteins which have been reported to harbor an extraordinary biotechnological potential as a biodegradable adhesive material is another promising field in which proteomics may play a significant role. It is noteworthy that the protein can be readily obtained from mussels, a marine source characterized by their wide availability and low price. Thus, proteomic tools should play a significant role when undertaking optimization strategies aimed at achieving the highest yields of such proteins, mainly by elucidating the biochemical modifications to be considered in the process design step. The investigation of marine bacteria by proteomic techniques is also still in its early days. Although the protein profiles of the thermophilic microorganism *Pyrococcus abyssi* were investigated when the organism was subjected to different levels of pressure and temperature, other authors applied 2-D PAGE to investigate the level of protein biosynthesis and expression in *Sphingomonas* sp. cultivated under different conditions. Earlier reports considered high-resolution 2-D PAGE to elucidate the mechanisms of UV protection in cyanobacteria, concluding that this technique had greater power of resolution than conventional SDS-PAGE (SDS: sodium dodecyl sulfate) to detect proteome changes. The relationship between symbiotic marine bacteria and their hosts is another field in which proteomics may represent a valuable tool. In this sense, 2-D PAGE has also been successfully applied to the elucidation of proteome changes in the squid *Euprymna scolopes* due to the presence of the symbiotic marine luminous bacterium *Vibrio fischeri*. These authors described the usefulness of 2-D PAGE in following up protein changes in the host soluble proteome in colonized organs of the squid specimens in comparison with uncolonized specimens.

10.11 Pharmacophore Model Hypo1, Virtual Screening for Identification of Novel Tubulin Inhibitors with Potent Anticancer Activity

Microtubules are cytoskeletal filaments consisting of R/β-tubulin heterodimers and are involved in a wide range of cellular functions, including shape maintenance, vesicle transport, cell motility, and division. In the mitotic phase of the cell cycle, microtubules are in dynamic equilibrium with tubulin dimers by assembling the tubulin into microtubules or, conversely, disassembling microtubules to tubulin. Disruption of the dynamic equilibrium can induce cell cycle arrest and ultimately lead to apoptosis. Therefore, compounds that could in-

hibit tubulin polymerization or interrupt microtubule depolymerization would be useful in the treatment of cancer. In recent decades, several compounds, mostly natural products, targeting tubulin have been discovered and developed; some of them are already in clinical use, such as epothilone, paclitaxel, and vindesine.

Recent studies reveal that the antitubulin agents targeting the colchicine sites could, in addition to their antimitotic action, shut down the blood supply to tumors by disassembling the microtubules of abnormal vasculatures and changing the endothelial cell morphology, which would consequently cause nutrient starvation of tumor cells and eventually lead to apoptosis. These vascular-disrupting agents, e.g., combretastatin (CA4), provide a promising new approach in the treatment of cancer and are currently under clinical evaluation for single-drug cancer therapy or multidrug treatment in combination with other cytotoxic drugs and antiangiogenic agents.

10.11.1 CBM4-2 Carbohydrate Binding Module from a Thermostable *Rhodothermus marinus* Xylanase

The family of CBM4-2 structures was calculated using hybrid distance geometry/simulated annealing protocol following methods similar to those used by *Sorimachi* et al. [10.30] and *Simpson* et al. [10.31]. The final set of restraints contained 1654 nonredundant unambiguous NOEs and ambiguous NOEs, 93 æ, 72 ø1, and 1 ø2 restraints, and 65 pairs of hydrogen bond restraints, plus 177 backbone dihedral restraints based on chemical shifts from TALOS. Almost all hydrogen bond restraints were limited to those expected from the regular secondary structure, except for Ala4-Ile164 and Asn79-Ala4, which contribute to tertiary structure, together with Trp28-Val25, Val36-Gly33, and Trp69-Asn67, which are involved in â-turns. The final restraint set had 12.8 restraints per residue. The first 12 models had a very similar energy and showed no NOE violations greater than 0.2 Å and no angle violations greater than 5°. Hence, these models were selected to represent the solution structure of CBM4-2. The average structure was calculated from these 12 structures (by selecting the lowest energy structure and superimposing the remaining 11 on this one) and was subsequently subjected to restrained energy minimization to yield the minimized average structure. The 12 best structures and the average structure have been deposited with the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB ID 1k42 (ensemble) and 1k45 (minimized average)).

10.12 The Polymerase Chain Reaction: A Marine Perspective

A major step in the widespread application and development of the polymerase chain reaction was undoubtedly the early use of the heat stable DNA polymerase Taq. Owing to commercial interest in manufacturing different heat stable polymerases, a larger number of enzymes from hot springs and marine environments were subsequently reported. The fidelity of these enzymes also differed, and one enzyme, that from the marine thermophile *Pyrococcus furiosus*, named Pfu, was found to have superior copying fidelity and is now a widely used polymerase chain reaction PCR reagent. *P. furiosus* was isolated from geothermally heated marine sediments on Vulcano Island, Italy, and is a good example of marine biodiversity providing useful solutions to analytical methods. The widespread use of PCR and high throughput sequencing has allowed us to realize that the vast majority of marine microbes remain to be isolated and studied even though their genes are now accessible.

10.13 Conclusions and New Frontiers

It is clear that once a significant advance has been made in physics, mathematics or chemistry, it is often decades before optimization, improved instrumentation, and increased awareness allow the use of these new techniques in biotechnology. It would, therefore, be interesting to look into the world of physics and mathematics today and asks our colleagues in those disciplines what the key breakthroughs have been in the last few years, so that we might deliberately accelerate their uptake into both mainstream biotechnology and biotechnology of the sea. Recent reports have highlighted the problems arising from the need to handle,

manage, and utilize so-called *big data* in all sectors of the economy, not just biotechnology. It is difficult to extract biological meaning from such data sets. Each of the many new analytical techniques described in this chapter will involve rapid data acquisition accelerating the problem still further, and it may, therefore, be in the fields of mathematics and computing where the most interesting breakthroughs will occur in the coming years.

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11. Microbial Bioprospecting in Marine Environments

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During evolution microorganisms accumulated a remarkable physiological and functional heterogeneity as a result of their adaptation to various selective environmental pressures. Marine microorganisms have been evolving and diversifying for billions of years in contrast microorganisms from terrestrial habitats, which possess a much shorter evolutionary history. In addition, the oceans harbor unique habitats that are mostly unexplored, making them exciting targets for bioprospecting of new microbial capabilities. Over the last years, novel and more complex approaches for the screening of molecules and activities from marine microorganisms with biotechnological potential have been developed and successfully applied. In this chapter, we explore methodological approaches that are currently being used for microbial bioprospecting, with emphasis in the marine environment. These include, among others, high-throughput and other advanced cultivation techniques aiming to recover a larger fraction of culturable microbes, as well as culture-independent, *omics* and *meta-omics* approaches for mining the microbial metabolic potential.

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11.1 Bioprospecting

The term bioprospecting refers to the systematic search for novel biological products and activities with biotechnological applications in natural habitats [11.1]. The course of search and discovery in biotechnology starts with the selection of the most appropriate environments and sampling methods from previous information, continues with the retrieval of the biological materials and their correct storage, moves through screening for desired attributes in the form of microbial assemblages, cells, macromolecules, metabolites, or bioactive compounds using an ever-growing toolkit, and culminates with the development of a commercial product or process ([11.2], Fig. 11.1). This workflow represents a value-adding chain that ends with the addition of products and services that respond to society's needs [11.3]. The biotechnological potential of microbial diversity can be further improved by tools such as enzyme engineering, metabolic engineering and directed evolution [11.4]. Due to their exceptional microbial biodiversity, marine habitats represent fertile grounds for bioprospecting. In this chapter, we will explore the state-of-the art and emerging approaches that can be used to search for biological products and activities with applications in all biotechnological fields.

11.2 Marine Microbial Habitats and Their Biotechnologically-Relevant Microorganisms

The marine environment covers more than 70% of the Earth's surface and contains 97.5% of the water of our planet. Marine habitats contain a rich variety of distinctive life forms, the majority of them represented by microorganisms. Salinity is the major environmental determinant of microbial community composition, clearly distinguishing marine habitats from terrestrial ones [11.5]. Moreover, marine sediments constitute the most phylogenetically diverse environments on Earth, in contrast with soil, which bears high species-level diversity but has below-average phylogenetic diversity [11.5]. Marine microorganisms are progressively recognized as a promising source of biotechnologically valuable products and capabilities. Over the last years, many biomolecules with unique structural features and unique molecular mode of action have been identified in marine environments [11.6]. However, many marine microbial habitats still remain largely unexplored, understudied, and underexploited in comparison with terrestrial ecosystems and organisms.

Through billions of years of evolution, marine microorganisms have developed unique metabolic and physiological capabilities to thrive in a variety of marine habitats. In fact, oceans include the greatest extremes of temperature, light, and pressure encountered by life [11.7]. In recent years, marine microorganisms living under extreme conditions have been the focus of bioprospecting efforts as novel sources of biomolecules with biotechnological potential [11.8, 9]. For example, hydrothermal vents comprise microorganisms with distinct metabolisms based on chemosynthesis. The high diversity and abundance of these communities are comparable to those found in shallow tropical seas, and thus they are recognized as potentially rich sources of biologically active natural products [11.10]. Piezophilic microorganisms inhabiting deep-sea habitats are also of interest, as they can provide enzymes for high-pressure bioreactors, among other applications [11.11]. Interestingly, these microorganisms can be either psychrophilic or thermophilic due to the cold temperatures of the deep ocean or to their proximity to hydrothermal vents, respectively.

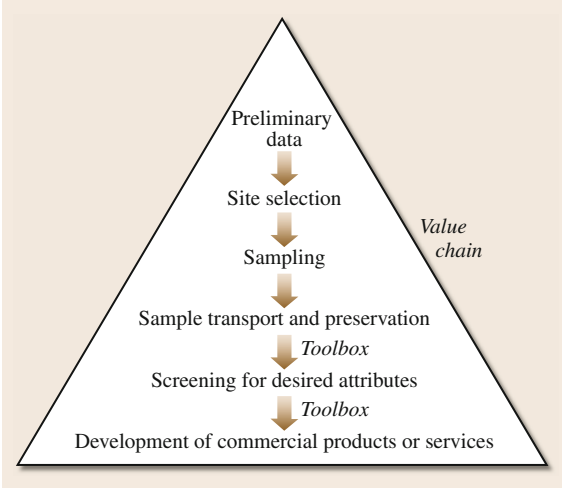

Fig. 11.1 Workflow of search and discovery in biotechnology

Other types of marine microorganisms with potential biotechnological capabilities include those living under epiphytic, epibiotic, and symbiotic lifestyles. Competition and defence strategies characteristic of surface-associated microorganisms, such as the production of toxins, signaling molecules, and other secondary metabolites, constitute an unparalleled reservoir from a biotechnological perspective [11.12, 13]. Bacteria living in symbiotic associations with marine invertebrates often produce complex metabolites as a consequence of coevolution with their host [11.14]. Sponges and corals are examples of habitats where symbiotic microorganisms with interesting capabilities have been found [11.15]. In many cases, microorganisms have been found to be the producers of metabolites previously assigned to their hosts [11.16].

Microorganisms from intertidal zones must be able to tolerate rapid and repeated fluctuations in environmental conditions. These include temperature, light, and salinity, as well as wave action, ultraviolet radiation, and periods of drought [11.17]. Intertidal microbial communities preferentially grow as biofilms on natural and artificial surfaces. Within these protective microenvironments, they are subjected to intense biological and chemical interactions, leading to the production of various interesting secondary metabolites [11.18]. For example, in response to intense solar radiation, cyanobacteria and other microorganisms inhabiting intertidal or supratidal zones produce UV-absorbing/screening compounds, which present potential for the development of novel UV blockers for human use [11.19].

There are certain phylogenetic groups which per se constitute interesting targets for bioprospection. Actinomycetes (within the phylum Actinobacteria) are widely known for their capabilities of producing metabolites, which include antibiotics, antitumor and immunosuppressive agents and enzymes, among others [11.20]. Novel compounds with biological activities have already been isolated from marine actinomycetes [11.6]. Culture-independent studies have shown that the majority of actinomycetes from marine environments are not recovered by cultivation-based methods, and that marine actinomycetes are very different phylogenetically from their terrestrial counterparts [11.21, 22]. Deep-sea sediments and marine flora and fauna allow the access of true marine actinomycetes, away from the influence of land wash-offs [11.6]. Given the fact that 45% of all microbial bioactive secondary metabolites currently derive from actinomycetes and that only 10% of these metabolites is estimated to have been discovered so far, it becomes evident that advances in the ability to access this yet unexploited diversity will provide a new source for the discovery of secondary metabolites [11.23].

11.3 Methods for Microbial Bioprospecting in Marine Environments

Both culture-dependent and independent methods have uncovered an incredible diversity of microorganisms whose metabolisms largely have yet to be characterized [11.24, 25]. These methods have been further empowered by genomic-level information, which in turn is supported by sequencing technologies and bioinformatics [11.26, 27]. In the next sections, traditional, state-of-the-art and emerging approaches used for bioprospecting marine microorganisms with biotechnological potential will be reviewed.

11.3.1 Culturing Techniques

Microbial bioprospection and biodiscovery is currently severely limited by the lack of laboratory cultures [11.28]. Although culture-independent approaches have revolutionized environmental microbiology, the development of biotechnological applications from the genetic potential of microbial communities as well as fundamental environmental research must be anchored by the corresponding study of pure cultures. Furthermore, this novel diversity needs to be deeply characterized and adequately preserved in order to guarantee its future availability [11.29]. Novel cultivation methods, fortunately, continue to emerge as alternatives to overcome culture limitations [11.30]. These methods rely on advances in basic biological and ecological knowledge in order to best simulate the natural environment, as well as on the development of new technologies for more efficient screenings (Table 11.1). With the aid of sophisticated high-throughput cultivation techniques, the proportion of microorganisms from marine environments represented in culture has increased significantly over the last years [11.31].

High-throughput dilution-to-extinction culture is one of the most powerful and sensitive approaches for the culture of marine microorganisms such as bacterioplankton. This technique led to the cultivation of the first member of the widespread but yet uncultured marine SAR11 clade [11.40]. This method consists in dilution of bacteria up to 1–10 cells per well in microtiter plates, using low-nutrient filtered seawater. High-throughput screening based on fluorescence microscopy clearly improved the technique over conventional methods, allowing rapid and sensitive detection of growing cells [11.35]. In later studies, this approach was coupled to long-term incubation at low temperatures to allow the recovery of new microbial variants [11.39].

The diffusion chamber [11.32] is a device in which microbial cells are inoculated in an agar matrix separated from the source environment by membranes, isolating the cells but allowing nutrients and growth factors to pass through. The use of this device greatly improved the proportion of culturable bacteria from marine sediments [11.32]. Another version of this approach is the microbial trap, which selectively enriches for filamentous bacteria (e.g. actinomycetes) by allowing the filament colonization of the sterile agar through membranes with 0.2 µm pores [11.34]. Microdroplet encapsulation in an agarose matrix, combined with growth detection by flow cytometry, led to the recovery of new clades from the marine environment [11.36, 37]. This approach is similar to the diffusion chamber in the sense that the agarose is porous, and nutrients and signaling molecules can diffuse into the growing colony and waste metabolites can diffuse out. Another advantage of the approach is that the microdroplets are physically separated and, because they are much larger than bacterial cells, they can be manipulated [11.28, 36].

Currently, second-generation high-throughput automated methods are being developed from these environ-

Table 11.1 Limitations encountered with the culturing of marine bacteria and solutions implemented through novel culturing techniques

Limitation	Solution	References
Lack of knowledge on physiological needs	Culture in situ or in simulated natural media	[11.32–34]
Oligotrophy (particularly bacterioplankton)	Use of low-nutrient filtered seawater	[11.35]
Overgrowth of fast growers	Dilution, encapsulation	[11.35–37]
Slow growth	Long-term incubation	[11.38, 39]
Poor cell density	Highly sensitive screening techniques (e.g. fluorescence microscopy, flow cytometry)	[11.35–37, 40]
Dormancy	Long-term incubation, repeated short-term incubations	[11.41]
Special needs of chemical or physical parameters	Chemical gradient systems, high-pressure reactors, flow-through devices, gas-lift reactors	[11.42–44]
Need of attachment to a solid surface	In situ colonization carriers	[11.44–46]
Loss of interactions between interdependent microorganisms (e.g. syntropy, cell-to-cell signaling)	Diffusion devices, microdroplet encapsulation, co-culture of *helper* strains, addition of growth factors	[11.30, 32, 36, 47, 48]
Complexity of bacterial communities	High-throughput cultivation and screening	[11.40, 49–51]

mental cultivation devices. One example is the development of the isolation chip (Ichip), a culture/isolation device composed of several hundreds of miniature diffusion chambers, each inoculated with a single environmental cell [11.50]. Another example is the micro-Petri dish, a device supported by porous material and reaching a million growth compartments [11.49]. An interesting method that couples high-throughput culture to rapid chemical screening was recently developed to identify symbiotic microbes producing secondary metabolites [11.51]. The screening is chemically based, by means of the use of ultra high-performance liquid chromatography/mass spectrometry. In this approach, a 96 multi-well plate format is utilized in rounds of successive culturing steps [11.51]. This strategy prevents spending significant resources on isolating, culturing, and analyzing microbes that do not possess the capability to produce the compounds of interest.

Various factors are thought to contribute to the low rate of culture recovery of environmental microbes (Table 11.1). The lack of knowledge of the environmental and nutritional requirements of yet unknown microorganisms is the most obvious. Another is the loss of biological cell-to-cell interactions in the isolation process [11.44]. For example, most of the strains able to grow on Petri dishes after recovery in diffusion chambers were, indeed, mixed cultures, highlighting the importance of chemical signaling for microbial growth [11.32]. The co-culturing with *helper strains*, followed by the identification of an oligopeptide signal, allowed previously uncultured strains to be successfully isolated in the laboratory [11.30, 48]. These authors have also reported problems in the successful adaptation to laboratory conditions or *domestication* of the cultured strains, as many of the strains forming microcolonies in diffusion chambers could only undergo a limited number of divisions in Petri dishes [11.32]. In further experiments, successive rounds of in situ cultivation in the chambers allowed for a larger recovery of isolates [11.30].

Advances in the understanding of basic microbiological principles can greatly help us to overcome the limitations of culturing environmental microbes. The theory of *scouting* of dormant cells proposes that any microbial population consists of a mixture of active and dormant cells [11.41, 52]. Individual cells periodically exit dormancy, although these events are not related to the onset of favorable environmental conditions, but are rather essentially random. Moreover, this happens independently of the nature of the microbial species (sporulating vs. nonsporulating, or fast versus slow growers). Indeed, the importance of the slow growers in environmental samples may be lower than previously thought, as many of the microbes regarded as slow growers in culture are, in fact, late awakening events [11.52]. One practical implication of this theory is that the success in discovering novel species depends on the overall amount of cultivation effort rather than the length of in-

cubation (i. e., the same amount of effort focused either on many short-term or fewer long-term cultivation experiments) [11.41]. This theory could explain previous findings, such as why, for example, representatives of the phylum Verrucomicrobia that had been considered unculturable for many years, were eventually cultivated using rather conventional techniques. Dilution cultures were used to separate the very fast growers, but long incubation times were not necessary [11.41, 53].

11.3.2 Culture Independent Gene-Targeted Methods

In spite of the recent advances in microbial culturing, the majority of environmental microorganisms are still unculturable. Out of the more than 100 bacterial divisions that have been proposed to date, only 30 possess a cultivated representative [11.29]. Moreover, marine microbes are at the top of the list of those unculturable by conventional methods [11.26]. Since the landmark studies of *Woese* and *Pace* [11.54, 55], which stated the basis for molecular phylogeny, culture-independent methods have revolutionized our understanding of microbial communities [11.56] and currently stand on their own as a valid alternative for bioprospection. These methods are based on the information provided by biomolecules, mainly deoxyribonucleic acid (DNA), bypassing the need of cultivation by extracting these biomolecules directly from the environmental sample. Although they are not exempt from biases [11.57], they are still the best way to gain access to the overwhelming biodiversity of environmental microbes.

Culture-Independent Phylogenetic Approaches

Among culture-independent methods, the approach based on the molecular phylogeny of rRNA (ribosomal ribonucleic acid), particularly the small subunit (16S rRNA for archaea and bacteria), continues to be one of the most widely used. This gene has two properties that have positioned it as a building block for a universal molecular phylogenetic framework: its presence in all forms of life and a domain structure with variable evolutionary rates, which enables phylogenetic reconstruction at various levels. Fingerprinting techniques, polymerase chain reaction (PCR) clone libraries, and microscopy-based techniques like fluorescence in situ hybridization (FISH) have been routinely utilized over the last decades to describe and compare the structure and composition of microbial communities [11.58]. More recently, large-scale sequencing of 16S rRNA gene hypervariable regions has brought new strength to the classical phylogenetic approaches, which suffered a number of limitations associated with low coverage and cloning biases [11.59]. These approaches can be used as a guide for phylogenetically-driven biodiscovery. For example, analysis of community 16S rRNA by sequencing or fingerprinting can be used to select the most diverse sampling sites, thus maximizing novel taxa recovery in culture (Fig. 11.2, [11.23]). This approach is based upon the premise that taxonomic diversity is coupled to chemical diversity due to the role that secondary metabolism plays in speciation [11.23].

Functional Gene-Based Approaches

Approaches based on functional genes, which focus on the potential of the community to perform an activity of interest, can give a complementary view to the phylogenetic approach. Gene coding for key enzymes participating in different environmental processes, such as sulfate reduction [11.60], denitrification [11.61], nitrogen fixation [11.62], ammonia oxidation [11.63], hydrocarbon biodegradation [11.64, 65] among others, have been studied in the marine environment. Targets include not only bacterial but also archaeal populations and subgroups within these, by means of the use of primers with different specificities. Due to its highly focused nature, this approach is very powerful. However, one of its major drawbacks is the relative lack of database sequence information for functional genes, with respect to the 16S RNA gene. Another shortcoming is the lack of accuracy in taxonomic assignment due to lateral gene transfer [11.66].

Functional genes have the potential to be used as biomarkers in assays developed for the environmental biotechnology field, including wastewater treatment [11.67], and environmental remediation (Fig. 11.2, [11.68]). These molecular biological tools have been applied in the marine environment, for example, for the study of hydrocarbon degrading bacterial populations [11.64, 65]. In particular, quantitative polymerase chain reaction (qPCR) is a promising technique due to its quantitative nature, high sensitivity and the possibility of high throughput analysis [11.68]. However, this tool is still in its infancy for field applications in marine environments.

Linking Phylogeny and Function: Labeled Isotopic-Based Approaches

One of the long-standing goals of environmental microbiology is the possibility to link the phylogenetic identity of an uncultured microorganism with its function in

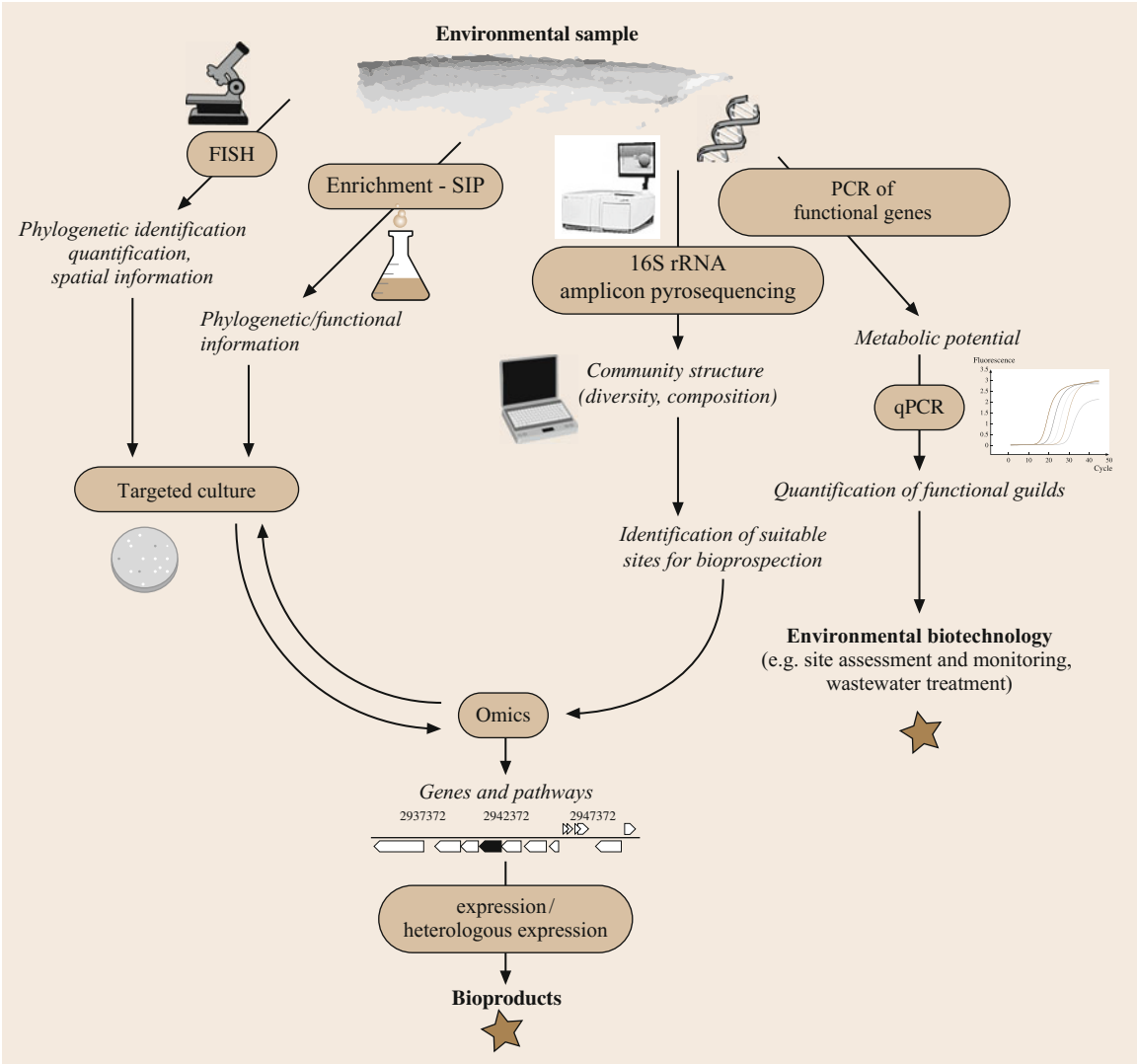

Fig. 11.2 Culture-independent gene-targeted approaches relevant for biotechnology. These approaches can aid in the selection of suitable sites for bioprospection and in inferring possible culturing conditions. They are also relevant in environmental biotechnology

the environment. The achievement of this goal can have far-reaching consequences for our understanding of microbial communities and ecosystems. Furthermore, it can fuel our efforts in the identification of new biotechnologically sound microbes and activities. Advances in isotope labeling have started to contribute over the last years to this goal [11.69]. The basic principle of this approach is that the labeling of substrates with stable or radioactive isotopes allows for the differentiation of metabolically active populations that incorporate the substrate. When this approach is coupled to some form of identification tool, the result is an experimental evidence of both the functional role and the phylogenetic identity of a previously unknown population. Stable isotope probing (SIP) was one of the first methods to be developed [11.70]. Microbial populations utilize the substrates offered labeled with a stable isotope and assimilate the heavier source into cell components, which in turn become labeled. The heavier and lighter molecules are then physically separated and analyzed.

The most widely used method is DNA-SIP, in which DNA is separated in caesium chloride gradients and further purified and analyzed by cloning and sequencing [11.70]. The RNA-based SIP approach maintains the sequence-based phylogenetic resolution of DNA-SIP, but focuses directly on the RNA molecule itself rather than its gene, with the advantage of a high copy number and a turnover that is independent of cell replication [11.71]. Marine environments studied by this method include marine and estuarine sediments [11.72–74] and seawater samples [11.75]. Biotechnological applications of SIP have mainly addressed issues related to environmental biotechnology [11.76].

SIP depends upon the availability of stable isotopes (^{13}C, ^{15}N, ^{18}O) and of substituted substrate compounds [11.69]. However, they have the advantage of generating de novo information about the identity of the populations associated with a certain metabolic process. Interestingly, different incubation times can be used to follow the carbon flow in the different members of the community. The results can then be further tested experimentally, for example, by means of imaging techniques or targeted culture (Fig. 11.2).

Microscopy provides information about spatial arrangement and physical interactions of cells, which is applicable to spatially complex environments such as biofilms, consortia and symbiotic assemblages (Fig. 11.2). The development of fluorescence in-situ hybridization (FISH) enabled the detection and identification of single microbial cells in environmental samples by means of rRNA-targeted gene probes [11.77]. Microscope-based enumeration of cells makes this method an excellent approach for quantitative estimations, which is more accurate than conventional PCR. Furthermore, the technique is suitable for the use of multiple hierarchical probes in the same sample, which reduces the possibility of false positives. This powerful method has been coupled with the microautoradiography technique (FISH-MAR), which offers the possibility to directly observe the incorporation of substrates labeled with a radioactive isotope into single microbial cells [11.78]. As in SIP, the main limitation of this technique is the availability of radiolabeled substrates, with the additional concern of safety issues. In addition, some environmental samples bearing cells with low ribosome content (e.g., marine oligotrophic environments) can have detection problems with FISH. Horseradish peroxidase (HRP)-labeled oligonucleotide probes and tyramide can be used to enhance the signal intensities of hybridized cells. This approach is sometimes called catalyzed reporter deposition FISH (CARD-FISH, [11.79]), which can also be coupled to microautoradiography, further increasing its potential [11.80]. Raman microspectroscopy and nanometer-scale secondary-ion mass spectrometry (nanoSIMS [11.81, 82]), are other techniques that are currently under development and may potentially be useful in the future for bioprospecting.

11.3.3 *Omics* and *Meta-Omics* Approaches

The large-scale study of genes (genomics), transcripts (transcriptomics), proteins (proteomics), metabolites (metabolomics), lipids (lipidomics), and interactions (interactomics) are globally defined as *omics* in the study of individual species and are often referred to as *meta-omics* approaches when microbial communities are analyzed [11.83]. These are rapidly evolving fields, which are highly dependent on the development and improvement of technologies and analysis tools. Besides their critical role for understanding the structure and function of microbial communities, they represent powerful approaches for the bioprospection of biological products and activities with biotechnological potential in marine environments.

Genomics

Since the publication of the genome of the bacterium *Haemophilus influenzae* Rd in 1995 [11.84], the number of sequenced genomes has expanded quickly [11.85]. By the end of 2012, the Genomes Online Database (GOLD, Table 11.2) listed more than 4000 completed genome projects, 90% of them belonging to bacteria. Approximately 60% of these genomes where *finished*, that is, all segments obtained after the assembly were ordered, all gaps were closed, and any ambiguities or discrepancies were resolved after a series of rigorous quality-control steps [11.86, 87]. As the finishing step increases the cost and time required to sequence a genome, often the final goal is to obtain a draft genome, represented by a number of contigs or scaffolds [11.87]. Although there are limitations for the use of draft sequences in some applications [11.88], draft assemblies are a powerful resource for bioprospecting, as the majority of the genes of an organism are usually represented in its draft genome [11.86].

Not only the number of genomes sequenced so far represents a minimal proportion of the microbial diversity present in our planet [11.89], but also some phylogenetic groups of microorganisms (such as members of the Proteobacteria and Firmicutes) are greatly over-represented, while other groups have no represen-

Table 11.2 Websites of initiatives of genomic and metagenomic data generation, repository and/or analysis tools, useful for marine microbial bioprospecting

Initiative/Website	Description
Genomic Encyclopedia of Bacteria and Archaea (GEBA) project www.jgi.doe.gov/programs/GEBA	It aims at systematically filling in the gaps in sequencing along the bacterial and archaeal branches of the tree of life
Marine Microbial Genome Sequencing Project http://camera.calit2.net/microgenome	Its aim is to increase the number of whole genome sequences of ecologically relevant marine microorganisms
Genomes Online Database (GOLD) www.genomesonline.org/cgi-bin/GOLD/index.cgi	Resource for comprehensive access to information regarding genome and metagenome sequencing projects, and their associated metadata
Integrated Microbial Genomes and Metagenomes System (IMG and IMG/M) http://img.jgi.doe.gov	Community resource for analysis and annotation of genome and metagenome datasets in a comprehensive comparative context
National Center for Biotechnology Information (NCBI) Genome and Sequence Read Archive (SRA) www.ncbi.nlm.nih.gov	NCBI Genome organizes information on genomes including sequences, maps, chromosomes, assemblies, and annotations. The NCBI SRA stores raw sequencing data from the next generation of sequencing platforms
Metagenomics Analysis Server (MG-RAST) http://metagenomics.anl.gov	The MG-RAST server is an automated analysis platform for metagenomes providing quantitative insights into microbial populations based on sequence data
Microbial Ecological Genomics DataBase (MegDB) www.megx.net	MegDB is a collection of publicly available georeferenced marine bacterial and archaeal genomes and metagenomes
Earth Microbiome Project (EMP) www.earthmicrobiome.org	Massive multidisciplinary effort to analyze microbial communities across the globe, using metagenomics, metatranscriptomics, and amplicon sequencing
Microme www.microme.eu	Resource for bacterial metabolism, whose aim is to support the large scale inference of metabolic flux directly from genome sequence
Microbial Genome Annotation & Analysis Platform (MicroScope) www.genoscope.cns.fr/agc/microscope/home/index.php	Web-based platform for microbial comparative genome analysis and manual functional annotation
Text Mining for Bacterial Enzymes (TeBactEn) http://tebacten.bioinfo.cnio.es	Tool designed to facilitate the retrieval, extraction and annotation of bacterial enzymatic reactions and pathways from the literature
Characterized Protein Database (CharProtDB) www.jcvi.org/charprotdb/index.cgi/home	Resource of expertly curated, experimentally characterized proteins described in published literature
Natural Product Domain Seeker (NaPDoS) http://napdos.ucsd.edu	Bioinformatic tool for the rapid detection and analysis of secondary metabolite genes
MetaBioME: A Comprehensive Metagenomic BioMining Engine http://metasystems.riken.jp/metabiome	Web resource to find novel homologs for known commercially useful enzymes in metagenomic datasets and completed bacterial genomes

tative sequences [11.85]. This bias has a negative effect on gene discovery and annotation in both genomic and metagenomic data, as microbial genomes provide scaffolds for the interpretation of sequence information. With the aim of systematically filling in these existing gaps, the US Department of Energy's Joint Genome

Institute created the initiative *Genomic Encyclopedia of Bacteria and Archaea* (Table 11.2). Similarly, the Microbial Genome Sequencing Project of the Gordon and Betty Moore Foundation's Marine Microbiology Initiative has sequenced the genome of hundreds of ecologically relevant microorganisms isolated from diverse marine habitats (Table 11.2).

The phenotypic analysis of isolates can severely underestimate its genetic potential, as genes may not be expressed or may be expressed at very low levels under laboratory conditions. For example, it has been proposed that a unique combination of environmental factors may be required for the expression of biosynthetic genes [11.6]. Therefore, the mining for genes or gene clusters in microbial genomes could uncover *hidden treasures* that could be exploited, for example, using heterologous gene expression [11.90]. Moreover, the use of sequence information has assisted in the determination of the chemical structure of new compounds by a combination of bioinformatics and chemistry [11.91]. The potential for biodiscovery of this approach has led to the explosion of interest in genome mining as a tool for bioprospection, which has been aided by the development of new bioinformatic tools for the analysis of the growing volume of DNA sequence data [11.92]. However, as sequencing efforts are rarely followed by the biochemical characterization of the putative gene products, many genes emerging from these studies have unknown functions, and basic local alignment search tool (BLAST)-based protein functional assignments can easily propagate annotation errors [11.93]. Recently, a database of experimentally characterized proteins was created (CharProtDB, Table 11.2), enabling to link experimental characterizations of protein functions with computationally accessible protein sequences [11.94].

The mining of the ever-increasing amount of genomic data from marine microorganisms has led to the bioassay-independent discovery of gene clusters with important biotechnological applications [11.95]. In recently published work, *Wargacki* and collaborators [11.96] used a public database to identify a genome fragment from the *Vibrio splendidus* strain 12B01, containing genes for alginate degradation, transport, and metabolism. This gene cluster was used to construct a microbial platform that enables bioethanol production from macroalgae via a consolidated process [11.96]. In silico mining of genomes combined with molecular biology approaches has resulted in the discovery of novel gene clusters with potential use for the development of peptide-based drug candidates [11.95]. In addition, a search of sequenced bacterial genomes showed that marine cyanobacteria present an extraordinarily efficient strategy for generating many cyclic peptide secondary metabolites [11.97].

Other *Omics* Approaches

Currently, only one-third of an annotated bacterial genome corresponds to information that is well known [11.98]. However, in order to understand how a cell operates and, therefore, for the successful application of its genetic potential, a better knowledge of the two ignored parts is required. Molecular approaches that can be used in combination with genome sequencing to study a marine microbial isolate include, for instance, transcriptomics, proteomics, and metabolomics [11.99]. Concerning transcriptomics, the development first of microarray technology and later of whole transcriptome shotgun sequencing using next-generation sequencing technologies has provided valuable insight into gene function and regulation [11.100]. Importantly, this information also serves as a basis for genome re-annotation. In addition to its function as information carrier, RNA can present various regulatory functions in bacteria [11.101]. Although this information is still in its infancy, it could be highly valuable for the biotechnological application of pure cultures.

Technological advances in the field of mass spectrometry (MS) have enabled us to obtain information regarding a significant proportion of the proteome of microbial isolates [11.102]. Several proteomic studies of cyanobacterial strains have been published, which are microorganisms that have interesting biotechnological applications [11.103]. For example, the analysis of the proteome of *Synechocystis* sp. PCC 6803 rendered evidence of the mechanisms used by this microorganism for gaining resistance against the biofuel hexane [11.104]. In a second study, the complex response of this model microorganism to ethanol was analyzed using a quantitative proteomics approach [11.105]. Ethanol sensitivity of cyanobacteria currently restricts efforts to increase biofuel production levels in metabolic engineered strains for autotrophic ethanol production, and this study provided a list of potential gene targets for engineering ethanol tolerance.

Although highly challenging due to the extremely fast turnover times of the small molecules of the cell, metabolomics is fundamental for the understanding of metabolic reaction networks and their regulation, as well as to link the genotype of an isolate to its phenotype [11.106]. Interactomics, on the other hand, attempts to resolve the whole set of molecular in-

teractions in cells and fluxomics establishes dynamic changes of molecules within a cell over time [11.99]. No single approach is sufficient to characterize the complexity of biological systems [11.99]. In fact, it is only through the integration of multiple layers or dimensions of information (provided by different *omics* approaches) that a proper understanding of the whole cell operation can be obtained [11.98]. The integration of the overwhelming amount of information obtained from multiple datasets can only be accomplished with the use of mathematical modeling and computational tools and results in a dynamic map of all cellular functions and regulatory circuits with spatiotemporal resolution [11.107]. The advances of the field of systems biology are empowering the engineering of industrial microorganisms, allowing the development of more robust strategies and moving the field toward a design-based engineering of biological systems [11.108].

Single-Cell Analyses

The analysis of single cells is an approach with multiple biotechnological applications and presents both unprecedented challenges and opportunities [11.83]. Individual cells can be physically separated from each other and/or from the environmental matrix material before further analysis, through a technique called single-cell isolation [11.109]. In addition, targeted cells can be individually recognized and distinguished from background populations through cell-sorting techniques, although some cell-sorting instrumentation also allows cell isolation [11.109]. The fundamentals, advantages, and drawbacks of the different devices used for cell sorting and cell isolation were recently reviewed in detail [11.107, 109] and therefore they will not be covered in this chapter.

One of the applications of single-cell analysis is the study of cell-to-cell variations within an isogenic cell population, delivering functional biological information beyond the statistical average of a microbial population [11.107]. For example, through the use of total transcript amplification, the heterogeneity of transcript levels among individual cells within a bacterial population can now be studied [11.110]. A second application of single-cell analysis is to individually study yet-to-be cultured microorganisms using *omics* approaches. Single-cell genomics (SCG) involves the isolation of single cells from an environmental sample, the purification of its DNA, followed by whole-genome amplification and sequencing [11.26]. Methodological difficulties of SCG include background contamination during the amplification of single-cell DNA, biases during amplification and sequencing, as well as difficulties in sequence assembly [11.83, 111]. Different strategies have been tested in order to improve SCG, such as reagent decontamination before amplification [11.112], artificially inducing polyploidy in single cells [11.113], improving the efficiency of genome amplification [11.83], as well as using more efficient assembly algorithms [11.111]. In spite of its limitations, it is currently possible to obtain a high percentage of de novo genome sequences from yet-to-be cultured microorganisms.

SCG is considered a powerful complement of both cultivation and metagenomics, as it allows us to link the metabolic potential of an uncultured microorganism with its taxonomic identity, as well as to define possible strategies for the isolation of the microorganism [11.114]. In addition, SCG allows us to study in situ interactions among organisms and is particularly suited for the analysis of symbiotic systems, for example, the biotechnologically-relevant bacterial symbionts of marine sponges [11.115, 116]. In a recent work, *Bayer* et al. [11.117] identified novel enzymes involved in halogenation reactions in marine sponge-associated microbial consortia using a combination of *omics* approaches, including SCG. In another study, *Martinez-Garcia* and collaborators [11.118] sequenced five *Verrucomicrobia* cells, identifying genes encoding a wide spectrum of glycoside hydrolases, sulfatases, peptidases, carbohydrate lyases, and esterases. In addition, the analysis of partially assembled genomes of only ten cells of *Prochlorococcus* increased the pan-genome of this genus by 4.6%, highlighting the potential of this approach for bioprospecting [11.119]. Information concerning which proteins are being expressed in a particular environmental condition, their abundance, as well as post-translational modifications could be obtained through single-cell proteomics. However, further development of MS and micro/nanofluidic based technologies is needed for the analysis of the proteome from single cells. Although still not widely used in biotechnological applications, the combination of single-cell *omics* approaches has the potential to significantly contribute to this field [11.107, 114].

Metagenomic Approaches

Metagenomics, the direct analysis of the genomes contained in a microbial community, nowadays represents a key tool for microbial marine bioprospecting, as it allows access to the genetic potential of a microbial community. Metagenomic analyses typically start with the purification of DNA from an environmental sam-

ple, which is called metagenomic DNA [11.120]. This DNA can be used for the construction of a metagenomic library [11.121] or alternatively, it can be randomly sequenced using next-generation sequencing technologies [11.122]. Due to the uneven distribution and high species richness of most microbial communities, metagenomic analyses are very challenging. Despite the difficulties still affecting metagenomics, in recent years this discipline has been fundamental in increasing our understanding of microbial communities and has become an important tool for mining novel biomolecules or activities with biotechnological potential [11.123, 124]. For instance, the construction and screening of metagenomic libraries have resulted in the identification of many novel biocatalysts, including lipases/esterases, cellulases, chitinases, DNA polymerases, proteases, and antibiotics [11.123, 125]. Marine sediments, microbial communities from marine invertebrates, and cold marine environments are among the most commonly studied habitats, due to their high biotechnological potential [11.126–128].

The cloning of fragments of metagenomic DNA using the appropriate vectors and suitable hosts allows us to store and mine the genetic potential contained in a microbial community [11.121]. The selection of the vector for library construction (plasmids, cosmids, fosmids, or BACs – bacterial artificial chromosomes) depends mainly on the desired insert length. For example, large-insert libraries are required for recovering large gene clusters [11.123]. Other factors to consider are the desired cell copy number, the quality of the metagenomic DNA, the genes that are being targeted, the chosen host, as well as the selected screening strategy [11.121, 123]. In order to reach sufficient coverage of metagenomes of highly diverse microbial communities, such as those from soils or sediments, metagenomic libraries need to contain a large number of clones [11.129]. To increase hit rates, enrichment cultures were used prior to metagenomic DNA extraction, although this approach can result in an overall loss of diversity [11.130]. Another possible strategy is the use of stable-isotope-labeled substrates to enrich the functionally-relevant fraction of the microbial community. In this case, density centrifugation of metagenomic DNA is performed after labeling, before the construction of the metagenomic library [11.121, 131].

Two different strategies can be used for the screening of a metagenomic library: a function-based approach (detection or selection for metabolic activity) or a sequence-based approach (detection of a specific target gene). The first strategy, called functional metagenomics, does not require previous knowledge of sequence information and can, therefore, result in the identification of entirely novel classes of genes [11.123]. It presents the additional advantage that the identified gene or gene cluster is already being functionally expressed in the host. However, functional-based screenings can be problematic due to low-level gene expression, lack of post-translational modifications, the formation of insoluble aggregated folding intermediates, as well as detrimental effects that the products can have on the host cell [11.132, 133]. Currently, the most commonly used vector and host for constructing metagenomic libraries are fosmids and *Escherichia coli* [11.121]. The use of other hosts and the development of vectors able to replicate in various species are particularly useful for expression-based analyses of metagenomic libraries [11.129, 134, 135]. In addition, hosts can be engineered to improve gene expression [11.136].

Different function-driven approaches can be used for the screening of metagenomic libraries. One of them is the detection of the desired phenotype in agar-plate assays, for instance, an enzymatic activity or colony pigmentation [11.124]. Agar-plate based screenings have the advantage of not requiring expensive devices. However, they are usually labor intensive and they tend to have low hit rates due to the generation of weak signals [11.129]. In addition, this strategy depends on the availability of assays able to detect the desired metabolic function, of which there are, unfortunately, very few [11.131]. In order to increase the sensitivity of the assays, the enzymatic activity can be measured in cell lysates [11.124, 129]. This is usually performed using colony picking robots and microplate readers to shorten the processing time. Another strategy, called heterologous complementation, significantly simplifies functional screening by taking advantage of gene targets for which the desired phenotype is required for the survival of the host, such as genes that confer resistance to metals or antibiotics [11.129, 137]. On the other hand, the ability of some substrates to induce gene expression through closely located regulatory elements has been used to engineer vectors containing reporter genes [11.124]. Interestingly, *Uchiyama* and collaborators [11.138, 139] have created specific reporter assays based on fluorescent proteins in order to screen for enzyme-encoding genes in metagenomic libraries.

In contrast to functional metagenomics, molecular screenings involve the use of primers or probes that have been designed based on conserved regions of already-known genes or protein families to mine

a metagenomic library [11.123]. The main advantage of this approach is that the target can be identified even if it is not being expressed by the host [11.128, 140]. Subcloning using the appropriate host and vector can result in the functional expression of the gene or gene cluster of interest, allowing the functional characterization and biotechnological application of the product. A critical drawback of molecular screenings is that they depend on sequence database information, which is currently biased, and as a consequence usually results in the retrieval of variations of previously known genes. In order to maximize the discovery process in molecular screenings, sequences of genes identified in a metagenomic library by a functional approach or gene fragments retrieved by PCR from the same environment can also be used as a source of de novo, unbiased genetic information for primer design [11.141]. An interesting approach to mine for gene clusters involved in the biosynthesis of bioactive molecules is to perform a retrobiosynthetic analysis on the structure of these compounds in order to predict the enzymes involved in the biosynthetic pathway [11.128]. This information is then used to design degenerate primer sets for the retrieval of gene fragments by PCR [11.128].

Besides the construction of metagenomic libraries, DNA isolated from environmental samples can be sequenced directly using next-generation sequencing technologies, resulting in the random generation of sequence information from the genomes contained in the microbial community [11.142]. This approach has been extensively used for the analysis of microbial communities from marine environments, providing unprecedented insights into their genetic potential [11.120]. Continuous improvements in sequencing technologies is resulting in longer read lengths, larger sequence outputs, as well as lower costs, allowing a deeper analysis of the microbial communities. As individual reads are usually assembled, genome fragments containing whole operons, and even draft genomes from uncultured bacteria can be obtained [11.143, 144]. This information is critical in bioprospecting efforts, as it can be used as a basis for the recovery of the genome fragment from the same community, or alternatively, for its synthesis. The term *synthetic metagenomics* has been proposed to define the discovery approach that involves in silico identification of hypothetical target sequences followed by automated chemical DNA synthesis and heterologous expression [11.145]. This approach has recently been used to obtain de novo functional methyl halide transferases using information from the GenBank database, enzymes that are useful for biofuel production [11.145]. This approach allows the exploitation of existing sequence databases, currently underexplored and underexploited [11.146]. Synthetic metagenomics has the additional advantage of allowing codon optimization, which may significantly improve gene expression [11.145]. *Sharma* and collaborators [11.147] have developed a resource called MetaBioME with the goal of facilitating the discovery of novel commercially useful enzymes from metagenome information (Table 11.2).

Other *Meta-Omics*

Next-generation sequencing technologies can also be used to analyze the subset of genes in a microbial assemblage that is being transcribed under a particular environmental condition [11.148, 149]. For this approach, total RNA is extracted from the environmental sample, rRNA is removed in order to enrich for the mRNA fraction, and copy DNA (cDNA) synthesis is performed before sequencing [11.148]. When cDNA yield is insufficient for analysis, however, an amplification step can be included. Metatranscriptome sequencing represents a powerful tool to analyze microbial communities, albeit with considerable challenges. Not only environmental cells present low mRNA contents, but also their half-lives are very short, in the range of a few minutes [11.150]. In addition, mRNA constitutes a very small fraction of the total RNA in bacterial cells and the enrichment of the mRNA fraction in prokaryotes is challenging [11.151]. Limitation in environmental sample quality and quantity, as well as low mRNA integrity and purity can also affect metatranscriptomic analyses [11.149, 151]. Despite these methodological challenges, this approach has been increasingly applied in fundamental research on microbial communities from various marine habitats [11.152–157].

Like shotgun sequencing metagenomics, sequence-based metatranscriptomics presents no significant bias towards known sequences, and is considered highly informative concerning ongoing ecologically relevant processes [11.149, 150]. Other advantages of metatranscriptomics over metagenomics are that only ecologically relevant information is retrieved and fewer resources are required for this analysis [11.148]. However, sequence reads are still too short for bioprospecting efforts. Metatranscriptomics represents a powerful approach for the discovery of metabolically relevant enzymes that are actively involved in particular biochemical pathways [11.148, 158]. Another application is the analysis of community-specific variants of functional genes; this information is highly relevant to the

environmental biotechnology field and may lead to the discovery of genes or processes with biotechnological potential [11.148, 149]. For instance, in a recent study, a combination of *omics* approaches that included metatranscriptomic analysis was able to elucidate which hydrocarbon degradation pathways were actively expressed in the deep sea after an oil spill and to ascribe these pathways to particular taxa [11.154].

Another approach that can be used for the identification of novel enzymes is to directly analyze the proteins of a microbial community using metaproteomics. This approach consists in the extraction of the proteins from an environmental sample, followed by the separation of the proteins (or peptides) by two-dimensional polyacrylamide gel electrophoresis or liquid chromatography, and lastly MS analysis and the identification of the proteins by in silico spectral matching against sequence databases [11.159]. The main challenges of metaproteomics are the large complexity of protein species expressed by the members of the microbial community and the large dynamic range of protein levels [11.159]. However, faster and more sensitive mass spectrometers and advances in *omics* datasets and data handling facilitate the analysis of increasingly complex environments [11.160]. Most marine metaproteomic studies performed so far have focused on the analysis of planktonic microorganisms, providing clues concerning key metabolic processes such as those involved in ocean biogeochemical cycles [11.161–164]. More recently, *Kleiner* and collaborators [11.165] used metaproteomics and metabolomics to investigate metabolic interactions in the association between a gutless marine worm and its bacterial symbionts, revealing highly efficient pathways for the uptake, recycling, and conservation of energy and carbon sources.

Molecular systems biology at the ecosystem level, also known as eco-systems biology, attempts to build models that are able to predict the behavior of a community, through the integration of *omics*, *meta-omics*, and single-cell approaches, as well as the use of mathematical models [11.166–168]. Although still in its infancy, this discipline has the potential to provide a comprehensive understanding of the functioning of microbial communities, and, therefore, to facilitate their management, which is a long-term goal of environmental biotechnology [11.168]. For example, improving the mechanistic understanding of biodegradation processes may facilitate the development of knowledge-based bioremediation strategies and the design of biosensors for the detection of pollutants [11.169].

11.4 Conclusions

Over the last years, the development of a broad array of methodologies for the analysis of environmental microorganisms has profoundly altered bioprospecting efforts, thus significantly increasing our access to the genetic potential contained in microbial communities. However, finding properties of interest in the prospected environments is only the first stage in a series of value-adding steps, which ends in the development of products or services with applications in human health, industry, renewable energy, etc. Importantly, strategies that are able to maximize the biotechnological potential of environmental microorganisms, for example, microbial engineering and synthetic biology, have been matching the evolution of bioprospecting tools.

As microorganisms from marine habitats are increasingly being recognized as particularly promising resources for bioprospecting, both academia and biotechnology industry sectors are increasing their investments in marine biotechnology research and development. This is evidenced by an increment in the number of publications in marine microbial bioprospecting, as well as the development of new products from marine biodiversity. Furthermore, marine biotechnology has been recognized in many parts of the world as having an enormous development potential, and the furthering of this discipline is considered as strategic not only for reaching key societal needs but also for economic growth [11.170].

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12. Novel Bioreactors for Culturing Marine Organisms

Debashis Roy

This chapter considers a wide range of novel bioreactor configurations for cultivation of marine organisms for purposes of biomass harvesting/enrichment or synthesis of target metabolites or wastewater treatment. It begins by analyzing biofilm reactors that promote surface-attached growth, including the niche-mimicking types viz. modified roller bottles, air membrane surface bioreactor, and ultralow speed rotating disk bioreactor as well as the small-scale extended surface shaken vessel. Photobioreactors (PBR), used mainly for phototrophic algal growth, are discussed next – these include the tubular, plate/panel and stirred tank types on the one hand and vertical column PBRs on the other, the latter mainly comprising airlift (AL) and bubble column (BC) PBRs. Important AL/BC configurations have been described. Membrane bioreactors (MBR) are then taken up, which include, e.g., the anaerobic MBR, ion-exchange MBR, etc. Immobilized cell bioreactors – primarily packed bed bioreactors (PBBR) and their hybrids (e.g., with PBR, airlift bioreactor (ALBR), MBR etc.), are reviewed next, followed by hollow fiber bioreactors (HFBR) (e.g., HF–submerged MBR, HF–PBR, etc.) which, technically, are also a class of immobilized-cell bioreactors. This is followed by a brief overview of fluidized bed and moving bed bioreactors, used primarily for wastewater treatment. Finally, the different classes of high-pressure and/or high-temperature bioreactors are considered, which are practically wholly devoted to cultivation of extremophiles (barophiles and/or thermophiles) isolated from the deep sea.

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12.1 Biofilm Reactors (BFR)

In their natural habitat, most bacteria exist within biofilms that are anchored to surfaces and are inherently different from bacteria existing in a planktonic state. Many bacterial strains upon attaching to a surface reportedly produce exopolysaccharides (EPS) which mediate the attachment of the bacteria to the surface culminating in the formation of a biofilm. Marine microbial communities often occur as biofilms which are high-density surface-attached aggregates embedded in extracellular biopolymer (EPS) matrices. The microbial biofilm is a common adaptation of natural bacteria and other microorganisms. In the fluctuating environment of intertidal systems, biofilms form protective microenvironments and may structure a range

of microbial processes. Surface attachment and biofilm formation possibly also induce metabolic changes resulting in the production of different metabolites under attached growth conditions. Furthermore, many bacteria, including Bacillus species, are known to produce signal molecules that are used by the cells to monitor cell density, a phenomenon termed *quorum sensing*, which also controls cell metabolism. Cells in biofilms usually grow at much higher cell densities (up to 10 000 times higher) than in liquid suspension cultures, and quorum-sensing mechanisms could affect the production of secondary metabolites by these biofilm cells. Biofilm Reactors essentially promote surface-attched growth of biofilms (Table 12.1).

Yan et al. [12.1] examined the possibility whether allowing biofilm forming bacteria to grow under conditions that mimicked their marine ecological niche could elicit the production of antimicrobial compounds that they do not synthesize in the planktonic state, using modified roller bottle cultures (Fig. 12.1). Now, roller bottles provide a (typical) growth environment to cells anchored directly or indirectly to the inner wall of the bottles, allowing the attached cells to form a biofilm periodically in contact with both the gas and the liquid phase, (i. e., culture medium) – conditions similar to the alternating wetting and air exposure experienced by bacteria growing on seaweeds or other surfaces in an intertidal environment. However, the challenge lay in devising a method for anchoring bacterial cells, directly or indirectly, to the inner wall of the roller bottles. During preliminary studies it was noted that the epibiotic marine bacterial strains, EI-34-6 (*Bacillus licheniformis*) and II-111-5 (*Bacillus subtilis*) isolated from the surface of the seaweed *Palmaria palmata*, when cultivated on agar medium remained attached to the agar surface even when mixed or washed with the corresponding liquid broth. Based on this finding, the authors devised a modified roller bottle cultivation method wherein an agar coating on the inner wall of the roller bottles provides the surface necessary for the bacteria to attach and form a biofilm matrix. Subsequently, the effect of surface attachment/biofilm formation on antibiotic production was examined.

To prepare a surface-attached roller bottle culture, an agar coating was deposited on the inside wall of a 500 mL Duran bottle during solidification of the agar (after autoclaving) by rolling on ice, with one part of the coating intentionally thickened. The thickened portion of the agar coating was intended to serve as a baffle and help in enhancing oxygen transfer to the surface-attached culture. The cell inoculum was spread on the surface of the agar coating using a swab. The Duran bottles were incubated under the static condition to initiate an agar surface culture (for biofilm formation), then 50 mL of the corresponding liquid medium was added and the bottles rolled horizontally at 1 rpm; thus allowing the biofilm developing on the agar surface to be periodically exposed to air, and remain submerged in the liquid media.

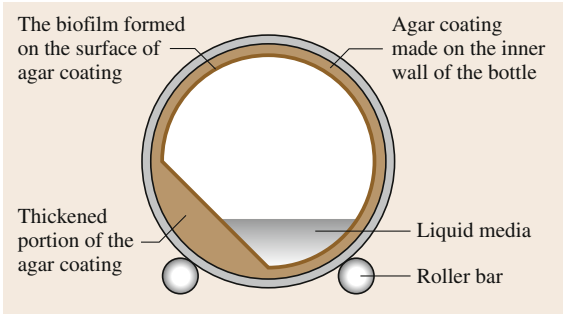

Fig. 12.1 Roller bottle cross section. A layer of agar coating was made on the inner wall of a 500 mL Duran bottle with one fraction thickened. The cell inocula were spread on the surface of the agar coating by a swab and cultivated statically for different times (after *Yan* et al. [12.1])

In suspension cultures, either in a shake flask or in standard roller bottle cultivation, the marine bacteria did not form biofilms on glass surface, which evidently was not conducive for mimicking the ecological niche of the studied strains, i. e., seaweed surface. *B. licheniformis*, strain EI-34-6, characteristically forms colonies that are usually strongly attached to agar surface; thus the agar-coated roller bottle culture makes good use of this phenomenon, facilitating the formation of a strongly bound biofilm. Although the liquid medium provided sufficient nutrients, the cells adhered to the surface of the agar coating, preferring growth in the biofilm even when in the presence of a liquid nutrient source. Furthermore, although the liquid medium provided the same nutrients, cells attached to the agar surface-produced antibacterial compounds that planktonic cells could not. Rolling cultivation for 2 h after the addition of liquid medium showed that antibacterial compounds were produced by surface-attached cells during biofilm growth – in fact detectable antimicrobial activity in the medium was always associated with observed biofilm formation.

Both isolates EI-34-6 and II-111-5 reached very high cell densities (of the order of 10^9 CFU mL^{-1}) even in shaken suspension cultures but without any detectable production of antibiotics, thus pointing to

Table 12.1 Biofilm reactors (BFR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Modified roller bottle cultivation (MRBC)	Antimicrobial compound production by bacterial strains *Bacillus licheniformis* EI-34-6 and *B. subtilis* II-111-5	*Yan* et al. [12.1]
2	Air-membrane surface bioreactor (AMSBR)	Antimicrobial compound production by bacteria *B. licheniformis* EI-34-6	*Yan* et al. [12.2]
3	Ultralow speed rotating disk bioreactor (UL-S-RDBR)	Antimicrobial synthesis by three estuarine/intertidal actinobacteria MS 310, MS 3/20, MS 1/7	*Sarkar* et al. [12.3]
4	ULS-RDBR	Actinomycin-D production by estuarine isolate *Streptomyces* sp. MS 310	*Sarkar* et al. [12.4]
5	ULS-RDBR	Antimicrobial synthesis by estuarine *Streptomyces* sp. MS 1/7	*Sarkar* et al. [12.5]
6	Extended surface shaken vessel (ESSV)	Protease production by intertidal estuarine gamma proteobacterium (DG II)	*Sarkar* et al. [12.6]
7	ESSV	Melanin synthesis by *S. colwelliana* and antibiotic production by *P. rubra* (both bacteria)	*Mitra* et al. [12.7]
8	ESSV	Cellulase and xylanase production by two intertidal filamentous fungi, *C. crispatum* and *G. viride*, respectively	*Mitra* et al. [12.8]

the possibility of either induction mechanisms other than quorum-sensing regulating antibiotic production by the attached cells or mere sensing of the physical attachment by these cells triggering changes in gene expression associated with antibiotic synthesis. The periodic exposure of the growing biofilm to the liquid medium and to air mimics the marine ecological niche of the biofilm-forming microbes on intertidal seaweed, hence the term *niche-mimic bioreactor* for the modified roller bottle cultures.

Yan et al. [12.2], designed a novel bioreactor – the air membrane surface (AMS) bioreactor, that allows the growth of bacteria as biofilms attached/anchored to the surface of a semipermeable membrane disk in contact with air, to investigate the production of antimicrobial compounds by the marine bacterium *B. licheniformis, strain EI-34-6* (used earlier by *Yan* et al. [12.1]) isolated from the surface of a marine seaweed *P. palmata*. The AMS bioreactor (Fig. 12.2) consists of a small, shallow dish filled with sterile liquid medium, with a semipermeable membrane disk placed on top of the dish such that the membrane remains in contact with the medium on one side and with air on the other, is held in place by surface tension. Bacteria were inoculated onto the membrane surface by swabbing, then the inoculated AMS bioreactor was placed in a sterile petri dish during cell growth. *B. licheniformis* produced antimicrobial compounds (the major component identified as bacitracin) when it grew in surface-attached condition as a biofilm at an air–membrane interface in the AMS-BR but not when it was grown planktonically in shake-flask cultures. An unidentified red pigment was also produced by surface-attached cells but not

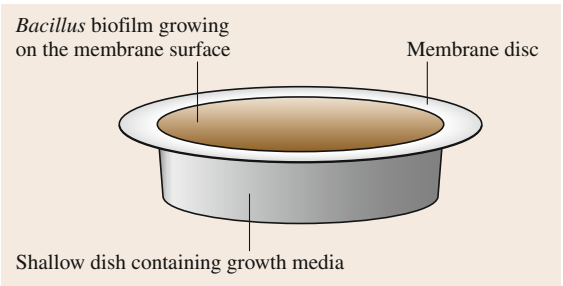

Fig. 12.2 AMS bioreactor. The small chamber beneath the membrane is filled with liquid medium, and the membrane disk is held in place by surface tension. Bacteria were inoculated onto the surface of a semipermeable nylon membrane. The AMS bioreactor was placed in a sterile petri dish during growth to maintain sterility (after *Yan* et al. [12.2])

by suspended cells. Different types of semipermeable membranes with widely varying pore sizes (viz., nylon, cellophane, and flat dialysis membranes) gave similar results, indicating that antibiotic production was not due to the chemical composition of the membrane but rather due to the niche-mimicking environment provided by the AMS bioreactor. Thus, it was established that biofilm formation as well as periodic direct exposure to air are necessary for eliciting production of antimicrobial compounds by the surface-attached cultures of *B. licheniformis*.

Sarkar et al. [12.3], developed an ultralow speed rotating disk biofilm reactor (ULS-RDBR) operated at a rotational speed of one revolution per day at 50% submergence of the rotating discs to mimic the intertidal

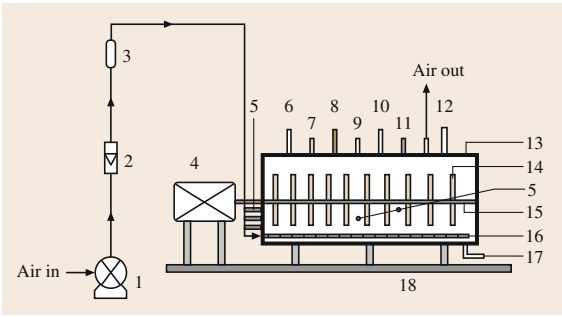

Fig. 12.3 Schematic of the ultralow-speed rotating disk bioreactor (RDBR) (1 – air pump; 2 – rotameter; 3 – air filter; 4 – electrical motor and reducing gear train for speed reduction; 5 – sampling port; 6 – temperature sensor; 7 – antifoam port; 8 – inoculation and medium addition port; 9 – acid port; 10 – pH sensor; 11 – alkali port; 12 – DO sensor; 13 – reactor vessel; 14 – rotating coaxial disks; 15 – shaft; 16 – sparger; 17 – drain; 18 – base plate) (after *Sarkar* et al. [12.4])

estuarine ecological niche of three marine actinobacteria viz. MS 310 (*Streptomyces* sp.), MS 3/20, and MS 1/7. The ULS-RDBR (Fig. 12.3), which facilitates microbial growth in the form of surface-attached biofilms, was designed on the concept of a rotary biological contactor (RBC). The maximum volume of the RDBR is 25 L, and the shaft (on which 10 disks are coaxially mounted) is rotated at an ultralow speed of one revolution per day. When operated with half the volume of the tank filled with liquid medium (i. e., at 50% submergence level), any given point on the disks would remain exposed to air and submerged in the medium alternatively for 12 h; thus mimicking the intertidal conditions of the location from where the microorganism was collected. The ULS-RDBR with its much higher surface-to-volume ratio (compared to a STBR) and niche-mimicking ability, supported good biofilm formation on the extended surface in the reactor (i. e., the rotating disks) and faster attainment of the peak level of antimicrobials metabolized by these isolates. It was also noted that antimicrobial synthesis was growth associated.

The ULS-RDBR (volume 25 L) was employed by *Sarkar* et al. [12.4] for detail investigation of the production of a potentially novel antimicrobial compound by the biofilm-forming marine *Streptomyces* sp. MS 1/7, in particular the effects of pH, aeration rate and disk submergence level (varied at 25, 50 and 75%) on the peak antimicrobial activity (PAMA), peak activity attainment rate (PAAR), and biofilm density (BD). PAMA, PAAR, and BD were all maximized at the intertidal niche-mimic operating condition (1 rev d^{-1}, 50% disk submergence) along with the highest aeration rate provided in the experiment. Furthermore at any aeration rate, PAMA was always highest under the niche-mimic condition – 12 h. alternating cycles of inundation and aerial exposure as in the intertidal conditions characterizing the biofilm-forming microbe's marine ecological niche.

The ULS-RDBR is a horizontal, rectangular parallelepiped shaped vessel (42 cm × 27 cm × 27 cm, volume 25 L), comprising 10 coaxial, equispaced disks (each of 16 cm diameter and 5 mm thickness) mounted on a single rotating shaft. The reactor vessel, the disks as well as the shaft are all made of acrylic (polymethyl methacrylate), which is transparent, corrosion resistant to high salt concentrations, and generally provides a surface conducive to attachment of biofilm. For 50% disk submergence level, i. e., 12.5 L working volume, the ratio of surface area to working volume of the RDBR is 342 $cm^2\,L^{-1}$, considering total surface area of 10 disks only. If, however, submerged wall surface and floor of the tank are also considered together with the disks, then the ratio increases to 552 $cm^2\,L^{-1}$. It was observed (in trial runs) that the roughened acrylic surface allowed substantially more biofilm formation than the smooth surface. Due to this reason, the disk surfaces were roughened using sand paper (Grade 50) to facilitate surface attachment by the biofilm-forming microorganisms. A reducing gear train was designed to reduce the speed of a 7 rpm motor (for driving the rotating shaft) by ca. 10 000 times to the ultralow rotational speed of 1 rev d^{-1}. Air was supplied into the RDBR using an air compressor, passed through a sterilizing air filter, and then uniformly distributed into the fermentation medium using a rectangular sparger (also made of acrylic) having uniformly spaced holes in the downward direction. Ports on the top lid of the RDBR are available for sampling, addition of medium/inoculum/antifoam, pH sensor, DO sensor, temperature sensor, and air exhaust. The fermentation medium was sterilized ex situ and added aseptically to the reactor disinfected both chemically (i. e., by repeated washing with sodium hypochlorite) and by UV radiation.

Sarkar et al. [12.5] found almost similar results in their investigation of actinomycin-D production by the biofilm-forming estuarine isolate MS 310 cultivated in the ULS-RDBR operated at 1 rev d^{-1}. The niche-mimic condition along with maximum permissible aeration was found to be most favorable for antibiotic produc-

tion – peak antibiotic activity (PAA) and peak activity attainment rate (PAAR) simultaneously attaining their highest values at this operating condition – 50% disk submergence. Both PAA and PAAR are observed to increase with increasing aeration at all operating conditions examined. At the niche-mimic condition, a threefold increase in the aeration rate causes PAA to increase by 33%, whereas PAAR increases by 2.5 times, underlining the strong aeration dependence of this actinomycin-D producer. Again, compared to the highest values obtained for antimicrobial production in flask (500 mL) experiments, the corresponding RDBR values were 16% higher for PAA and more than five times higher for PAAR – strong evidence for employing these novel bioreactors for cultivation of antibiotic-producing marine microbes.

It follows from the above discussion that for microorganisms which synthesize metabolites/enzymes at the highest rates only when growing in surface-attached condition, i. e., as biofilms anchored to solid surfaces, special culture conditions are necessary that are conducive to surface attachment and biofilm formation. For this purpose, several typical niche-mimic bioreactors have been developed (e.g., modified roller bottle cultures, AMS-BR, ULS-RDBR, etc.) as described earlier in this section. However, a lack of small-scale shaken vessels with high surface/volume ratio and surface properties favoring attachment of biofilm-forming microbes was noted by *Sarkar* et al. [12.6]. They developed a novel small scale, extended surface shaken vessel (ESSV) in the form of a PMMA acrylic made conico-cylindrical flask (CCF) (volume 500 mL) in which enhanced surface for microbial attachment and biofilm formation was provided by eight equidistantly located vertical rectangular strips radially mounted on the base of the vessel with the base diameter close to that of a 500 mL Erlenmeyer flask (EF) for easy placement in a rotary shaker. The small-scale vessel was designed to allow the use of different internal surface materials – hydrophilic (glass) or hydrophobic (acrylic). Furthermore, protease production by two strains in the ESSV were examined, of which one was marine – an intertidal gamma – Proteobacterium (DG II). Relative to a standard EF with no additional surface, growth and protease synthesis by the marine isolate DG II were 20 and 30% higher, respectively. Again, compared to glass, the use of acrylic surface (hydrophobic) resulted in more than 200% increase in protease production and a dramatic increase (i. e., by 19, 275%) in microbial growth.

Mitra et al. [12.7] cultivated two biofilm-forming marine bacteria in the novel ESSV described above, viz. *Shewanella colwelliana* for melanin production and *Pseudoalteromonas rubra* for antibiotic synthesis. The design allowed comparison of production between (1) CCF with hydrophobic surface (PMMA), (2) ESSV with hydrophilic glass surface, and (3) standard unbaffled EF. Growth and melanin production by *S. colwelliana* were highest in the ESSV with (hydrophilic) acrylic surface, further melanin synthesis increased with increase in surface (for attachment) and increase in biofilm formation and increase in planktonic growth. Growth of *P. rubra* was also highest in the acrylic ESSV but not antibiotic synthesis – it was maximum in the EF without any extended surfaces. Thus antibiotic production was favored by a hydrophilic vessel surface (glass).

Mitra et al. [12.8] examined cellulase and xylanase production in relation to biofilm formation by two intertidal filamentous fungi, viz. *Chaetomium crispatum* and *Gliocladium viride*, respectively, in the novel ESSV (described above) with either hydrophobic (acrylic) or hydrophilic (glass) surface and compared with that in an ordinary EF. Mixed results were obtained with regard to suitability of the EF or the ESSVs for enzyme production by the two filamentous fungi – surface properties as well as surface area of attachment of the cultivation vessel affected biofilm formation and enzyme production.

12.2 Photobioreactors (PBR)–Tubular, Plate/Panel and Stirred Tank Configurations

Photobioreactors, as the name indicates, are specialized bioreactors (Table 12.2) for phototrophic growth of microorganisms – mainly alga but also photosynthetic bacteria; as well as macroorganisms, i. e., macroalgae (*seaweed*). Application of PBRs for microalgal growth has been extensively reviewed [12.19, 20]. PBRs originated as open-air cultivation systems with natural sunlight as the source of illumination that are easy to build and operate – these include natural or artificial ponds/tanks, raceway-shaped culture ponds (which are basically closed-loop recirculation channels) and so-called *inclined surface ponds* driven by paddle wheels. However, open-air PBR systems are prone to evaporative losses and contamination problems and most im-

Table 12.2 Photobioreactors (PBR)-tubular, plate/panel, stirred tank etc.[a]

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Flat plate PBR	CO_2 removal by green alga *Chlorococcum littorale*	*Hu* et al. [12.9]
2	Double-phase flat panel-type PBR	Microaerobic biohydrogen production by the nonsulfur photosynthetic bacteria *Rhodovulum* sp.	*Matsunaga* et al. [12.10]
3	Flat alveolar panel PBR (FAP-PBR), Green Wall Panel PBR (GWP-PBR)	Oil production by algal strains (e.g., *Nannochloropsis* sp.)	*Rodolfi* et al. [12.11]
4	Tubular loop PBR[a]	Cultivation of red macroalga *Porphyridium* sp.	*Merchuk* et al. [12.12]
5	Tubular recycle PBR	Brown macroalga *L. saccharina* cultivation	*Rorrer* and *Mullikin* [12.13]
6	Stirred tank PBR	Biomass production of microalga *Acrosiphonia coalita*	*Rorrer* and *Zhi* [12.14]
7	Stirred tank PBR	Production of α-tocopherol by microalgae *Euglena gracilis*	*Ogbonna* et al. [12.15]
8	Three-stage serial column-type PBR (CPBR)	*Spirulina* sp. (cyanobacterium) for CO_2 biofixation through photosynthesis	*de Morais* and *Costa* [12.16]
9	Laboratory-scale and pilot-scale PBRs	Harvesting algal biomass of *Euglena gracilis* as potential animal food source	*Chae* et al. [12.17]
10	Serpentine PBR and hybrid flow through-PBR	Biogas production by anaerobic digestion of microalga *P. tricornutum*	*Zamalloa* et al. [12.18]

[a] Also compares the tabulated reactors with airlift and bubble-column reactors, restated in Table 12.4 with discussion in the Sect. 12.3 ALBR and BCBR

portantly, have significantly lower biomass productivity than closed PBRs. The common design configurations of the latter include plate/panel, tubular, stirred-tank and vertical column (including airlift and bubble – column reactors). Tubular PBRs consist of an array of straight, coiled, or looped transparent tubes through which the culture is circulated by pump or by airlift mechanism – the latter having the advantages of permitting exchange of CO_2 and O_2 between the liquid medium and the aeration gas, minimizing shear damage to the cells caused by mechanical pumping and achieving medium circulation without any moving parts. Increase in tube diameter (above 0.1 m) decreases the surface/volume ratio and as culture density increases with growth, the cells begins to shade one another (*self-shading effect*) which results in decreasing volumetric biomass productivity. Again, excessive increase in tube length causes accumulation of O_2 (produced photosynthetically) which, when in excess of the air saturation value, becomes inhibitory for photosynthesis (oxidative photoinhibition). Some common tubular configurations are horizontal/serpentine, near horizontal, helical, conical, and inclined. In general, tubular PBRs have a large illumination surface area but poor mass transfer characteristics.

Flat-plate-type PBRs probably originated from the laminar morphology of plant leaves which are well-evolved natural solar collectors with high surface/volume ratio. They can be horizontal/vertical and posses the advantage of high illumination surface area, high photosynthetic efficiency, and lower accumulation of dissolved oxygen (than tubular PBRs). A tilted flat-plate PBR (e.g., the flat inclined modular PBR – FIMP) may be angled to ensure maximum exposure of the culture to sunlight and facilitates change of light path as and when required. This flexibility is often crucial as individual photosynthetic microorganisms often have an optimum light path that is a compromise between growth inhibition in innermost layers due to insufficient lighting and self-shading, and photoinhibition of growth in outermost layers due to excessive illumination. Stirred tank PBRs have also been used for algal cultures but they are not as common as flat plate/tubular-PBRs. Vertical column PBRs – mainly AL and bubble column (BC) PBRs are discussed in the following section.

PBRs can be externally or internally illuminated. External illumination may be natural (i. e., direct sunlight) or artificial (e.g., fluorescent lamps), internal illumination is necessarily artificial. Optical fiber-based internally illuminated PBRs have the advantage of heat sterilizability and stability to withstand mechanical agitation stresses; however, low light delivery efficiency ($< 50\%$) is a concern.

Hu et al. [12.9] used a modified flat plate PBR (1.4 L) to investigate CO_2 removal by an ultrahigh-cell-

density culture (i. e., with optimal cell density > 10 g DCW/L) of the marine green alga *Chlorococcum littorale*, which shows extended linear growth under light limiting conditions and grows very vigorously in the presence of extremely high CO_2 levels. The remarkably high CO_2 fixation rate reported with this alga is due to the ultrahigh cell density attained in this PBR which has a narrow light path in which intensive turbulent flow is generated by streaming compressed air through perforated tubing into the culture suspension (that produces vigorous air-bubble mixing). The acrylic-made reactor (Fig. 12.4) (height 50 cm, length 38 cm) consists of an outer chamber (width 6 cm) that serves as a temperature regulator and an inner chamber (with a total illuminated area of about 0.16 m^2) of varying width corresponding to the length of the light path (1, 2, and 4 cm) which was optimized in this study for biomass productivity. An air-bubbling tube is placed along the bottom of the inner chamber, through which compressed CO_2-enriched air is streamed to produce turbulence in the culture suspension. The top opening of the inner chamber is covered by silicone rubber together with a thick acrylic lid with a number of openings/ports for various sensors and for air exhaust. A panel with a bank of white fluorescent lamps was installed on each side of the reactor for illumination.

Microaerobic biohydrogen production by the marine, nonsulfur, photosynthetic bacterium *Rhodovulum* species was examined by *Matsunaga* et al. [12.10] in a *double-phase* flat panel PBR consisting of *light* and *dark* compartments. Hydrogen production under microaerobic conditions was found to be four times higher than in anaerobic conditions, mainly due to the much higher ATP accumulation during respiration under microaerobic conditions – ATP accumulated in the dark compartment was utilized for hydrogen production in the light compartment. Double-phase and conventional flat-panel-type PBRs (2 cm × 10 cm × 30 cm) made from 3 mm polyacryl resin sheets, had an illuminated area of 250 cm^2 and a culture volume of 500 mL. Light and dark compartments in the double-phase PBR were obtained by positioning a flat mirror (18 cm × 10 cm). Six fluorescent lamps were used as the source of illumination in this PBR.

Rodolfi et al. [12.11] used two types of PBRs viz., a flat alveolar panel (FAP) PBR (volume 20 L) and green wall panel (GWP) PBR (volume 110 L) for outdoor mass cultivation of the oil-producing marine eustigmatophyte microalga *Nannochloropsis* species. The study was claimed to be the first report of an increase in both lipid content and areal lipid productivity attained in an outdoor algal culture, through nutrient deprivation.

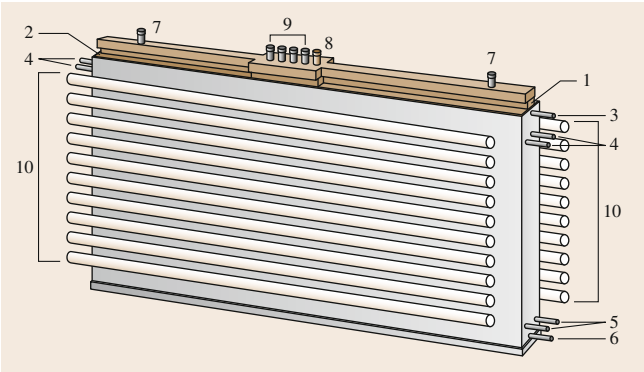

Fig. 12.4 Schematic diagram of the prototype flat-plate photobioreactor. 1 – inner culture chamber; 2 – outer temperature-regulation chamber; 3 – culture overflow; 4 – cooling-water inlets; 5 – cooling-water outlets; 6 – port for compressed-air tubing; 7 – air outlet; 8 – sampling port; 9 – ports for various sensors (pH, temperature, O_2, etc.); 10 – a bank of fluorescent lamps (after *Hu* et al. [12.9])

Merchuk et al. [12.12] studied the effect of light/dark cycles of different frequencies on growth of the red microalga *Porphyridium* sp. in a laboratory-scale tubular loop PBR and compared it with the performances of an AL-PBR and BC-PBR (both of volume 35 L). In the laboratory-scale PBR liquid is circulated by a peristaltic pump at a controlled rate whereas in the column PBRs, which are orders of magnitude larger, liquid movement is pneumatically driven by the injection of gas into the reactors. However, despite basic differences, the light/dark cycles generated, either by the pump of the laboratory-scale tubular bioreactor, or by gas flow in the column PBRs were found to be of the same order. By virtue of the small diameter of the loop and the low concentrations of the biomass used, the performance data from the laboratory-scale tubular loop PBR was free of the effect of self-shading, and therefore, this PBR could be considered as a *thin-film* PBR. The tubular loop PBR (total volume 0.43 L) consisted of a series of glass tubes (I.D. 0.007 m) connected to a small vessel into which 3% CO_2 was bubbled (to provide a carbon source and also to remove O_2) with the loop closed through a peristaltic pump. The cycle time could be varied by manipulating the flow rate in the pump. The ratio of light/dark zones was controlled by darkening different lengths of the tube – the ventilation vessel, being always covered, was part of the dark zone. A bank of fluorescent lamps provided the necessary illumination.

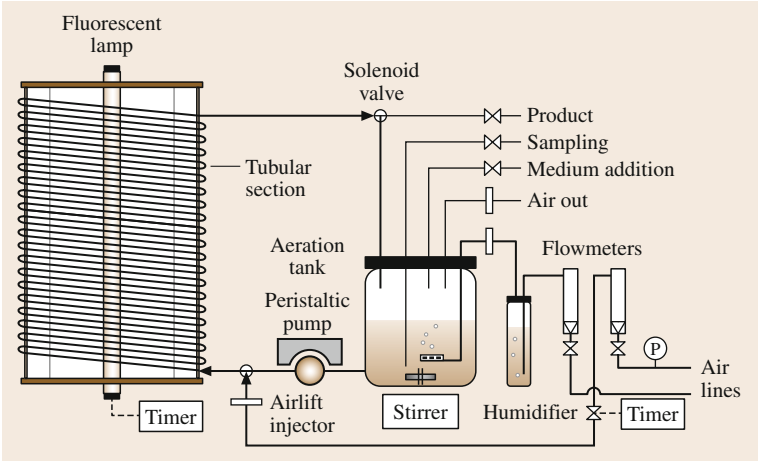

Fig. 12.5 Three-liter tubular photobioreactor, featuring coiled tubular section, aeration tank, and airlift injection system. In batch operation, the product outlet line is closed and the culture continuously recirculates between the aeration tank and the tubular section (after *Rorrer* and *Mullikin* [12.13])

Rorrer and *Mullikin* [12.13] cultivated cell suspension cultures obtained from the marine macrophytic brown alga (*seaweed*) *Laminaria saccharina* in a 3 L tubular recycle PBR (Fig. 12.5) where the culture is recycled between an unaerated coiled tubular section for culture illumination and a nonilluminated aeration tank where absorption of CO_2 (needed for photosynthetic biomass production) and stripping of dissolved O_2 evolved from photosynthesis take place. The tubular recycle PBR could be operated in batch, semicontinuous and continuous-recycle modes. A three-way solenoid valve located at the outlet of the tubular section is used for mode selection. In batch operation, the solenoid valve is closed, and the cell suspension culture leaving the tubular section is recycled back to the aeration tank (total recycle). In semicontinuous or continuous recycle operation, the solenoid valve opens and closes periodically, permitting a portion of the culture to be drawn off as product (while the rest is returned to the reactor), with concurrent feeding of fresh medium.

Rorrer and *Zhi* [12.14] compared the biomass productivities of a semidifferentiated tissue suspension culture of the marine cold-water green macroalga *Acrosiphonia coalita* (a source of oxylipins with potent antimicrobial properties), in two types of PBRs, a stirred tank PBR (ST-PBR) and a BC PBR (BC-PBR). Now, although the parent plant is highly branched and must be anchored to rocky substratum in its marine ecological niche, the tissue culture comprises mainly linear filaments growing in a homogeneous liquid suspension. Toward this end, the inoculum tissue was finely blended to 1–2 mm long filaments but it was observed that only the ST-PBR could provide the agitation necessary for uniform suspension of this tissue culture – in fact, of all the cultivation vessels considered, the highest biomass productivity was obtained in the ST-PBR.

The ST-PBR (Fig. 12.6) (volume 3 L) is actually a jacketed, round-bottomed, glass vessel (I.D. 13 cm, height 24 cm), equipped with a three-blade marine impeller (diameter 4.5 cm, height 6 cm) pitched at an angle of 45°. Ambient air was pumped through a sterile air filter, and then sparged to the culture through an air inlet pipe having seven holes (diameter 1 mm) drilled in a row. Optionally, CO_2 was metered separately and then mixed with the inlet air stream before passing through a sterilizing air filter. Cold water from a low-temperature circulator was pumped through the glass vessel jacket to maintain a constant cultivation temperature. The illumination stage consisted of two light banks positioned on opposite sides of the bioreactor, each comprising three fluorescent tube lamps (each 9 W), mounted horizontally in a parallel array. Each light bank was aligned with the bioreactor vessel so that the length of the lamp coincided with the vessel width, whereas the cumulative height of all the lamps matched the height of the liquid in the reactor vessel. A referencing plate was used to set the distance between the lamp and the vessel surface with high precision so that the desired light intensity (incident on the vessel surface) could be uniformly delivered to the culture. For the ST-PBR, the light sensor was positioned on the inside surface of the vessel to obtain the true incident light intensity to the culture. The light intensity incident on the inner surface of the vessel was fixed at nominally twice the saturation light intensity, to compensate for light attenuation through the culture.

Ogbonna et al. [12.15] studied the production of α-tocopherol by the microalgae *Euglena gracilis* in a con-

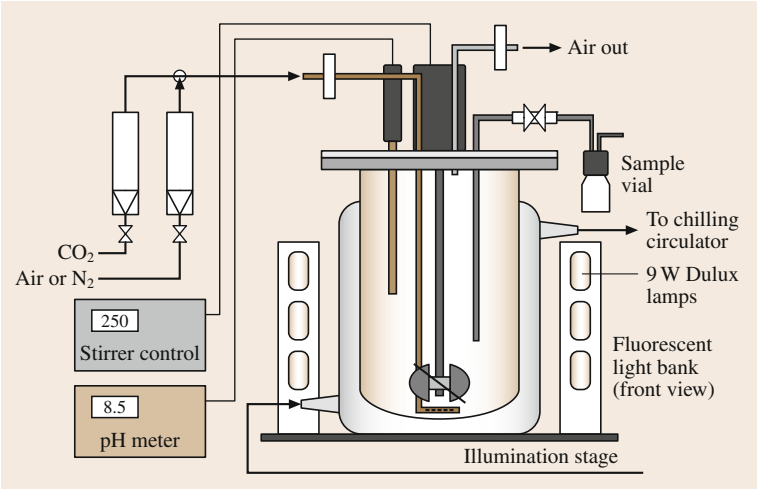

Fig. 12.6 Three-liter stirred-tank photobioreactor, including illumination stage and instrumentation (after *Rorrer* and *Zhi* [12.14])

tinuous sequential heterotrophic–photoautotrophic cultivation system. The cells were continuously cultured heterotrophically in a conventional aerated, stirred mini-jar bioreactor (volume 2.5 L, working vol. 2 L) and the effluent continuously passed through an internally illuminated photobioreactor for the photoautotrophic phase, with α-tocopherol production. The novel sequential continuous process resulted in α-tocopherol productivity (of 100 mg h^{-1}) which is $\approx$ 9.5 and 4.6 times higher than corresponding productivities obtained (individually) in batch photoautotrophic and heterotrophic cultures, respectively.

De Morais and *Costa* [12.16] designed a novel temperature-controlled, three-stage, serial column (i. e., tubular) photobioreactor (volume 2 L, working vol. 1.8 L for each CPBR) to investigate CO_2 biofixation by marine photosynthetic cyanobacteria *Spirulina* species. This study demonstrated the high CO_2 biofixation potential of the cyanobacteria cultivated in the three-reactor cascade CPBR1-CPBR 2-CPBR3. It was also noted that using multiple PBRs in series resulted in much lower CO_2 levels in the final gaseous effluent discharged to the atmosphere. Agitation and aeration were carried out using air from a compressor and a sintered sparger – the effluent air (with or without CO_2) from CPBR1 being fed to the sparger in CPBR2 and the effluent from CPBR2 being fed to CPBR3. The CPBRs were placed in a 30 °C growth chamber under a 12 h dark/light photoperiod with illumination provided by 40 W daylight-type fluorescent lamps during the light period.

Chae et al. [12.17] used two novel PBRs – one laboratory scale (working volume 100 L) (Fig. 12.7) and the other pilot scale (working volume 1000 L) to study single-cell protein production and atmospheric CO_2 biofixation by the microalga *Euglena gracilis*. Besides CO_2 fixation during photosynthesis, algal biomass may be used as a biofertilizer, soil conditioner, and also as feed for terrestrial and aquatic animals. Insofar as the last application is concerned, *E. gracilis* scores highly due to several reasons viz. (1) it has relatively high crude protein content (47% w/w of biomass) and thus higher nutritional quality compared to other microalgae like *Chlorella* and *Spirulina*, (2) its in vitro digestibility is slightly higher than that of casein making it an attractive animal fodder, (3) it grows well under acidic conditions with very little risk of culture contamination. *Euglena gracilis* growth is very sensitive to light intensity. The novel pilot-scale PBR (which uses sunlight as energy source and flue gas from an oil heater as a CO_2 source) minimizes the self-shading effect typical of dense microalgal cultures (that leads to increasing light attenuation with distance from the light source, and thereby to decreasing biomass productivity) and, expectedly, shows much higher biomass yields compared to the laboratory-scale PBR.

The laboratory-scale PBR (90 cm × 20 cm × 70 cm) was provided with a cover and baffles that induced plug flow of the culture medium. Pure CO_2 and air were fed at rates of 0.3 and 2.7 L min^{-1}, respectively, and the mixed gas was passed through a humidifier before entering the reactor. To minimize light attenuation, reactor width was fixed at 20 cm and fluorescent lamps were installed on both sides of the PBR as light sources in parallel. The pilot-scale PBR used sunlight as energy source and flue gas from an industrial oil heater as CO_2

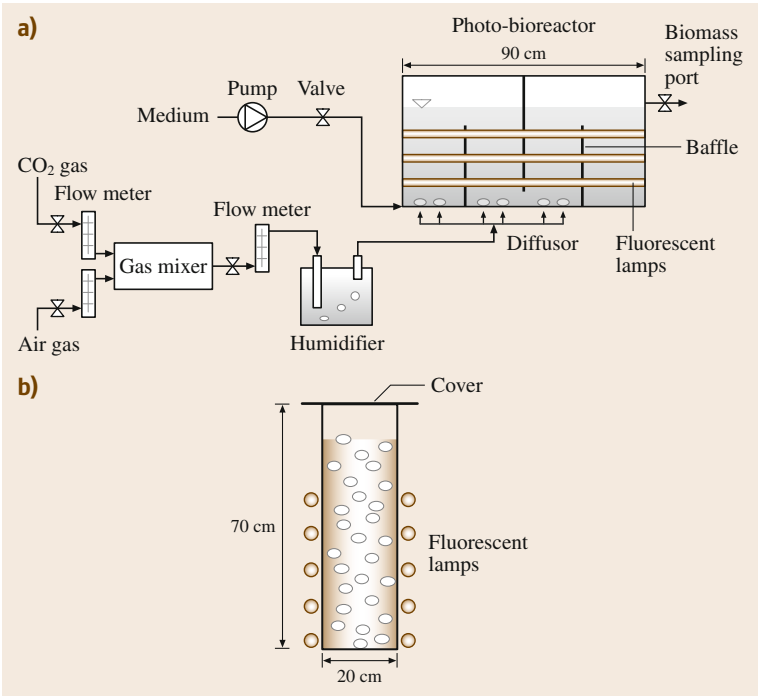

Fig. 12.7a,b Schematic (**a**) and cross-sectional diagrams (**b**) of a laboratory-scale photobioreactor for semicontinuous and continuous culture (after *Chae* et al. [12.17])

source (30 L min^{-1}). The L-shaped PBR (working volume 1000 L) was separated into a dark zone (1.8 m × 0.2 m × 1.4 m) and a light zone (1.8 m × 1.0 m × 0.4 m) of equal working volumes, each 500 L with a cover. To minimize light attenuation, effective height of the light zone was fixed at 20 cm. A scraper was installed for internal circulation of culture medium between dark and light zones.

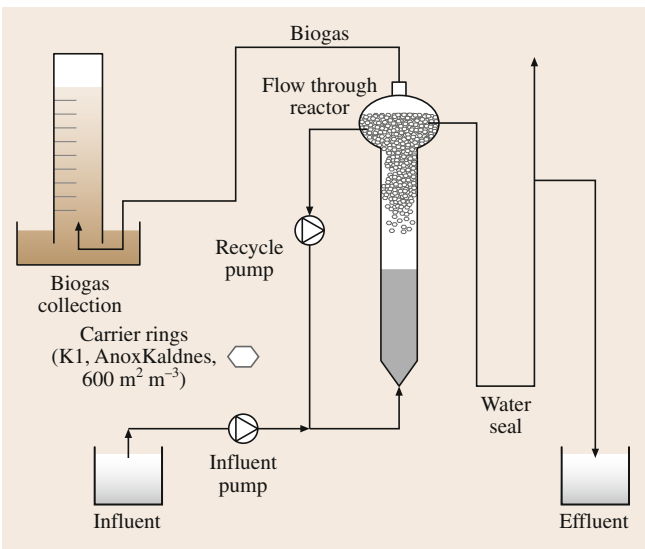

Fig. 12.8 Scheme of the laboratory-scale experimental setup of the hybrid flow-though reactor (after *Zamalloa* et al. [12.18])

Zamalloa et al. [12.18] examined the anaerobic digestibility of the marine microalga *Phaeodactylum tricornutum* for biogas production in a serpentine tubular PBR and a novel, hybrid flow-through anaerobic reactor (HFAR) – the latter essentially a cylindrical tube with a three-phase separator in the upper part (Fig. 12.8). The serpentine PBR comprises of a glass vessel (total volume 80 L, working volume of 65 L) and a transparent tube (diameter 19 mm, length ≈ 80 m). The culture broth was recirculated using a peristaltic pump (10 L min^{-1}). The PBR was installed in a greenhouse at 25 ± 2 °C with illumination provided continuously by fluorescent lamps. Culture pH was controlled (at 8) and regulated by on-demand, automated CO_2 injection. Mixing was achieved by bubbling air through diffusers at an aeration rate of ≈ 0.5 vvm. Each HFAR consisted of a cylindrical tube (diameter 5 cm) having a three-phase separator in the upper part, with a recirculating pump generating an upflow velocity of ≈ 1 m h^{-1}. Biogas production was measured by the liquid displacement method in 10 L airtight calibrated vessels containing water at pH 2 to prevent dissolu-

tion of CO_2. 150 g of plastic carrier rings per reactor were added as carrier material, and an anaerobic filter for increased retention of algal biomass particles, was installed.

12.3 Airlift Bioreactors (ALBR) and Bubble Column Bioreactors (BCBR)

12.3.1 Airlift Bioreactors

Airlift bioreactors (ALBR) (Tables 12.3 and 12.4) are pneumatically agitated gas–liquid or gas–liquid–solid contacting devices characterized by fluid circulation in a defined cyclic pattern through two vertical channels viz. the *riser* for gas–liquid upflow and *downcomer* for downflow) connected at the top (the *gas separator*) and bottom (the *base*). The driving force for recirculation of the fluid is the density difference between the downcomer and the riser which generates the pressure gradient necessary for liquid recirculation [12.21]. Air/gas is usually injected at the bottom of the riser and a portion of the gas disengages in the gas separator. The remaining portion is entrapped by the descending liquid and flows down the downcomer. If the gas residence time in the separator is substantially longer than the time required for disengagement of the gas bubbles, the fraction of gas recirculating through the downcomer would be minimized. In addition to agitation, the gas stream also facilitates exchange of material between the gas phase and the culture medium – oxygen is usually transferred to the liquid and often, metabolic products, from the liquid to the gas phase. ALBRs provide a relatively homogeneous low-shear field for microbial growth. They are further characterized by (a) their total lack of any moving parts and (b) their high aeration efficiency – the latter due to the high rates of oxygen transfer alongside minimal power consumption compared to conventional stirred bioreactor systems. ALBRs may be classified into two basic categories viz. (i) external loop ALBRs, in which fluid circulation occurs through separate and distinct channels, and (ii) internal – loop (baffled) ALBRs – where strategically installed baffles create the channels required for circulation. Configurations of both types may be further modified.

Jiang et al. [12.22] examined simultaneous carbon and nutrient removal from wastewater in a plexiglass bench-scale ALBR (working volume 22 L) by filamen-

Table 12.3 Airlift and bubble column bioreactors (ALBR and BCBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	ALBR	Simultaneous C and N removal from waste water by bacteria *Thiothrix* sp.	*Jiang* et al. [12.22]
2	ALBR	Rhamnolipid production by bacterium *Pseudomonus aeruginosa*	*Jeong* et al. [12.23]
3	Split column ALBR	Decolorization of textile waste water by marine fungi *A. niger*	*Assadi* and *Jahangiri* [12.24]
4	ALBR+ Fiber optics sensors	Filamentous callus induction and microplantlet culture propagation of macroalga *Kappaphycus alvarezzi*	*Munoz* et al. [12.25]
5	AL-PBR	Halogenated monoterpene production by red macroalga *O. secundiramea*	*Polzin* and *Rorrer* [12.26]
6	Concentric tube airlift PBR (AL-PBR)	Cultivation of microalga *P. tricornutum*	*Contreas* et al. [12.27]
7	Triangular configuration inclined tube – AL-PBR	CO_2 fixation from flue gas by green algae *Dunaliella* sp.	*Vunjak-Novakovic* et al. [12.28]
8	Outdoor airlift driven tubular PBR	Production of lutein by green unicellular microalga *Muriellopsis* sp.	*Del Campo* et al. [12.29]
9	BC-PBR	CO_2-fixation and H_2 production by green microalga *Platymonas subcordiformis*.	*Guo* et al. [12.30]
10	BC-PBR	Phototrophic cultivation of microplantlet suspension culture of the red macroalga *A. subulata*	*Huang* and *Rorrer* [12.31]
11	BC-PBR	Photolithotrophic cultivation of cell suspension culture from microscopic, filamentous gametophyte life phase of the complex brown macroalga *L. saccharina*	*Zhi* and *Rorrer* [12.32]

Table 12.4 ALBR and BCBR and/or other PBR – comparative studies

Sl	Bioreactors Compared	Marine strain and bioprocess	Reference
1	ALBR, BCBR	NOx removal by green microalga *D. tertiolecta*	*Nagase* et al. [12.33]
2	AL-PBR, BC-PBR	Harvesting diatom *S. costatum*	*Monkonsit* et al. [12.34]
3	BC-PBR, AL-PBR	Cultivation of red macroalga *Porphyridium* sp.	*Merchuk* et al. [12.12]
4	BC-PBR, AL-PBR	Cultivation of diatom *C. calcitrans*	*Krichnavaruk* et al. [12.35]
5	BC-PBR, split cylinder AL-PBR, draft tube sparged AL-PBR	Harvesting microalga *P. tricornutum*	*Sanchez-Miron* et al. [12.36]
6	BC-PBR, AL-PBR, AL-PBR + helical flow promoter (HFP)	Cultivation of red microalga *Porphyridium* sp.	*Merchuk* et al. [12.37]
7	BC-PBR, AL-PBR, externally illuminated stirred tank PBR, tubular recycle PBR	Culture of red macroalga *A. subulata*	*Rorrer* and *Chenny* [12.38]

tous marine bacteria *Thiothrix* species in a limited filamentous bulking (LFB) state – a repeatable and controllable state that brings about a balance between floc forming and filamentous bacteria. The ALBR with a low height-to-diameter ratio (overall height 1000 mm) consists of a reaction zone (I.D. 160 mm) and an upright settling zone (I.D. 220 mm). Two draft tubes, an upper tube (height 160 mm, dia 130 mm) and a lower tube (height 850 mm, dia 100 mm) were concentrically placed in the ALBR. For sparging gas, 120 holes (dia 0.5 mm) in a perforated pipe were positioned equidistantly around the circle at the middle of the riser. The flow rate of the mixed broth circulating between the annulus and the draft tube could be varied by changing the gas-flow rate. Influent was fed to the reactor through one of the three wastewater inlets located at the bottom and the middle of the riser and the upper part of the annulus, whereas effluent was withdrawn from the liquid in the settling zone. The ALBR and its component tubes were cleaned from time to time to prevent bacterial growth in the lines and on the vessel walls. The authors concluded that under an LFB state in the ALBR, the balance of aerobic and anoxic/anaerobic zones was achieved which is required for enhanced nutrient removal and effluent clarification. Furthermore, the LFB state, which is characterized by low DO levels, causes a reduction in the height-to-diameter ratio of the reactor and thereby in energy requirement for aeration.

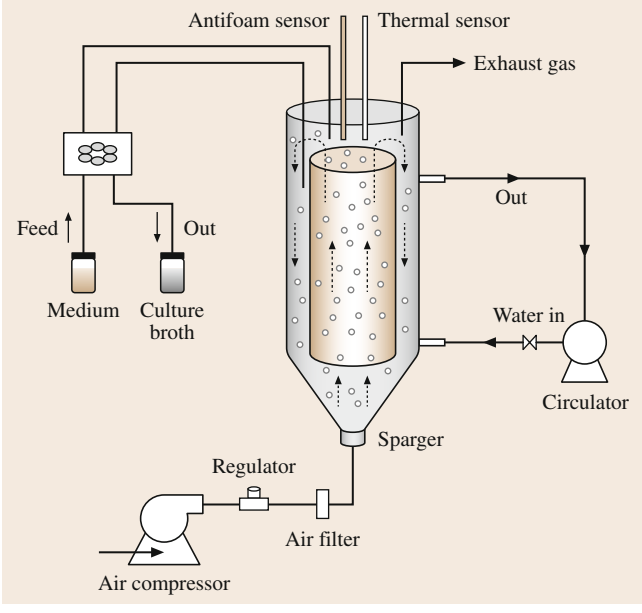

Fig. 12.9 Schematic diagram of an airlift bioreactor (after *Jeong* et al. [12.23])

Jeong et al. [12.23] demonstrated the continuous production of rhamnolipid-type biosurfactants in an ALBR (maximum volume 1.8 L, working volume 1.2 L, diameter 100 mm, height 320 mm) by a marine strain of *Pseudomonas aeruginosa* (isolated from the southern sea of Korea) immobilized, by entrapment in Ca-alginate modified PVA beads (Fig. 12.9). They noted that the medium-to-bead volume ratio is a key parameter for evaluating bioreactor performance and that an optimal value exists considering productivity and economics.

Assadi and *Jahangiri* [12.24] used a split ALBR to obtain a very high level (up to 97%) of decolorization of textile wastewater by a marine strain of *Aspergillus niger* (isolated from Gorgan Bay in the Caspian Sea). The jacketed glass-made ALBR is split by a PTFE strip

(800 mm × 100 mm × 3 mm) and is fitted with a glass condenser to arrest any outgoing moisture. Air is introduced through a chamber (length 100 mm) connecter by a flange to the distributer. 28 holes (1 mm diameter) were drilled (on a square pitch, 6 mm center-to-center) on half of the S.S. sparger that works as a riser. A condenser (area 0.2 m^2) is included to arrest any outgoing moisture.

Munoz et al. [12.25] employed an ALBR fitted with a fiber optic spectrophotometer, for filamentous callus induction and microplantlet culture propagation of the macroalga *Kappaphycus alvarezii* (Doty) which is the largest source of κ-carrageenan in the global phycocolloid industry. Now, ALBRs are usually preferred for macroalgal cell cultivation because they facilitate enhanced gas exchange and light transfer on the one hand and reduced shear damage on the other. Mixing in ALBRs is obtained by pneumatic and/or mechanical agitation in order to maintain a uniform concentration of chemical species in the bulk phase and enhance mass transfer. Since proper mixing is crucial for adequate distribution of cells and nutrients in the liquid phase, a realistic estimation of liquid-phase mixing times is necessary for effective ALBR design. For this purpose, a fiber optic spectrophotometer is used for mixing-time evaluation which has several advantages over other traditional methods viz. faster response without data loss, minimal measurement error, operational flexibility through use of several solutions as tracers, faster in situ measurements and reduced frictional resistance. It is noted that liquid circulation is very sensitive to bioreactor geometry and, on this count, the ALBR described here could provide adequate liquid circulation of the culture media.

The ALBR (effective working volume 1.5 L) externally illuminated with fluorescent lamps, consisted of an acrylic pipe (height 30 cm, I.D. 10 cm) and a draught tube (height 22.5 cm, I.D. 3.8 cm), with inlet air pumped through a flow meter. A fiber optic spectrophotometer was placed 5 cm below the water surface, and a tracer of 1.0 ± 0.3 mL saturated aniline blue aqueous solution was injected over the optical fiber through a Pasteur pipette. Mixing time (defined as the time required to attain a specified mixing intensity at a given scale), was measured from the time at which maximum absorbance was recorded, to the time of minimum absorbance, assuming a completely homogenized medium. It was concluded that filamentous callus production from axenic *K. alvarezii* explants was effectively promoted in the ALBR (with the tested plant growth regulator).

Polzin and *Rorrer* [12.26] used a perfusion airlift photobioreactor, i. e., with continuous liquid medium perfusion, for synthesis of halogenated monoterpenes by regenerated microplantlet suspension cultures of the macrophytic marine red alga (*seaweed*) *Ochtodes secundiramea*, claimed as the *first successful bioreactor production of halogenated monoterpenes from a marine organism*. The bioreactor (Fig. 12.10) is a glass jacketed vessel (working volume 2.1 L, 50 cm high, 7.6 cm I.D.) with the vessel jacket connected to a temperature controlled water circulation bath (maintained at 26 °C). Illumination was provided by four vertically mounted, timer-controlled (14 h on/10 h off) 15 W cool-white fluorescent lamps positioned at 1.0 cm from the vessel to provide a uniform incident light intensity along the reactor surface. CO_2 in the aeration gas served as the sole carbon source for algal growth, supplemental CO_2 (from a CO_2 tank) was mixed with the inlet air which passed successively through a filter (0.2 µm) and a humidifier before entering the reactor through a glass frit (diameter 4 cm, pore size 40–60 µm). Dissolved CO_2 speciates to bicarbonate ion (HCO_3^-) in seawater pH > 7. Fresh medium was continuously pumped into the bottom of the reactor during the 14 h light

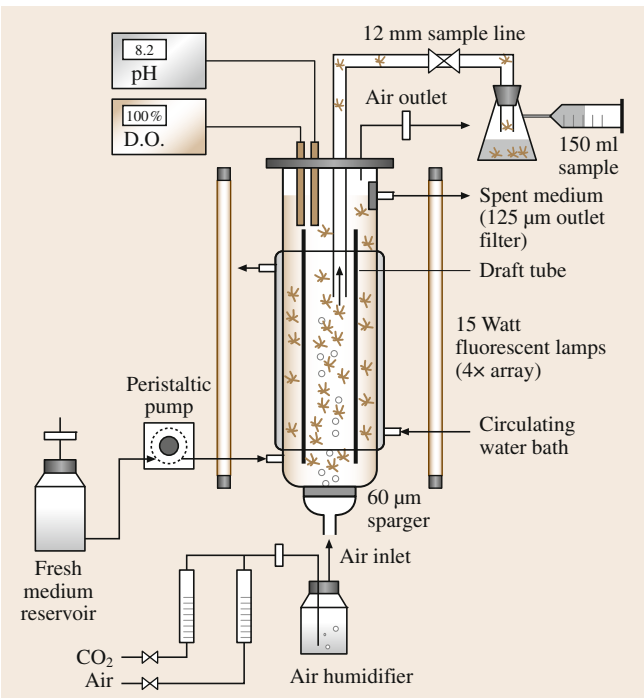

Fig. 12.10 Perfusion airlift photobioreactor (after *Polzin* and *Rorrer* [12.26])

phase. A nylon mesh (125 μm) was affixed on the medium outlet port for biomass retention within the reactor.

Contreras et al. [12.27] employed a plexiglass-made concentric tube airlift PBR (working volume 12 L) for culturing the photosynthetic microalga *P. tricornutum*. The AL-PBR (Fig. 12.11) consisted of a 2 m high outer tube (diameter 0.09 m) within which was installed a 1.5 m high concentric draft tube – the riser, with a cross-sectional area (2.8×10^{-3} m^2) same as that of the downcomer. Continuous illumination was provided by 10 fluorescent lamps, installed around the reactor with an illuminated surface area of 0.471 m^2. Compressed air was passed through an oil separator and a 0.5 mm sterile filter. A cylindrical, sintered glass sparger (diameter 0.02 m, height 0.03 m, pore size 60 mm) located in the riser, was used for aeration. It was noted that the AL-PBR adequately complied with the requirements of microalgal cultivation viz. high mass transfer rates, large surface-to-volume ratio, ease of control, safe sterile operation, and low mechanical shear forces on the cells. The existence of a maximum in μ_{max} of *P. tricornutum* was observed with respect to the gas flow rate as well as the shear rate which is indicative of the presence of a growth limiting or inhibitory effect in the reactor below and above these optimal values.

Vunjak-Novakovic et al. [12.28] designed a novel *triangular-configuration* inclined-tube AL-PBR for CO_2 fixation from flue gas by the marine green algae *Dunaliella* sp. When gas enters from the bottom of an inclined tube, the gas bubble travels along the inner upper surface of the tube – this renews the upper surface liquid layer making surface adherence difficult for the growing algae, thereby preventing fouling. As light penetration into the ALBR usually occurs through the upper surface, this self-cleaning feature substantially reduces the need for tube maintenance. Most of the solar radiation incident on the *triangular-configuration* AL-PBR enters through the hypotenuse (3.3 m long), with a circular cross-sectional area. Thus, the cross section has an *intensive light* region, corresponding to that in the annular region of a concentric-tube ALBR. The liquid flow rate is controlled mainly by the feed flow rate of the gas and can be adjusted to give a wide range (seconds to minutes) of retention times within each of the reactor zones. As the algae circulate through the inclined-tube segment, turbulence caused by two gas spargers creates microtrajectories that carry the suspended cells back and forth between zones with different illumination (i. e., closer to the illuminated surface or deeper into the liquid flow with less illumination). The desired throughput for flue gas purification is obtained simply by increasing the number of ALR *triangles* that are connected in parallel. Preliminary studies were carried out in a small-scale laboratory ALBR (volume 7 L) with no internal temperature control, housed in a wedge-shaped greenhouse (temperature-controlled) with a triangular side view resembling the shape of the reactor. A so-called *second*

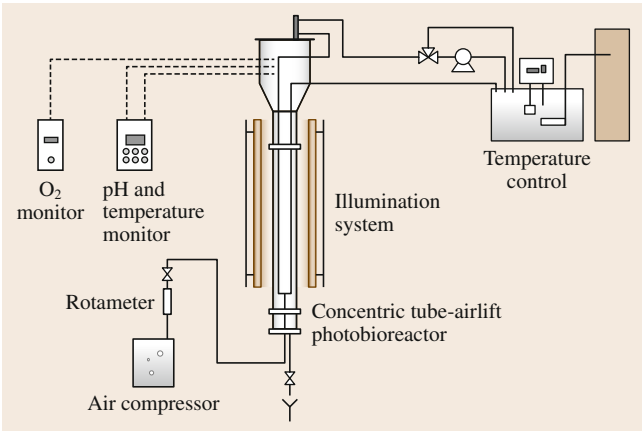

Fig. 12.11 Culture system (after *Contreras* et al. [12.27])

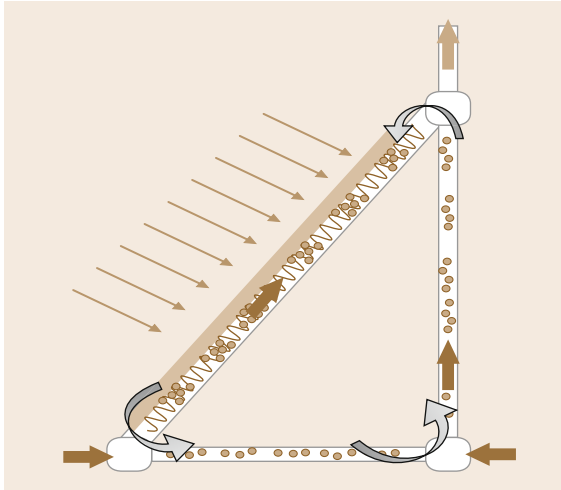

Fig. 12.12 Inclined-tube ALR configuration: Schematic presentation of one ALR triangle. *Solid arrows* indicate the direction of the gas flow, and *open arrows* indicate the direction of the liquid flow (after *Vunjak-Novakovic* et al. [12.28])

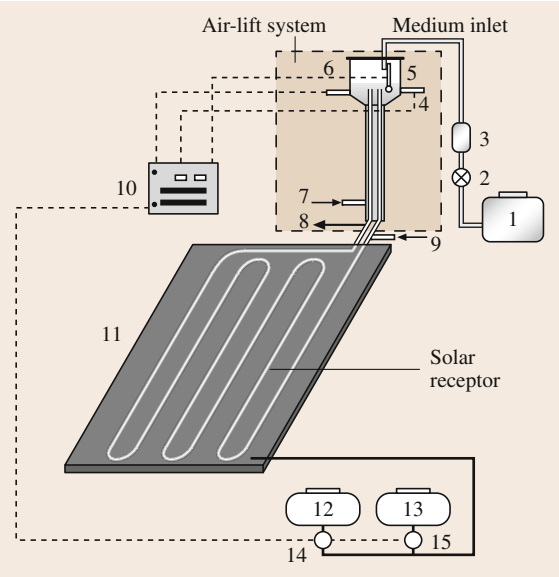

Fig. 12.13 Scheme of outdoor culture system. 1 – Fresh medium; 2, 14, 15 pumps; 3 – sterilization UV lamp; 4 – temperature sensor; 5 – level sensor; 6 – pH probe; 7 – air injection; 8 – sampler; 9 – CO_2; 10 – control unit; 11 – thermostatic water pool; 12 – cool water reservoir; 13 – warm water reservoir (after *Del Campo* et al. [12.29])

pilot-plant unit was also installed that comprised a cascade of 30 ALBRs (volume 30 L each) with a configuration as in Fig. 12.12. These ALBRs were continuously supplied with flue gas from a small power plant to demonstrate CO_2 removal from flue gas by growing algae.

Del Campo et al. [12.29] employed an outdoor airlift-driven tubular photobioreactor (volume 55 L, made of acrylic) for the production of lutein (a valuable carotenoid pigment with a wide range of uses) by *Muriellopsis* species, a chlorophycean microalga. The reactor (Fig. 12.13) has an airlift system to recirculate the cell culture and an external horizontal loop, consisting of tubes (length 90 m, I.D. 2.4 cm, surface area 2.2 m^2) that serve as solar receivers, immersed in a thermostatic pond of water. The airlift consisted of a degasser (in which the pH and temperature probes were inserted) and two 3 m high tubes (i. e., the riser and the downcomer). Compressed air was supplied into the riser to transport the cell suspension through the tubes and create turbulence. The reactor was operated in continuous-flow mode during daylight and in batch mode at night, to prevent culture washout.

12.3.2 Bubble Column Bioreactors (BCBR)

Bubble columns (BC) (Tables 12.3 and 12.4) are also pneumatically agitated vertical column reactors but the main difference with ALRs is in the nature of fluid flow, which depends on the geometry of the reactor. The bubble column is a rather simple vessel into which gas (air) is injected at the bottom and random mixing is produced by the rising bubbles. Now, in contrast to a BC where flow patterns are rather random, the ALR generates a more homogeneous flow pattern that moves suspended cells from the riser to the downcomer. Again cell sedimentation may occur in a BC, but cells remain more uniformly suspended in an ALR.

Guo et al. [12.30] developed an integrated process of CO_2 fixation and biohydrogen photoproduction by the marine green microalga *Platymonas subcordiformis*, grown photoautotrophically in a CO_2 supplemented air BCBR (Fig. 12.14) (volume 600 mL, diameter 50 mm, height 400 mm). CO_2 is required in photosynthesis for algal production of intracellular starch; the latter is then utilized for hydrogen production under anaerobic conditions by mitochondrial respiration to deplete oxygen. In fact, alga with higher starch accumulation shows a substantial increase in rate and duration of hydrogen production. Compressed air and CO_2 were mixed (up to 15 vol. % of CO_2 in air) and

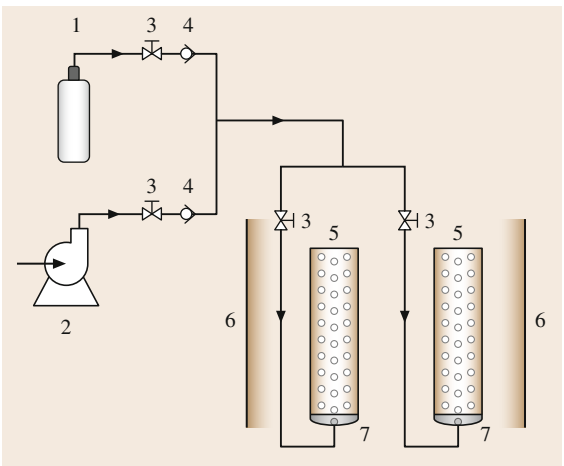

Fig. 12.14 Schematic diagram of the bubble column bioreactor system used for the growth of *P. subcordiformis* cultures under photoautotrophic conditions. 1 – CO_2 bottle, 2 – air compress pump, 3 – manual valve, 4 – gas flowmeter, 5 – bubble column bioreactor, 6 – cool-white fluorescent light, and 7 – porous sieve (after *Guo* et al. [12.30])

metered through calibrated flow meters, and sterilized using membrane filters (pore size 0.22 μm) before entering the reactor (at an aeration rate of 0.2 vvm). The bottom of the bioreactor was filled with a porous quartz sieve (diameter 10 mm), which dispersed the airstream. The cultures were illuminated from two sides with cool white fluorescent lamps under 14 h/10 h light/dark cycle. CO_2 was supplemented in air only during the light phase.

Seven-day-old cell cultures from the BCBR were concentrated by centrifugation and transferred into a 500 mL cylindrical glass bioreactor. They were subjected to the dark anaerobic induction of hydrogenase at 25 °C for 12 h, after continuous flushing for 10 min with N_2 (99.9% purity) through the culture to purge O_2. 15 1M CCCP (carbonylcyanide *m*-chlorophenylhydrazone) was then added, and the culture further incubated in darkness for 20 min, before initiating photobiological H_2 production under continuous illumination. It was demonstrated in this study that the marine green alga *P. subcordiformis* could efficiently convert CO_2 into intracellular starch through photosynthesis and improve H_2 photoproduction through increased accumulation of intracellular starch under CO_2 supplementation in an air bubble column PBR.

Huang and *Rorrer* [12.31] investigated the optimal values of cultivation temperature and diurnal photoperiod for the phototrophic growth of a microplantlet suspension culture derived from the macrophytic marine red alga *Agardhiella subulata* in a BC-PBR. Cultivation temperature and light delivery are two crucial process variables in the design of PBRs for culturing marine microalgae (seaweed). The latter has two components, viz., the light flux intensity and the diurnal photoperiod, i. e., the light/dark (L/D) illumination cycle in 24 h. It was observed in this study that biomass production increased with increasing photoperiod at low fractional photoperiods ($\leq$ 10 : 14 L/D) but at high fractional photoperiods approaching continuous illumination ($\geq$ 20 : 4 L/D) biomass production practically stopped, presumably due to photodamage that inhibits growth). The optimal photoperiod for biomass production was found to be 16 : 8 L/D.

An externally illuminated BC-PBR (same working principle as that used in *Zhi* and *Rorrer* [12.32], cited immediately afterward) (effective cultivation volume 250 mL) consisted of a 12.7 cm straight section (diameter 4.5 cm) and a 15.2 cm conical riser section (inner diameter 1.3 cm at the base). The small vessel diameter minimized light attenuation through the microplantlet suspension. Air was metered, humidified in a bubbler, sterilized through an autoclaved filter (0.2 μm pore size), and then introduced into the base of the riser section through a glass frit (diameter 1.3 cm, pore size 40–60 μm). The liquid suspension culture in the vessel was uniformly agitated and aerated by rising air bubbles (nominal diameter 1.0 mm). Ambient CO_2 in the aeration gas (normally 350 ppm) served as the carbon source for photosynthetic biomass growth. The illumination stage consisted of two 6.0 W cool-white fluorescent lamps vertically mounted on opposing sides of the glass vessel. A referencing plate set the distance between each lamp and vessel wall to deliver the desired incident light flux intensity to the culture and a programmable timer set the photoperiod for each lamp. The PBR was maintained at 24 °C within a temperature-controlled room. Two identical BC-PBRs described above were operated in parallel, each inoculated with microplantets from a common inoculum source.

Zhi and *Rorrer* [12.32] demonstrated the feasibility of the photolithotrophic cultivation of a cell suspension culture derived from the microscopic, filamentous gametophytic life phase of the brown marine macroalga *L. saccharina* in an illuminated BC-PBR, at 13 °C using CO_2 in air as the sole carbon source for growth. Two illuminated glass BCBRs were employed with effective cultivation volumes of 280 and 900 mL, respectively. Each BCBR system, comprising the bioreactor assembly, aeration unit, and illumination arrangement, was housed in a low-temperature incubator fitted with two auxiliary convection fans to maintain uniform temperature, and fresh air was supplied to maintain a constant ambient CO_2 concentration in the incubator gas space (vol. 500 L). Four identical 280 mL bioreactor systems and two 900 mL bioreactor systems were used for batch experiments under different conditions using a common inoculum. The bioreactors of a given volume were all located within the same low temperature incubator.

The 280 mL BCBR (Fig. 12.15) consisted of a 12.70 cm straight section (I.D. 4.45 cm) and a 15.24 cm conical riser section (base I.D. 1.27 cm), and was sealed to the head plate with two G-rings, one above and one below the flange of the glass body. The head plate had two ports, for the sampling assembly and for air outlet, respectively, with the latter connected to a sterilizing filter (0.2 μm). Agitation was provided by rising air bubbles introduced into the reactor base through the sparger assembly. Ambient air from an aquarium pump was metered, filter – sterilized (0.2 μm), and then bubbled through a ster-

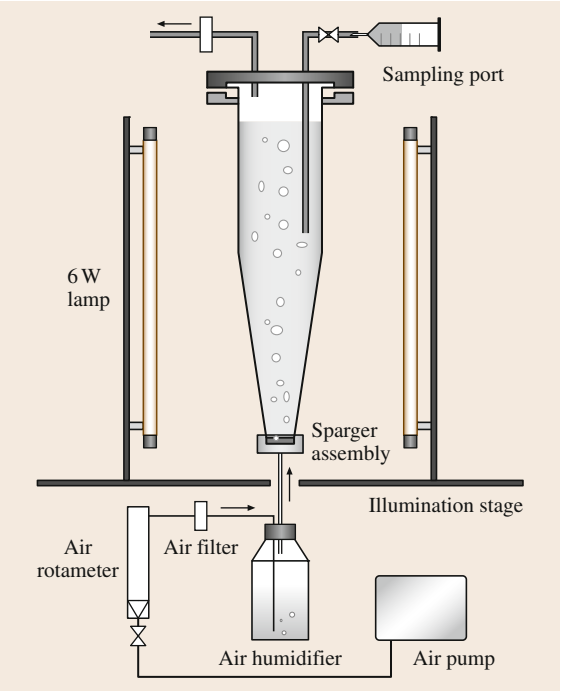

Fig. 12.15 Schematic of 280 mL bubble-column bioreactor (after *Zhi* and *Rorrer* [12.32])

ilized humidifier, before introducing into the culture through the sparger assembly fitted with a removable glass frit (pore size 40–60 µm, diameter 1.27 cm). The sparger generated air bubbles with diameter ranging from 0.2–0.7 mm. The conical riser section improved the fluid circulation provided by the rising air bubbles. CO_2 naturally present in the ambient aeration gas (nominally at 350 ppm) served as the sole carbon source for biomass growth. Illumination was provided by two vertically opposed, timer-controlled 6 W fluorescent lamps mounted on plexiglass plates. A referencing plate set the distance between each lamp and the vessel wall, so that the desired incident light intensity could be applied uniformly to both sides of the reactor. The larger BCBR had the same headplate assembly, sparger assembly, and aeration system as the smaller one but the straight section of the 900 mL glass reactor vessel was 48.26 cm long. Also, the light stage consisted of four 6 W fluorescent lamps mounted vertically, with two per side.

Initial cell density had a profound effect on the final biomass density of the clumped cell suspension, not on the specific growth rate. Increasing the aeration rate somewhat enhanced the specific growth rate and final biomass density, but the culture was not CO_2-transport limited. Initial nitrate concentrations above a certain threshold value had no significant effect on the specific growth rate and final biomass density.

12.3.3 ALBR and BCBR and/or other PBR – Comparative Studies

Nagase et al. [12.33] investigated the removal of NO (present in fossil fuel flue gas) by the marine green algae *Dunaliella tertiolecta* cultured in bubble column and air-lift-type bioreactors. Several alternative means of enhancing NO removal were examined in this study, the first of which was to reduce the bubble size in order to increase the gas–liquid contact surface and thus obtain a higher rate of NO dissolution. For this purpose, S.S. tubes of different gauges, and glass-ball filters (g.b.f.) of varying particle sizes were examined as spargers to give varying bubble diameters – of which the so-called No. 3 g.b.f. generated the smallest bubbles. However on actual use of No. 3 g.b.f in the parallel-flow ALBR, the algal cells became concentrated at the top of the reactor due to froth flotation and could not be cultured further. Secondly, for increasing the NO removal rate, the idea of using a longer column or a modified reactor configuration, viz., the counterflow ALBR, was considered, to increase the gas–liquid contact time. In fact, the counterflow ALBR improved gas liquid contact time by decreasing the rising rate of bubbles (i. e., by increasing gas holdup). With a No. 3 g.b.f. used in the counterflow ALBR, by which a simultaneous increase in gas–liquid contact area and gas holdup was accomplished, a remarkable enhancement in the NO removal rate was observed – there was no growth – inhibition by froth flotation in the reactor and the algal culture remained well mixed. The parallel-flow and counterflow-type ALBRs employed for algal NO removal (shown in Fig. 12.16) were fitted with a draft tube located centrally in the column. The NO containing flue gas was fed outside of the draft tube, where photosynthetic oxygen was produced vigorously. The draft tube was cut near the two parts connected by a fine-mesh stainless steel netting, thereby preventing the mixing of bubbles inside and outside the draft tube and facilitating circulation of the culture medium in the reactor column. The highest NO removal, i. e., 96%, was attained with a counterflow-type ALBR (when 100 ppm NO was aerated with smaller bubbles). The authors concluded that the NO removal ability of a counterflow-type ALBR was threefold higher than a simple BCBR.

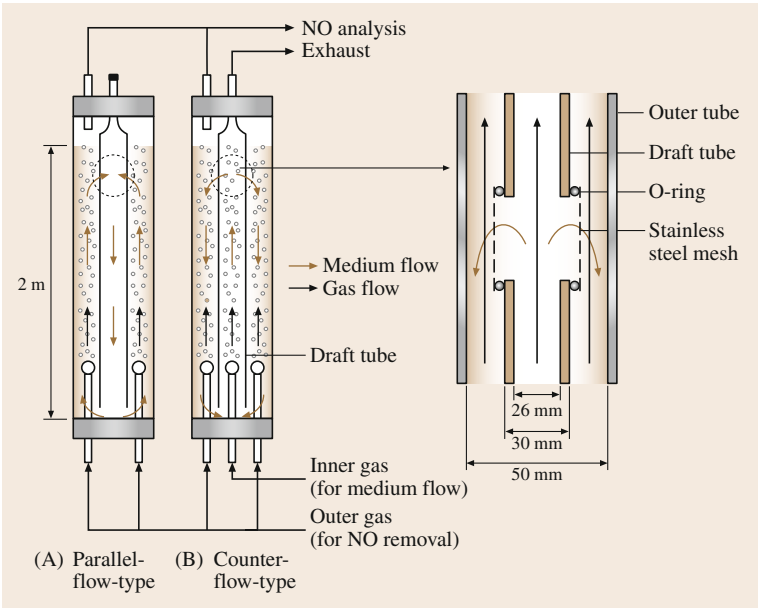

Fig. 12.16 Schematic diagrams of NO removal systems using airlift reactors (after *Nagase* et al. [12.33])

A comparative evaluation of the performance of an AL-PBR and BC-PBR (both 3 L volume, the same column diameter and height) was undertaken by *Monkonsit* et al. [12.34] for the cultivation of the marine diatom *Skeletonema costatum*, which is used as food for shrimp larvae in the first protozoea stage. Maximum cell concentration, specific growth rate, and biomass productivity were higher in the AL-PBR than in the BC-PBR, both having been operated under identical aeration rates and light intensity. The superior performance of the AL-PBR was attributed to its circulatory flow that prevents cell precipitation and improves light utilization efficiency. The optimal reactor operating parameters for cell growth in terms of:

1. Ratio of downcomer-to-riser cross-sectional area (3.27)
2. Superficial gas velocity (1.5 cm s^{-1})
3. Incident light intensity (34 μmol ph m^{-2} s^{-1}) were determined.

The AL-PBR (height 60 cm, column I.D. 9.4 cm) was equipped with a draft tube (height 40 cm) installed centrally in the column. For both reactors, compressed air was passed through a flowmeter and sterilized through a filter (0.45 μm) before entering the reactor bottom. Illumination was provided by 18 W fluorescent lamps installed at the side, along the length of each column (two lamps per column). The reactors were kept in an air-conditioned enclosure with temperature maintained between 25 and 30 °C.

Merchuk et al. [12.12] considered the effect of light/dark cycles of different frequencies on growth and polysaccharide production by the red microalga *Porphyridium* sp. in a laboratory-scale tubular loop PBR (total volume 0.43 L) and compared it with the performance of an AL-PBR and BC-PBR (both of volume 35 L). Whereas the loop device is a small-scale laboratory reactor in which liquid is circulated by a peristaltic pump at a controlled rate, the column reactors (bubble column and air-lift) are orders of magnitude larger, and the liquid movement is driven by the injection of gas into the reactor. However, despite basic differences, the light/dark cycles generated, either by the pump of the laboratory-scale tubular reactor, or by gas flow rate in the much larger BC and ALR, were found to be of the same order.

Under low light intensity and high gas flow rates, the BC and the ALR performed almost identically. However, with high light intensity and low gas flow rates, both growth and polysaccharide production were higher in the ALR. The interactions of photosynthesis and photoinhibition with the fluid dynamics in the bioreactors allowed interpretation of the differences in the performance of the BC-PBR and the AL-PBR. It was posited that the cyclic distribution of dark periods in the AL-PBR facilitates better recovery from the photoinhibition damage suffered by the cells. Due to the

small diameter of the loop and the low biomass concentration involved, the laboratory-scale tubular loop PBR performance data could be considered free of the effect of self-shading, and in this regard, the TL-PBR could be considered a *thin-film* photobioreactor. The larger column reactors were used to examine the effect of mixing under conditions of high optical density, where self-shading and fluid dynamics were responsible for the periodicity of exposure to light.

The tubular loop reactor (volume 0.43 L) consisted of a series of glass tubes (I.D. 0.007 m) connected to a small vessel into which air containing 3% CO_2 was bubbled (to supply a carbon-source and remove O_2); the loop was closed through a peristaltic pump. The ratio of illuminated/dark zones was controlled by darkening different lengths of the tube. The ventilation vessel, which was always covered, formed part of the dark zone. Illumination was provided from a bank of fluorescent lamps.

The column reactors (both 2 m high, column diameter 0.180 m) were also supplied with air containing 3% CO_2 and were housed in a temperature-controlled room at 25 °C. The difference in construction between the AL-PBR and the BC-PBR was that a draft tube (height 1.5 m, diameter 0.09 m) was installed coaxially in the AL-PBR. The draft tube plays a pivotal role in transforming the flow of the liquid, which is approximately random in the bubble column, into more ordered patterns in the ALR. The liquid rises through the central riser due to the difference in hydrostatic pressure, and descends through the annular downcomer, engaging part of the gas. The extent of this gas carryover depends on the operating conditions.

Krichnavaruk et al. [12.35] used a small-scale glass BC-PBR (2.5 L) as well as an AL-PBR and a BC-PBR (both 17 L volume) to investigate the growth of the chlorophyll containing marine diatom *Chaetoceros calcitrans* which is commonly used as feed for shrimp larvae. For the large-scale column reactors, run in batch mode, both maximum specific growth rate and maximum cell concentration were higher in the AL-PBR by about 18 and 16%, respectively. For the AL-PBR operated in semicontinuous mode, maximum specific growth rate increased further by 30%. Experiments to determine the optimal growth conditions of *C. calcitrans* were performed in a small-scale glass bubble column (volume 2.5 L). Compressed air (flowing 3.8 L min^{-1}) entered the bottom of the column. Illumination was provided through 250 W lamps, and incident light intensity was controlled by varying the distance between the lamps and the column. Both large-scale bioreactors were acrylic-made (diameter 15 cm) but the AL-PBR was equipped with a draft tube installed centrally in the column. The ratio between the cross-sectional areas of downcomer and riser was 2.63. Compressed air was supplied at the bottom of the draft tube and a 5 cm gap was left between the bottom of the draft tube and the column to allow liquid circulation. Aeration was controlled by a calibrated rotameter – superficial gas velocity in the riser was controlled in a range of 2–5 cm s^{-1}. Light was supplied through 12 fluorescent lamps placed at the side, along the length of the columns and temperature was controlled ≈ 30 °C (±2 °C).

The superior performance of the ALBR vis-a-vis the BCBR may be attributed to a well-defined flow pattern in the ALBR that allows more effective light utilization by the diatom. In the BCBR, proper recirculation of cells is not possible since aeration only superimposes random motion with no net liquid movement – whereas some cells are exposed to high light intensity in close proximity of the illuminated column walls, those centrally positioned in the column are exposed to much lower light intensity causing ineffective photosynthesis and consequently low biomass growth. However, uneven fluid density in the riser and downcomer sections of the ALBR, induces a well-defined flow pattern – upward liquid movement in the riser and downward in the downcomer. Consequently diatoms in the riser, would, after lapse of a certain time, flow to the downcomer where light is directly incident, implying exposure to more uniform light density than in the BC. Furthermore, liquid movement in the ALR prevents cell accumulation at the bottom of the column (and an uneven cell density along the length of the column arising therefrom) by facilitating cell circulation even at high cell density. Cell accumulation at the bottom of the column may potentially cause starvation, death, and even culture contamination, thereby resulting in an overall reduced growth rate.

Sanchez-Miron et al. [12.36] undertook a comparative evaluation (mainly in terms of hydrodynamics and transport phenomena) of three air-agitated photobioreactors, viz., a bubble column, a split-cylinder or split-column ALR and a concentric draft-tube sparged ALR (Fig. 12.17). Their focus was on fractional gas holdup, liquid circulation velocity, and the overall gas–liquid oxygen mass transfer coefficient and the interdependence of these variables in regimes relevant to microalgal cultures. Comparative evaluation of reactor performance was presented for the culture of the photo-

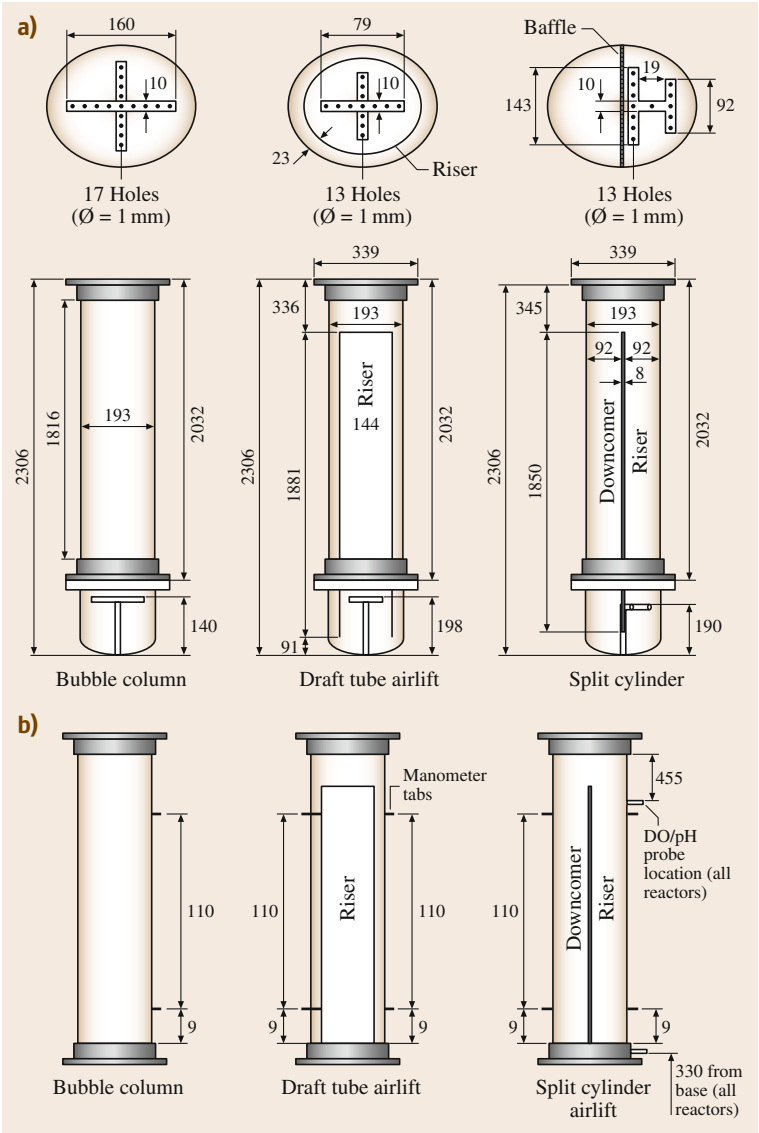

Fig. 12.17a,b Reactors: **(a)** vessel dimensions and air sparger details; **(b)** location of dissolved oxygen (DO) and pH electrodes. All dimensions in mm (after *Sanchez-Miron* et al. [12.36])

synthetic marine microalga *P. tricornutum*, a potential source of certain omega-3 polyunsaturated fatty acids of therapeutic value. Based on the findings of this study, it was noted that the performance of all three reactors were equivalent and that the results did not indicate a clear preference for any reactor – a maximum specific growth rate of 0.022 h^{-1} and a final algal biomass concentration of 4 kg m^{-3} was obtained for all the three reactors.

All the reactor vessels (diameter 0.193 m) were made of acrylic (3.3 mm thick) but for the lower 0.25 m sections, which were made of stainless steel. The ratio of the riser-to-downcomer cross-sectional area for the split cylinder ALBR and draft-tube ALBR were 1.0 and 1.24, respectively. The draft tube (I.D. 0.144 m) and the baffle were located at, respectively 0.091 m, and 0.096 m from the reactor bottom. The gas-free liquid level was about 2 m in all cases.

Merchuk et al. [12.37] considered three types of bench scale, pneumatically agitated photobioreactors for culturing the red microalga *Porphyridium* species – a BCBR, an ALBR, and a modified ALBR fitted with

helical flow promoters (ALBR + HFP). Now, in an earlier study *Merchuk* et al. [12.12] comparing BC and ALR-type photobioreactors for *Porphyridium* growth, it was concluded that the more ordered flow in the ALBR leads to the exposure of the growing cells to light and darkness in more homogenous cycles, resulting in the superior reactor performance.

The HFP is a static device consisting of several fins or baffles that causes fluid flow in a helical path along the downcomer. It effectively modifies the fluid flow path from a rectilinear trajectory along the axis to a helix. Although the HFP may be installed at any point along the riser or the downcomer, a much preferred location is the top of the downcomer – wherefrom a helical flow is generated in the downcomer, which becomes a swirl at the bottom and a corkscrew-like path in the riser. The helical fluid motion causes secondary flow which leads to an enhanced radial mixing, and thus more homogeneous distribution of light among liquid elements and suspended particles. This increases the likelihood of all fluid elements getting the same exposure to light. In this study, the HFPs were positioned at the upper rim of the downcomer. As the ALBRs were illuminated through the external walls, aeration from the bottom of the draft tube was preferred in order to minimize the quantity of bubbles in the downcomer, which would cause loss of efficiency due to light scattering. Installing the HFP at the downcomer inlet maximizes the fluid dynamic effects (i. e., secondary flow) in the photosynthesis zone of the reactor.

The reactors (I.D. 0.13 m, height 1.5 m) were made of flexible polyethylene sleeves with a conical bottom. For operation as an ALBR, an acrylic draft tube (external diameter 0.09 m, height 1.05 m) was inserted. For the ALBR + HFP the same draft tube was used but with the HFP installed. Selection of the draft tube diameter was based on the fluid residence time in the riser as time spent in the dark zone. The HFP comprises three fins at 45° from the axis, placed at the upper 0.05 m of the downcomer, with a width equal to the gap between draft tube and the external wall. Gas entered the reactors through a sparger installed 0.03 m above the bottom. The sparger was a ring of 0.05 m diameter made of a stainless steel tube of 0.005 m diameter, with 15 holes (diameters 0.001 m) equidistantly positioned on its upper face. Now, the gas flow rate to the reactor strongly influences the mixing of medium, the distribution of cells, their nutrient availability, and CO_2 absorption. It was observed for the ALR + HFP that the more ordered flow and the additional secondary circulation due to the HFP provide sufficient mixing and better cell distribution at a lower gas flow rate, without too many cells adhering to the reactor walls. Operability at lower gas flow rates leads to lower consumption of air and CO_2 resulting in lowered costs of air compression and CO_2, thereby improving the efficiency of CO_2 uptake for algal photosynthesis – the basis for superior performance of the ALR + HFP relative to the other reactors considered.

Rorrer and *Cheney* [12.38] have compared four major photobioreactor configurations for microplantlet suspension cultures of photolithotrophic marine macroalgae *A. subulata*, viz., bubble column, airlift (internal draft tube), externally illuminated stirred tank and tubular recycle (helical array) photobioreactors in both batch and medium perfusion modes of macronutrient delivery. Of these four reactors considered, the ST-PBR and tubular recycle PBR have been used earlier by *Rorrer* and *Zhi* [12.14] and *Rorrer* and *Mullikin* [12.13], respectively, and have already been discussed at length in Sect. 12.2. These four PBRs were compared on four major parameters, viz., mixing and biomass suspension, aeration, and gas exchange, light transfer and potential for shear damage. Both BC and ALR-type PBRs have low shear damage potential and very good aeration capabilities, and while mixing and light transfer are rated as adequate for BC, they range from good to very good for ALR. The stirred tank PBR, on the other hand, has high shear damage potential and poor light transfer facilities but with regard to mixing and aeration it is rated as excellent. Finally, for the tubular recycle PBR, while light transfer is excellent and aeration adequate, mixing and shear damage are problem areas. Certain essential common features of these PBR systems are worth noting: the bioreactor control volume being externally illuminated by artificial light source(s), transparency of at least a part of the bioreactor vessel is necessary – this requires the reactor to be made out of glass/polycarbonate for BC/ALR and translucent silicone tubing for tubular PBR; also, the nonfriable nature of the macroalgal cell and tissue precludes continuous culture, because dilution of the culture suspension by continuous addition of fresh medium will dilute out these cell clumps. It may be noted that in microplantlet tissue cultures, since the biomass is compacted into ball-like nonfriable multicellular tissue and not dispersed in the liquid medium as single cells, light attenuation through the culture suspension is low relative to microalgal suspension cultures at the same cell mass density. Again, microplantlets are easily suspended but also easily separated from the culture broth thus facilitating biomass harvesting.

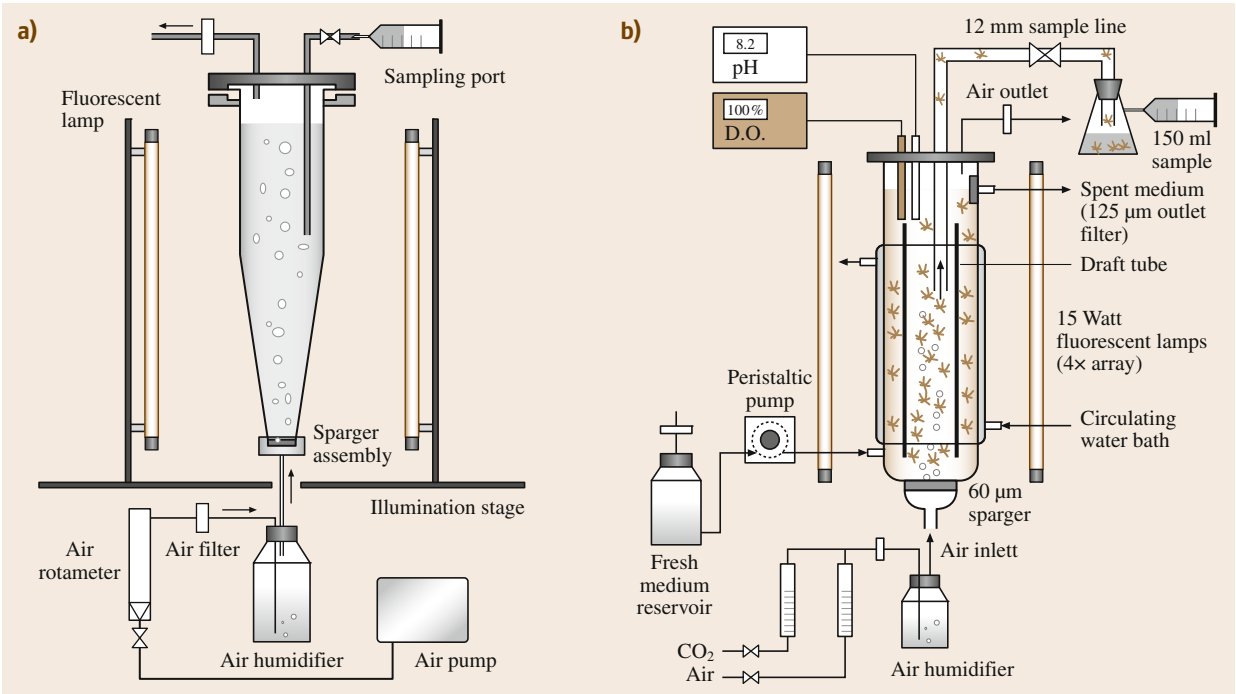

Fig. 12.18a,b Representative bench-scale bubble-column and airlift photobioreactors for macroalgal cell and tissue culture: **(a)** 250 mL bubble-column photobioreactor and **(b)** 3.0 L airlift photobioreactor equipped for continuous medium perfusion (after *Rorrer* and *Chenny* [12.38])

The PBRs discussed (Fig. 12.18), are designed to provide mixing, illumination, gas exchange, and dissolved macronutrients to the macroalgal suspension culture under controlled conditions. In a BCBR, the culture is mixed and suspended by the rising air bubbles, whereas in an ALBR biomass suspension is improved by a *draft tube* (of diameter d_i) which fits within a cylindrical vessel of width d ($d > d_i$). The draft tube provides a defined liquid circulation pattern where the suspension ascends within the draft tube (riser) and descends outside of the draft tube (downcomer). Draft tube ALBRs also provide enhanced light transfer to the culture broth as it moves down the downcomer because the suspension remains in close proximity to the illuminated reactor wall and is confined within a short light path ($d - d_i$). Aerated stirred tank bioreactors improve mixing and biomass suspension. Cell-lift and marine-blade impellers provide axial circulation patterns which help to suspend macroalgal cell clumps and tissues. Moreover, the rotating impeller breaks up and disperses the air bubbles to improve interphase mass transfer for CO_2 delivery. In general, cell cultures derived from terrestrial plants are prone to damage by hydrodynamic shear forces generated by the stirred tank impeller rotation. However, the nonfriable cell and tissue cultures of marine macroalgal plants are not susceptible to shear damage in stirred tank photobioreactors. Light delivery is an important design parameter for PBRs made for macroalgal cell and tissue suspension culture, where growth becomes light saturated at relatively low light intensities compared to microalgae because in the rocky, intertidal, benthic ecological niche microalgae are exposed to much low light intensity. Therefore, as long as the light path through the culture is short and the reactor vessel is transparent, external illumination of the bioreactor with cool-white fluorescent lamps is generally adequate for bench-scale or pilot-scale reactors for macroalgal culture.

For macroalgal culture suspensions that can be pumped, the tubular recycle photobioreactor (TR-PBR) offers excellent light transfer and low light attenuation, because the path length for light transfer is reduced down to the diameter of the coiled tubing, and the light source is placed within the center of the coil. TR-PBR was found to successfully cultivate *L. saccharina* gametophyte cell clump suspensions. It is not suitable for

microplantlet cultures, however since their large tissue size makes it difficult for them to pass through the tubing without settling. Continuous bubbling aeration of the culture with CO_2 in air simultaneously serves four purposes, viz. (1) transfer of CO_2 to the culture; (2) maintenance of the dissolved inorganic carbon level in the culture medium; (3) pH control; and (4) removal of dissolved O_2 produced by photosynthesis.

Although macroalgal suspension cultures grow rather slowly (with specific growth rates less than 0.20 day^{-1}) and k_La values in aerated PBRs are high, it is challenging to supply CO_2 at flow rates that can avoid the CO_2 limited growth rate condition at high cell density unless CO_2 is added to the aeration gas. This requires CO_2 to be present in the aeration gas at 10 times its normal ambient concentration. If the culture suspension is continuously supplied with light and CO_2, then the cumulative biomass production will be limited ultimately by available macronutrients dissolved in the liquid medium, mainly N in the form of nitrate, and P in the form of phosphate. Macronutrient delivery depends on the mode of cultivation. In batch mode, macronutrients are initially provided in the growth medium at culture inoculation. Nitrate and phosphate concentrations in the medium decrease with time until one (or both) becomes zero. At this point, the *limiting nutrient* is exhausted, and cell growth stops even if light and CO_2 are still being continuously supplied. In perfusion cultures, liquid medium containing dissolved macronutrients is continuously supplied to the bioreactor culture suspension. The spent medium leaves the bioreactor but the biomass is retained inside. Under these conditions, cumulative biomass production continues, but its rate is ultimately limited by the rate of CO_2 delivery, or the attenuation of light through the dense culture suspension. The final limit to biomass production is space. If the tissues completely fill up the bioreactor control volume, then biomass production will stop even in the presence of an infinite supply of nutrients (light, CO_2, and macronutrients).

12.4 Membrane Bioreactors (MBR)

An MBR is basically a membrane filtration unit (membranes ranging between microfiltration, and ultrafiltration) coupled or integrated with a suspended phase bioreactor (Table 12.5). Depending on whether the membrane module is located inside or outside the bioreactor, MBRs are categorized as internal (i. e., *submerged*-type) or external (i. e., *cross-flow* type). In a cross-flow MBR medium is recirculated between the membrane module and the BR by pumping it through the membranes. In a submerged MBR, the membrane filtration unit is immersed inside the culture medium in the BR (or sometimes in a separate tank connected to the BR). Some common membrane configurations employed in MBRs are:

i) Hollow fiber
ii) Spirally wound
iii) Plate-and-frame (i. e., flat sheet)
iv) Tubular.

Insofar as wastewater treatment applications are concerned, MBRs provide a host of advantages compared to traditional activated sludge processes that include the total separation of hydraulic retention time (HRT) and solids retention time (SRT), high efficiency of pollutant removal and no requirement of a secondary clarification unit.

Zamalloa et al. [12.39] employed an anaerobic membrane bioreactor (AnMBR) for biomethanation of the marine microalgae *P. tricornutum*. In fact, this report was claimed as the pioneering study to demonstrate the feasibility of an AnMBR for digestion of algal biomass. In conventional, continuously stirred anaerobic digesters, where the SRT is identical to the HRT, a high SRT required for effective destruction of volatile solids necessitates a large, often prohibitively high reactor volume. Now, in an AnMBR, the solids are separated from the sludge suspensions by a membrane so that biomass wasting rates are low – therefore, an AnMBR routinely allows operation at SRTs as high as 50 d or even higher. This facilitates the growth of slow-growing microorganisms, e.g., methanogens, and also increases the fraction of fermented organic matter. The AnMBR employed in this study consists of (a) a reactor (working volume 8 L) made of acrylic panels and (b) a microfiltration membrane module arranged in parallel. Two peristaltic pumps were used to feed influent into the anaerobic reactor and separately withdraw permeate from there. For mixing and membrane scouring to control cake formation, biogas recirculation was carried out using a diaphragm gas pump through a diffuser located just below the membrane module. Biogas production was measured

Table 12.5 Membrane bioreactors (MBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Anaerobic membrane biorector (AnMBR)	Biomethanation of microalgae *P. tricornutum*	*Zamalloa* et al. [12.39]
2	AnMBR	Biohydrogen production by bacterial consortia	*Oh* et al. [12.40]
3	Ion-exchange MBR (IEMBR)	Nitrate removal from saline water by bacterial consortium	*Matos* et al. [12.41]
4	Submerged-MBR (sMBR)	Harvesting anaerobic methanotrophic archaea (ANME)	*Meulepas* et al. [12.42]

by means of a gas meter. Transmembrane pressure (TMP) was measured with an analogical gauge installed between the membrane module and the permeate pump.

Oh et al. [12.40] studied biohydrogen production by bacterial consortia dominated by marine (viz., *Clostridiaceae*) and nonmarine (viz., *Flexibacteraceae*) bacterial families in an anaerobic MBR (AnMBR) – basically a cross-flow membrane coupled to a chemostat. According to the authors, this was the pioneering study on biohydrogen production in an MBR. The facilities/accessories of the reactor (Fig. 12.19) (volume 2 L, working volume 1 L) include constant stirring, flow level controller, pH controller, three peristaltic pumps (feed, waste, and recirculation – bioreactor side), a centrifugal pump (recirculation – membrane side), a membrane housing, liquid flow meter, three pressure gauges, and a backpulse unit with a control cabinet. The feed tank was pressurized using nitrogen gas. The recycle loop around the membrane module was operated at a flow rate of 378 L h^{-1} producing a membrane cross-flow velocity of 2.8 m s^{-1}. The recycle flow rate from the reactor to the high recycle loop was set at 51 mL min^{-1}. The working volume of the reactor was maintained by a level probe connected to the feed pump. To maintain the SRT, solids were purged from the MBR intermittently using a timer and the peristaltic pump, and medium (without glucose) was simultaneously fed into the reactor at the same rate to maintain a constant liquid level. Three alumina membranes of various pore sizes were used. Pressures were measured at the inlet, outlet, and permeate side of the membrane to determine the TMP. A constant permeate flow was obtained by adjusting the peristaltic pump on the permeate side of the membrane reactor. To limit membrane fouling, intermittent backpulsing (every 10–30 s) was done using a piston that injected nitrogen gas into the permeate side of the membrane module for a very short period of time (0.5–1 s). This backpressure caused a reversal in flow through the membrane thereby removing solids off the membrane surface.

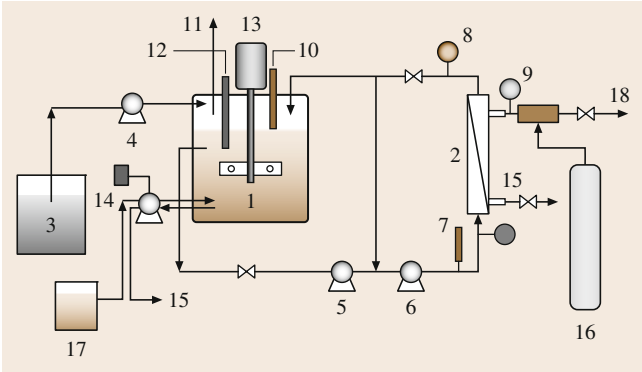

Fig. 12.19 Schematic diagram of the membrane bioreactor for hydrogen production. 1 – anaerobic reactor; 2 – cross-flow membrane; 3 – influent purged with nitrogen; 4 – feed pump; 5 – recirculation pump; 6 – high recirculation pump; 7 – flow meter; 8 – manometer; 9 – backpulsing; 10 – level controller; 11 – gas monitor; 12 – pH-controller; 13 – motor; 14 – timer; 15 – waste; 16 – nitrogen gas; 17 – medium w/o organics; 18 – effluent (after *Oh* et al. [12.40])

A common problem in biohydrogen production is that some of the bacteria in the consortium may consume hydrogen thereby lowering the overall hydrogen productivity, e.g., methanogens that convert H_2 to methane (which has less than half of the specific energy content of H_2). Of the several strategies to control or arrest the growth of methanogens, which are typically slow growing, the one that was deemed most effective was to maintain short residence times in continuous-flow reactors. However, this, in turn, reduces the efficiency of substrate utilization by hydrogen-producing bacteria and thereby that of the overall process.

Matos et al. [12.41] employed the ion exchange membrane bioreactor (IEMBR) for nitrate removal from highly saline water in a closed marine system, viz., a marine aquarium (oceanarium). Ammonia released by catabolism of reduced nitrogen compounds by aquatic animals is converted by nitrifying organisms into nitrate, the latter accumulating in a closed system like an aquarium up to levels potentially toxic for many marine species (which are generally much more sensitive to nitrate than freshwater ones) as well

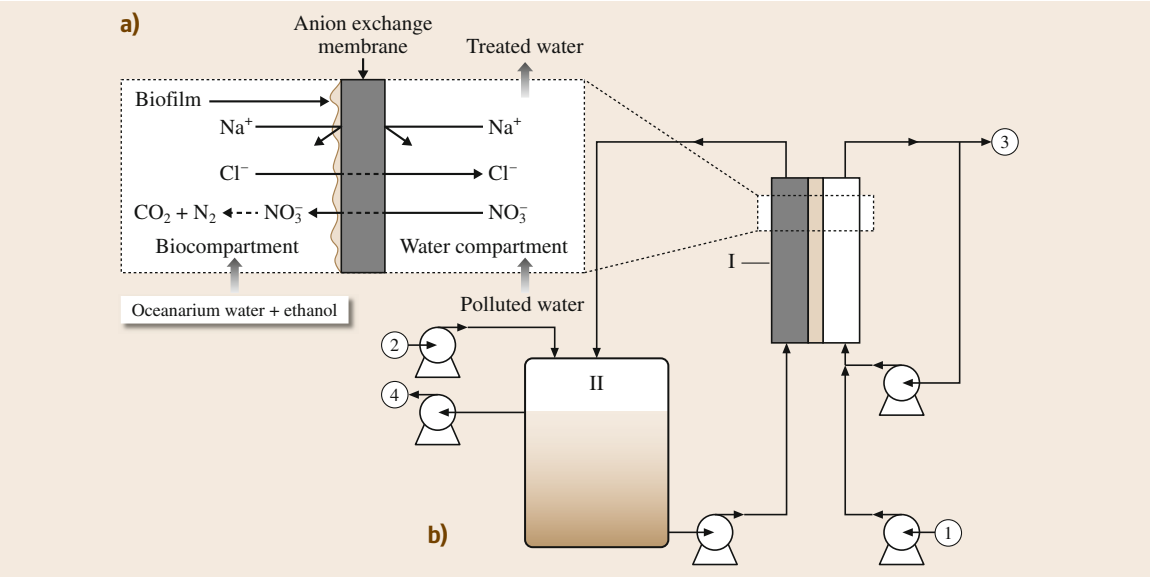

Fig. 12.20 **(a)** Schematic diagram of nitrate transport and bioreduction in the IEMB. **(b)** IEMB setup: 1 – feed water; 2 – biofeed; 3 – treated water; 4 – biocompartment effluent; aI, membrane module with two compartments separated by an anion-exchange membrane; bII – bioreactor vessel (after *Matos* et al. [12.41])

as posing problems related to its discharge into the external environment. The IEMBR concept is based on the integration of membrane separation technology with biological denitrification – nitrate is transported through a dense, nonporous anion-exchange membrane and subsequently converted into molecular nitrogen by a microbial consortium in a separate *biocompartment* isolated from the water stream (by the nonporous membrane *barrier*). This isolation allows for independent adjustment of the HRT in the biocompartment without affecting the production rate of the treated water and prevents the potential risk of secondary contamination of the treated water by excess carbon source (ethanol in this study). The transport across the membrane follows the principles of Donnan dialysis and may be increased by adding a suitable counter-ion (viz., chloride) to the biocompartment. An IEMBR is thus based on biological wastewater treatment avoiding secondary contamination of the treated water by complete isolation of the microbial culture through use of a membrane while preserving the water composition with respect to other ions.

The IEMBR (Fig. 12.20) consists of a membrane module with two identical rectangular channels, separated by a mono-anion permselective membrane (working area 39 cm^2). One channel was connected to an external loop where the aqueous phase was recirculated (at a flowrate of 97.2 $L\,h^{-1}$, $N_{Re} = 3000$). This *water compartment* was continuously fed with oceanarium water containing high levels of nitrate. The other module channel was connected to a stirred vessel through another recirculation loop ($N_{Re} = 3000$). This vessel, inoculated with 100 mL of the enriched microbial culture, was continuously fed (at a flow rate of 0.0048 $L\,h^{-1}$) with nitrate-free saline water from the oceanarium to which ethanol was added as a carbon source. This *biocompartment* was operated at a HRT of 5 d. To lower the volume of effluent produced in the biocompartment, a relatively high HRT was chosen. Lower HRTs could be set without affecting the nitrate reduction rates, which would, however, increase the volume of liquid waste discharged, which is undesirable.

Vis-a-vis other membrane treatment technologies for wastewater, e.g., reverse osmosis (RO), it was noted that the IEMBR provides for conversion of nitrate into harmless N_2 whereas in RO-based processes, the nitrate removed accumulates in a brine stream which usually requires further treatment. Again, in the IEMBR, the ion balance of the treated water is unaffected (other than nitrate) but in RO, demineralized water is obtained and all required salts have to be added to the treated water for it to be reused.

Meulepas et al. [12.42] used a novel, well-mixed, ambient-pressure, submerged-membrane bioreactor

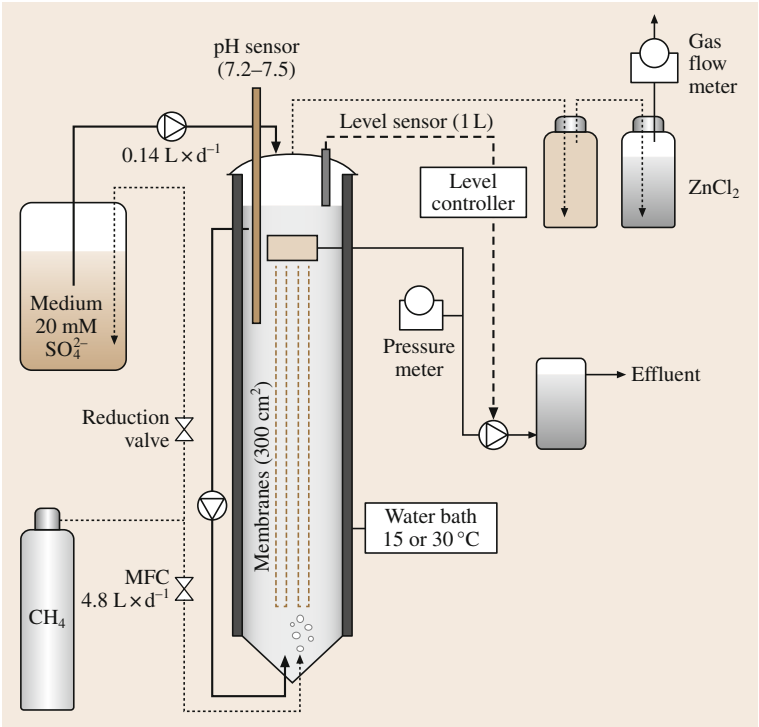

Fig. 12.21 Schematic overview of submerged-membrane bioreactor used for the enrichment experiments (after *Meulepas* et al. [12.42])

(sMBR) for enrichment of anaerobic methanotrophic (ANME) archaea that mediate anaerobic oxidation of methane (AOM) in marine sediments, which is coupled to sulfate reduction (SR) mediated by sulfate-reducing bacteria (SRB) that exist in consortia with the ANME. The sMBR (shown in Fig. 12.21) consists of a cylindrical glass vessel (total volume: 2.0 L; covered with opaque plastic to prevent phototrophic conversions), equipped with sampling ports for the headspace and the culture suspension, and connected to diaphragm metering pumps that continuously feed medium to the reactor. Each reactor was equipped with 4 polysulfone membranes, through which the effluent was extracted by means of a peristaltic pump. The membrane pore size guaranteed complete cell retention. Transmembrane pressure was monitored using a pressure sensor. The effluent pump was controlled by a level switch, which maintained the working liquid volume at 50% of the total volume (1.0 L). Each reactor was equipped with a water-jacket, through which water, cooled or heated in a thermostatic water bath, was recirculated to maintain a constant reactor temperature. CH_4 gas (purity 99.9995%), was supplied through a gas sparger at the reactor bottom to (a) aid the growth of the microorganisms, (b) to promote reactor mixing, (c) to strip off the sulfide, and (d) to prevent membrane fouling. The influent CH_4 flow was measured and controlled by a thermal mass flow controller. The exit gas from the reactor contained hydrogen sulfide (H_2S) and carbon dioxide (CO_2) stripped from the liquid, it was passed through two gas cleaning bottles, the first meant for collecting liquid entrained in the reactor exit gas, whereas the second (filled with a 0.5 M zinc chloride solution), intended to selectively retain H_2S was placed on a magnetic stirrer. The reactor suspension was recirculated from top to bottom to provide additional mixing and to suspend the sediment/biomass. Notably, the reactors in this study were not operated at *niche-mimicking* conditions but rather at conditions that supported high conversion rates. It was found that the AOM rate increased exponentially and a very high enrichment 1.0 mmol (g VSS d)$^{-1}$ was attained – the sMBR could thus be deemed an excellent system for growth of microorganisms mediating AOM-coupled-SR. In contrast to their marine ecological niche, the microorganisms grown in the sMBRs were continuously exposed to high shear forces due to liquid recirculation and gas sparging, and further, were suspended in the liquid phase. However, these factors did not prevent the observed exponential increase in AOM rate.

12.5 Immobilized-Cell Bioreactors

An immobilized cell may be defined as one that, by natural or artificial means is constrained in space such that it cannot independently be in motion relative to cells in its immediate vicinity. The common immobilization techniques are covalent coupling, affinity immobilization, adsorption, confinement in liquid–liquid emulsion, retention by semipermeable membranes and entrapment, of which the last named is the most frequently adopted technique for all immobilization. In immobilization by entrapment, cells are confined in a three-dimensional gel lattice made of either synthetic (e.g., acrylic, polyurethane, polyvinyl etc.) or natural polymer (e.g., collagen, agar, agarose, cellulose, alginate, carrageenan etc.). Substrate/nutrients and products can diffuse through the lattice to/from the cells. The microorganisms are entrapped onto the surfaces of the gel *beads* or *carrier particles* which are normally cross-linked (to obtain a stable immobilization matrix) by polymerization or by other means, e.g., carrageenan droplets are typically cross-linked with K^+.

The most common configuration of an immobilized cell bioreactor (Table 12.6) is the so-called PBBR, where cells immobilized on *carrier particles* or *beads* are *packed* into a matrix or bed that fills the reactor, usually a cylindrical column, and flow of nutrients/gases/products etc. usually occurs longitudinally through the beds. An alternative to cell entrapment is immobilization of cells in semipermeable membranes, which confine and thereby shield the enclosed microorganisms while facilitating transport of soluble matter (nutrients/products) to and from the cells. This technology is employed in HFBR (considered in the following section) where the growing microbial cells are constrained to one side of porous fiber strands and media containing nutrients and products on the other. However, pressure buildup due to excessive accumulation of biomass could lead to membrane rupture/fiber leakage, therefore cell growth in such membranes/fiber immobilized systems must be carefully controlled.

Table 12.6 Immobilized-cell bioreactors

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Packed bed bioreactor (PBBR)	Photo-evolution of hydrogen by polyvinyl alcohol immobilized cyanobacterium *Phormidium valderianum* (in combination with other strains)	*Bagai* and *Madamwar* [12.43]
2	PBBR	Hydrogen production by Ca-alginate/agar-agar immobilized cyanobacterium *Phormidium valderianum* (in combination with other strains)	*Patel* and *Madamwar* [12.44]
3	PBBR	Continuous production of L-glutaminase by bacteria *Pseudomonas* sp. *BTMS-51* immobilized on Ca-alginate gel	*Kumar* and *Chandrasekaran* [12.45]
4	PBBR	Continuous production of L-glutaminase by Ca-alginate-immobilized fungus *B. bassiana* BTMF S-10	*Sabu* et al. [12.46]
5	PBBR	Nitrification by NBC immobilized on polystyrene beads	*Kumar* et al. [12.47]
6	Serially connected activated PBBR	Nitrification by NBC comprising mainly *Nitrosococcus* sp. immobilized on polystyrene and low-density polyethylene	*Kumar* et al. [12.48]
7	Packed bed external loop ALBR (PBEL-ALBR)	Nitrogen compound removal from marine aquaculture wastewater by mixed (bacterial) consortia immobilized on plastic bioballs	*Silapakul* et al. [12.49]
8	Immobilized Cell Continuous ALBR	Nitification by nitrifying bacterial consortia NBC immobilized on polyvinyl alcohol PVA	*Seo* et al. [12.50]
9	Continuous-flow packed bed-photobioreactor (PB-PBR)	Nitrate removal from water by cyanobacterium *P. laminosum* immobilized on polyurethane foams	*Garbisu* et al. [12.51]
10	PB-PBR	Nitrate removal from water by microalga *S. obliquus* immobilized on polymeric foams	*Urrutia* et al. [12.52]
11	Immobilized-cell tubular PBR	Continuous synthesis of the blue-green pigment marennin by the diatom *H. ostrearia* immobilized in an agar gel layer	*Lebeau* et al. [12.53]
12	Free-cell and immobilized cell membrane photobioreactor (M-PBR)	Production of pigments marennin by the diatom *H. ostrearia*	*Rossignol* et al. [12.54]

The nonheterocystous marine cyanobacterium *Phormidium valderianum* (which is known to produce hydrogen under argon atmosphere) was immobilized in combination with two other nonmarine cells (viz., the extreme halophile *Halobacterium halobium* and salt tolerant *Escherichia coli*) for stable, long-term biohydrogen photo-evolution under an alternating light/dark illumination cycle (6 h on/18 h off) in a PBBR by *Patel* and *Madamwar* [12.44], as well as by *Bagai* and *Madamwar* [12.43].

The former workers used Ca-alginate or agar-agar as the immobilization matrix for cell entrapment in a 50 mL working volume PBBR (basically a packed column) continuously fed with N-free growth medium – comparatively better results were obtained with alginate. The problem of cell and enzyme leakage from alginate beads (leading to loss of H_2 production) was overcome by using glutaraldehyde as a cross-linking agent. H_2 photoevolution rate increased with medium feed rate up to a maximum, and then declined markedly. Stable, long-term H_2 production for 11 d was demonstrated.

Using the same combination of microorganisms, in a similar PBBR, but with a different immobilization matrix, viz., PVA cross-linked with glutaraldehyde, *Bagai* and *Madamwar* [12.43] demonstrated continued H_2 production for over 60 d. In both these studies for photohydrogen production, compared with a free-cell system, the immobilized system was much more productive, stable, and long lasting.

Sabu et al. [12.46] and *Kumar* and *Chandrasekaran* [12.45] all reported the production of the therapeutically and industrially important enzyme L-glutaminase by Ca-alginate immobilized marine microorganisms in glass-column PBBRs, but whereas the former employed the fungus *Beauveria bassiana* the latter used the bacterium *Pseudomonas* for that purpose. Although the PBBRs used in these studies differed in dimensions (i. e., column height and diameter) with the one used by *Kumar* and *Chandrasekaran* [12.45] being larger (height 45 cm and internal diameter 3.6 cm), the basic principle of reactor construction was the same – immobilized viable cell beads were aseptically packed into the column with a perforated Teflon disk placed at the top of the packing to prevent bed expansion during operation.

Sabu et al. [12.46] pumped the medium from the bottom of the packed column and collected the effluent from the top. This was, however, reversed by *Kumar* and *Chandrasekaran* [12.45]. The inlet and outlet flow rates were kept equal to maintain a constant liquid level at just above the bed level. Now *B. bassiana* being aerobic with strong aeration dependence, air was introduced through a sparger at the bottom of the packed column after passing through a bacterial filter. Remarkably, *Sabu* et al. [12.46], noted that the maximum enzyme production rate was observed in the absence of forced aeration, and any increase in aeration rate caused a decrease in enzyme productivity. In order to explain this finding, the authors posited that with external aeration, air bubbles fill the void spaces of the packing beads, thus reducing the contact between the cell-laden beads and the liquid medium. They also found that enzyme productivity increased with increasing bed height/decreasing flow rate, leading them to conclude that increase in residence time either with increase in bed height or with decrease in flow rate, in turn increases the contact time between the immobilized beads and the medium, thereby causing an increase in productivity.

Kumar and *Chandrasekaran* [12.45] reported that enzyme production declined with an increase in dilution rate, irrespective of the substrate concentration used. Again, at all dilution rates examined, enzyme yield increased with increase in substrate concentration. It was noted that their continuous-flow PBBR system could be operated for 120 h without any decline in enzyme productivity.

Kumar et al. [12.47] developed a nitrifying PBBR immobilized with an indigenous NBC (nitrifying bacterial consortium) (comprising predominantly marine species, e.g., *Nitrosospira*) for rapid nitrification in brackish water and marine hatchery systems. They examined nitrification performances of the PBBR integrated into a recirculating aquaculture system (RAS) viz., a *Penaeus monodon* (a marine crustacean – shrimp) recirculating maturation system. The RAS (Fig. 12.22) comprises a shrimp maturation tank (MT), an overhead tank (OHT), Six PBBRs (designated as R1 through R6) and a collection tank (CT). Fluid from the MT was pumped into the OHT from where it flowed through the reactors (R1–R6) connected serially by gravitation and finally collected in the CT, from where the treated water was recirculated back to the MT. Pumping was controlled by an automated water level controller installed in the OHT. A regulator valve was connected to the OHT to maintain the flow through the reactors.

All six PBBRs (R1–R6) (working volume 20 L each) were constructed identically, consisting of a fiberglass shell with a perforated Perspex plate carrying nine equidistantly fixed polyvinylchloride (PVC) pipes (air-

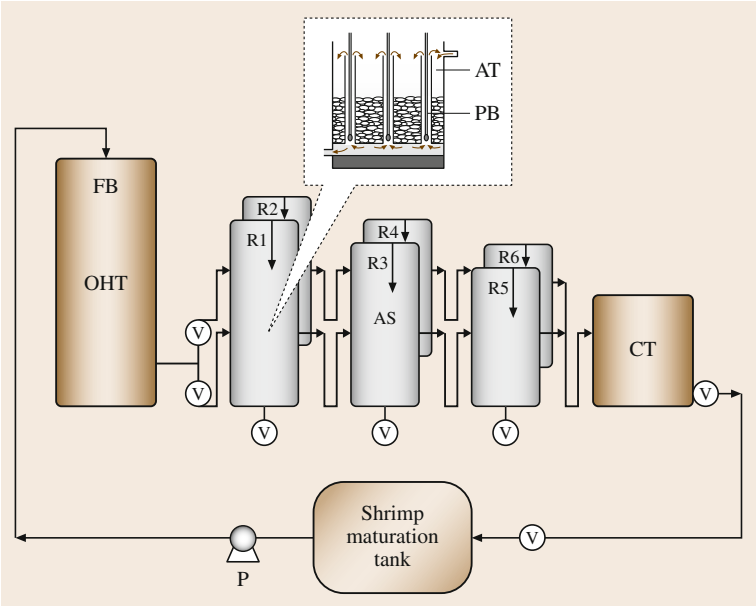

Fig. 12.22 Packed bed bioreactor connected to a shrimp maturation system. AS – aeration supply; AT – aeration tube; CT – collecting tank; FB – filter bags; OHT – overhead tank; PB – polystyrene beads; P – pump; R1–R6 – reactor R1–R6; V – valves (after *Kumar* et al. [12.47])

lift pumps) installed at the base of the reactors. On passing air, the area filled with the support medium surrounding each airlift pump works as an aeration cell. The baseplate is at an elevated location (5 cm) from the bottom, supported by PVC pipes (length 5 cm, diameter 3 cm). An inlet pipe is fixed at a water discharge height of 35 cm from the reactor base. The outflow pipe, on emerging from the base of the reactor, bends upward at a water discharge height of 35 cm from the base to the next reactor. Polystyrene beads (diameter 5 mm, surface area 0.785 cm^2) with spikes on the surface were used as the immobilization matrix – each reactor was packed with 60 000 beads. The reactors were equipped with a valve for periodical backwashing, located at the bottom. A bag filter placed in the OHT was used to filter debris from the incoming water from the MT. Significant nitrification was observed with the immobilized PBBRs integrated into the marine RAS for shrimp (*P. monodon*) maturation, over a wide range of substrate concentrations and flow rates examined. Nitrification was effective even after 70 d of operation as evident from the consistently reducing levels of total ammoniacal nitrogen (TAN).

Kumar et al. [12.48] employed two serially connected activated immobilized-cell PBBRs – one an ammonia oxidizing bioreactor (AOB) activated by ammonia-oxidizing bacterial consortia immobilized on polystyrene (PS) beads and the other a nitrite-oxidizing bioreactor (NOB) activated by nitrite-oxidizing bacteria immobilized on low-density polyethylene (LDPE), for rapid nitrification in a RAS – a brackish water hatchery system for larva production of the marine crustacean *Macrobrachium rosenbergii* (Fig. 12.23). Upon integration of the activated PBBRs with the hatchery system (thereby enabling it to operate in closed recirculating mode), ammonia removal was obtained up to BDL (beyond detectable limit) levels and consequently a substantially higher percentage survival of larvae was

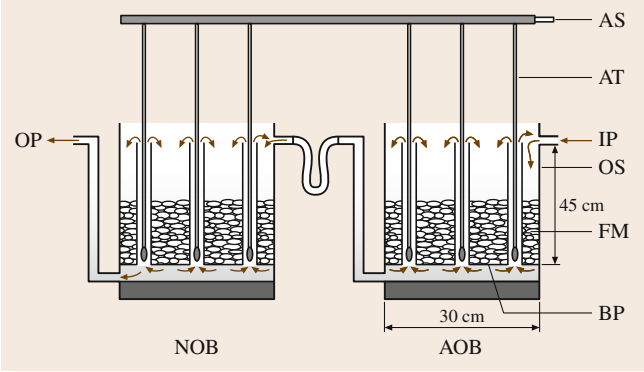

Fig. 12.23 Cross-sectional view of the bioreactors connected serially (AOB – ammonia-oxidizing bioreactor, NOB – nitrite-oxidizing bioreactor, BP – base plate, FM – filter media, OS – outer shell, IP – inlet pipe, OP – outlet pipe, AT – aeration tubes, AS – air supply) (after *Kumar* et al. [12.48])

observed. The PBBR system was energy optimized for continuous operation by limiting energy input to a single stage pumping of water and aeration to the aeration cells.

Both the PBBRs have the same configuration and are identical to the PBBRs used in *Kumar* et al. [12.47], as are the PS beads used for immobilization. The AOB and NOB reactors were connected in series. Water flowed from a large OHT into the AOB and therefrom into the NOB by gravity. The outflow from the NOB flows into a CT and from there into the larval rearing tank (LRT). From the LRT, water is pumped out into the OHT. The major components of the nitrifying bacterial consortia (NBC) were the marine genus, viz., *Nitrosococcus* and *Nitrobacter*. A noteworthy operational flexibility of the PBBRs was that they were interchangeable between prawn (salinity $15\,g\,L^{-1}$) and shrimp (salinity $30\,g\,L^{-1}$) larval rearing systems simply by changing the NBC based on salinity.

Silapakul et al. [12.49] examined a novel packed bed external loop airlift bioreactor (PBEL-ALBR) (volume 60 L) as an integrated system with simultaneous nitrification/denitrification for treatment of marine aquaculture wastewater containing ammonia and nitrate compounds. The PBEL-ALBR consisted of both aeration and nonaeration zones in the same unit, that served as nitrification and denitrification compartments (where ammonia and nitrate were biodegraded, respectively), which were packed with plastic bioballs to increase the surface area for microbial attachment, on the surface of which nitrifying and denitrifying microorganisms were immobilized.

The reactor (Fig. 12.24) consists of one aerated column (riser) interconnected by conduits with two unaerated columns (downcomer) – the cross-sectional area of the downcomers being almost 10 times larger than the riser to ensure adequate retention time for denitrification which is known to require $\approx 5-10$ times longer reaction time than nitrification. The aerated and unaerated columns were packed with 200 and 2000 bioballs (per column), respectively. A porous gas sparger for air dispersion was located at the bottom of the aerated column. The airflow rate was determined as a minimum that could induce liquid circulation between the aerated and unaerated sections. The recirculation of water was driven from the aerated riser where water moved up as aeration was provided and down through the unaerated downcomer. In the riser, nitrifying bacteria fed on wastewater containing high dissolved oxygen, oxidizing ammonia to nitrate. The low dissolved oxygen effluent from the riser flowed through the downcomer where denitrifying bacteria removed the nitrate. The authors noted that no nitrite/nitrate accumulation occurred in any experiment, indicating rapid and effective denitrification. Overall, the reactor performance was satisfactory and comparable with other treatment systems, however, where the PBEL-ALBR stands out is in providing nitrification and denitrification in a single setup without requiring a two-reactors-in-series cascade.

Seo et al. [12.50] examined the nitrification performance of an NBC (comprising mainly *Nitrosomonas* species) immobilized in boric acid treated PVA beads, for a marine RAS, by employing a continuous immobilized-cell ALBR (volume 45 L) for acclimation of the NBC from activated sludge and estimation of ammonia removal rate. In addition, four three-phase, completely mixed, fluidized bed-type bioreactors (FBBR) (volume 2.5 L each) were also employed for acclimation of the immobilized nitrifiers from freshwater to seawater system, with increasing salt concentration. Immobilization in support gel is an effective technique for maintaining high cell density and preventing washout of slow-growing autotrophic nitrifiers (ATN) under a low water temperature or high water flow rates. Although ATNs are sensitive to low temperatures, immobilization (a) improves tolerance to low temperatures and also, (b) provides protection against various toxic agents. In the operational range of values for the HRT, an optimum HRT was found for the marine nitrification process at which the highest ammonia removal rate was reached.

The four reactors were used to determine nitrification activity at four different salt concentrations. Each FBBR consisted of two separate compartments – one for beads settling and the other for airlift of the beads. Air was supplied through acrylic pipes (0.1 vvm). Influent NH_3 concentration was maintained at $10\,mg\,L^{-1}$. Nitrification activity decreased during the immobilization process due to centrifugation, transportation by pump, shear stress arising out of stirring, toxicity from the immobilization support material, and low pH of the boric acid solution. However, nitrification activity gradually recovered with operating time. It was observed that, nitrification activity of recovered nitrifier beads was higher than that of free nitrifiers due to high ammonia loading.

Polysaccharide-based gels and reticulate foams are usually considered as the preferred immobilization matrices for photosynthetic cell systems. Although five major immobilization techniques (viz., entrapment, microencapsulation, covalent coupling, aggregated cells, and adsorption) are in vogue, for growth of photosyn-

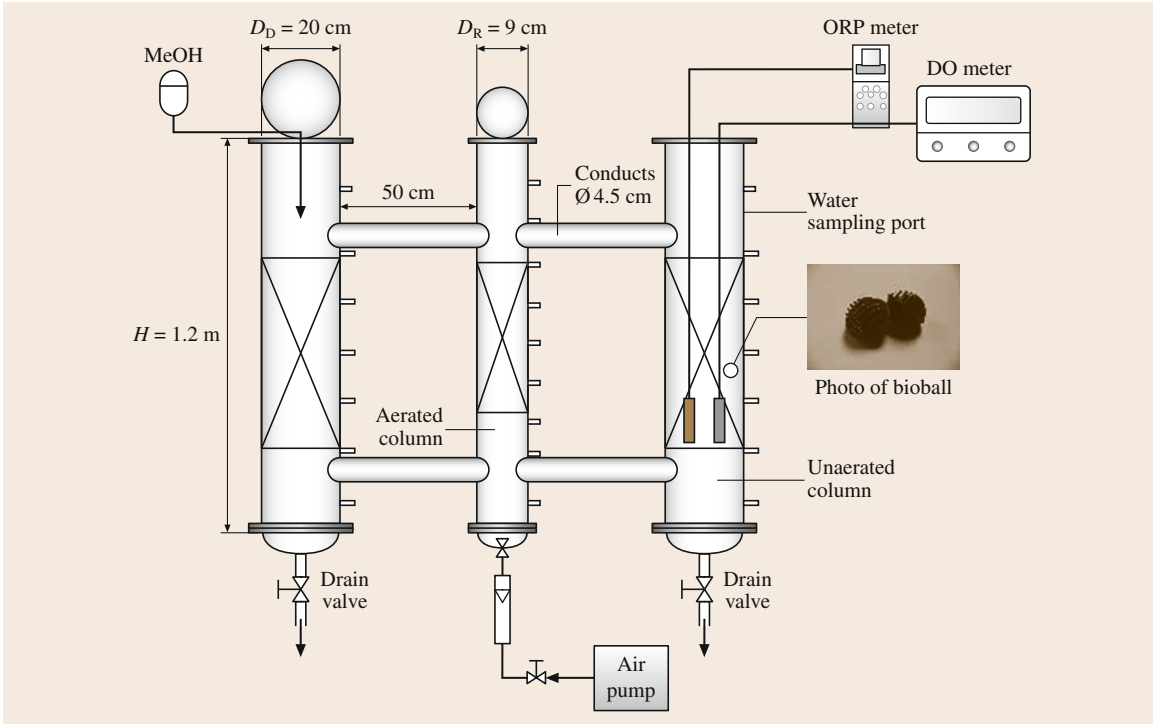

Fig. 12.24 Experimental setup (after *Silapakul* et al. [12.49])

thetic cyanobacteria in foam pieces, immobilization is often carried out by adsorption of cells on the foam surface in addition to entrapment in foam cavities. A continuous-flow packed bed photobioreactor (PB-PBR) was employed by *Garbisu* et al. [12.51] to study nitrate removal from water by cells of the non-N_2-fixing, filamentous, thermophilic cyanobacterium *Phormidium laminosum* immobilized in (a) polyurethane (PU) foams either by (i) absorption, or by (ii) entrapment in the PU prepolymer followed by polymerization, as well as in (b) polyvinyl (PV) foams by adsorption. Although entrapment repeatedly caused toxicity problems leading to rapid cell death, cells could be effectively immobilized by adsorption onto PU/PV foams and maintained their photosynthetic electron transport activities for at least 7 weeks. *P. laminosum* being thermophilic grows at 45 °C, a temperature too high for most other contaminating species, and further, has the ability to utilize either nitrate, nitrite or ammonium as the sole N-source – thus affording great flexibility and efficiency in the water treatment process. It was demonstrated in this study that *P. laminosum* immobilized on polymer foams is of potential value for biological nitrate removal (BNR) from water in a continuous-flow packed bed column PBR.

Urrutia et al. [12.52] obtained almost similar results (similar to *Garbisu* et al. [12.51]) in investigating BNR from water in a continuous packed bed column PBR with immobilized cells, but employing the green, mesophilic, unicellular, chlorophycean microalga *Scenedesmus obliquus*. Cells were immobilized by (a) entrapment, using urethane prepolymer as well as by (b) adsorption in preformed PU/PV foams, the latter deemed more convenient and effective, particularly when cells were subjected to nitrogen starvation, which also caused substantial increase in N uptake rate of immobilized cells. The study established the effectiveness of *S. obliquus* cells immobilized on hydrophilic polymeric foams grown in packed column photobioreactors, in N removal from water. Notably, polymeric foam immobilization has the particular advantage that at any point, after deimmobilizing the cells by simply squeezing the foam, it can be recycled and reused for further cell immobilization almost indefinitely.

Lebeau et al. [12.53] reported the continuous synthesis of the blue-green pigment marennine (with potential anticancer applications) by the marine diatom

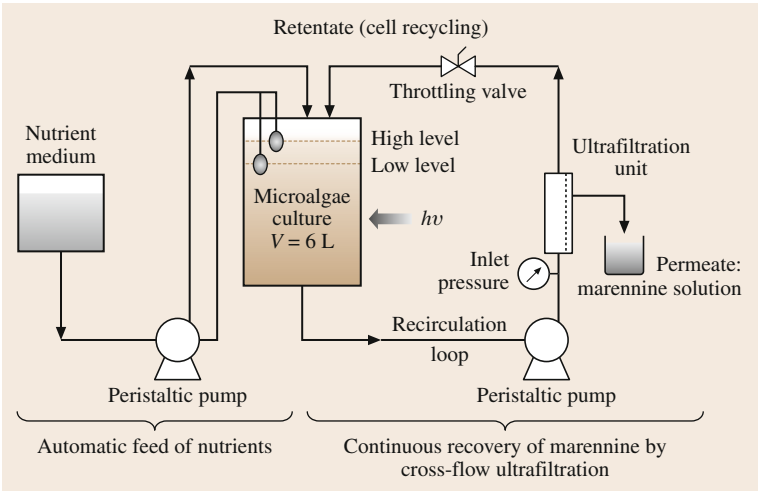

Fig. 12.25 Schematic of the free-cell M-PBR (after *Rossignol* et al. [12.54])

Haslea ostrearia in an immobilized-cell tubular membrane PBR (with internal illumination by optical fiber), described by *Rossignol* et al. [12.54]. Now, in its marine ecological niche, as the diatom migrates from the planktonic to the benthic phase, the number of pigmented cells increase, becoming maximum in the benthic phase where the cells are immobilized in a natural matrix formed from their own exopolysaccharides. Artificial immobilization, as reported in this study, was essentially an attempt to mimic this ecological niche.

The PBR was operated with 10 L of media. Agar immobilized cells were fed continuously (at a dilution rate = $0.025\,d^{-1}$ during the first experiment and at $D = 0.25\,d^{-1}$ during the other two experiments). The PBR was placed in an air-conditioned room with the temperature maintained at 15 °C. The immobilized-cell layer was illuminated by a barrel of optical fibers connected to a 150 W light generator, with a 14 h/10 h light illuminated cycle. The reactor has a large surface/volume ratio and efficient lighting is provided by the evenly distributed optical fibers. The *cold* light source is particularly suitable for *H. ostrearia* whose cultivation temperature is quite low.

Rossignol et al. [12.54] have compared two membrane photobioreactors (MPBRs), with free and immobilized cells, respectively, for the exocellular production of the blue-green, hydrosoluble pigment, marennine by the marine diatom *H. ostrearia*. In the former, cells are free and grown in a recycle PBR with total biomass recycling coupled to a membrane ultrafiltration system (external loop). In the latter, i. e., the immobilized cell tubular membrane PBR, essentially a glass and S.S. cylinder (volume 10 L) cells are immobilized in a tubular agar gel layer (surface area 955 cm^2, volume 550 cm^3) placed in liquid culture medium with an optical-fiber barrel (as a source of artificial illumination) running through the inner annular space of the tubular agar gel and an external microporous membrane forming an outer sheath around the gel layer. Major drawbacks of the free cell continuous recycle system are membrane fouling and hydrodynamic shear stress on the shear-sensitive microalgal cells, which may be somewhat overcome by immobilization that essentially mimics the marine ecological niche of these cells – the number of pigmented cells increase as the algae migrate from the planktonic to the benthic phase becoming maximum as the cells are immobilized in a biofilm matrix formed by their excreted exopolysaccharides. PBRs were housed in a 15 °C air-conditioned room and illumination followed a 14 h/10 h light/dark cycle. The respective reactor setups are shown in Figs. 12.25 and 12.26. The free-cell photobioreactor (FCB) consisted of a glass cylinder integrated with an external flat – membrane module (to perform tangential filtration) comprising a plane polyacrylonitrile (PAN) membrane (MWCO 40 kDa, area 100 cm^2). Although the retentate loop was closed, sampling could be done as scheduled. To maintain a constant volume, sterile nutrient solution was fed into the bioreactor, as the permeate was extracted, using a peristaltic pump. Product (marennine) concentrations and volumetric productivity were found to be much higher for the free-cell system, however specific (i. e., cell number based) productivity was larger for the immobilized cell system due to the decreased cell numbers in the latter at the end of the culture.

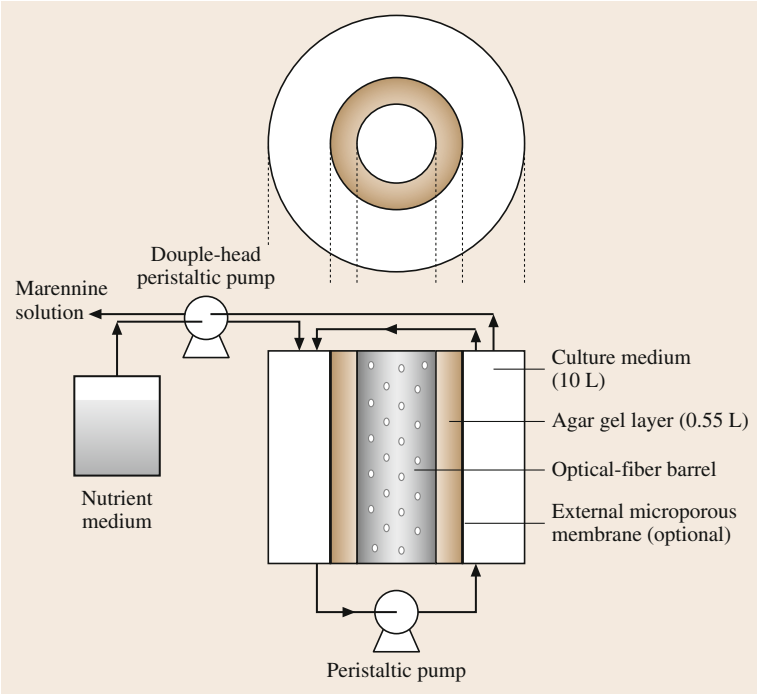

Fig. 12.26 Schematic of the immobilized-cell M-PBR (after *Rossignol* et al. [12.54])

12.6 Hollow Fiber Bioreactors (HFBR)

In an HFBR (Table 12.7), hundreds of thousands of hair-like hollow fibers (which are essentially semipermeable membranes) are bundled together within a tubular/cylindrical casing to form a cartridge. The cartridge is then linked to a perfusion system which circulates nutrient media for cell growth continuously through the fibers. Cells are usually inoculated into the so-called *extra capillary space* or shell-side space external to the fibers within the cartridge, whereas culture media is circulated through the *lumen* of the fibers (i. e., the middle of the fibers), allowing nutrients, dissolved gases and metabolic wastes to diffuse across the fiber walls. HFBRs are characterized by extremely high surface-to-volume ratio (> 150 cm^2) and support surface-attached growth of cells bound to a porous matrix whose MWCO (molecular weight cut-off) may be varied. Hollow-fiber reactors may be operated in direct mode, where growth media is passed through the fiber lumen, or in the transverse mode as done by *Lloyd* et al. [12.55], where growth medium is passed over immobilized cells, and spent medium with products collected after passage of the liquid through the semipermeable membrane of the hollow fibers. Cells are retained in the HFBR unless the hollow fibers rupture or the end-seals fail.

Table 12.7 Hollow fiber bioreactors (HFBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	HF-MBR	Production of recombinant toxoid by bacteria *Vibrio* sp. immobilized on hollow-fiber membrane.	*Lloyd* et al. [12.55]
2	HF-sMBR	Growth of marine rotifer *Brachinus plicatilis* immobilized on Ca-alginate	*Rombaut* et al. [12.56]
3	HF-sMBR	Oily water treatment by bacterial consortium	*Soltani* et al. [12.57]
4	Microfiltration HFBR	Enhancing cell yield of thermoacidophillic archaeon *S. solfataricus*	*Schiraldi* et al. [12.58]
5	HF-PBR	Nitrate and phosphate removal by non-N_2-fixing cyanobacterium *P. laminosum* immobilized on hollow fibers	*Sawayama* et al. [12.59]

An HFBR permits high cell densities to be attained in a practically nonshear environment with mild nutrient perfusion. Bacterial cell densities between 100 and 200 g L^{-1} have been commonly attained in HFBRs. However, the exceptionally high density of 550 g L^{-1} was reported [12.60] for a rifamycin-producing strain of *Nocardia mediterranei* (later renamed *Amycolatopsis rifamycinica*) grown in a dual hollow-fiber bioreactor. It is this ability to establish high cell densities while operating with continuous nutrient supply and product removal, that gives rise to higher product yields. HFBRs have been shown to work well with bioprocesses that result in the synthesis of products from major metabolic pathways, e.g., glycolysis, but, they do not work very well with processes that involve metabolic pathways of apparently less significance to the producer microbe.

Lloyd et al. [12.55] used an HFBR for production of a recombinant toxoid by a marine *Vibrio* species and compared it with recombinant protein synthesis in traditional batch and chemostat cultures. The type of fiber used significantly affected productivity, both with regards to maintenance of reactor integrity and by allowing passage of the toxoid through the selectively permeable membrane. A hollow-fiber-based system was selected for this comparative study because it offers easy immobilization and growth of recombinant cells, in addition to the potential to select fibers with permeability properties that could help improve the purity of the product obtained in the outflow from the reactor, thus maximizing the exploitation of the secretory capabilities of the *Vibrio* species cultured. Fibers were embedded in polyacetal end plugs, but spe-

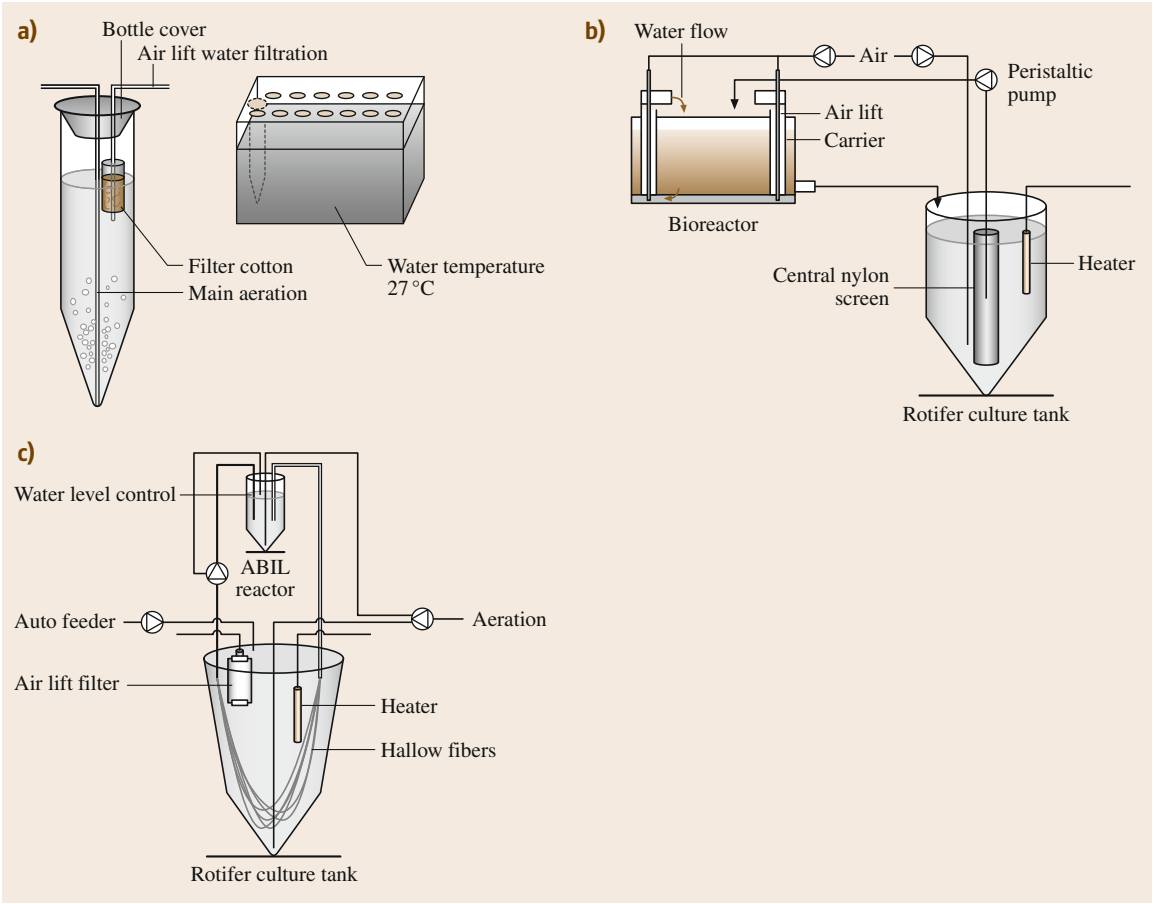

Fig. 12.27a–c Schematic of the experimental setup: **(a)** Batch reactor to test ABIL for rotifers. **(b)** Bioreactor setup with four different carrier materials. **(c)** Integration of an ABIL reactor with hollow fibers to the rotifer culture system (after *Rombaut* et al. [12.56])

cific microfibers were embedded in S.S. tubes (length 2 cm, I.D. 10 mm) using adhesive resin for holding the fibers in place during reactor operation. Medium supply was through the shell-side port. Air was supplied in a transverse mode through the fiber lumen. (It may be noted that air supply may also be done in direct mode through the shell side space.) Growth was evaluated by using a pressure transducer, which monitors the pressure differential between the inside and outside of the fibers and by measuring the protein content of fibers at the end of an incubation.

Rombaut et al. [12.56] used a dense, nitrifying inoculum culture (ABIL) for stimulating growth of the marine rotifer *Brachionus plicatilis* in several bioreactor systems, viz., batch culture, continuous bioreactors packed with carrier materials (a PVC matrix, gravel and $CaCO_3$ were evaluated separately) and a submerged hollow-fiber membrane bioreactor (HF-MBR).

A series of rectangular shaped bioreactors (Fig. 12.27) (total volume of one tank, 10 L) were each filled with 4 dm^3 of carrier material and 4 L of artificial seawater (25 $g\,L^{-1}$ salinity). The bottom of each tank was fitted with a cover (with holes drilled on it) for retaining the carrier material, through which two airlifts were installed for oxygen supply and for recirculation of the aqueous phase. $CaCO_3$ stones (2.0 ± 0.7 mm), gravel (16 ± 2 mm), a mixture of both types of stones, and a PVC-based carrier material (viz., Bionet) were examined as biomass carrier materials, however for the rotifer production periods, only the crushed $CaCO_3$ stones were used. After the start-up period of 11 d, water from the different bioreactors was drained out of the system and replaced by new diluted seawater. Except for the reactor packed with Bionet, the bioreactors were connected to a cylindro-conical culture tank of 10 L, well aerated to ensure good oxygenation and uniform distribution. The culture-rearing tank was filled with artificial seawater and maintained at a constant temperature ($25 \pm 1\,°C$). A central nylon screen (mesh size 33 µm) sieve was installed in the center of each tank to retain the rotifers and also to enable pumping of the culture water (using a peristaltic pump) to the bioreactors. The water returned by gravity to the rotifer culture tanks.

For the experiments with HF-MBR, conical PVC tanks were used for rotifer culture which were filled with 10 L of artificial seawater. Hydrophilic hollow fibers (nominal pore size 0.2 µm; wall thickness 450 µm; inner diameter 1800 µm), adjusted to pieces of 1 m and 10 fibers (total surface area = 0.078 m^2), were installed in each culture tank. The ends of the hollow fibers were connected to the ABIL reactor, inside which filter cotton was placed at the bottom to collect large debris. Water and bacteria from the ABIL reactor were pumped through the hollow fibers (using a peristaltic pump). The rotifer feed was stored at temperatures $< 10\,°C$ and was fed (15 $min\,h^{-1}$) by a peristaltic pump. The rotifer culture tanks were continuously aerated and the temperature was controlled by a heater at 28 °C. Results showed that concentrations of the major nitrogenous waste, viz., TAN in the control batch culture was much higher than that in the HF-MBR recirculating system. Since the principal objective of the bioreactor systems evaluated is the removal of TAN, thereby facilitating vigorous rotifer growth, the improved water quality obtained with the HF-MBR system resulted in much higher rotifier densities compared to the normal batch culture.

Soltani et al. [12.57] demonstrated that a hollow-fiber submerged MBR (HF-sMBR) could be used very effectively for oil removal from oilfield wastewaters using a marine bacterial consortium isolated from sea sediments and adapted to growth environments containing high amounts of salt and oil. The reactor setup (Fig. 12.28) consisted of a hollow fiber membrane module (made from polypropylene with pore size ≈ 0.2 µm) submerged in a feed tank. Permeate was withdrawn by a vacuum pump, its flow rate monitored by a rotameter and adjustable by operating a valve placed before the pump. Oxygen necessary for microbial growth and aeration of the membrane were supplied by two air diffusers, placed below the membrane module. Remarkably, 100% oil removal in the permeate was accomplished. Furthermore, although isolated from a typically saline marine ecological niche, the bacteria didn't lose their oil-degrading ability even in the total absence of salt in the culture medium – leading the authors to classify them as halotolerant.

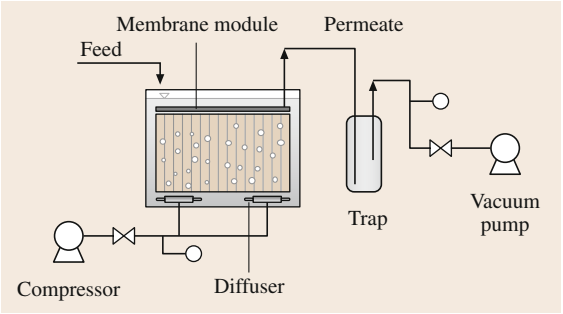

Fig. 12.28 A schematic of the HFBR experimental setup (after *Soltani* et al. [12.57])

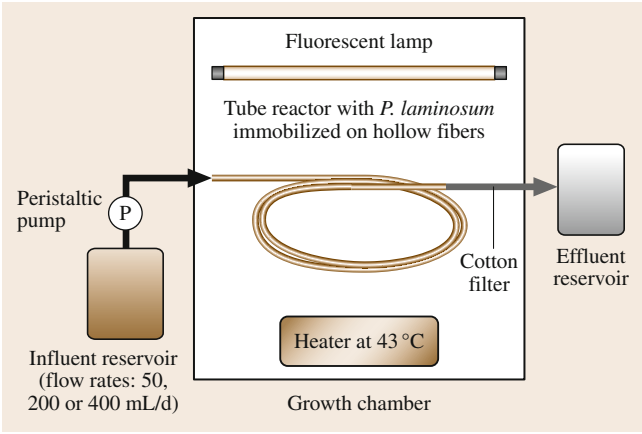

Fig. 12.29 Schematic diagram of the hollow-fiber photobioreactor (after *Sawayama* et al. [12.59])

Schiraldi et al. [12.58] used a hollow fiber microfiltration (MF) bioreactor (HF-MFBR) for high cell density growth of the thermoacidophilic archaeon *Sulfolobus solfataricus* – with the MF-hollow fiber module located inside a traditional fermentation vessel. They obtained biomass yields about 15–20 times higher than in batch culture, and about 6 times that of fed-batch culture. Furthermore, the MF module was found to be highly resistant to the extreme environments typical of thermoacidophile fermentation. Despite the high cell densities attained, transmembrane flux was consistently maintained (with repeated backflushing) at 75% of the maximum which is quite remarkable compared to usual crossflow filtration where flux declines to 20% of the maximum within 30 h of operation. To obtain the same amount of biomass, whereas at least 2000 h of batch time is necessary (ignoring the turnaround time between batches) only about 15% of this time is required with the HF-MFBR.

The polypropylene membrane used consisted of a long capillary (5–10 m) with an inner diameter of 0.2 mm and a cutoff of 0.22 µm. A number of 15 cm-long capillaries were obtained from the original membrane and were assembled together with a silicon adhesive; one side was closed and the other was sealed to a connector. The module was connected to a peristaltic pump equipped with a silicon tube (I.D. 2 mm), which provided the driving force for transmembrane flux. Considering the geometry of the module, the total filtering area measured 1.47×10^{-2} m^2. Before operating the module, the membranes were treated with a 70% ethanol solution for 1 h to make them hydrophilic. The microfiltration module was then placed inside the fermentation vessel and fixed vertically to a baffle in order to expose it (i. e., the module) to high turbulence near the filtering surface for minimizing fouling.

Sawayama et al. [12.59] studied nitrate and phosphate removal from water by the non-N_2-fixing cyanobacterium *P. laminosum* immobilized on cellulose hollow fibers in a tubular photobioreactor at 43 °C, continuously supplying dilute growth medium for 7 d and then secondarily treated sewage (STS) for 12 d. Cyanobacteria can grow much faster than higher plants under appropriate conditions, therefore, cyanobacteria-based inorganic nutrient removal systems are worth exploring. Furthermore, with thermophilic cyanobacteria, contamination can be avoided since it is tolerant of high-temperatures permitting sewage treatment at temperatures (≈ 45 °C), which cannot be tolerated by most other contaminating microbial species. Hollow fiber-immobilized cyanobacterial systems are easy to construct and immobilization is relatively simple. The bioreactor (Fig. 12.29) comprised a transparent PVC tube containing cellulose hollow fibers (used as the immobilization matrix), a peristaltic pump, a heater and two cool, white fluorescent lamps. Cells were immobilized on 600 cellulose hollow fibers (length 0.5 m, internal diameter 0.18 mm, total surface area 0.19 m^2) and placed in a PVC tube (length 0.5 m, I.D. 16 mm) with a total reactor volume of 100 mL. Immobilization was carried out by addition of cell culture (2 mg total chlorophyll) to the reactor using a peristaltic pump. Two days after cell inoculation, diluted medium (at a flow rate of 200 or 400 mL d^{-1}) or STS (at a flow rate of 50 mL d^{-1}) was passed through the HFBR at 43 °C (±1 °C) continuously illuminated with fluorescent light. The quantity of immobilized cyanobacterial chlorophyll in the bioreactor was 1.7–1.9 mg after 24 h of influent flow. *P. laminosum* cultured in the continuous HF-PBR removed nitrate and phosphate ion from water quite efficiently, however nitrate ions present in STS could not be removed satisfactorily. Again, compared to chitosan immobilized cells used earlier, P removal was higher (more than double) in this HF-PBR but N removal was significantly lower (less than half).

12.7 Fluidized Bed and Moving Bed Bioreactors (FBBR and MBBR)

12.7.1 Fluidized Bed Bioreactors (FBBR)

An FBBR (Table 12.8) is a reactor consisting of a *bed* of particles/carriers such that the particles are not in constant contact with each other (i. e., they are in motion resembling that of fluid particles) due to the flow of a fluid through them. If the component particles of the *bed* are indeed in continuous contact with each other, the bed is referred to as a *packed bed*. Often, the term *expanded bed* is used to describe a bed that has expanded just marginally above the corresponding *packed bed* height, i. e., above the *settled height* – the condition when the bed particles have *settled down* and are NOT in motion. The term fluidized bed is usually used for beds that have undergone significant expansion, so that *fluidization* of the bed has occurred – i. e., the bed particles are in continuous motion as in case of a fluid. Fluidization involving liquids and solid particles is commonly particulate characterized by smooth expansion of the bed, as opposed to *aggregative* characterized by violent bubbling and particle motion typical of gas–solid fluidization. FBBRs are essentially immobilized-cell reactors. Individual cells being very small and light and with a settling velocity less than the superficial liquid velocity are washed out, whereas carrier particles with cells immobilized on them have much larger settling velocity and are retained in the bed, which allows operation of FBBRs at dilution rates much higher than the cell specific growth rate. Compared with stirred-tank immobilized reactors, the shear at the particle surface is much lower in an FBBR, which actually poses a problem with bacteria and yeast because they can continue to grow as a biofilm around the particle. Increasing biofilm volumes forces the bed to expand and the steady state, where cell growth equals the rate that cells are washed off by surface shear is attained, only with a very large bed height.

Cytryn et al. [12.61] investigated the diversity of microbial communities correlated to physiochemical parameters in a digestion basin (DB) of a zero-discharge experimental mariculture system that integrated conventional nitrification with a combination of sludge digestion and denitrification. The integrated system comprises an intensively stocked fish basin with two parallel biofiltration loops (Figs. 12.30 and 12.31): (1) a trickling filter, where ammonia is oxidized to nitrate; and (2) a digestion/sedimentation basin connected to an FBBR for organic degradation and denitrification. Surface water from the basin was pumped over the trickling filter in one loop while bottom water was recirculated through a sedimentation basin followed by the FBBR in the other. Ammonia oxidation to nitrate in the trickling filter and organic matter digestion coupled with nitrate reduction in the combined sedimentation basin–FBBR loop, allowed zero-discharge operation of the integrated system. Chemical activity and microbial diversity were higher in the sludge layer than in the overlying aqueous layer of the basin. Chemical parameters in sludge samples close to the basin inlet indicated enhanced microbial activity relative to other sampling areas with evidence of both nitrate and sulfate reduction.

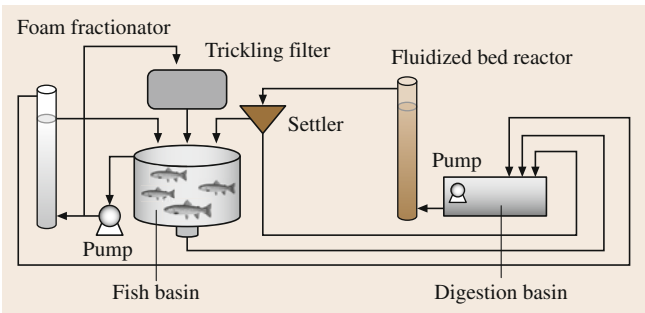

Fig. 12.30 Schematic diagram of the recirculating zero-discharge mariculture system (after *Cytryn* et al. [12.61])

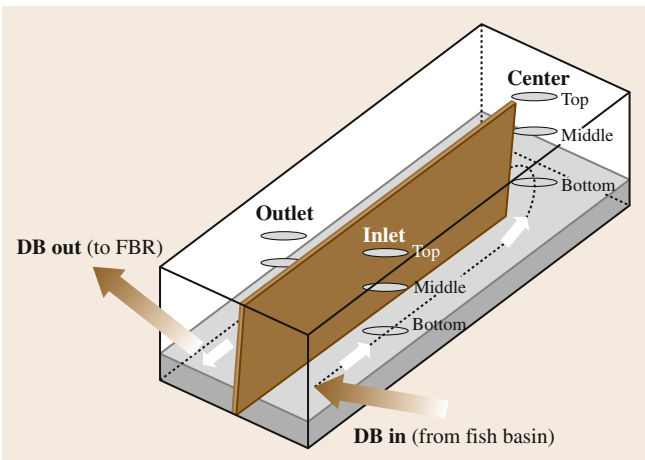

Fig. 12.31 Schematic diagram of the digestion basin. Sampling stations (inlet, center, and outlet) and sampling depths (top, middle, and bottom) are depicted by *ellipses*. *Gray area* indicates the underlying sludge layer of the basin (bottom 5 cm) and the *clear area* indicates the overlaying aqueous phase (top 10 cm). *Arrows* and *dotted lines* indicate the flow direction (after *Cytryn* et al. [12.61])

Cytryn et al. [12.62] integrated microscopic and molecular microbial analyses to characterize rapidly developing white filamentous tufts in an FBBR (used earlier by *Cytryn* et al. [12.61]) for nitrate removal from a marine recirculating aquaculture (fish culture) system – RAS. The dominant constituents of these tufts were identified as filamentous, gram-negative bacteria with densely packed intracellular sulfur granules belonging to the marine *Thiothrix* genus. Water from the upper part of a double-drain fish basin (2.3 m^3), stocked with Gilthead Seabream (fish) to a final density of 20–50 $kg\,m^{-3}$, was recirculated at a rate of 1–2 fish basin volumes per hour over a trickling filter. Simultaneously, water from the bottom center of the fish basin flowed into a digestion basin (DB) (working volume 0.4–0.5 m^3) at a rate of 0.1–0.25 fish basin volumes per hour. Water from the upper layers of the DB outlet was pumped (5–7 $L\,min^{-1}$) into the cylindrical FBBR through a vertical pipe that extended from the top center to ≈ 3 cm above the base of the FBBR. The FBBR (working volume 6.26 L) was filled with sand (average diameter 0.7 mm), as carrier material for growth of microbial biofilm. Upflow from the inlet pipe caused the flocs (biofilm-attached sand grains) to float within the column. Water from the FBBR outlet at the top of the cylinder was drained back to the fish basin after passing through a settler for removal of particulate matter, which was funneled back into the DB.

Schramm et al. [12.63] analyzed the change of activity and abundance of *Nitrosospira* and *Nitrospira* spp. along a bulk water gradient in a nitrifying FBBR, by a combination of microsensor measurements and fluorescence in situ hybridization (FISH), which facilitated a detailed analysis of the in situ structure and function of the nitrifying bioreactor on a microscale. The conical, continuous-upflow FBBR (originally developed by *de Beer* et al. [12.64]) (Fig. 12.32) (volume 360 mL, height 0.8 m, I.D. 1 cm at the bottom, 3 cm at the top) was covered with black paper to prevent phototrophic algal growth. It was fed with mineral medium containing 72 mM NH_4^+ and the liquid phase was recirculated at a rate of 1.8 $mL\,s^{-1}$. The conical shape of the vertical reactor column generates a flow velocity gradient that stabilizes nitirifying bacterial aggregates of different diameters and densities at fixed positions in the column according to their settling velocity. Nitrification occurred in a narrow zone of 100–150 mm on the surface of the aggregates, that contained a very dense community of nitrifying bacteria. The central part of the aggregates was inactive, containing significantly lesser number of nitrifiers. Since conditions in the reactor were ammonium limiting, it was completely oxidized to nitrate within the active layer of the aggregates, the rates decreasing with increasing reactor height. Thus, this study, as well as the one carried out by *de Beer* et al. [12.64] demonstrated the successful operation of a two-phase (liquid–solid) fluidized-bed nitrification reactor with an external aerator, for development of nitrifying bacterial aggregates. Most importantly, the much lower shear forces generated, relative to those in three-phase systems, facilitated development of bacterial aggregates without addition of carrier materials.

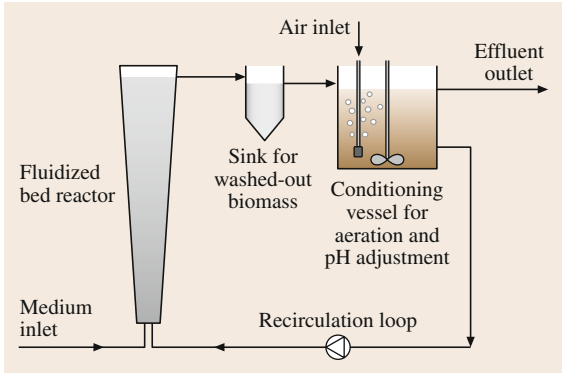

Fig. 12.32 Scheme of the nitrifying reactor setup (after *de Beer* et al. [12.64])

Garbisu et al. [12.65] used free and polyvinyl foam-immobilized cells of the cyanobacteria *P. laminosum* for phosphate removal from water in batch and flow-type FBBRs as well as in continuous flow PBBR. Phosphate uptake by nitrogen starved free-living cyanobacteria showed that N-starvation led to lower uptake rates, but addition of nitrate significantly increased the uptake. Phosphate uptake by free as well as immobilized cyanobacteria was inhibited in the dark and simulated by the presence of calcium ions or bicarbonate. Addition of a chelating agent, such as ethylenediaminetetraacetic acid to the cultures, led to zero phosphate uptake. Although high phosphate uptake was not observed with cyanobacteria immobilized on polymer foams, concurrent removal of nitrate and phosphate from water using nitrogen-starved immobilized cyanobacteria showed sufficient potential for future development.

12.7.2 Moving Bed Bioreactors

Although FBBRs ensure homogeneous environmental conditions for all biofilm surfaces and enhance mass transfer rates, nevertheless, both for upward and down-

ward fluidization, the active volume is low and fluidization performance may deteriorate over time and may not remain at the optimum level, primarily due to the changing density of the carriers with biofilm growth on their surfaces. An effective alternative to an FBBR is an MBBR (Table 12.8), which has carrier particles that are continuously in motion. As the liquid circulates, the carriers move through the liquid between two retention grids at two ends of the reactor and are agitated either mechanically or hydrodynamically by liquid jets. High shear stress from water circulation coupled with interparticle collision prevents uncontrolled proliferation of biofilm on the carrier surface and thereby stabilizes the reactor system.

An MBBR is thus *self-cleaning* and has low head loss. In fact, MBBRs with up to 60% of the working volume occupied by carriers have been used and found to perform satisfactorily.

A pilot scale, submerged MBBR was installed at the Montreal Biodome (a 3.25 million closed-circuit mesocosm) to deal with the constraints of seawater denitrification by an attached-growth process, with an aim to optimize a large-scale MBBR. *Labelle* et al. [12.66], evaluated the seawater treatment performance of the reactors, using methanol as carbon source at different C/N ratios and examined its efficiency in preventing sulfate reduction by limiting biofilm thickness, dead mixing zones and residual substrate in the outflow.

Two well-mixed bioreactors in series (Fig. 12.33) (working volume 110 L each, diameter 43 cm and with conical bottom) were used for deoxygenation and denitrification, respectively. Methanol was fed to both reactors (C-source) and a trace element solution to the second one, the latter intended to maintain iron, manganese, and copper concentrations over 150, 80 and 10 $\mu g\,L^{-1}$, respectively. Indigenous biomass in seawater was used to colonize carriers in the two-stage bioreactor system. The first stage, a pretreatment deoxygenation unit, was actually a packed bed biofilter packed to 80% of its total volume with 63 mm random plastic carriers (specific surface area 180 $m^2\,m^{-3}$, void fraction 91%, specific gravity $\approx$ 0.90). The second stage, a denitrification unit, consisted of a submerged MBBR configured to minimize media fouling and dead

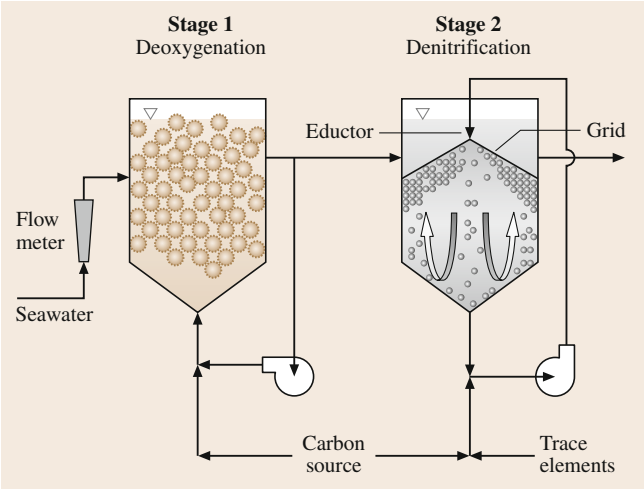

Fig. 12.33 Schematic diagram of experimental setup: Two CFSTR in series for deoxygenation and denitrification, respectively (after *Labelle* et al. [12.66])

Table 12.8 Fluidized bed and moving bed bioreactors (FBBR and MBBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	FBBR	Digestion of marine waste by mixed bacterial consortium	*Cytryn* et al. [12.61]
2	FBBR	Cultivation of a novel filamentous bacteria *Thiothrix* sp.	*Cytryn* et al. [12.62]
3	FBBR	Nitrification by *Nitrosospira and Nitrospira* sp., immobilized in bacterial aggregates.	*Schramm* et al. [12.63]
4	Batch and continuous-flow FBBR	Nitrate removal from water by cyanobacterium *P. laminosum*	*Garbisu* et al. [12.65]
5	Submerged MBBR	Denitrification of seawater in a closed circuit mesocosm	*Labelle* et al. [12.66]
6	Downflow MBBR	Denitrification of seawater by mixed bacterial consortia from a marine mesocosm	*Dupla* et al. [12.67]
7	MBBR	Harvesting anaerobic ammonium-oxidizing (anammox) bacteria in biological filter and waste sludge compartments of a marine RAS	*Tal* et al. [12.68]
8	Sequencing MBBR	Denitrifying biological phosphorus removal (BPR) in wastewaters by mixed bacterial consortia	*Vallet* et al. [12.69]

mixing zones. The 2.5 cm spherical polyethylene carriers used in the second stage had a fill ratio of 25%, a surface area of 278 $m^2\,m^{-3}$, a void fraction of 90%, and a specific gravity of 0.89. Effective surface area for biofilm attachment was estimated at ≈ 35% of the total surface area (i. e., 97 m^2/m^3) since no growth was observed on the outer parts of the moving carriers. Carriers were kept submerged by a conical welded stainless steel wire mesh (5 mm openings, wire diameter of 0.56 mm) and mixing was ensured by the downflow jet of a 9.5 mm glass-filled polyethylene eductor positioned vertically over the conical grid. The single-jet configuration was designed to generate adequate hydrodynamic shear to prevent carrier fouling and to create a hydraulic movement minimizing the surface diffusion of oxygen in the MBBR. High sulfate concentration in seawater complicates denitrification in attached-growth processes. Natural, anoxic marine environments where sulfate reduction occurs are associated with N_2O production. However, the study demonstrated that a pilot-scale sMBBR could efficiently denitrify seawater/saline water with a high sulfate concentration.

In seawater aquariums, nitrates are produced in the biological aerobic treatment units by nitrification of excreted ammonia, which is toxic in its unionized form. The recommended level of nitrates in an artificial seawater ecosystem is 50 mg NO_3-N L^{-1}, which is low compared to its generation rate in a closed system. *Dupla* et al. [12.67] investigated the hydrodynamic behavior and denitrification capacity of a downflow self-cleaning moving bed bioreactor (MBBR) using floating packed-bed carriers. A deoxygenation unit was installed upstream from the denitrification unit, consisting of an MBBR (Fig. 12.34) (working volume 110 L) packed with plastic carriers placed randomly. The denitrification unit comprised two identical cylindrical reactors made of PVC (height 40 cm, diameter 30.5 cm, height of conical section 15 cm). Each reactor (including pipes) contained 33 L of water and was equipped with a recirculation loop and piping that allowed tangential recirculating water inflow, causing rotational movement of the liquid. Welded stainless-steel grid cones (10°, 45° or 60° inclination) were used to retain the carriers (5 mm grid openings, wire diameter 0.56 mm). Two jet nozzles with outlet diameter of 4.8 mm (small) and 8 mm (large) were installed to generate different water recirculation velocities at the nozzle i. e., 18.5 and 6.6 m s^{-1}, respectively. Eleven types of plastic packed-bed carriers commonly used in biological processes were considered and carrier selection was based on specific surface area, density, and size. The MBBR appeared to control biofilm development and could easily be scaled up to denitrify seawater or freshwater systems. Due to hydrodynamic control by means of the jet nozzle, the carriers did not clog and a thin biofilm was maintained throughout.

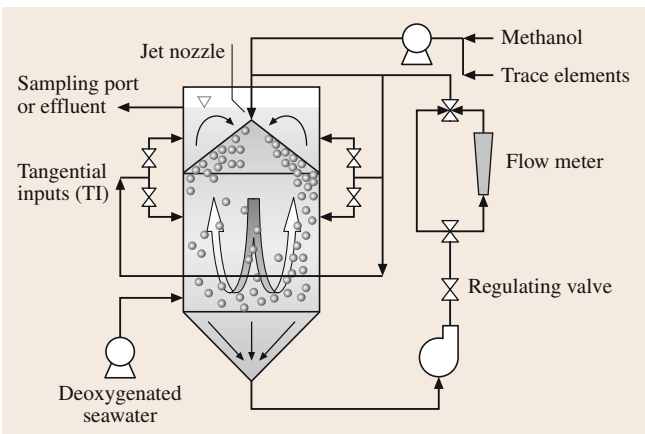

Fig. 12.34 Schematic diagram of the MBBR used in denitrification experiments (*arrows* show water circulation) (after *Dupla* et al. [12.67])

Tal et al. [12.68] examined the microbial communities in the biological filter and waste sludge compartments of a marine RAS to determine the presence and activity of anaerobic ammonium-oxidizing (anammox) bacteria. Fluorescence in situ hybridization (FISH) studies using an anammox-specific probe confirmed the presence of anammox *Planctomycetales* in the microbial biofilm from the denitrifying biofilters, and anammox activity was observed in these biofilters, as detected by the ability to simultaneously consume ammonia and nitrite.

Two tanks (vol. 4.2 m^3 each) containing the fish gilthead seabream (*Sparus aurata*) were operated at a density of 10–50 kg m^{-3} and a feeding rate of 1–1.6% body weight d^{-1}. The tanks were connected to a 2 m^3 nitrifying MBBR filled with 1 m^3 of polyethylene beads (diameter 1 cm, surface to volume ratio 500 $m^2\,m^{-3}$). A flow rate of 16 $m^3\,h^{-1}$ was used to obtain two exchanges of tank water per hour through the nitrifying biofilter. Attached to each tank as a denitrification side loop was a cylindrical up-flow fixed-bed biofilter (volume 0.3 mL) filled with 0.2 m^3 of polyethylene beads. The flow rate for this anaerobic biofiltration compartment was 0.1 $m^3\,h^{-1}$, and the water was supplied by two sources, direct water from the fish tank and water recovered from the sludge tank. Sludge was collected with a drum screen filter using a backwash system in

which tank water was used. Sludge and backwash water were collected in a rectangular tank (volume 0.3 m^3) packed with 0.1 m^3 beads that provided a means for solids removal, as well as a substrate for bacterial colonization. Water from the sludge tank was pumped back into the system through the anaerobic biofilter, and high-density sludge was collected and removed. In view of stricter environmental regulations for effluent discharge from aquaculture facilities there is increasing emphasis on closing the nitrogen cycle in the water treatment system by utilizing the anammox process as a preferred alternative to heterotrophic denitrification, especially in marine recirculating systems in order to reduce effluent volumes as well as nitrogen loads. The results of this study show that anammox bacteria and their activity are associated with the consortia of a denitrifying biofilter, which makes this approach feasible for implementation.

Enhanced biological phosphorus removal (EBPR) in wastewater treatment is based on the fact that phosphorus accumulating organisms (PAOs) can accumulate intracellular phosphorus as polyphosphate (poly-P). In EBPR, the biomass is exposed to cyclically occurring anaerobic and aerobic/anoxic conditions. Under anaerobic conditions, PAOs store rapidly biodegradable substrate in their cells as poly-β-hydoxyalkanoates (PHA) by using intracellular poly-P and glycogen as energy sources. Under aerobic/anoxic conditions, PAOs use PHA as a carbon source to refill poly-P and glycogen stocks. Poly-P synthesis allows bulk phosphate to accumulate in the biomass. The phosphate is then removed with the biomass during aerobic/anoxic conditions. Another group of bacteria, the glycogen accumulating organisms (GAOs), also grow under cyclic alternation of anaerobic and aerobic/anoxic conditions, using only glycogen as energy source and therefore consuming substrate but not removing phosphorus. Thus to optimize EBPR, anaerobic conditions must be strictly controlled, poly-P storage maximized, and GAOs growth minimized. Poly-P storage is maximized by using acetate as a carbon source because it increases the production of poly-P compared to other volatile fatty acids during aerobic periods. Environmental conditions known to favor PAOs over GAOs are temperature $< 25\,^{\circ}C$ and pH > 7.5. Aquariums and fish farms run in closed circuit face major accumulation of phosphate and nitrate which cause, respectively, excessive algal growth and toxicity to aquatic fauna. As an alternative to regularly changing the water that causes highly polluted discharge to the surroundings, denitrifying biological phosphorus removal (DN-BPR) may be applied to this system without producing excessive sludge.

The Montreal Biodome operates a 3250 m^3, closed circuit, cold (10 °C) marine mesocosm named St. Lawrence Mesocosm (SLM) containing very high nitrate and phosphate levels. Earlier workers (e.g., *Labelle* et al. [12.66], *Dupla* et al. [12.67]) have demonstrated the feasibility of denitrifying the SLM water using an MBBR with methanol as the C-source. *Vallet* et al. [12.69] modified the process to implement DN-BPR to promote the anaerobic growth of PAOs in the reactor – and additional C-source was required in view of low concentration of organic matter in the SLM water (2–4 mg C/L). The process developed is based on the alternation of anaerobic and aerobic/anoxic conditions in the MBBR with biofilm growth occurring on the surfaces of plastic carriers. A sequential complete exchange of two waters thus takes place in the MBBR – (1) the influent containing nitrate and phosphate and (2) the anaerobic water stored in a stock tank. Therefore, the environmental conditions in the MBBR selectively favor the growth of PAOs only. An additional advantage is the accumulation of phosphate in a stock tank thereby allowing an easy recovery of P for use as fertilizer.

Two identical reactor setups (used earlier by *Labelle* et al. [12.66]) were operated in parallel under identical conditions. Each setup comprised a well-mixed bioreactor (volume 110 L) filled with 35 L of plastic carriers, and a stock tank (volume 220 L). The packed bed was moved using a circulation pump thereby allowing operation as an MBBR. Water was pumped from the stock tank to the MBBR through a three-way transfer valve, while the MBBR was filled with the pressurized influent through a solenoid valve. A pH probe, a redox-potential probe, an oxygen probe, and a UV nitrate analyzer were installed on the recirculation line. This study demonstrated the inhibition of BPR by seawater presumably due to the high calcium levels in seawater. However, in freshwater, a biofilm was developed with a phosphorus removal efficiency rate of 20% of which 80% was attributed to biological removal and 20% to chemical precipitation.

12.8 High-Temperature and/or High-Pressure Bioreactors (HP-/HTBR)

High-pressure and/or high-temperature bioreactors (Table 12.9) are practically wholly devoted to cultivation of extremophiles (barophiles and/or thermophiles) isolated from the deep sea. Most, though not all, HP/HT bioreactor systems may be expected to fall into one of the following categories:

i) One-phase batch systems without gas enrichment (e.g., *Canganella* et al. [12.70], *Kallmeyer* et al. [12.71]).
ii) Processing of microbial samples without depressurization (e.g., *Parkes* et al. [12.72]).
iii) Two-phase batch systems with gas enrichment from free gas (includes studies by the group of Prof. Douglas Clark at the University of California, Berkeley e.g., *Miller* et al. [12.73], *Miller* et al. [12.74], *Miller* et al. [12.75], *Nelson* et al. [12.76], *Nelson* et al. [12.77], *Hei* and *Clark* [12.78], *Park* and *Clark* [12.79]).
iv) High-pressure fed-batch/continuous incubation (e.g., *Girguis* et al. [12.80], *Houghton* et al. [12.81]).
v) High-pressure fed batch/continuous incubation with gas-enrichment medium without free gas (e.g., *Deusner* et al. [12.82], *Zhang* et al. [12.83]).

Besides the above HP/HT bioreactor systems, *Marteinsson* et al. [12.84, 85] used pressurized gas-

Table 12.9 High-temperature and/or high-pressure bioreactors (HP-/HTBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	HPHTBR	Cultivation of thermophilic archaebacterium *T. peptonophilus*	*Canganella* et al. [12.70]
2	High-pressure thermal gradient system (HPTGS)	Cultivation of sulfate-reducing bacteria (SRB)	*Kallmeyer* et al. [12.71]
3	Deep-IsoBUG System	Enrichment and isolation of deep subsea floor prokaryotes	*Parkes* et al. [12.72]
4	High-pressure-high-temperature bioreactor (HPHTBR)	Effect of pressure on methanogenic archaea *M. jannaschii*	*Miller* et al. [12.73]
5	HPHTBR	Study of pressure–temperature relationship on growth and productivity of methanogenic archaea *M. jannaschii*	*Miller* et al. [12.74]
6	HPHTBR + fiber optics sensors	Assay of thermostable hydrogenase from methanogenic archaea *M. jannaschii*	*Miller* et al. [12.75]
7	HPBR	Effect of hyperbaric He pressure on the growth of archaeon ES 4	*Nelson* et al. [12.76]
8	HPBR	Comparing effects on hyperbaric and hydrostatic pressure on thermophile archaeon ES4	*Nelson* et al. [12.77]
9	Anaerobic HTBR	Pressure stabilization of protein from methanogenic archaea *M. jannaschii*	*Hei* and *Clark* [12.78]
10	HPHTBR	Cultivation of methanogenic archaea *M. jannaschii*	*Park* and *Clark* [12.79]
11	Continuous-flow anaerobic methane incubation system (AMIS)	Growth and methane oxidation of anaerobic methanotrophic archaea in marine sediment samples	*Girguis* et al. [12.80]
12	HPLC column flow-through bioreactor	Cultivation of nitrate and sulfate reducing thermophiles	*Houghton* et al. [12.81]
13	HPBR	Anaerobic oxidation of methane (AOM) by bacterial consortium	*Deusner* et al. [12.82]
14	Continuous HPBR	Anaerobic oxidation of methane in deep-sea sediments	*Zhang* et al. [12.83]
15	Sterilized gas-tight glass syringe in HPHT incubation system	Cultivation of a hyperthermophillic species (archaea) *P. abyssi* GES	*Marteinson* et al. [12.84]
16	Glass Syringes in HPHT incubation system	Cultivation of a novel strain archaeon *Theemococcus barophilus*	*Marteinson* et al. [12.85]
17	High-pressure bioreactor (HPBR)	Study on pressure tolerance of marine bacterial strains isolated from surface water	*Wright* et al. [12.86]

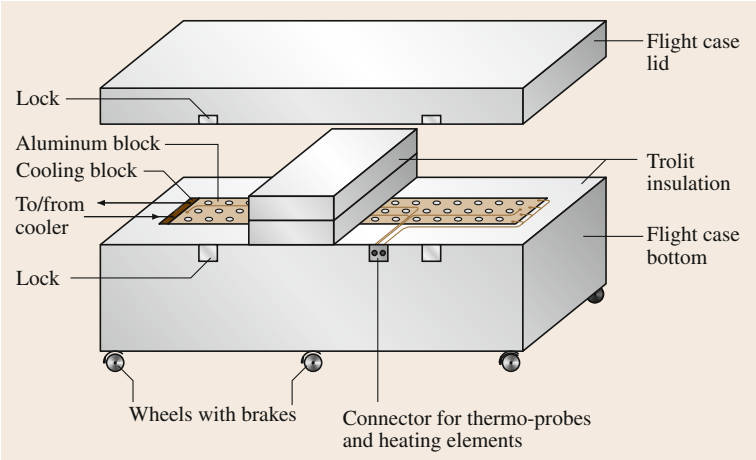

Fig. 12.35 Schematic view of the thermal gradient system. For better viewing, only parts of the Trolit blocks that cover the top are shown. The case has eight heavy-duty handles (not shown) (after *Kallmeyer* [12.71])

tight syringes for cell cultivation, placed in an HPHT incubation system. Again, *Wright* et al. [12.86] investigated the pressure tolerance of barotolerant (rather than barophilic) strains isolated from surface waters (and not from the deep sea).

Canganella et al. [12.70] investigated the effects of high temperatures and elevated hydrostatic pressures on the physiological behavior and viability of the extremely thermophilic deep-sea archaeon *Thermococcus peptonophilus* (isolated from deep-sea hydrothermal vents in the western Pacific ocean at a depth of 1380 m) in a high-pressure/high-temperature bioreactor (HPHTBR). Growth was fastest at 30 and 45 MPa without any noteworthy increase in cell yields, growth at 60 MPa was slower. The optimal growth temperature shifted from 85 °C at 30 MPa to 90–95 °C at 45 MPa. Cell viability during the stationary phase was also enhanced under high pressure. A trend toward barophily at pressures greater than those encountered in situ at the sea floor was observed at increasing growth temperatures. Relative to that at atmospheric pressure, the viability of cells during starvation, at high temperature (90, 95 °C), as well as at low temperature (10 °C) increased at 30 and 45 MPa. These results established that the extremely thermophilic archaeon *T. peptonophilus* was a barophile. The HPHTBR used in this study was designed to work within a temperature range of 0–300 °C and up to 68 MPa pressure and was suitable for continued sampling without any perturbation of the culture.

Kallmeyer et al. 2003 [12.71] developed a high-pressure thermal gradient system (HPTGS) consisting of a thermal gradient block (TGB) and a high-pressure unit (HPU) for simultaneous incubation of multiple microbial samples over a wide range of temperatures and pressures. To maintain the pressure independent of the thermal expansion of sample vessels, a back-pressure system (BPS) with a constant small leakage was also incorporated into the HPTGS. The HPTGS was used to study the pressure–temperature characteristics of sulfate-reducing bacteria (SRB) isolated from hydrothermal vent sediments (from Guyamas Basin, Gulf of California) and measure bacterial sulfate reduction rates which were found to increase with increasing pressure and showed maximum values at pressures higher than in situ.

The HPTGS consists of two main units: the TGB and the high-pressure unit (HPU). The TGB (Fig. 12.35) consists of an aluminum block (Al Cu Mg Pb F34; 1185 × 150 × 150 mm) with three rows of 15 equidistant holes (140 mm deep × 36 mm diameter) insulated by a 20 cm thick layer of Trolit (proprietary solid material, made from mineral fibers) with very good thermal insulation characteristics, temperature resistant to > 1000 °C, also robust and mechanically stable. The temperature gradient is almost linear, independent of the minimum and maximum temperatures. At incubation temperatures > 200 °C, the outside of the unit is at ≤ 25 °C. For ease of transportation, the whole block is housed in a custom-made case on wheels. The Trolit and the aluminum block are held in place only by the case to allow for thermal expansion of the material and easier dismantling. At one end of the block, a cooling element is attached, milled from a solid piece of aluminum (150 × 150 × 60 mm) with a tortuous internal channel where coolant is circulated. The inlet and outlet are on top of the unit and connected to a cooling unit mechanically fixed to the block with four screws, and

sealed with heat-resistant elastic sealant. The coolant was chosen according to the desired temperature and for very low temperatures, the hoses connecting the cooler and the TGB were thermally insulated to avoid precipitation of moisture and buildup of ice. At the opposite end of the TGB, four electrical heating elements (6.4 mm diameter × 88.9 mm, 200 W) were placed into 6.5 mm diameter holes in the block. The maximum temperature attainable is 220 °C and the steepest thermal gradient is 160 °C, providing ≈ 10 °C temperature difference between each row of containers. Over the length of the block, four thermo-probes (PT 100, −60 to +400 °C) were installed to continuously monitor the temperature, which were connected to an electronic multicontroller for on–off switching of the heating elements.

The HPU (Fig. 12.36) is essentially a back-pressure system with a constant small leakage. Pressure is created by two HPLC pumps that can operate in a higher pressure range (from 400 to 600 bar). The pumps run in constant pressure mode with variable flow rate. In case of any thermal expansion during the warm-up, pressure remains below the specified threshold. The back-pressure system comprises five used HPLC columns (250 × 4.6 mm) in line. At 450 bar pressure, ≈ 0.7–0.8 mL leaks through. During the warm-up period, thermal expansion may cause the pressure to rise faster than the back-pressure system can release it, therefore opening of one of the connections between the HPLC columns smoothly increases the outflow of the system in a controlled manner. After thermal equilibrium is achieved, the connection is fastened again. The outflow of the back-pressure system flows back into the eluant vessel for subsequent reuse.

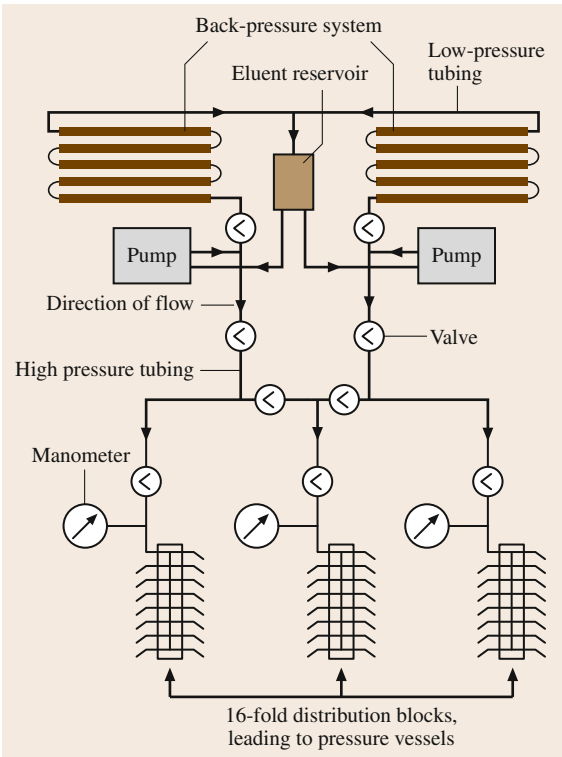

Fig. 12.36 Schematic view of the high-pressure system. The *thin lines* are high-pressure connections; the *bold lines* are low-pressure connections (after *Kallmeyer* et al. [12.71])

The pressure distribution system consists of pumps connected to a manifold of valves which allows connection of each pump to any of the three lines of pressure vessels, or for use of the pumps in parallel. Each line has a Bourdon-type manometer (16 cm diameter) to dampen the pulsation of the pump. A custom-made 16-fold distribution block connects the pressure containers of each line to the valve manifold. Small pockets in the Trolit insulation house the distribution blocks. Pressure vessels consist of large standard S.S. HPLC columns (I.D. 20 mm, 120 mm long), one end having a normal HPLC $\frac{1}{16}$ in fitting, the other end closed with a steel cap. The pressure containers are sealed with copper rings that last for several incubations but must be replaced periodically. Custom-made screw-cap tubes (O.D. 18 mm, 50 mm long) are used as sample vials, two of which fit in each pressure vessel, thereby providing exact duplicates in pressure and temperature.

The pressure vessels are placed in a bucket filled with demineralized water and the sample vials dropped into them. The caps are screwed on the pressure vessel under water to avoid trapping of air bubbles. After all pressure vessels are connected to the distribution block, ≈ 50 bar is applied to check for leaks. When no leak is noted, the vessels are inserted into the TGB and allowed to attain thermal equilibrium. The buildup of pressure is carefully monitored and released if necessary, then, after 1–2 h, the pressure is set to the desired value.

Parkes et al. [12.72] developed the novel DeepIsoBUG system for handling sediments (that contain depressurization sensitive anaerobic, piezophilic prokaryotes) under elevated pressure (up to 25 MPa) for enrichment, growth, and isolation of prokaryotes at pressures up to 100 MPa. When coupled with acquisition of pressurized subsurface cores using the HYACINTH pressure retaining drilling and core storage system and the PRESS core-cutting system, Deep-

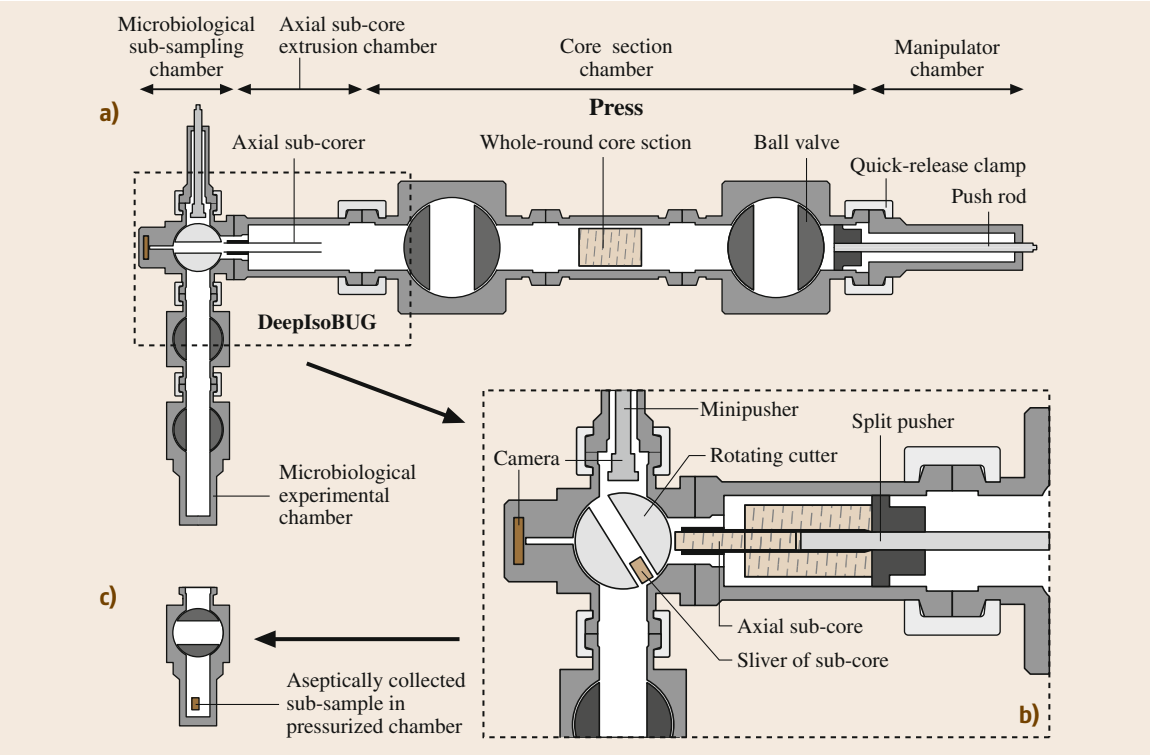

Fig. 12.37a–c Schematic of subcoring and slicing procedure using the coupled PRESS and DeepIsoBUG system to obtain an uncontaminated central core slice for prokaryotic enrichments and other experiments. **(a)** Complete system connected after cutting and transfer of core section. **(b)** Blow-up showing the subcoring and slicing operation. **(c)** Sediment slice isolated in pressure vessel (after *Parkes* et al. [12.72])

IsoBUG enables the recovery and handling of cores at in situ pressures (up to 25 MPa) and subsequent enrichment and isolation of prokaryotes at a range of pressures without depressurization. Using subsurface gas hydrate containing sediments (obtained from the Indian Continental Shelf, the Gulf of Mexico, and the Cascadia Margin), it was examined whether enrichment and isolation of subseafloor anaerobic prokaryotes under elevated pressure, without depressurization, would enable acquisition of different culturable prokaryotes compared with the normal procedure of using depressurized sediments and atmospheric pressure handling and isolation. They found that, in general, highest cell concentrations in enrichments occurred close to in situ pressure (14 MPa) in various growth media, although growth was observed at pressures up to 80 MPa.

The DeepIsoBUG system consists of a subcoring and slicing system (Fig. 12.37), a transfer chamber, an isolation chamber and pressure vessels, described below. The subcoring and slicing system (made of S.S. 316) enables a central subcore (20 mm) to be obtained from a core section and to be sequentially sliced (using a manually rotated blade), with each slice being transferred (using a rotating central section) to a low pressure (up to 25 MPa) vessel (with a large ball valve) containing anaerobic, mineral salts medium, vigorous shaking of which produces a sediment slurry for use as inocula. Two cameras behind sapphire windows allowed viewing of the extrusion process.

Through the transfer chamber, the sediment slurry is transferred from the low-pressure vessel (5 mL slurry aliquots) to a number of high-pressure (up to 100 MPa) culture vessels containing enrichment medium. The chamber also contains a filter ($\approx$ 100 mm) to prevent transfer of large particles that could damage ball valves.

The isolation chamber has 12 agar plates attached to an electric motor-driven chain allowing individual plates to be selected. The chain drive lifts out of the isolation chamber and plates detach so that anaerobic

media can be prepared and the system assembled in an anaerobic chamber and transferred (via an anaerobic bag) to the presterilized and gas-flushed chamber body. The chamber also contains eight detachable cells (up to 15 mL in volume) that allows microbial growth in liquid medium within the chamber and also transfer in and out of the isolation chamber. Enrichments for isolation were transferred into an individual growth cell within the isolation chamber. A motorized robotic arm in the isolation chamber enabled a sterile inoculation loop (electrically heated in situ) to be dipped into the growth cell and its contents to be streaked onto an agar plate. After incubation, individual colonies could then be picked off and transferred to other cells containing sterile media for further growth. Transfer of these purified cultures out of the isolation chamber into high-pressure incubation chambers permitted further subculture and physiological tests to be conducted on isolates. All manipulations in the isolation chamber were viewed through a sapphire window via an endoscope attached to a digital camera and monitor.

Pressure vessels (100 cm^3 total volume, 80 cm^3 medium) made of titanium, used for enrichment and cultivation have a body and a lid, attached together with a screw thread. They are sealed by a male cone in the lid and a complementary female cone in the body, plus O-ring seals. The low-pressure enrichment vessels have a small-bore ball valve (4 mm) on the lid for gas input and liquid transfer, as well as a large-bore ball valve (32 mm) for sediment slice transfer. The high-pressure, incubation vessels have just two small-bore ball valves. Preprepared sterile anaerobic media were transferred into sterile pressure vessels within an anaerobic chamber. For high-pressure liquid transfer between systems, a small pressure differential ($\approx$ 2 MPa) between vessels was maintained. Ball valves were used to prevent shear stresses and possible cell rupture during culture transfer by pressure differential. For routine high-pressure sampling of pressure vessels and subculture into vessels with fresh medium, a bored-out *T*-piece and a series of valves were used, that enabled transfers without depressurization.

The study accomplished the following, viz.:

a) The direct enrichment of subseafloor prokaryotes under elevated pressure.
b) Enrichment of sedimentary, anaerobic prokaryotes without depressurization.
c) High-pressure cultivation from subsurface gas hydrate sediments.

Results showed that many subseafloor prokaryotes from gas hydrate containing sites could grow (anaerobically) up to at least 80 MPa in various media which is a much higher pressure than that previously recorded for subseafloor prokaryotes. As 70% of the ocean is at a pressure of 38 MPa or higher and the average depth of sediments is 500 m, growth at 80 MPa would enable the majority of the subseafloor environment to be populated by prokaryotes. This study was claimed as the first report on the isolation of *Carnobacterium*, *Marinilactobacillus*, *Acetobacterium*, *Clostridium*, and *Bacteroidetes* species from deep, subseafloor, gas hydrate deposits.

Miller et al. [12.73] developed a novel HPHTBR to investigate pressure–temperature relationships (up to 260 °C and 350 bar) in the growth and productivity of the extremely thermophilic methanogenic archaea *Methanococcus jannaschii* isolated from a deep sea hydrothermal vent. The corrosion-resistant reactor system permits direct sampling (of both liquids and gases) from a transparent culture vessel without affecting the internal growth conditions. It was found that increasing the pressure from 7.8 to 100 bar accelerated the production of methane and unicellular protein at 90 °C; raised the maximum temperature for growth from 90 to 92 °C but further increase in pressure did not affect growth. The reactor vessel or pressure cell (internal volume $\approx$ 43 mL) is a transparent synthetic single crystal sapphire tube (I.D. 0.7″, O.D. 1.25″, length 6″) which allows direct visual observation of the growth medium. It may be sealed by two different configurations – one for working at 100 bar consisting of a spring-loaded Teflon gasket held onto the tube ends by flanges which, again, are held in place by six spacer rods. In the other sealing technique, the distance between the flanges is reduced, thus compressing the Teflon rings which, on application of pressure, are further compressed thereby maintaining the seal.

The pressure cell is connected to a magnetic pump to recirculate vapor through the liquid, which serves two purposes, viz. (1) agitation of the liquid and (2) to promote vapor–liquid equilibrium. The pump (I.D. 0.375 in, internal length 2 in) is actuated by a stack of ceramic ring magnets located around the pump body that are oscillated up and down through a motor driven crank and cable. A piston with a plug travels the length of the pump. On the upstroke, gas is pushed out of the pump into the pressure cell, on the down stroke, gas fills the pump for the next stroke. A crank valve at each end of the pump prevents flow in the opposite direction.

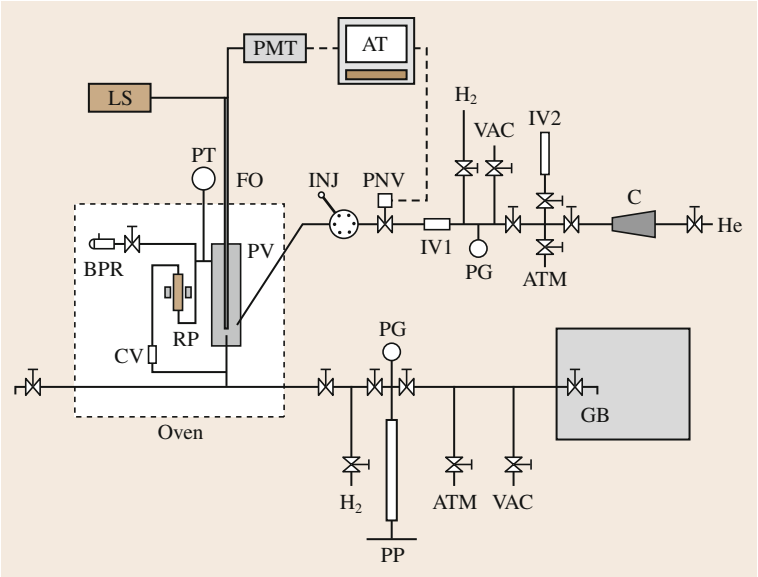

Fig. 12.38 Schematic diagram of the apparatus: PV – stainless steel pressure vessel, RP – magnetically driven vapor recirculation pump, CV – check valve, BPR – back pressure regulator, PT – pressure transducer, FO – fiber optic probe, LS – light source, PMT – photomultiplier tube, water-cooled housing, and photometer, AT – computer, PNV – pneumatic valve, INJ – six-port valve, IV1 – 1 mL injection vessel, HJ – hydrogen supply, PG – pressure gauge, IV2 – 100 mL injection vessel, C – two-stage compressor, He – helium supply, GB – anaerobic glove box, and PP – piston pump. The abbreviations ATM and VAC refer to atmosphere and vacuum, respectively (after *Miller* et al. [12.75])

The pressure cell and magnetic pump are housed in an oven (maximum temp. 260 °C) equipped with a viewing window. The oven temperature is measurable by a thermocouple and a digital thermometer. Pressure is maintained by a triple range strain gauge transducer coupled to a digital voltmeter for display.

All gas entering the high-pressure system is passed through a trap to strictly exclude molecular O_2. For experiments performed at pressures above the cylinder pressure of the gases, a two-stage diaphragm compressor is used to raise the gas pressure. For liquid supply to the reactor vessel, the microbial suspension is drawn (from the anaerobic glove box) into the screw-actuated piston pump, which is operated to raise the pressure on the microbes to the value in the reaction vessel. When the pressure in the pump equals that in the vessel (20 psi initially), the valve is opened for solution inflow to the vessel. In case of methanogenesis, a pressure drop accompanies methane formation – this necessitates use of a pressure regulator for injecting inert gas into the reaction vessel to maintain constant pressure. The regulator also maintains constant pressure when samples are withdrawn. Both the liquid and gas lines are connected to a vacuum system so that lines can be evacuated or purged before material is passed through them into the pressure cell. In this way, molecular oxygen is excluded from the pressure vessel. To prevent corrosion and H_2 embrittlement, all metal parts in contact with high-pressure fluid are made of S.S. 316.

Miller et al. [12.74] cultivated the deep-sea thermophilic archaea *M. jannaschii* in a high-temperature, high-pressure bioreactor (HTHPBR) at high temperatures and hyperbaric pressures of helium. The bioreactor used was similar to a prototypical device described earlier in *Miller* et al. [12.73] modified as follows – the synthetic sapphire pressure vessel (volume 90.63 cm^3) was replaced with a larger S.S. 316 vessel (internal volume, 167 cm^3) capable of withstanding pressures up to 1000 atm; the screw-actuated piston pump was replaced with a higher throughput pneumatic pump to allow rapid addition of fresh medium to the reactor vessel. High hyperbaric pressure of He up to 750 atm was observed to increase the growth rate of *M. jannaschii* at both 86 and 90 °C but did not extend the upper temperature limit for growth. However, increased pressure did extend the maximum temperature for methanogenesis by *M. jannaschii* from < 94 °C at 7.8 atm to 98 °C at 250 atm, indicating a possible pressure-stabilizing effect on enzymes facilitating methane synthesis.

Miller et al. [12.75] designed a HPHTBR for measuring enzyme activity (by UV-visible spectrophotometry/gas chromatography) at temperatures up to 260 °C and pressures up to 667 atm. Initial studies focused on the pressure-enhancement of methyl viologen-reducing hydrogenase activity in crude extracts of *Methanococcus jannaschii*. The oven of this reactor system (Fig. 12.38) contains a S.S. 316 cylindrical

reaction vessel and a magnetically driven pump to recirculate gas through the liquid phase. A fiber optic probe is inserted into the reaction vessel for spectrophotometric measurements (Fig. 12.39). A six-way valve is used to inject substrate or enzyme into the vessel. The rest of the setup is used for transferring fluids and for maintaining constant pressure. The reaction vessel is a custom-built S.S. 316 cylinder (O.D. $3'' \times$ I.D. $\frac{5}{8}'' \times$ length $5\ \frac{1}{2}''$) topped by a high pressure female opening (I.D. $1\ \frac{1}{2}''$, length $\frac{1}{2}''$, threaded 1 in deep, 12 threads in^{-1}) above a $\frac{3}{8}$ in long $60''$ taper. In addition, there is a high-pressure female opening (O.D. $\frac{1}{8}''$) at the bottom of the vessel. Two side arms extend from the vessel: one leads from the injection valve to the liquid phase, and the other from the gas phase to the magnetically driven pump. The pump draws vapor from the headspace and bubbles it up through the liquid. Pressure in the vessel is maintained by a back pressure regulator and measured by a Bourdon tube transducer coupled to a digital voltmeter for display. Temperature is controlled by the oven, which may be operated up to 260 °C.

Nelson et al. [12.76] investigated the effects of hyperbaric helium pressures on the growth and metabolism of the deep-sea hyperthermophilic archaeon, strain designate ES4 (that grows up to 110 °C) in a HPHT bioreactor. Growth studies performed at pressures up to 500 atm in a stainless steel vessel with and without a glass lining indicate that the behavior of ES4, specifically, its ability to grow and its response to elevated hyperbaric pressure, depends overwhelmingly on its environment. ES4 was grown at hyperbaric pressures in an HPHTBR originally developed by *Miller* et al. [12.73] but modified in this study as detailed below. Three pneumatically actuated valves were installed in the gas sampling line for automated sampling. The compressed-air supply to these valves was controlled by electronically actuated solenoid valves, which were in turn controlled by an integrator through a sample/event control module. The integrator was programmed to withdraw samples at any desired interval with less than a 0.75% drop in total system pressure at 500 atm. (The pressure drop was $\approx 2.5\%$ with each sample at 8 atm.) The sample loop had an internal volume of 0.20 cm^3 for pressures up to 10 atm; for higher pressures, its volume was 0.03 cm^3. The larger sample volume at lower pressures was necessary to purge all lines. At each sampling time, two samples were taken: the first to purge the sample lines, and the second for analysis. The lines were evacuated with a vacuum pump between

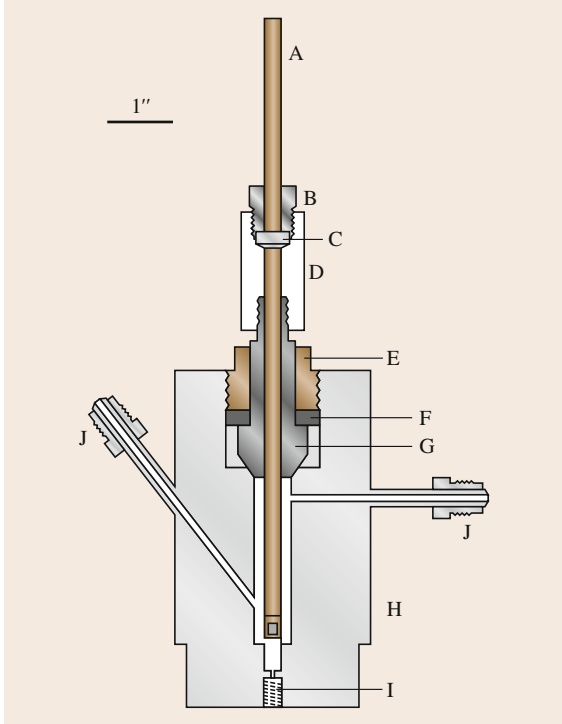

Fig. 12.39 Reaction vessel and fiber optic probe assembly: A – SS316 tube containing optical fibers, B – $\frac{1}{4}$ in. O.D. tube taper-seal gland, C – $\frac{1}{4}$ in. O.D. tube taper-seal sleeve, D – modified $\frac{1}{4}$ in. O.D. tube taper sealto- $\frac{1}{4}$ in. NPT coupling, E – custom $\frac{7}{8}$ in. high-pressure gland, F – washers, G – $\frac{7}{8}$ in. O.D. $\times \frac{1}{4}$ in. I.D. high-pressure plug with $\frac{1}{4}$ in NPT connector (H) SS316 reaction vessel, (I) $\frac{1}{8}$ in. high-pressure female opening, and J – $\frac{1}{4}$ in. high-pressure collar and gland (after *Miller* et al. [12.75])

all samples. For pressures > 10 atm, a 20 cm^3 reservoir was included between the gas chromatograph and the reactor to allow decompression of the gas sample before it entered the G.C. A back-pressure regulator maintained the gas sample pressure at 120 kPa while the temperature was constant at 100 °C. Helium was used to pressurize the system, and the concentrations of CO_2 and H_2S were measured with a G.C. The final modification was the incorporation of a liquid sampling device that allowed the slow decompression of liquid samples before their removal from the reactor system.

An S.S. 316 high-pressure syringe (internal volume 2.5 mL) was used with a maximum working pressure > 1000 atm. A polycarbonate piston equipped with two

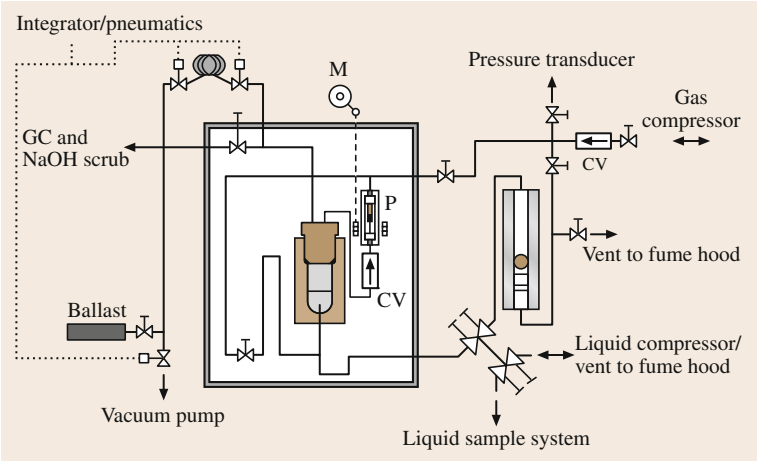

Fig. 12.40 Schematic diagram of modified high-pressure system. CV – check valves; P – gas recirculation pump; M – motor-driven crank; GC – gas chromatograph (after *Nelson* et al. [12.77])

O-rings, separated the culture broth from the hydraulic fluid. A minimum pressure drop of 100 psi across the piston is required for its movement. A second high-pressure syringe (maximum working pressure 250 atm, internal volume 1 mL) was equipped with polycarbonate windows for direct viewing of the sample during decompression.

Nelson et al. [12.77] designed a bioreactor for precise determination of the growth rate as a function of hyperbaric and hydrostatic pressures, simultaneously. The effects of hyperbaric and hydrostatic pressures of up to 500 atm on the growth rate of the deep sea isolate ES4 (*Nelson* et al. [12.76]) were compared at 95, 100, and 105 °C. Results indicate that the microbial growth rate at elevated pressures could have a remarkable dependence on the mode of pressurization.

A previously used high-pressure reactor system (*Nelson* et al. [12.76]) was modified in this study to facilitate studies on hydrostatic pressure concurrently with hyperbaric experiments (Fig. 12.40). A second high-pressure vessel was connected to the gas and liquid lines feeding the hyperbaric system. The temperature and pressure of each vessel were controllable independently, or the pressure could be equilibrated between the two. Temperature was microprocessor-controlled with two types of J thermocouples (one internal and one external) as the input source for the controller. Although He is used as a pressurizing gas, a Teflon piston equipped with O-rings provided a barrier against the direct liquid–gas contact. A ferromagnetic steel ball enabled magnetic mixing of the liquid as required and also marked the location of the piston. The ball could be magnetically lifted to promote mixing prior to sampling, or a stack of ceramic ring magnets could be oscillated up and down by a motor-driven crank and cable if continuous mixing was desired. The hydrostatic vessel (total internal volume 58 mL) has a working volume of 50 mL. The reactor consists of an S.S. 316 tube (length 18″, O.D. 1″, I.D. $\frac{1}{2}$″), with a maximum working pressure of 15 000 psi and a 24 karat gold plated interior surface to reduce corrosion and consequent leaching. The interior of the hyperbaric system was also treated similarly to minimize differences between the two systems. All wetted thermocouples and the steel ball were also gold plated. To minimize erosion of the gold due to piston motion, subsized O-rings (nominal O.D. $\frac{3}{8}$″) were used on the piston, which were stretched to provide a seal. Since the pressure drop across the piston was less than 1 atm when static (up to 10 atm during sampling), little leakage across the piston was detected. It was also possible to use standard $\frac{1}{2}$″. O-rings with deeper-than-standard grooves in the piston to minimize compression of the O-ring. This approach has an advantage of longer O-ring life but could reduce the lifetime of the piston at high pressure. Vertical alignment of the vessel facilitated the removal of trapped gases and containment of sulfur when required.

Hei and *Clark* [12.78] studied the pressure stabilization of enzymes, including a hydrogenase from *M. jannaschii*, an extremely thermophilic deep-sea methanogen, in an anaerobic HTHPBR. *M. igneus, M. jannaschii, M. thermolithotrophicus, and M. maripaludis* (all thermophilic archaea) were grown in artificial seawater medium with a continuous anaerobic gas phase of 20% CO_2 and 80% H_2. The bioreactor (internal volume 10 mL) and all tubings and fittings were constructed of S.S. 316, pressurized with oxygen-

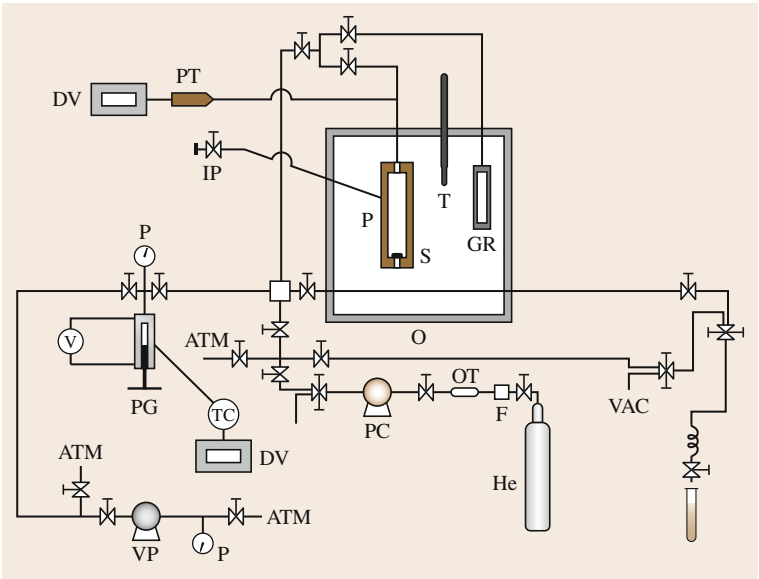

Fig. 12.41 Schematic diagram of the high-pressure reactor used in pressure stability studies. Components shown: P – stainless-steel pressure vessel; GR – gas reservoir; S – water-driven magnetic stirrer; T – ASTM thermometer; O – oven; IP – injection port; PT – pressure transducer; DV – digital voltmeter; P – pressure gauge; PC – pneumatic compressor; F – filter; OT – oxygen trap; PG – pressure generator; TC – thermocouple; V – power supply to heating tape; VP – vacuum pump; ATM – line to atmosphere; VAC – line to vacuum (after *Hei* and *Clark* [12.78])

free helium compressed with a pneumatic compressor and pressure controlled with a manually operated pressure generator (Fig. 12.41). The reactor and the reservoir holding the pressurizing gas were enclosed in a forced-air oven that allowed temperature control to $\pm 0.1\,^{\circ}C$. Agitation of reactor liquid was done by a magnetic stir bar driven by a water-powered magnetic stirrer.

The effect of decompression on the structure of *M. jannaschii*, an extremely thermophilic deep-sea methanogen, was examined in a novel HPHTBR by *Park* and *Clark* [12.79]. The cell envelope of *M. jannaschii* appeared to rupture upon rapid decompression (ca. 1 s) from 260 atm of hyperbaric pressure. However, when decompression from 260 atm was performed slowly over 5 min, the proportion of ruptured cells decreased significantly. In contrast to hyperbaric decompression, decompression from a hydrostatic pressure of 260 atm did not cause cell lysis.

A confined gasket-closure reactor (Fig. 12.42) used as the hyperbaric bioreactor (volume 1.15 L) could be operated up to 200 °C and 590 atm. A thermocouple inserted into an S.S. 316 well extending three-fourths of the way into the bioreactor provided the signal to a PID controller for precise temperature control through a heating belt. A diaphragm compressor was used to supply H_2, CO_2, and He at high pressures. An oxygen trap was installed between the diaphragm compressor and the gas cylinder. The gas atmosphere in the hyperbaric bioreactor was H_2/CO_2 (4 : 1) up to 7.8 atm, and He was used for pressurization > 7.8 atm. For precise pressure control, an air-actuated backpressure regulator was placed in the exit line of the gas loop.

A tubular reactor (volume 65 mL) was used as the hydrostatic bioreactor which was first pressurized with H_2/CO_2 (4 : 1) to 7.8 atm, then pressure was increased to 260 atm hydrostatically by pumping in cells and growth medium. The medium (45 mL) was anaerobically inoculated in a serum bottle with 20 mL of exponentially growing *M. jannaschii* cells, followed by transfer into the hydrostatic reactor through the liquid pump until internal pressure reached 260 atm. Temperature control was achieved by incubating the pressurized hydrostatic bioreactor in an oven. The reactor was reconnected to the system and repressurized to 260 atm every 2 h throughout the incubation period, during which the pressure did not fall by > 10%. After 10 h of cultivation, the hydrostatic bioreactor was depressurized in 1 s and the culture was withdrawn for further analysis.

Girguis et al. [12.80] incubated both marine hydrocarbon seep sediments and marine nonseep (i. e., aerobic) sediments in a continuous-flow anaerobic methane incubation system (AMIS) that simulates the majority of in situ conditions and supports the metabolism and growth of anaerobic methanotrophic archaea. Methane oxidation rates and population growth of MOA (methane oxidizing archaea) were measured over the course of the incubation (24 weeks), thus allowing the evaluation of growth and metabolism of MOA in

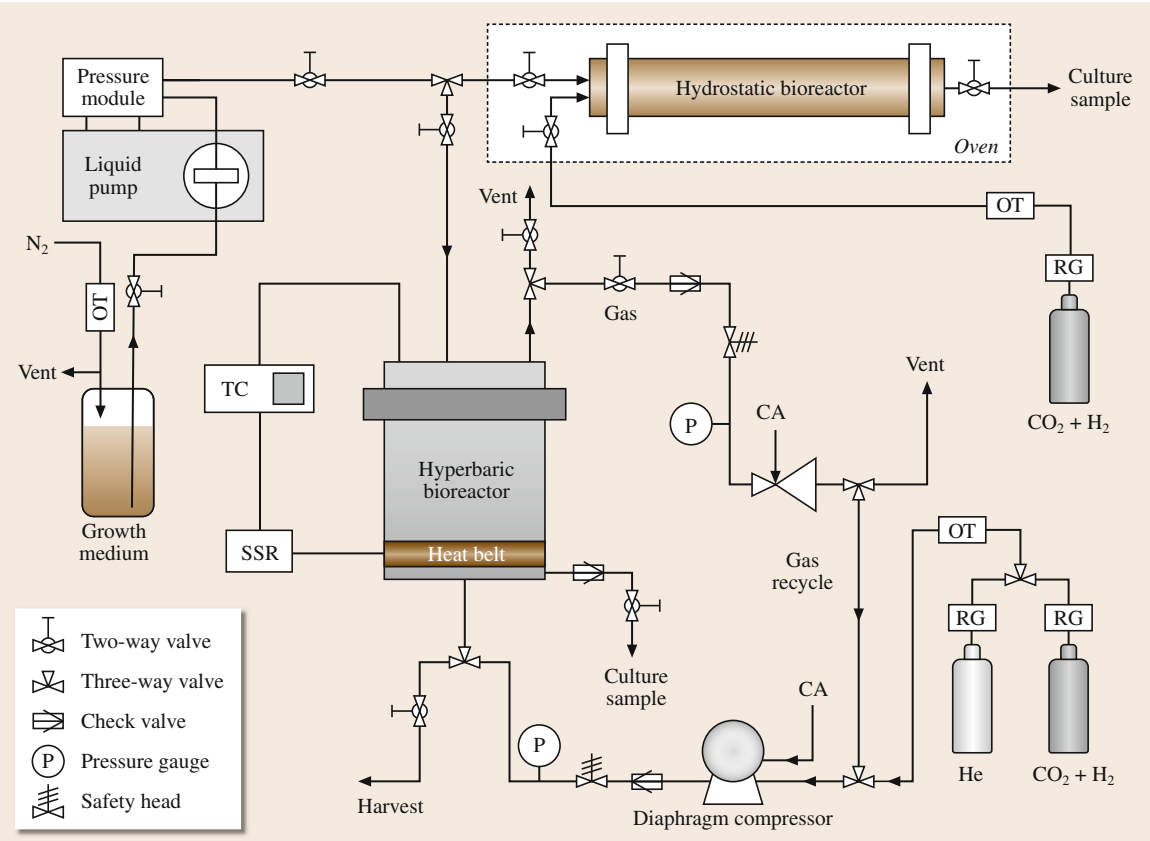

Fig. 12.42 Schematic diagram of a high-pressure, high-temperature bioreactor system. The *dotted lines* signify an oven into which the pressurized hydrostatic bioreactor was placed after being disconnected to control the temperature. SSR – solid-state relay; TC – temperature controller; OT – oxygen trap; CA – compressed air; RG – regulator (after *Park* and *Clark* [12.79])

both seep and nonseep sediments both prior and subsequent to incubation on AMIS.

Conditioned seawater was pumped out of the conditioning column by a diaphragm-metering pump through a capsule filter and rigid polypropylene tubing (I.D. 1.5 cm, O.D. 2.5 cm) into a schedule 80 (PVC) manifold (the *lower* manifold) on which four sediment cores were incubated with a gas- and water-tight seal (using double O-ring seals) maintained on the bottom of each sediment core sleeve. Gas-tight PVC ball valves were installed on both the inlet and the outlet, allowing the manifold to be sealed to maintain anaerobic conditions even during servicing. During operation, the backpressure in the lower PVC manifold was maintained at (200 kPa) by a PVC backpressure valve or by running a length of PVC pipe vertically to create a seawater pressure head at the outlet. After circulating through the manifold, seawater returned to the 25 L reservoir and was recirculated through the conditioning column. The *upper* PVC manifold consisted of PVC tees connected by rigid PVC tubing – here each sediment core sleeve was also sealed to a PVC tee fitting with O-ring seals. The upper PVC manifold was connected to a chilled seawater system (5 °C) and could be sealed at both the inlet and outlet by gas-tight PVC ball valves. During operation, the entire manifold assembly and seawater reservoir were maintained in a cold room at 5 °C. The AMIS system thus successfully stimulated the maintenance and growth of anaerobic methanotrophic archaea, and possibly their syntrophic, sulfate-reducing partners as well.

Houghton et al. [12.81] developed a *PEEK-HPLC column flow-through bioreactor* for continuous enrichment of nitrate and sulfate reducing thermophiles under

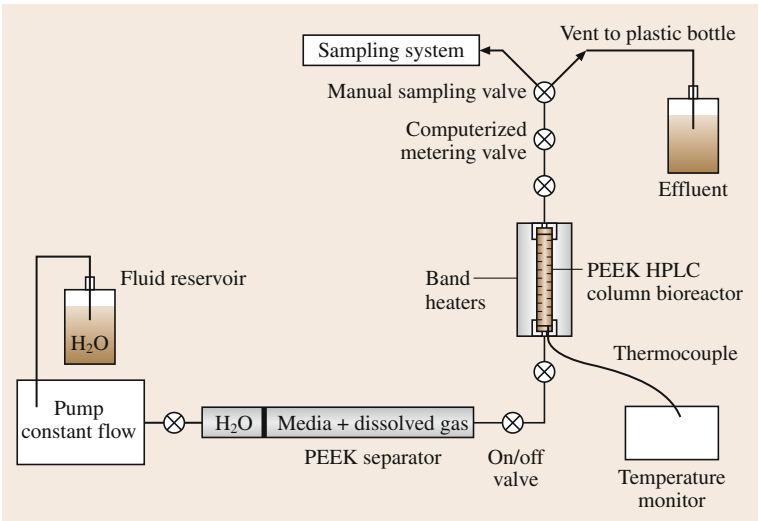

Fig. 12.43 Schematic drawing of the flow-through apparatus designed for this study. All parts were constructed of PEEK plastic, with the exception of the control valves that were composed of stainless steel. The bioreactor in line with the fluid-delivery system contains the sulfide-chimney material used for the experiments. Two thermocouples were attached to the reactor, which in conjunction with a fully automated control system, maintained temperature and pressure within very narrow limits during the 100–180 h experiments (after *Houghton* et al. [12.81])

in situ deep-sea hydrostatic pressures, in order to investigate microbial activity at conditions similar to seafloor hydrothermal vents. The experimental setup (Fig. 12.43) was designed to promote the growth of microbial populations at temperatures $< 100\,°C$ and pressures of ≈ 250 bar while permitting dynamic monitoring of changes in fluid chemistry and microbial diversity.

An HPLC pump and linked *separator* continuously provided fluid reactants and dissolved gases at high pressure to bioreactor columns made of PEEK (I.D. 0.75 cm, length 10 cm, volume 4.41 cm^3), which were maintained at a constant temperature by a series of band heaters external to the reactor and enclosed in an insulated tube furnace. Time proportioning controllers provided temperature control in response to input from a series of thermocouples directly attached to the reactors in the furnace. Except flow control valves, made from S.S. 316, all wetted parts of the fluid delivery and control system were made of PEEK, although buna O-rings were used as pressure seals throughout. Moreover, all tubing, PEEK reactors, and stainless valves were replaced between experiments to minimize contamination effects. Fluid samples could be taken throughout experiments by activating manual and computer-controlled valves, which not only diverted sample fluid into gas-tight containers for processing, but also maintained the system at the desired pressure. The media, mainly artificial seawater was prepared under anaerobic conditions, which were also maintained by bubbling CO_2 and H_2 through the media while filling the separator, which, by piston action, was used to achieve high-pressure conditions. The separator permitted appropriate amounts of gas to be dissolved in the media by filling a headspace with gas before fully pressurizing the system. The media used for all experiments was thus saturated with dissolved CO_2 and contained ≈ 2.2 mM H_2. Water delivered at high pressure to the input side of the piston in the separator ensured complete dissolution of the gas in the media as well as complete pressurization to the operational condition of 250 bar.

The study achieved growth and culture enrichment of thermophiles under conditions of temperature and pressure in a flow-through bioreactor that provided an effective means for investigating hydrothermal systems at temperatures, pressures, and chemical conditions similar to seafloor chimney (vent) deposits and their associated microbial communities.

Deusner et al. [12.82] developed a novel two-stage high-pressure bioreactor system for gas-phase free continuous incubation of enriched, highly active marine microbial consortia (prepared from microbial mats obtained from the Black Sea at a depth of 213 m) performing anaerobic oxidation of methane (AOM). The systems developed, viz., High-pressure continuous incubation system (HP-CI) and high-pressure manifold incubation system (HP-MI) were used to demonstrate the feasibility of investigating bioprocesses in high-pressure fed-batch or continuous incubation systems with gas-enriched medium without free gas in the incubation stage. In continuous operation in the HP-CI system, initial methane-dependent sulfide production increased 10–15 times on increasing methane par-

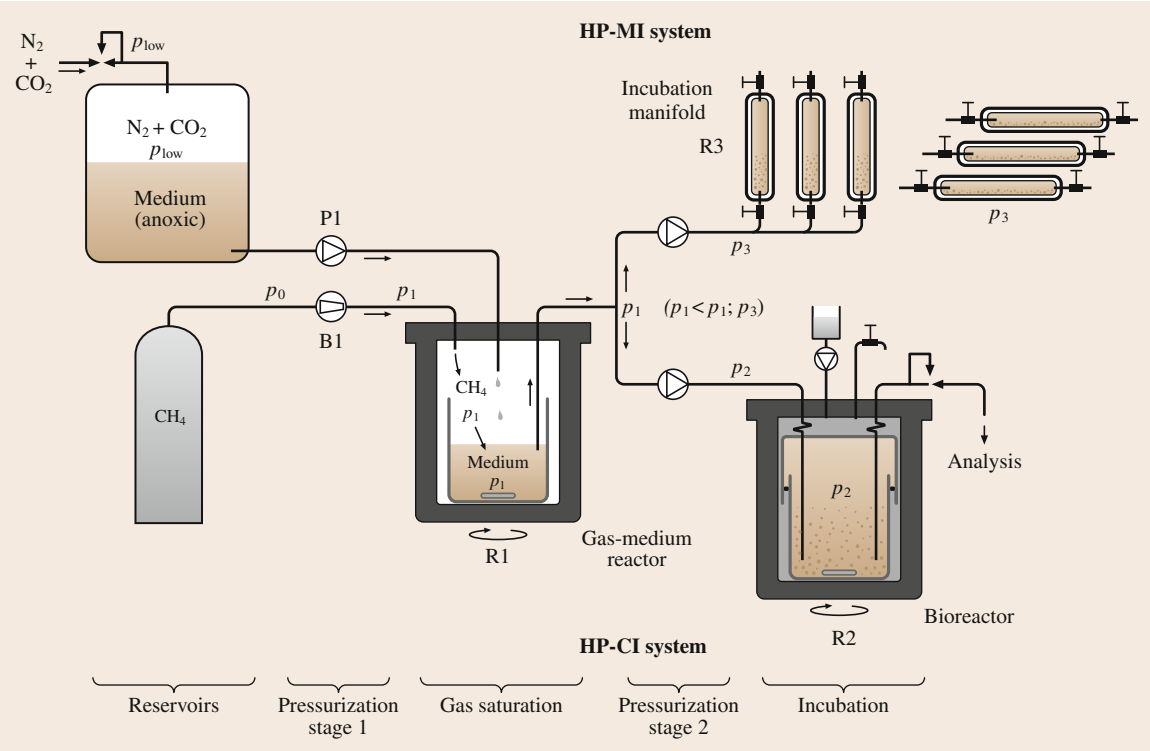

Fig. 12.44 Incubation principle. In two-stage operation, medium is enriched with methane under high-pressure conditions in the continuously stirred gas-medium reactor R1. The gas-enriched medium is further transferred to the bioreactor R2 or R3. Pressure p2 and p3 within R2 and R3, respectively, is increased compared to p1 in R1. In R1, R2, and R3 medium and culture fluid are contained in internal vessels to avoid contact to the metal surfaces. Incubation is carried out without free gas in R2 and R3. To compensate pressure/volume changes in R2 the internal vessel is adjustable in volume. Pressure vessels in R3 can be detached and manipulated separately. Sensors for electronic regulation are not indicated (after *Deusner* et al. [12.82])

tial pressure from near ambient pressure (0.2 MPa) to 10 MPa at a hydrostatic pressure of 16 MPa in the incubation stage. A methane partial pressure of 6 MPa and a hydrostatic pressure of 12 MPa in manifold fed-batch incubation in the HP-MI system gave a sixfold enhancement in the volumetric AOM rate.

The HP-CI system (Fig. 12.44) consists of a two stage high-pressure reactor, viz., a gas-medium reactor (R1) and a bioreactor (R2), along with two high-pressure pumps (P1, P2), and a gas compressor station (B1). Continuous enrichment of medium with methane in R1 occurs at pressures up to 20 MPa. In R1, while gas transfer from the headspace into the medium is expedited by agitation using a magnetic stirrer, liquid remains confined within an internal vessel (made of inert polymeric materials e.g., poly-ether-ether-ketone (PEEK) or poly-oxy-methylene (POM)) that prevents corrosion of the pressure vessel as well as contamination of the biomass. In R1 the fluid flow and the liquid level are automatically adjusted with constant – flow inflow and outflow pumps (P1 and P2) and is also monitored gravimetrically. The pressure P1 is adjusted with the gas compressor unit B1, operated in constant pressure mode, which is linked to R1 through a gas-distribution system comprising of a shut-off valve, a pressure-regulating valve, and a metering valve for fine regulation of gas flow. Pressurized gas can be supplied either batchwise or continuously, which facilitates continuous exchange of the gas phase in R1 and may be utilized for supply of low-concentration gaseous substrates during continuous operation.

Incubation in R2, may be performed up to a hydrostatic pressure of 35 MPa. The gas-enriched medium is transferred to R2 via pump P2 which is set to increase

the hydrostatic pressure relative to the pressure in R1 from P1 to P2 and allows incubation at increased hydrostatic pressure compared to the methane partial pressure in R1. The fluid flow in R2 is also automatically adjusted with pump P2 working in constant flow mode. R2 is also equipped with an internal vessel (made of PEEK or POM) to prevent potential chemical contamination of the biomass. Due to the mode of headspace-free incubation, the internal vessel is designed as a closed vessel. For compensating pressure or volume changes it is has a top and a bottom part and its volume is adjustable. The bottom part is equipped with an O-ring sealing that permits axial movement of the two parts relative to each other. The internal vessel is housed inside the pressure vessel surrounded by a hydraulic fluid that can be pressurized independently. Under standard operating conditions, the free volume for incubation is 420 mL within R2. Medium transport upstream and downstream of R2 is carried out through a tubing that is directly connected to the internal vessel through flexible lead-through systems. The internal vessel can be heat-sterilized and inoculated. The culture broth inside R2 is agitated with a magnetic stirrer. Liquid samples were taken from downstream section of both R1 and R2 at the sampling ports, directly into glass vials closed with butyl stoppers or into glass syringes after opening the shut-off needle valves in the sampling lines.

The HP-MI system (R3), originally developed (*Kallmeyer* et al. [12.71]) for high-pressure/high-temperature (HPHT) batch incubation, was modified and adapted in this study to be operated for manifold fed-batch incubation. Gas enrichment of liquid medium and delivery is attained in a similar method as in the HP-CI system. Each pressure vessel is equipped with shut-off valves at the inlet and outlet, respectively, and can be manipulated independently. Gas-enriched medium is transferred separately to each of the high-pressure vessels and each vessel is filled in upstream mode. Like R2, pressure vessels in R3 are operated without free gas and are equipped with internal vessels (free volume for incubation, 7 mL) (made of PEEK or POM), which are constructed as open vessels that seal against the steel by a polytetrafluoroethylene (PTFE) plate that presses into the cap of the steel vessel. While the medium is delivered into an internal vessel via lead-through tubings (similar to R2 in the HP-CI system), the upper outlet of an internal vessel has a built-in glass filter plate which allows permeation by both gas and liquid medium. Before sampling the biomass was allowed to settle to the bottom of the internal vessel; subsequently, an aliquot of the supernatant was collected through the bottom capillary while the sampled liquid volume was replaced with gaseous methane through the top capillary at the pressure corresponding to the saturation methane partial pressure applied during incubation. On commencing the fed-batch mode of operation, the design of the internal vessels ensured that the gas introduced during sampling was completely removed during filling of the vessels under high-pressure conditions.

This study demonstrated that biomass-specific activity increases substantially as a function of methane partial pressure in both continuous and fed-batch incubation. The systems could be used to control incubation parameters and to simulate environmental conditions with regard to constant substrate supply and product removal in continuous incubation. Stability with respect to sulfide production in continuous operation and reproducibility of results from manifold fed-batch incubation indicate that both the system components and the experimental procedure could be successfully applied for the study and incubation of AOM-performing marine microbial consortia.

Zhang et al. [12.83] developed a novel, continuous high-pressure bioreactor (HPBR) system to mimic a diverse methane-rich deep-sea ecological niche, to stimulate in vitro sulfate reduction (SR)-coupled- anaerobic oxidation of methane (AOM) activity; and to examine the kinetics of the SR-AOM process. The HPBR system (Fig. 12.45) comprises three main parts: (a) the conditioning vessel, (b) the high-pressure pump, and (c) the incubation vessel. The conditioning vessel (volume 1 L) made of S.S. 316 is directly connected to two pipettes – a liquid pipette (to charge liquid medium at high-pressure) and a gas pipette (to charge methane gas at high-pressure) both of which are pressurized by the methane gas bottle. Artificial seawater medium is saturated with methane at a determined pressure (up to 8 MPa) in the conditioning vessel and transferred to the incubation vessel by a high-pressure pump. In the conditioning vessel, built-up gas pressure determines the amount of methane saturated into the medium and available as substrate. A motor-mixer (40 rpm) is used to achieve a homogeneous dissolved methane concentration in the whole vessel. The high-pressure pump is piston based with a separated chamber to rinse the piston while it is moving – this self-flushing mechanism allows feed of saline water without damage to the piston and its seals. The incubation vessel (volume 0.6 L), made of titanium to prevent potential microbial corrosion, is placed inside a thermal regulated incubator (5–55 °C) to control the incubation temperature. The pressure inside this incubation vessel could be in-

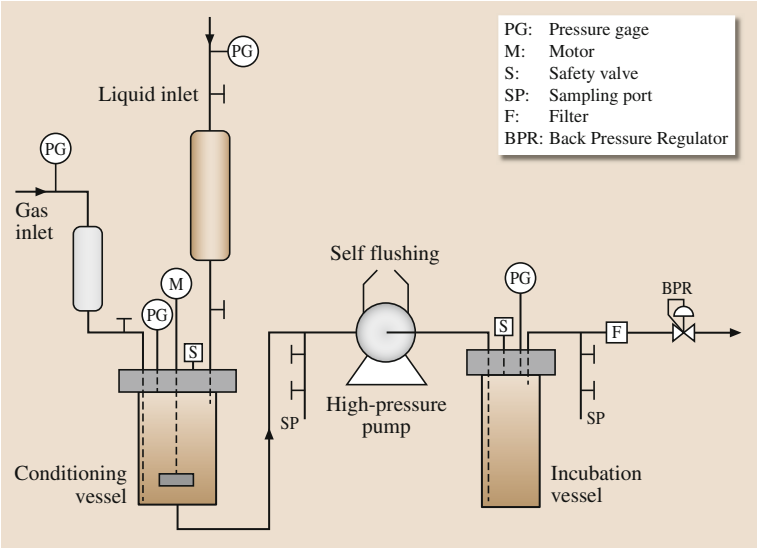

Fig. 12.45 The continuous high-pressure bioreactor system. PG – pressure gage; M – motor; S – safety valve; SP – sampling port; F – filter; BPR: back pressure regulator (after *Zhang* et al. [12.83])

creased to 16 MPa, and is regulated by the pump flow and a backpressure regulator. Two sampling ports were provided – one between the conditioning vessel and the high-pressure pump to take influent samples; the other between the incubation vessel and the backpressure regulator to take effluent samples.

Marteinsson et al. [12.84] examined the growth and survival of the deep-sea hyperthermophilic archaeon *Pyrococcus abyssi* GE5, isolated from hydrothermal vents in the North Fiji Basin at a depth of 2000 m, in sterile gas-tight glass syringes with cut pistons incubated in a high-pressure high-temperature (HPHT) cultivation system (Fig. 12.46) consisting of four S.S. pressure vessel incubators heated in four vertically positioned ovens (300 °C maximum). Pressure was generated with a hydraulic pump (with cold tap water as the hydraulic fluid) and monitored by Bourdon gauges (100 MPa) (maximum working pressure 60 MPa). Thermocouples, one internal and one external, were used for each pressure vessel, independently connected to a microprocessor in a temperature controlling unit, and the vessels could be heated from room temperature to 100 °C in < 30 min. The authors concluded that the effect of growth temperatures and pressure on the deep sea hyperthermophilic archaeon *P. abyssi* was associated with changes in the level of phospholipids more than the level of core lipids or proteins. The resistance shown by *P. abyssi* to lethal temperatures under in situ pressure was considered as the principle reason for its surviving tremendous spatial variability in temperature.

Marteinsson et al. [12.85] studied the effect of pressure, in pressurized syringes, at the temperature range for the growth of *Thermococcus barophilus* sp. nov. (Strain MP^T), a barophilic and hyperthermophilic, anaerobic, sulfur-metabolizing archaeon, isolated under high hydrostatic pressure (40 MPa) and temperature (95 °C) from a deep-sea hydrothermal vent (depth 3550 m). All culturing procedures before the high-pressure experiments were carried out under anaerobic conditions. Samples, stored at 4 °C under 40 MPa hydrostatic pressure, were depressurized and 0–5 mL suspension/fluids were transferred into 10 mL syringes which were sealed (by plunging needles into rubber stoppers), before media/samples were dispensed in them. Finally, the pistons were positioned and the gas phase was purged out before fastening the seal on each syringe. The syringes were then transferred into the HPHT incubation system (*Marteinsson* et al. [12.84]) pressurized to 40 MPa and heated to 95 °C. The archeaon was found to be barophilic at 75, 80, 85, 90, 95 and 98 °C, and was identified as an obligate barophile between 95 and 100 °C. For growth > 95 °C, a pressure of 15.0–17.5 MPa was necessary. It grew at 48–95 °C under atmospheric pressure. The optimal temperature for growth was 85 °C at both high (40 MPa) and low (0.3 MPa) pressures. The growth rate at 85 °C under in situ hydrostatic pressure was double that noted at low pressure.

Wright et al. [12.86] demonstrated the potential of high pressure as a stressing agent for marine bioprocess intensification by evaluating and confirming the

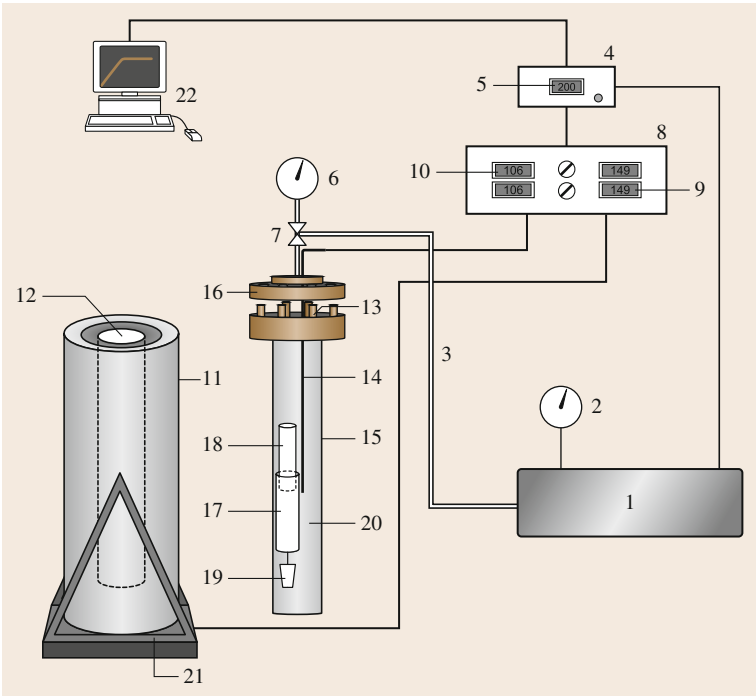

Fig. 12.46 Schematic representation of the high-pressure apparatus *hot bucket* for cultivating microorganisms at high temperature and pressure. One unit of four is shown. Each can be loaded or unloaded separately while the others are kept under constant temperature and pressure conditions. Key: 1 – hydraulic pressure generator; 2 – Bourdon gauge (100 MPa); 3 – water tube (inox); 4 – pressure indicator; 5 – digital pressure indicator; 6 – Bourdon gauge; 7 – valve; 8 – computerized independent thermoregulators; 9 – digital temperature indicator in oven; 10 – digital temperature indicator inside the pressure vessel; 11 – heating element (oven); 12 – bucket for the pressure vessel; 13 – O-ring; 14 – thermocouple in jacket; 15 – stainless steel pressure vessel; 16 – the head for the pressure vessel; 17 – culture syringe; 18 – cut piston; 19 – needle embedded in rubber stopper; 20 – water; 21 – thermocouple for heating unit; 22 – computer (after *Marteinsson* et al. [12.84])

barotolerance (at 12 MPa) of several marine bacteria isolated from shallow surface waters (< 1.5 m depth, pressure < 50 Pa) rather than from the deep sea. For this purpose, they used a high-pressure batch bioreactor (HPBBR) to create a maximum hydrostatic pressure of 120 MPa and to survey the pressure tolerance of the marine isolates. The HPBBR (maximum working volume 3.9 mL) was made of stainless steel and hydrostatic pressure was applied to the culture broth using an electrically driven piston. High-pressure cycling was employed since it is known to lower the tolerance of the microorganisms to high pressure. Interestingly, it was observed that barotolerance was much higher in bacteria obtained from biofilms anchored to the surface of seaweed than those isolated from open waters. It was noted that barotolerant rather than barophilic microorganisms is much more preferable for most bioprocess engineering operations as isolation and culture enrichment of barophiles is technologically more challenging as the barophilic strains must be continuously maintained at the remarkably high-pressure levels (> 50 MPa) for viability.

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13. Transgenic Technology in Marine Organisms

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Marine organisms into which a foreign gene or non-coding deoxyribonucleic acid (DNA) fragment is artificially introduced and stably integrated in their genomes are termed transgenic marine organisms. Since the first report in 1985, a wide range of transgenic fish and marine bivalve mollusks have been produced by microinjecting or electroporating homologous or heterologous transgenes into newly fertilized or unfertilized eggs and sperm. In the past few years, rapid advances of gene transfer technology has resulted in the production of many genetically modified organisms (GMOs) such as fish, crustaceans, microalgae, macroalgae, and sea urchins. These GMOs are valuable in assisting the advances of basic research as well as biotechnological application. In this chapter, the principle of producing transgenic marine organisms and the application of the technology to produce genetically modified marine organisms in the hope of improving the quality of human life as well as the earth environments will be critically reviewed.

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13.1 Synopsis

Marine organisms into which a foreign gene or non-coding DNA fragment is artificially introduced and integrated in their genomes are termed transgenic marine organisms. Since 1985, a wide range of transgenic fish species and marine bivalve mollusks have been produced mainly by microinjecting or electroporating homologous or heterologous transgenes into newly fertilized or unfertilized eggs and sometimes, sperm [13.1–5]. More recently, transgenic *Artemia* [13.6], microalgae [13.7], macroalgae [13.8], and sea urchins [13.9] have also been produced. Among the many transgenic marine organism species developed, transgenic fish species are the most abundant. To produce the desired transgenic marine organisms, several factors should be considered. First, an appropriate marine organism species must be chosen. This decision should depend on the nature of the study and the ability to maintain the organism in a biosafe containment facil-

ity. Second, a specific gene construct must be designed, based on the special requirement of each study. For example, the gene construct may contain an open reading frame encoding, a gene product of interest, and regulatory elements that regulate the expression of the gene in a temporal, spatial, and/or developmental stage-specific manner. Third, the gene construct must be introduced into sperm or embryos in order for the transgene to be stably integrated into the genome of embryonic cells. Fourth, since not all instances of gene transfer are efficient, a screening method must be adopted to identify transgenic individuals.

Since the development of the first transgenic fish in the mid-1980s, techniques to produce transgenic marine organisms have improved tremendously, resulting in the production of many GM marine species. In recent years, transgenic marine organisms have been established as valuable models for different disciplines of biological research, as well as human disease modeling. In addition, the application of transgenic technology to produce fish and other marine organisms with beneficial traits, such as enhanced somatic growth, disease resistance, and production of products for biotechnological applications, is also rising. In this chapter, the principle of producing transgenic marine organisms, especially transgenic fish, as well as the application of the technology to produce GM marine organisms to benefit humans will be critically reviewed.

13.2 Production of Transgenic Marine Organisms

13.2.1 Transgene Constructs

A transgene is a piece of DNA containing a non-self-origin or self-origin gene that is introduced into marine organisms for the production of transgenic marine organisms. Transgenes are usually constructed in plasmids that contain the desired structural genes and appropriate promoter/enhancer elements for proper expression of transgenes in marine organisms. At the beginning of the development of the technology for producing GM marine organisms, a variety of promoters from non-marine species were employed to control the expression of transgenes (Table 13.1). More recently, an increasing number of promoter/enhancer sequences of marine species origin (Table 13.1) have also been used for the purpose of generating *all-marine organism* expression cassettes. Among these all-marine-organism expression cassettes, the carp β-actin promoter from common carp [13.10, 11] has been shown to drive strong expression of transgenes in various fish cell types. Tissue-specific promoters and inducible promoters such as zebrafish *heat shock protein 70* (*hsp70*) have also been used to control the expression of transgenes in the desired tissue(s) under the desired conditions(s). For example, activation of the *hsp70*-controlled transgene can be achieved by heat shock and, intriguingly, by focusing a sublethal laser microbeam onto individual cells carrying the transgene [13.12]. The targeted cells appear normal after treatment.

Depending on the purpose of the gene transfer studies, transgenes can be grouped into three major types: i) *gain-of-function*, ii) *reporter-function*, and iii) *loss-of-function*. The basic feature and the function of these transgenes are discussed below.

Gain-of-function transgenes: transgenes of this type are designed to add new functions to the transgenic individuals or to facilitatey the identification of the transgenic individuals if the genes are expressed prop-

Table 13.1 Examples of representative promoters used in the production of transgenic fish

Origin of species	Promoter
Non-fish origin	
Rat	GAP43[a] ([13.12])
Chicken	β-actin ([13.13])
Xenopus	Elongation factor 1α ([13.14])
Cytomegalovirus	CMV intermediate-early ([13.15])
Moloney murine	MoMLV LTR ([13.16])
Leukemia virus	
Fish origin	
Carp	β-actin ([13.10, 11])
Goldfish	α 1 tubulin[a] ([13.10])
Medaka	Elongation factor 1α ([13.17]), β-actin ([13.18])
Zebrafish	H2A.F/Z ([13.19]), α-actin[a] ([13.20]), heat shock protein 70[b] ([13.21]), VTG[c] ([13.22]), Cyp19a1b[d] ([13.23])

[a] Tissue-specific promoters-GAP43: nervous system, α1 tubulin: nervous system, α-actin: muscle specific, [b] Heat inducible promoters; LTR: long terminal repeat; H2A.F/Z: histone H2A.F/Z, [c] VTG, estrogen inducible vitellogenin promoter, [d] estrogen inducible/tissue specific aromatase gene promoter

erly in the transgenic individuals. The coding region of the transgenes is usually homologous or heterologous genomic, or complementary DNA (cDNA) sequences which encode polypeptide products (Fig. 13.1a). The expression of transgenes is usually driven by promoter/enhancer sequences of homologous or heterologous sources. If transgenes a contain sequence of functional signal peptides, the gene products will be secreted out of cells once they are synthesized. Transgenes containing the structural gene of the mammalian or fish growth hormone (GH) or the respective cDNA fused to a functional promoter/enhancer of chicken or fish β-actin gene, are examples of the gain-of-function transgene constructs. Expression of such transgenes in transgenic individuals has been shown to result in growth enhancement [13.13–16, 24]. Winter flounder antifreeze protein gene or chicken δ-crystalline genes with their respective promoter/enhancer sequences are other commonly used gain-of-function transgenes in various gene transfer studies [13.18, 19].

Reporter-function transgenes: the prototypes of a reporter-function transgene are shown in Fig. 13.1b. Genes such as bacterial chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), neomycin phosphotransferase II (*neo*^{R}), luciferase, and green fluorescent protein (GFP) [13.20] are frequently used to produce a gene construct with a reporter-function (Fig. 13.1b). These reporter genes serve as a convenient marker for showing the success of gene transfer into target cells, since the translational products of these genes can be easily monitored. Furthermore, because these gene products can be measured quantitatively, they are also routinely adopted to identify the sequence of an undefined promoter/enhancer or to measure the relative activity of the promoter/enhancer element in question. A less frequently used reporter type is one with target-specificity. One such example is cameleon, a fluorescent calcium sensor, which was originally used to detect calcium transients in culture cells and pharyngeal muscles of *Caenorhabditis elegans*. Recently, cameleon and several other indicator genes have been used to establish transgenic zebrafish to monitor neuronal activity [13.21, 22].

Among the commonly used reporter genes, GFP and its variants identified in jellyfish (*Aequorea victoria*) have become the popular choice of markers for transgenic fish because of the easy and sensitive detection of GFP signals [13.20]. There are numerous GFP variants available, including those producing green, blue, and yellow fluorescent light. Together with red fluorescence protein (RFP), these reporters can be easily detected in the live transgenic fish such as zebrafish and medaka, whose embryos and bodies are transparent without the need to sacrifice the fish [13.20]. Additionally, these reporters can be used to study multiple transgenes simultaneously in the same fish. Since the first GFP transgenic fish produced in 1995 [13.20], GFP-tagged transgenic embryos and fish have become the most convenient and widely used tools in basic biological research, particularly in the regulation of gene expression, morphogenetic movements of cells, and organogenesis during embryonic development [13.17, 21, 23, 25]. More recently, the transgenes of GFP and their variants driven by tissue-specific promoters have also been transferred into medaka, zebrafish, and other

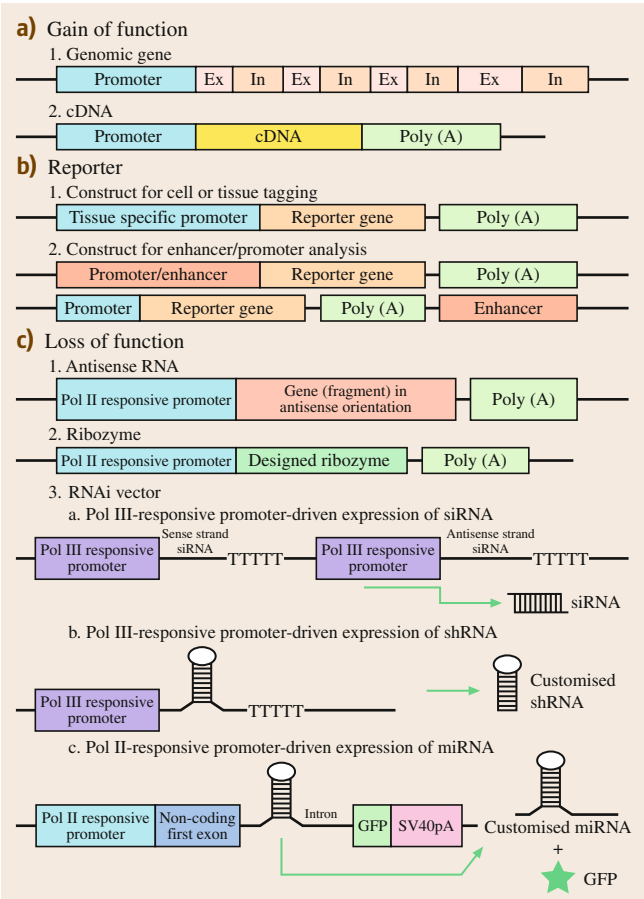

Fig. 13.1a–c Prototypes of transgenes for transgenesis of marine organisms. Depending on the experimental purpose, the promoter used in the transgene construct can be either a constitutive or an inducible promoter. Ex: exon; In: intron; pol III: RNA polymerase III promoters; pol II: RNA polymerase II promoter

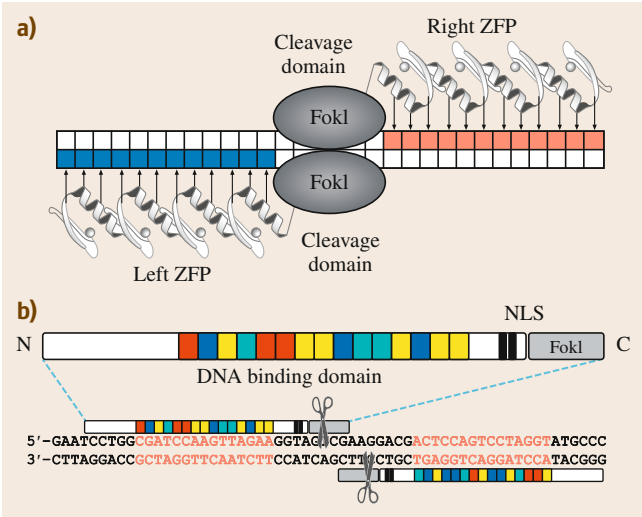

Fig. 13.2a,b Schematic presentation of recognition end cleavage by zinc-finger nucleases (ZFNs) and transcription of activator-like effector nuclease (TALEN). **(a)** Sketch of a ZFN dimer bound to a DNA target ZFNs; **(b)** Sketch of a TALEN bound to a DNA target (after [13.28, 29])

ornamental fish for commercial application [13.26, 27]. Similar applications have also been used with the other reporter genes mentioned above. However, the detection of these reporters is indirect and requires additional steps, such as enzymatic reaction with substrate or immunohistochemical staining with specific antibody to view the reporter gene products.

Lost-of-Function Transgenes: the loss-of-function transgenes are constructed for interfering with the expression of host genes at the transcriptional or translational level. This type of transgene construct is commonly used to inactivate the functions of genes through the mechanism of knocking down the expression of genes or knocking out the gene in various organisms (for a review, see [13.30, 31]). The prototypes of loss-of-function transgenes are shown in Fig. 13.1c. There are at least five types of loss-of-function transgenes. The first type of loss-of-function transgenes may encode antisense RNAs or oligonucleotides that selectively hybridize to an endogenous target messenger RNA (mRNA) of a gene, resulting in degradation of the mRNA by RNase H, or interference with translation of the mRNAs via preventing the binding of ribosomes to mRNA (Fig. 13.1c1, [13.31, 32]). A variation of the antisense RNA approach is to introduce a group of chemically modified oligonucleotides known as morpholino phosphorodiamidate oligonucleotides (MOs) into fish embryos [13.33–37]. In contrast to ordinary antisense molecules, MOs exhibit longer-term stability with less toxicity to living cells and appear to function through translation blockage only. If the MO target site contains bases flanking the intron junction, this MO antisense ribonucleic acid (RNA) can also block the splicing of respective precursor mRNA and thus knock down the gene activity [13.38]. The second type of loss-of-function transgenes express catalytic RNAs (ribozymes) that can cleave specific target mRNAs at a specific site and thereby *knock down* the normal level of gene expression (Fig. 13.1c2, [13.39–41]). The third group of *loss-of-function* transgenes expresses a group of small RNAs known as small (short) interfering RNAs (siRNAs). The prototypes of these genes are shown in Fig. 13.1c3a–c. Since the siRNAs contain sequences homologous to endogenous mRNAs to be regulated, they will guide a ribonuclease known as the RNA-induced silencing complex (RISC) to recognize the respective mRNAs and result in degradation of the mRNAs [13.42–47]. In recent years, several stable transgenic zebrafish lines carrying siRNA or miRNA transgenes have been produced [13.47].

The forth type of loss-of-function transgenes (Fig. 13.2a) is designed to express mRNAs that encode three zinc figure domains linking to the cleavage domain of *Fok*1 restriction endonuclease (ZFN). By introducing transgene expressing specific ZFN mRNA into cells or newly fertilized egg, incision of the gene to be inactivated will occur; and via the error-prone cellular repair enzymes, additional bases will be incorporated into the site of incision and result in creating a gene knockout without using embryonic stem cell lines that are not yet available in fish system [13.48–53]. By varying the number and type of zinc finger peptide (ZFP) in each ZFP-*Fok1* chimera, a cleavage component dimer may be targeted to a user-chosen site of sufficient length (18−36 bp) for a single occurrence in a gene. The ZFN transgene has been used to inactivate genes in zebrafish [13.54–56] and yellow catfish [13.57]. While ZFN is a very powerful technique for gene editing in fish, there exist some complication issues with the designing and application of ZFN. These complications are: i) some ZFNs have been associated with cytotoxicity due to cleavage at the off-target site, and ii) in some cases it is difficult to engineer ZFNs with the desired binding specificity [13.57]. To avoid these problems, scientists have turned to the use of the technique of TALEN (the fifth type of loss-of-function transgenes, Fig. 13.2b) for gene inactivation in zebrafish with greater success [13.58–60]. TALENs are novel

fusion proteins that are engineered by assembling the DNA-binding motif of the transcription activator-like effector (TALE) protein from *Xanthomonas* with *Fok1* nuclease. These molecules function similarly to ZFNs. Since TALEN-binding sites are expected to occur about once in every 35 base pairs on random DNA sequences, they will provide a greater flexibility in the selection of the target sites [13.61]. Very recently, another new method of gene inactivation has emerged from studies conducted on bacteria and archaea. In bacteria and archaea, there exists arrays of clustered, regularly interspersed short palindromic repeat (CRISPR) and CRISPR-associated (CAS) genes that can provide acquired resistance against phages by degrading the phage DNA or RNA [13.28, 62, 63]. From further studies on the structural features of the CRISPR and CAS systems, rational designing of CRISPR RNAs, and the CAS protein have been worked out for direct in vivo cleavage of RNAs or DNAs in zebrafish or other organisms [13.64].

13.2.2 Selection of Marine Organism Species

Gene transfer studies have been conducted in several different aquatic organisms including fish, shrimp, bivalves and algae. Since 1985, transgenic fish have been produced for many fish species such as salmon, rainbow trout, common carp, goldfish, loach, channel catfish, northern pike, seabream, tilapia, walleye, zebrafish, and Japanese medaka [13.1–3]. Depending on the purpose of the transgenic studies, the embryos of some fish species prove to be more suitable than others. In selecting a fish species for gene transfer studies, for instance, a series of parameters should be considered. These parameters are:

i) Life cycle length
ii) Year round supply of eggs and sperm
iii) Culture conditions
iv) Size of the adult at maturity, and
v) Availability of background information on genetics, physiology, and endocrinology of the fish species [13.5].

Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) are regarded as ideal fish species for conducting gene transfer studies. Both fish species have short life cycles (3 months from eggs to mature adults), produce hundreds of eggs on a regular basis without exhibiting a seasonal breeding cycle, and can be maintained in the laboratory for 2−3 years. Their eggs are relatively large (1.0−1.5 mm in diameter) and possess very thin and semi-transparent chorions. These features allow easy injection of DNA into the newly fertilized eggs if appropriate glass needles are used. Furthermore, inbred lines and various morphological mutants of both fish species are available. These fish species are ideal candidates for producing transgenic fish for:

i) Studying developmental regulation of gene expression and action
ii) Identifying *cis*-acting elements that regulate the expression of a gene
iii) Measuring the activity of promoters
iv) Producing transgenic models for human diseases and environmental toxicology. While the advantage of using zebrafish or medaka as transgenic model animals is due to the wealth of genetic and genomic databases, a major drawback of these two fish species is their small body size, which makes them less suitable for endocrinological and biochemical studies.

The handicap of using medaka or zebrafish as experimental animals in gene transfer studies can be overcome by the use of fish species such as loach, killifish (*Fundulus*), goldfish, and tilapia. The body sizes of these fish species are big enough to collect sufficient amounts of blood or tissue samples from individual fish for biochemical and endocrinological studies. Another important attribute of these fish species is their shorter maturation time compared to rainbow trout or salmon. In particular, tilapia is a very appropriate medium-sized fish species for gene transfer studies, since it only requires about 4 months to reach reproductive maturation. It is possible to produce almost three generations of transgenic tilapia in 1 year. Unfortunately, the lack of a well-defined genetic background and the asynchronous reproductive behavior of these fish species present some problems for the collection of a sufficient number of newly fertilized eggs to conduct gene transfer, unless a large number of reproductively mature fish is available.

Rainbow trout, salmon, channel catfish, and common carp are commonly used as large body size model fish species for transgenic fish studies. Since an extensive knowledge of the endocrinology, reproductive biology, and physiology of these fish species is available, they are well suited for studies on comparative endocrinology, as well as aquaculture application. However, the maturation time for each of these fish species is relatively long. For example, rainbow trout and salmon require 2–3 years to reach reproductive maturity, and common carp or channel catfish require

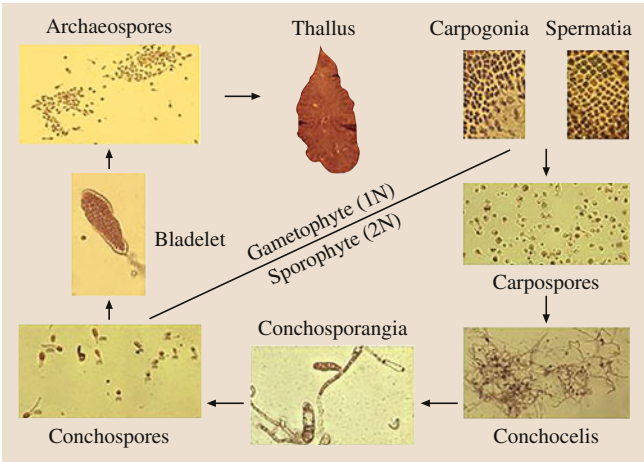

Fig. 13.3 Life cycle of Porphyra

about 1–2 years. Thus it will prolong inheritance studies. In addition, since these fish species have only a single spawning cycle per year, this restricts the number of gene transfer attempt that can be conducted per year.

Marine macroalgae, such as *Porphyra* and *Laminaria*, have a biphasic life cycle that alternates between macroscopic, foliose, gametophytic phase, and microscopic, shell-boring, sporophytic filaments referred to as the sporophytic phase (Fig. 13.3; [13.29, 65]). Since the genetic make-up of the gametophytic phase of *Porphyra* and *Laminaria* are haploid, they are suitable for gene transfer studies for biotechnological applications.

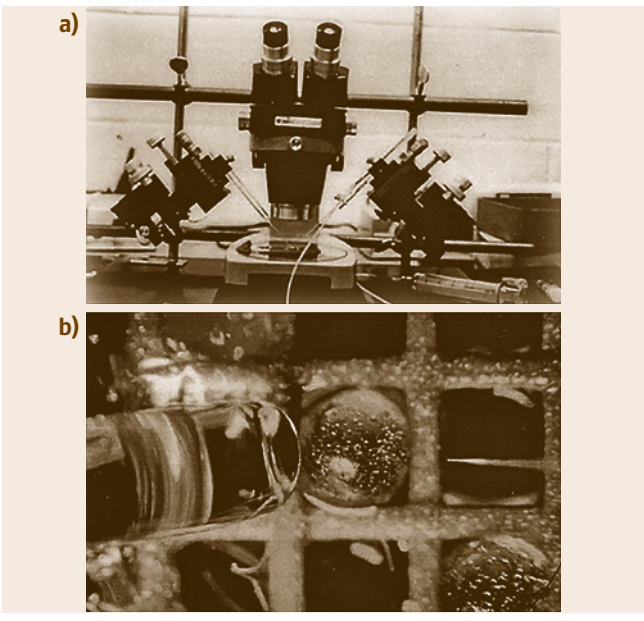

Fig. 13.4 (a) Microinjection setup; (b) microinjection of a medaka egg

13.2.3 Methods of Gene Transfer

Techniques such as calcium phosphate precipitation, direct microinjection, electroporation, retrovirus infection, lipofection, and particle gun bombardment have been widely used to introduce foreign DNA into animal cells, plant cells, and germ-lines of mammals and other vertebrates. Among these methods, direct microinjection and electroporation of DNA into newly fertilized eggs were proven to be the most reliable methods of gene transfer in fish systems in the early days. Methods such as transfection with pantropic retroviral vectors or liposomes have also been used to introduce genes into marine organisms in recent years.

Microinjection

microinjection of foreign DNA into newly fertilized eggs was first developed for the production of transgenic mice in the early 1980s [13.66]. Since then, the technique of microinjection has been adopted to introduce transgenes into Atlantic salmon, common carp, catfish, goldfish, loach, medaka, rainbow trout, tilapia, and zebrafish (for a review, see [13.1–4]). The gene constructs that were used in these studies include human or rat growth hormone (GH) genes, rainbow trout or salmon GH cDNA, the chicken δ-crystalline protein gene, the winter flounder antifreeze protein gene, the *E. coli* β-galactosidase gene, and the *E. coli* hygromycin resistance gene [13.1–4]. Gene transfer in fish by direct microinjection is described below, following the parameters summarized in Table 13.2. Eggs and sperm are collected in separate, dry containers. Fertilization is initiated by mixing sperm and eggs, then adding water, with gentle stirring to enhance fertilization. Eggs are microinjected within the first few hours after fertilization. The injection apparatus as shown in Fig. 13.4 consists of a dissecting stereo microscope and two micromanipulators, one with a micro-glass-needle for injection and the other with a micropipette for holding fish embryos in place. Routinely, about 10^6–10^8 molecules of a linearized transgene or transgene without plasmid DNA are injected into the egg cytoplasm. Following injection, the embryos are incubated in water until they hatch. Since natural spawning in zebrafish, medaka, or tilapia can be induced by ad-

Table 13.2 Parameters of fish transgenesis by microinjection and electroporation

Parameters	Gene transfer method	
	Microinjection	Electroporation
Developmental stage	1–2 cells	1–2 cells
DNA size	< 10 Kb	< 10 Kb
DNA concentration	10^{6-7} molecules/ embryo 100 µg/mL	
DNA topology	linear	linear
Chorion barrier	dechorionated/ micropyle	intact chorion
Electrical field strength	N/A[a]	500–3000 V
Pulse shape	N/A[a]	exponential/ square
Pulse duration	N/A[a]	ms[c] to s
Temperature	RT[b]	RT[b]
Medium	PBS/saline	PBS/saline

[a] N/A, not applicable, [b] RT – room temperature (25 °C), [c] ms – millisecond. These parameters were extracted from the following references [13.5, 11, 13, 15]

justing the photo-period and the water temperature, precisely staged newly fertilized eggs can be collected from the aquaria for gene transfer. If medaka eggs are maintained at 4 °C immediately after fertilization, the micropyle on the fertilized eggs will remain visible at least for 2 h. The DNA solution can be delivered into the embryos by injection through the micropyle during this time period.

Depending on the fish species, the survival rate of injected fish embryos ranges from 35 to 80%, while the rate of DNA integration ranges from 10 to 70% in the survivors [13.1, 2]. The tough chorions of the fertilized eggs in some fish species, e.g., rainbow trout and Atlantic salmon, can make insertion of glass needles difficult. This difficulty has been overcome by employing any one of the following methods:

i) Inserting the injection needles through the micropyle
ii) Making an opening on the egg chorions by microsurgery
iii) Removing the chorion by mechanical or enzymatic means
iv) Preventing chorion hardening by initiating fertilization in a solution containing 1 mM glutathione
v) Injecting the unfertilized eggs directly.

Electroporation

Electroporation is a successful method for transferring foreign DNA into bacteria, yeast, plant, and animal cells in culture [13.67–69]. This method has become popular for transferring foreign genes into fish embryos (or sperm) or newly fertilized eggs of dwarf surf clam in the past few years [13.5, 11, 70, 71]. The principle of electroporation is utilizing a series of short electrical pulses to permeate cell membranes, thereby permitting the entry of DNA molecules into embryos or sperm. The patterns of electrical pulses can be emitted in a single pulse of exponential decay form (i. e., exponential decay generator) or high frequency multiple peaks of square waves (i. e., square wave generator). Some commercially available electroporators are depicted in Fig. 13.5. The basic parameters of electroporation are summarized in Table 13.2. Studies conducted in our laboratory [13.11, 70] and those of others have shown that the rate of DNA integration in electroporated embryos is of the order of 20% or higher in the survivors [13.70, 71]. Although the overall rate of DNA integration in transgenic fish produced by electroporation was equal to or slightly lower than that of microinjection, the actual amount of time required for handling a large number of embryos by electroporation is orders of magni-

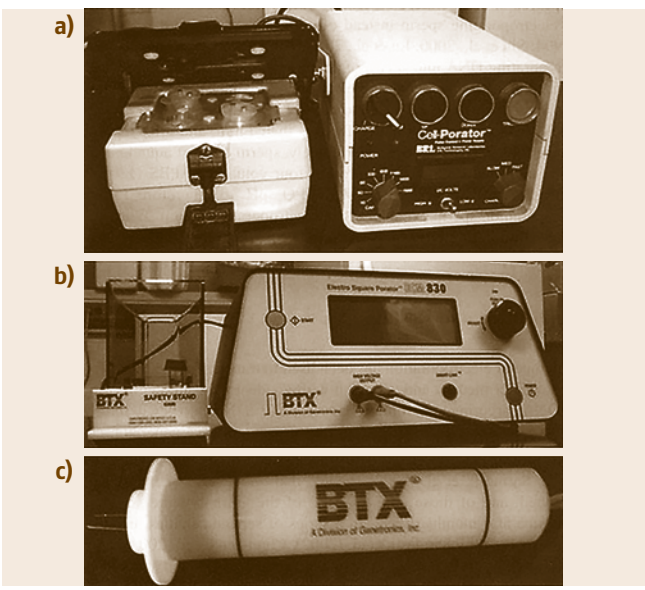

Fig. 13.5a–c Commercially available electroporator. **(a)** Exponential decay generator (cell porator from Gibco-BRL); **(b)** square wave generator (BTX); **(c)** hand-held electrode attachment to the square wave generator of BTX

tude less than that required for microinjection. Several reports have also appeared in the literature that describe successful transfer of transgenes into fish by electroporating sperm instead of embryos [13.11, 70–72]. Fish sperm remain un-activated in the presence of 100% seminal fluid (i. e., dry sperm). Upon dilution of the dry sperm with any aqueous buffer, the sperm will be activated and the window that sperm remain active is very brief (< 30 s). Out of the active window sperm are non-motile and unable to fertilize eggs. Therefore, electroporation of fish sperm needs to be conducted under a buffer condition that sperm remain un-activated. In our laboratory, we found that the dry sperm of rainbow trout can remain un-activated by making a 1 to 4 dilution with a phosphate buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L, KH_2PO_4, pH 7.6). With this condition we have successfully transferred foreign genes into rainbow trout via sperm-mediated gene transfer by electroporation.

Like insects, the sperm produced by male shrimp are stored in the spermatophore. Upon copulation, the spermatophore is transferred from the reproductive tracts of males to the reproductive tracts of females to fertilize eggs. By the time that eggs are spawned by reproductively mature females, the embryos are already in multiple cell stage, which is not suitable for conducting gene transfer by electroporation or microinjection. Recently, we found that Harvard Apparatus, Inc. has developed a hand-held electrode (two-needle array handle) for the BTX Medel 532 electroporator (Fig. 13.5c). We have used this hand-held electrode to introduce transgenes into the spermatophore of the American white shrimp, *Lipopenaeus vannamei*, following the protocol depicted in Fig. 13.6 [13.73]. In this procedure, the intact spermatophores were extracted from males, injected with linearized transgenes (20 ng in a final volume of 20 nL) and electroporated in a BTX square Wave Porter attached to a hand-held electrode (two-needle array handle). The conditions of electroporation are: voltage, 800 V; pulse length, 0.1 ms; pulse #, 2; pulse interval, 0.1 s. Following electroporation, the sperm were squeezed out from the electroporated spermatophore with a pair of forceps and transferred to the thylecum of the gravid females (Fig. 13.6). Results of polymerase chain reaction (PCR) analysis of genomic DNA samples isolated from pleopods of 8-month old presumptive transgenic shrimp showed that about 25% of the surviving animals contain the transgene under these electroporation conditions (Table 13.3). J.K. Lu at the National Taiwan Ocean University has also succeeded in producing transgenic tiger shrimp using the same device to electroporate the spermatophore (personal communication). In recent years there have been

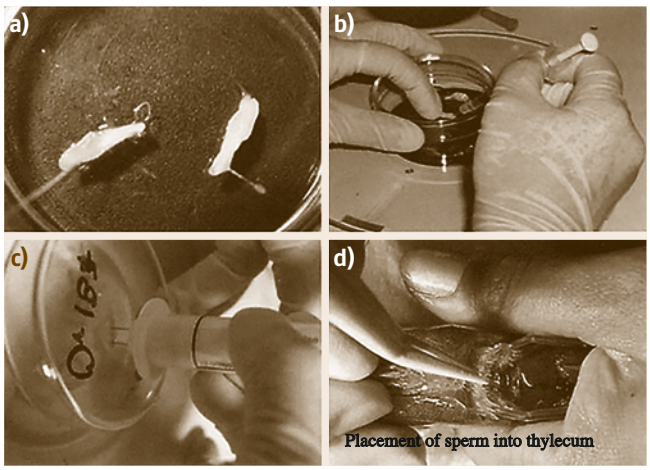

Fig. 13.6a–d Production of transgenic American white shrimp via electroporating transgene into spermatophore. **(a)** Intact spermatophore extracted from mature male shrimp; **(b)** injecting transgene into spermatophore; **(c)** electroporating transgene into sperm with hand-held electrode; **(d)** extracting sperm from electroporated spermatophore and inserting into the thylecum of gravid females

Table 13.3 Percent of transgenic American white shrimp produced by different gene transfer methods

Methods of gene transfer	% Transgenic shrimp
In situ electroporation[a]	25
Pantropic viral vector	27
Transfection[b]	
Lipofection[c]	22

[a] In situ electroporation: intact spermatophores were injected with linearized transgene (20 mg in 20 mL) and electroporated in a BTX square Wave Porter attached with a hand-held electrode (two-needle array handle) under the following conditions: voltage, 800 V; pulse length, 0.1 ms; pulse #, 2; pulse interval, 0.1 s. Following electroporation, the sperm were squeezed out from the electroporated spermatophore with a pair of forceps and transferred to the thylecum of the gravid females. [b] Pantropic viral vector transfection: pantropic viral vector (10^7 pfu/50 mL) was injected into each spermatophore, and 10 min after injection, the sperm were squeezed out from the injected spermatophore with a pair of forceps and transferred to the thylecum of the gravid females. [c] Lipofection: Each spermatophore was injected with 50 µl of a mixture containing 2 µg of linearized transgene construct and 3 µg of Lipofectamine (In Vitrogen, CA). The detail procedure of artifical insemination is as described in the text.

increasing numbers of publications appearing in the literature that report transfer of foreign genes into micro and macroalgal species by electroporation with good success [13.7, 74, 75].

By microinjection or electroporation, plasmid-encoded transgenes can be introduced into many fish species to produce transgenic offspring with satisfactory efficiency. However, the resulting P_1 transgenic individuals are almost always mosaics as a result of delayed transgene integration. In addition, neither methods is applicable for transfer of foreign DNA into embryos of life-bearing fish. In order to increase the efficiency of gene transfer by microinjection or electroporation, *Hsiao* et al. [13.76] and *Thermes* et al. [13.77] took the following approaches: i) using the inverted terminal repeat DNA from the *Adeno*-associated virus to increase the stability of transgene integration; and ii) employing *I-SceI* meganuclease to mediate high transportation efficiency of the transgene into the nucleus. While these modifications have resulted in improved efficiency of transgene integration, they are still not applicable for the production of transgenic life-bearing fish species.

Transfection with the Pantropic Retroviral Vector

A replication-defective pantropic retroviral vector containing the long terminal repeat (LTR) sequence of Moloney murine leukemia virus (MoMLV) and transgenes in a viral envelop with the G-protein of vesicular stomatitis virus (VSV) was developed by *Burns* et al. [13.78]. Since entry of VSV into host cells is mediated by interaction of the VSV-G protein with a phospholipid component of the host cell membrane, this pseudotyped retroviral vector has a very broad host range and is able to transfer transgenes into many different cell types. Using the pantropic pseudotyped defective retrovirus as a gene transfer vector, transgenes containing neo^R or β-galactosidase have been transferred into zebrafish [13.79], medaka [13.80, 81], and dwarf surf clams [13.5]. More recently, *Sarmasik* et al. [13.80, 81] and *Chen* et al. (unpublished results) also used a pantropic retroviral vector to transfer genes into the immature gonads of crayfish, desert guppy (Fig. 13.7; [13.81]), and shrimp (Table 13.3), respectively. They found that by using the pantropic retroviral vector as a gene transfer vector, the problem of transgene mosaicism in P_1 transgenic fish is eliminated when transgene-containing pantropic retroviral vectors were introduced into immature male gonads. Very recently, *Core* et al. [13.9] reported that the pantropic retroviral vector has been used as a tool for transducing sea urchin embryos.

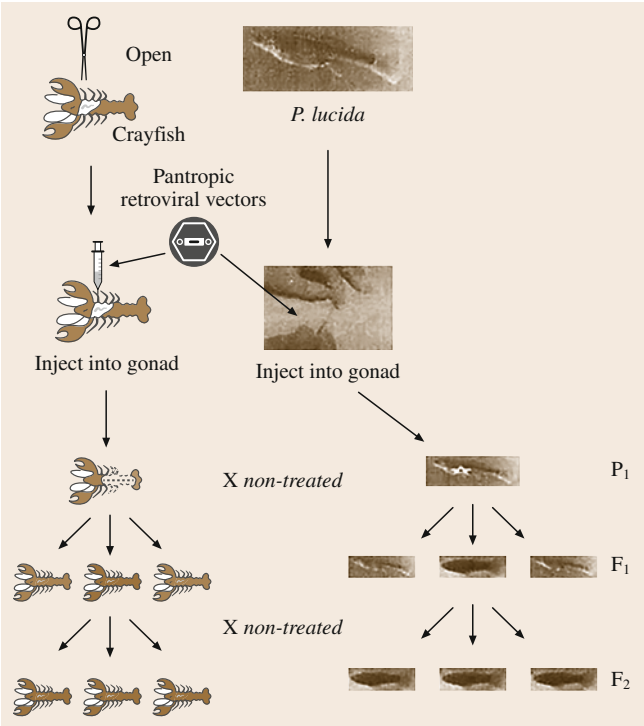

Fig. 13.7 Strategy of producing transgenic crustaceans and live-bearing fish by direct transformation of immature gonads with replicative defective pantropic retroviral vectors (after [13.82])

Transfection with Liposomes (Lipofection)

Cationic liposomes have been shown to be an effective vehicle for delivering DNA, mRNA, antisense oligomers, and proteins into living cells by fusion with cell membranes, since both are made of a phospholipid bilayer [13.83–86]. The efficiency of gene delivery by cationic liposomes has been further enhanced by modifying the compositions of the cationic lipids [13.87]. *Harel-Markowitz* et al. [13.88] have produced transgenic chicken by sperm-liposome mediated gene transfer. *Lu* et al. [13.11] have also succeeded in producing transgenic silver seabream by introducing liposome encapsulated transgenes into gonads of male silver seabream, followed by mating to mature females. In our laboratory, we have also produced transgenic white shrimp by injecting liposome encapsulated transgenes into intact spermatophores, after which we extracted the sperm from the treated spermatophore to inseminate the reproductive females (Table 13.3).

13.3 Characterization of Transgenic Marine Organisms

Once the gene transfer procedure has been conducted, the resulting organisms need to be characterized by i) identifying the presence of the transgene, ii) the expression of the transgene, and iii) the integration of the transgene in the transgenic organisms.

13.3.1 Identification of Transgenic Individuals

Identification of transgenic individuals is the most time-consuming step in the production of transgenic marine organisms. Traditionally, dot blot and Southern blot hybridization techniques were the common methods used to detect the presence of transgenes in presumptive transgenic individuals [13.89]. These methods involve isolation of genomic DNA from tissues of presumptive transgenic individuals, digestion of DNA samples with restriction enzymes, and Southern blot hybridization of the digested DNA products. Although this method is expensive, laborious, and insensitive, it offers a definitive answer as to whether or not a transgene has been integrated into the host genome. Furthermore, it also reveals the pattern of transgene integration if appropriate restriction enzymes are employed in the Southern blot hybridization analysis. In order to handle a large number of samples efficiently and economically, a polymerase chain reaction (PCR)-based assay has been adopted [13.13, 89]. The strategy of the assay is depicted in Fig. 13.8. It involves the isolation of genomic DNA from a very small piece of tissue, PCR amplification of the transgene sequence, and Southern blot analysis of the amplified products. Although this method does not differentiate between whether the transgene is integrated in the host genome or exists as an extrachromosomal unit, it serves as a rapid and sensitive screening method for identifying individuals that contain the transgene at the time of analysis. This method has been used in our laboratory as a routine preliminary screen for the presence of transgenes in thousands of presumptive transgenic fish.

13.3.2 Determination of Transgene Integration

Studies conducted in many fish species have shown that following transfer of linear or circular transgene constructs into fish embryos, the transgenes are maintained as an extrachromosomal unit through many rounds of DNA replication in the early phase of the embryonic development. At a later stage of embryonic development, some of the transgenes are randomly integrated into the host genome, while others are degraded, resulting in the production of mosaic P_1 transgenic fish [13.3]. A founder line of transgenic fish (F_1) should be established by crossing the P_1 transgenic individual with a non-transgenic counterpart. To determine the pattern of transgene integration in F_1 transgenic fish, genomic DNA extracted from PCR positive fish is digested with a series of restriction endonucleases and the resulting products resolved in agarose gels for Southern blot analysis. In many fish species studied to date, it is found that multiple copies of transgenes are integrated in a head-to-head, head-to-tail, or tail-to-tail form, except in transgenic common carp and channel catfish, where a single copy of transgene was integrated at multiple sites on the host chromosomes [13.3, 11, 81].

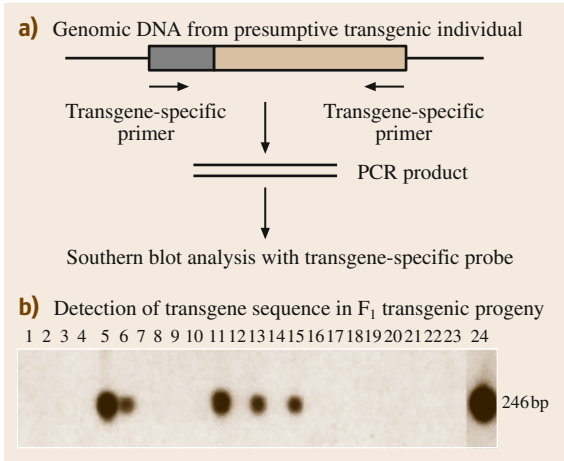

Fig. 13.8a,b Identification of transgene in transgenic individuals by PCR analysis of the genomic DNA. **(a)** Strategy of identifying transgene by PCR analysis of genomic DNA; **(b)** an example of detecting the presence of cecropin transgene in the genomic DNA of transgenic medaka. Genomic DNA samples extracted from the fin tissues of presumptive transgenic individuals were PCR amplified using a pair of transgene specific synthetic oligonucleotides as amplification primers. The PCR-amplified products were analyzed by Southern blot analysis. Lanes 1–22, genomic DNA from presumptive transgenic fish; lane 23, genomic DNA from non-transgenic fish; lane 24, transgene DNA (after [13.15])

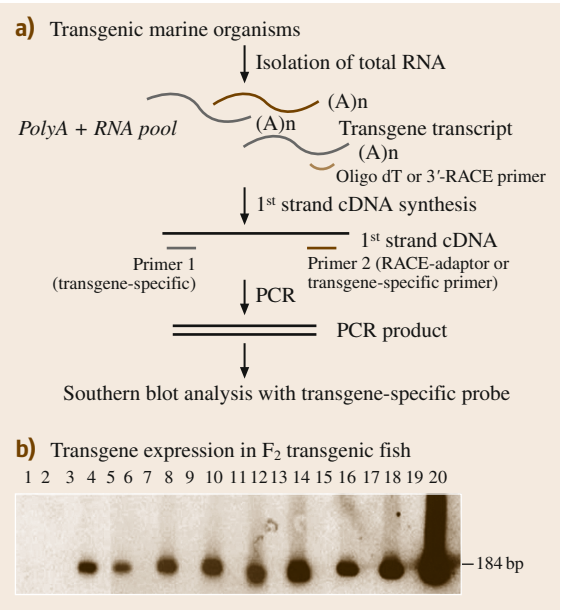

Fig. 13.9a,b Determination of transgene expression by RT-PCR or rapid amplification of cDNA ends-PCR (RACE-PCR). **(a)** Strategy of RT-PCR or RACE-PCR. **(b)** An example of detecting cecropin transgene expression in transgenic fish. Total RNA was isolated from fin tissues of F_2 transgenic medaka and the expression of cecropin transgene was detected by reverse transcribing poly A^+ RNA into cDNA and PCR amplifying the cecropin cDNA using cecropin-specific primers. The amplified product was confirmed by Southern hybridization using a cecropin-specific probe. RT-PCR was conducted with mRNA from transgenic fish without prior reverse transcription (lanes 5, 7, 9, 11, 13, 15, 17, 19) or with first cDNA transcribed from mRNA isolated from the transgenic fish (lanes 4, 6, 8, 10, 12, 14, 16, 18). Lane 1: RT-PCR with no mRNA input; lane 2: RT-PCR with mRNA from negative control fish; lane 3: PCR with mRNA of negative control fish without prior reverse transcription; lane 20: PCR with control plasmid containing cecropin gene (after [13.90])

Stable integration of transgenes is an absolute requirement for continuous vertical transmission to subsequent generations and establishment of a transgenic fish line. To determine whether the transgene is transmitted to subsequent generations, P_1 transgenic individuals are mated to non-transgenic individuals, and the progeny are assayed for the presence of transgenes by the PCR assay method described earlier [13.13, 14, 19, 91]. Although it has been shown that the transgene may persist in the F_1 generation of transgenic zebrafish as extrachromosomal DNA [13.92, 93], a detailed analysis of the rate of transmission of transgenes to F_1 and F_2 generations in many transgenic fish species indicates true and stable incorporation of gene constructs into the host genome [13.1, 3]. If the entire germline of the P_1 transgenic fish is transformed with at least one copy of the transgene per haploid genome, at least 50% of the F_1 transgenic progeny will be expected in a backcross involving a P_1 transgenic with a non-transgenic control. In many of such crosses, only about 20% of the progeny are transgenic [13.13, 19, 81, 89, 91]. When the F_1 transgenic is backcrossed with a non-transgenic control, however, at least 50% of the F_2 progeny are transgenics. These results clearly suggest that the germlines of P_1 transgenic fish are mosaic as a result of delayed transgene integration during embryonic development. For this reason, it is essential to establish a founder line of the transgenic organism by crossing the P_1 transgenic individual with its non-transgenic counterpart before proceeding to characterizing the transgenic organism in details.

13.3.3 Determination of Transgene Expression

An important aspect of gene transfer studies is the detection of transgene expression. Depending on the levels of transgene products in the transgenic individuals, the methods listed in the following are commonly used to detect transgene expression:

a) RNA northern or dot blot hybridization
b) RNase protection assay
c) Reverse transcription-polymerase chain reaction (RT-PCR)
d) Immunoblotting assay
e) Other biochemical assays for determining the presence of the transgene protein products.

Among these assays, RT-PCR is the most sensitive method and it only requires a small amount of sample. The strategy of this assay is summarized in Fig. 13.9. Briefly, the assay involves the isolation of total RNA from a small piece of tissue, synthesis of single-stranded cDNA by reverse transcription, and PCR amplification of the transgene cDNA by employing a pair of oligonucleotides specific to the transgene product as amplification primers. The resulting products are resolved on agarose gels and analyzed by Southern blot hybridization using a radio-labeled transgene as the hybridization probe. Transgene expression can also be quantified by a quantitative RT-PCR

method [13.94] or a quantitative real-time RT-PCR method [13.95].

13.3.4 Breeding Homozygous Transgenic Organisms

Almost all transgenic marine organisms produced by microinjection, electroporation, or transfection with pantropic retroviral vector or lipofection are heterozygous with respect to transgenes. For practical application, the transgenic lines need to be bred to be homozygous to maintain the brood stocks. This purpose can be achieved by self-crossing the heterozygous transgenic individuals and identifying the genotypes of the progeny by performing backcrosses. While this approach may be time consuming, depending on the length of the life cycle, it will obtain homozygous strains. In rainbow trout, since methods of chromosome manipulation are applicable, all male homozygous transgenic strains can be produced by the technique of androgenesis developed by *Parsons* and *Thorgaard* [13.82]. The strategy of this method is depicted in Fig. 13.10 [13.82]. Briefly, eggs from non-transgenic rainbow trout were irradiated with ^{60}Co γ-radiation to inactivate the nuclei and then fertilized with sperm from the heterozygous transgenic fish. Diploidy of the androgenic haploid zygotes was restored by suppression of the first cleavage division with hydrostatic pressure and the hatched fry were reared to adulthood. The YY transgenic males were identified by PCR amplification of the transgene and the Y chromosome specific sequence [13.96].

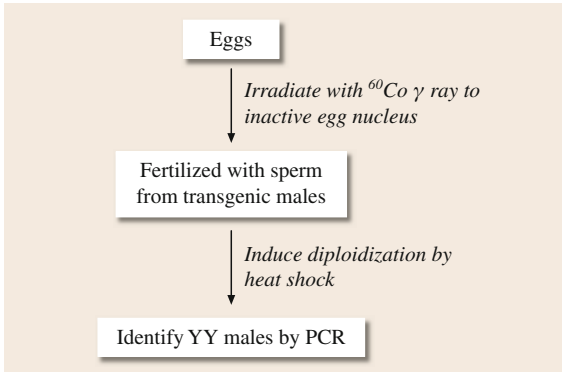

Fig. 13.10 Strategy for producing all male transgenic rainbow trout by androgenesis (after [13.97])

13.4 Biotechnological Application of Transgenic Marine Organisms

As techniques of producing various species of transgenic marine organisms have become mature in the last three decades, there has been a rapid boom in applying transgenic technology to different disciplines of basic research and biotechnological applications. Fish serve as extremely valuable models for basic research in vertebrate biology, as they are functionally similar to mammalian species. In addition, evolutionarily fish species are sufficiently close-distant to mammals; they are well suited for comparative genomics studies. Since transgenic fish can be easily and economically produced and reared in large quantities, this grants them superiority over the transgenic mouse model, especially in studies requiring a large number of animals. In recent years, transgenic fish technology has made major contributions in the areas of the study of vertebrate development, the analysis of promoter/enhancer elements of genes, the dissection of signal transduction pathways, and the development of human disease models. Transgenic fish technology research has also been driven toward biotechnological applications in recent years and it is hoped that it will be of high economic value to humans. Some of the advances in biotechnological applications will be reviewed in the following section.

13.4.1 Enhancement of Disease Resistance

Disease is one of the most severe bottlenecks in aquaculture. For the past few decades, efforts to control infectious diseases in commercially important fish species have primarily focused on the development of suitable vaccines for fish and selection of fish strains with robust resistance to infectious pathogens. Although effective vaccines have been developed for several important fish pathogens, current vaccination practice is expensive, laborious, and time consuming. Genetic selection based on traditional cross-breeding techniques is time consuming, and the outcome is frequently unpredictable, and sometimes disappointing due to a lack of the desired genetic traits. More effective approaches for controlling fish disease in aquaculture are highly desirable.

Transgenic fish technology can facilitate the genetic selection process by directly modifying the undesir-

able genetic traits that confer vulnerability to pathogens or introducing specific genes that are related to disease resistance into fish. The introduced transgenes can be fish-originated or characterized genes from other species. What genetic trait may confer resistance to infection by bacterial or viral pathogens? Upon exposure to invading bacterial, viral, or parasitic pathogens, fish, like higher vertebrates, will produce a family of polypeptides of small molecular weight, called antimicrobial peptides (AMPs), as part of the innate immunity response (Fig. 13.11). This family of peptides exhibits rapid killing of a broad spectrum of targets such as Gram-negative and Gram-positive bacteria, enveloped viruses, parasites, and even tumor cells [13.98–101]. Manipulating the AMP gene in fish may make them resistant to infection by pathogens. We have recently demonstrated the feasibility of introducing the antimicrobial peptide genes, cecropin B and cecropin P1, into fish to enhance disease resistance. Cecropins, first identified in the *Cecropia* moth, are members of the antimicrobial peptide family that are evolutionarily conserved in species from insects to mammals [13.101]. These peptides possess activities against a broad range of microorganisms, including bacteria, yeasts, and even viruses [13.101]. In vitro and in vivo studies conducted by *Sarmasik* et al. [13.90] showed that recombinant cecropin B inhibited the propagation of common fish pathogens such as *Pseudomonas flourescens, Aeromonas hydrophila*, and *Vibrio anguillarum*. Furthermore, *Chiou* et al. [13.102] showed that cecropin P1 and a designed analog, CF-17 peptide, effectively inhibited the replication of fish viruses, such as the infectious hematopoietic necrosis virus (IHNV), the viral hemorrhagic septicemia virus (VHSV), the snakehead rhabdovirus (SHRV), and the infectious pancreatic necrosis virus (IPNV). More recently, our laboratory has shown that synthetic peptide CF-17 is also effective in inhibiting the propagation of insect baculovirus (*Khoo* et al. unpublished result). Additionally, *Jia* et al. [13.97] demonstrated the enhanced resistance to bacterial infection in fish, which were continuously transfused with a cecropin-melittin hybrid peptide (CEME) and pleurocidin amide, a C-terminally amidated form of the natural flounder peptide. These results led to the hypothesis that production of a disease resistant fish strain may be achieved by manipulating AMP genes.

To test this hypothesis, we introduced gene constructs containing prepro-cecropin, procecropin, mature cecropin B, and cecropin P1 into the embryos of medaka by electroporation [13.90]. The F_2 transgenic medaka were subjected to bacterial challenges at an LD_{50} dose with *Pseudomonas fluorescens* and *Vibrio anguillarum*, respectively. The resulting relative percent survival (RPS) of the tested transgenic F_2 fish ranged from 72 to 100% against *P. fluorescens* and 25 to 75% against *V. anguillarum* [13.90]. To confirm the feasi-

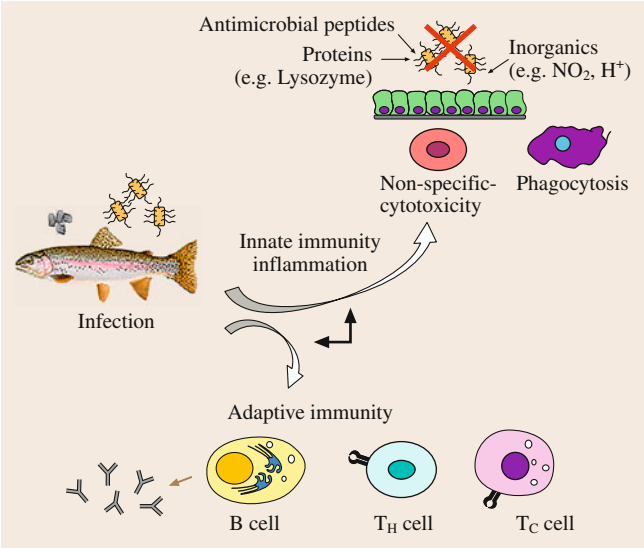

Fig. 13.11 Innate immunity and adaptive immunity exhibited by fish when encountering infection by bacterial and viral pathogenes

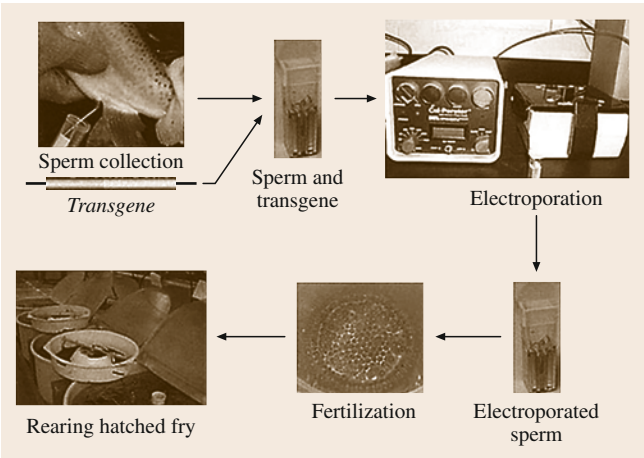

Fig. 13.12 Schematic presentation of the sperm-mediated gene transfer method. Sperm collected in a dry tube, diluted with sperm extender, mixed with insert of transgene DNA to give 10^4 DNA molecules/sperm, and electroporated in a cell porator under the following conditions: capacitance, 110 μF; voltage, 250–300 V; pulse number, 2. The electroporated sperm is used to fertilize rainbow trout and rear the hatched fry to adulthood (after [13.83])

bility of applying this technique to an aquaculture fish species, we transferred transgenes with cecropin P1 and CF-17 into rainbow trout by the sperm-mediated gene transfer method (Fig. 13.12). A total of nine families of cecropin P1 transgenic rainbow trout was established and bred to all male homozygous. Repeated challenge studies revealed that these transgenic trout exhibited resistance to the bacterial pathogen, *Aeromonas salmonicida* and the viral pathogen, infectious hematopoietic necrosis virus (IHNV) [13.103]. Via DNA microarray and quantitative real-time RT-PCR analyses, the expression of many genes in the innate/adaptive immunity pathways in transgenic trout was up or downregulated. This is the first time that a true disease resistant fish is available for detailed analysis of differential expression of genes in the innate/adaptive immunity pathways. All together these results clearly demonstrate the potential application of transgenic fish technology in producing fish with more robust resistance to infectious pathogens for aquaculture.

13.4.2 Enhancement of Somatic Growth

The initial drive of transgenic fish research came from attempts to increase the production of economically important fish for human consumption. The worldwide supply of fishery products has traditionally depended upon commercial harvest of finfish, shellfish, and crustaceans from freshwater and marine water sources. In recent years, while the worldwide commercial catch of fish has experienced a sharp reduction, the worldwide demand for fish products has risen steeply. In order to cope with the demand for fish products, many countries have turned to aquaculture. Although aquaculture has the potential to meet the world demand for fish products, innovative strategies are required to improve its efficiency. What can transgenic fish technology offer in this regard?

There are three aspects of fish growth characteristics that could be improved for aquaculture: i) increasing the initial growth rate of fry for an early head-start, ii) enhancing the somatic growth rate in adults to provide a larger market body size, and iii) improving feed conversion efficiency of fish to achieve effective utilization of feed. Among these three, enhanced somatic growth rates via manipulation of the GH gene show considerable promise. Studies conducted by *Agellon* et al. [13.104] and *Paynter* and *Chen* [13.105] showed that treatment of yearling rainbow trout and oysters with recombinant rainbow trout GH resulted in significant growth enhancement. Similar results of growth enhancement in fish treated with recombinant GH have been reported by many investigators [13.106–108]. These results point to the possibility of improving the somatic growth rate of fish by manipulating fish GH or its gene.

Zhu et al. [13.109] reported the first successful transfer of a human GH gene fused to a mouse metallothionein (MT) gene promoter into goldfish and loach. The F_1 offspring of these transgenic fish grew twice as large as their non-transgenic siblings. Since then, a similar enhanced growth effect has been observed in GH-transgenic fish (carrying the fish origin GH gene or its cDNA) of many other species, such as tilapia [13.110, 111], common carp [13.24, 89], catfish [13.16], sea bream [13.11], and salmon [13.15]. These studies have shown that the expression of a foreign human or fish GH gene could result in significant growth enhancement in P_1, F_1 and F_2 transgenic fish. Additionally, *Dunham* et al. [13.24] have demonstrated that transgenic common carp carrying rainbow trout GH transgene display, consistently in two consecutive generations, a favorable body shape, better dress-out yield, and better fresher quality compared with non-transgenic fish. These studies have demonstrated that GH-transgenic fish may be beneficial to worldwide aquaculture, and hence may help to alleviate starvation in many economically poor countries by providing more efficient, cheaper and yet high-quality protein sources. A strain of fast growing Atlantic salmon (AquAdvantage Salmon), which was initially developed by *Du* et al. [13.15] has been bred by AquaBounty Technologies (Maynard, MA) for approval by the Food and Drug Administration (FDA) of USA for commercialization.

13.4.3 Increase of Body Color Variation in Ornamental Fish

There are two main motives for producing fish with altered body color: i) to generate novel varieties of ornamental fish with rare colors for the purpose of rearing as pets, and ii) to use the change of body color as reporters for detecting environmental changes. The jellyfish green fluorescence protein (GFP) and several GFP variants driven by tissue-specific promoters such as the skin-specific keratin gene (*krt8*) and the muscle-specific gene (*mylz2*) are the genes that are used most for such purposes. Additionally, the melanin-concentrating hormone (MCH) isolated from chum salmon has also been exploited to generate transgenic medaka fish with altered body color [13.112]. So far, researchers have successfully produced several color-

ful transgenic zebrafish displaying whole-body green, red, yellow, or orange fluorescent colors in daylight, dim light. or UV light [13.113, 114]. Some of these fish have been sterilized to avoid contaminating the wild population if they should accidentally be released from aquaria to the environments [13.115]. For research purposes, mutant zebrafish and medaka with less interfering pigmentation for optical observation have also been developed. One such example is a line of *see-through* transgenic medaka that are transparent throughout their entire life, thus allowing clear visualization of GFP that is introduced into the fish as a reporter [13.116].

While studies on enhancing body color variation in ornamental fish were traditionally conducted in medaka and zebrafish, very little has been accomplished in mid-size or larger ornamental fish species. By co-injecting equal volumes of *Tol2* transposase mRNA (50 ng/μL) into one-cell stage angelfish embryos with a *Tol2* transposon-mediated transgene construct containing a muscle-specific zebrafish *CKMb* 2.4 kb promoter/enhancer linked to Acropora coral red fluorescent protein cDNA with SV40 polyA tail, we succeeded in producing strains of pink body color transgenic angelfish expressing the Acropora coral red fluorescent protein (Fig. 13.13). Furthermore, a strain of pink body color transgenic lionhead fish, another strain of mid-size ornamental fish, has also been produced by J.Y. Chen and J.L. Wu in the Institute of Cellular and Organismal Biology, Academia Sinica, Taiwan (personal communication).

Fig. 13.13 Four-month old F_1 transgenic anglefish (*Pterophyllum scalare*) expressing the Acropora coral (*Acroporo millepora*) red fluorescent protein gene. This research project was carried out in a collaboration with H.-Y. Gong and the Jy Lin Company in Taiwan

13.4.4 Models for Studying Human Diseases

In order to provide a general proof of principle or to reproduce specific aspects of human diseases, animal models have long been used to identify disease-associated factors or genes, or to screen new treatments for these diseases [13.117–119]. In addition to primate and murine models, zebrafish have been used to study human diseases for decades, particularly in cancer research and drug discovery [13.120, 121]. In recent years, transgenic zebrafish are emerging as a valuable model for studying human diseases, especially cardiac and neurodegenerative diseases and cancers [13.122–125]. In humans, tau proteins function to stabilize microtubules in neurons of the central nervous system. When tau proteins are defective, they result in dementias such as Alzheimer's disease. However, the early functional consequences of tau protein alterations in living neurons are incompletely understood. The development of transgenic zebrafish expressing mutated human tau in neurons may help to fill in the knowledge gap. By introducing a transgene construct harboring a mutated *tau* gene (FTDP-17 mutant) driven by the promoter of GATA-2 gene into the 1–2-cell stage zebrafish embryos, *Tomasiewicz* et al. [13.126] produced transgenic fish exhibiting characteristics resembled the neurofibrillary tangles (NFT) in human disease. The development of transgenic zebrafish with an approach similar to that described by *Tomasiewicz* et al. [13.126] will have a broad usefulness in the study of functional consequences, as well as in the genetic analyses of the introduction of other molecules involved in the pathogenesis of neurodegenerative diseases in vivo [13.123, 124].

Medaka and zebrafish can be induced to develop tumors in various organ sites that bear a striking resemblance to human malignancies, both histologically and genetically. With the significant advantages of in vivo imagining and well-established transgenic technology of high efficiency, these fish species will serve as powerful model organisms to study cancer biology. Stable lines of transgenic zebrafish expressing a fusion gene of green fluorescence protein and *c-myc* controlled by the *Rag2* promoter have been established [13.127]. Visualization of leukemic cells expressing the chimeric transgene revealed that leukemia arose in the thymus, spread locally into gill arches and retro-orbital soft tissue, and then disseminated into skeletal muscle and abdominal organs in transgenic individuals. The homing of leukemic T cells back to the thymus was also illustrated in irradiated fish transplanted with GFP-tagged leukemic lymphoblasts. This transgenic model provides a platform for drug

screening or for genetic screening aimed at identifying mutations that suppress or enhance *c-myc* induced carcinogenesis.

TP53 is the most frequently mutated tumor suppressor gene in human cancer, with about 50% of all tumors exhibiting a loss-of-function mutation. To elucidate the genetic pathways involving p53 and cancer, *Berghmans* et al. [13.128] developed transgenic zebrafish lines harboring missense mutation in the *tp53* DNA-binding domain. Transgenic zebrafish expressing the *tp53* missense mutation transgene developed malignant nerve sheath tumors. These transgenic lines can provide a unique platform for modifier screenings to identify genetic mutations or small molecules that affect *tp53*-related pathways, including apoptosis, cell cycle delay, and tumor suppression [13.129]. Very recently, a transgenic zebrafish model was established by *Liu* et al. [13.130] to study intrahepatic cholangiocarcinoma (ICC) associated with infection by hepatitis B (HBV) and C (HCV) viruses. These transgenic lines coexpress HBV X and HCV core protein genes in the liver and cause eventual ICC formation in the liver. Results of transcriptome sequencing analysis revealed that gene expression profiles of ICC formation in the transgenic fish are similar to those of human ICC formation. Thus, this in vivo model can serve as an ideal platform for identifying the molecular events leading to fibrosis and ICC caused by HBV and HCV infection. It may also serve as a platform for screening drugs to control hepatic fibrosis and ICC.

13.4.5 Transgenic Fish as Environmental Biomonitors

Fish have long been used as models in environmental toxicology studies. Prompted by concerns of human health, tissues of wild-caught fish have been used as indicators for the presence of dangerous pollutants such as polycyclic hydrocarbons, oxidants, and heavy metals in the waters or fish itself [13.131, 132]. Different assays have been employed to measure the biological parameters impacted by toxic pollutants, including DNA damage, defense enzymes (e.g., glutathione peroxidase and superoxide dismutase), genes inducible by toxic chemicals (e.g., cytochrome P450 1A1 and 1A2), and factors that regulate redox potential (e.g., glutathione and ascorbic acid) [13.131]. Although these assays are very sensitive, they require specialized techniques and equipment, and thus cannot be performed in the field where the samples are collected. In addition, the data obtained from wild-caught fish samples give no indication of the exact time when the fish were exposed to the toxicants.

Transgenic fish can potentially serve as sensitive sentinels for aquatic pollution, with the benefit of avoiding the inconveniences mentioned above. The principle of such fish biomonitoring is to introduce reporter genes that are easy to observe and are under the control of promoter/enhancer elements of pollutant responsive genes. GFP and its variants are thus the best reporters of choice for easy optical observation, whereas luciferase is the most sensitive. So far, researchers have made significant progress in establishing transgenic zebrafish carrying luciferase or GFP reporter genes under the control of aromatic hydrocarbon response elements (AHRE), electrophile response elements (EPRE), or metal response elements (MREs) [13.131]. The specificity of each type of response elements is as follows: AHREs respond to numerous polycyclic hydrocarbons and halogenated coplanar molecules such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls; EPREs respond to quinones and numerous other potent electrophilic oxidants; and MREs respond to heavy metal cations such as mercury, copper, nickel, cadmium, and zinc. In the zebrafish cell line, ZEM2S, these three types of elements can drive the expression of luciferase reporters in a dose-dependent, chemical-class-specific manner in response to more than 20 environmental pollutants [13.131].

Many environmental chemical pollutants possess endocrine disrupting activities; these compounds present a significant threat to human health. In recent years, several transgenic zebrafish and medaka lines have been produced for detecting environmental endocrine disruptors [13.132–138]. *Lee* et al. [13.136] developed a transient expression assay for detecting environmental estrogen via introducing a pair of plasmids (pRER_Gal4ff; pUAS_GFP) by microinjection into 1−2-cell stage embryos of zebrafish or medaka. The resulting transgenic embryos expressed GFP in response to exposure to pollutants containing estrogenic activity. This assay system will allow easy testing of environmental oestrogens, as well as detecting the oestrogenic target sites in developing embryos. However, severe drawbacks of the assay are: i) big fluctuations among embryos due to variations of the amount of transgene plasmid in each embryo and ii) cumbersome of microinjection. To overcome these problems, stable lines of transgenic zebrafish or medaka carrying reporter systems can be developed. *Chen* et al. [13.133] and *Brion* et al. [13.134] developed transgenic zebrafish carrying *ere-zvtg1-gfp* and *cyp19a1b-gfp* transgenes,

respectively, for in vivo screening of environmental estrogenic chemicals. Furthermore, by introducing a transgene carrying the *chgH-rfp* reporter system, *Cho* et al. [13.137] developed stable lines of estrogen-responsive transgenic marine medaka for assessing water born estrogenic compounds under a wide range of salinity conditions. Using a similar approach, transgenic zebrafish lines carrying the *gfp* transgene driven by the TH/bZIP promoter (promoter of thyroid hormone responsive genes) were developed by *Terrien* et al. [13.138] for detecting environmental toxicants that may disrupt thyroid function.

13.4.6 Other Biotechnological Applications

Transgenic marine organisms can potentially serve as bioreactors for producing proteins for therapeutic and other applications [13.114]. The advantages of fish as bioreactors include:

i) Relatively low cost as compared to mammals such as cows.
ii) Easy maintenance.
iii) Production of a large quantity of progenies.
iv) Most importantly, no known viruses or prions have been reported to infect both humans and fish.

Successful expression of bioactive human proteins such as coagulation factor VII, IGF-I, and IGF-II in fish embryos [13.139, 140] demonstrate this application of transgenic fish to produce therapeutic chemicals for humans in the future. Another application of transgenic organisms is the production of transgenic microalgae e.g., *Nannochloropsis oculata*) [13.7] and zooplankton (e.g., *Artemina*) [13.6] expressing biologically active fish growth hormones to serve as feed in aquaculture.

Hydrogen metabolism has been extensively studied in green alga, *C. reinhardtii*, resulting in significant advances in both the fundamental understanding of H_2 metabolism in this organism, as well as the improvements of overall H_2 yields. Thus, transgenic manipulation of genes involved in hydrogen production in *Chlamydomonas reinhardtii* would provide a great potential for biofuel production in the future [13.141].

Significant amounts of marine macroalgae (e.g., kelp and *Porphyra*) have been used as food for human consumption, feed for aquaculture of finfish and shellfish, and fine chemicals for industrial and pharmaceutical applications worldwide [13.142]. According to a report from the Food and Agriculture Organization of the United Nations (FAO) in 2005 [13.143], the annual harvests of kelp and *Prophyra* grown on shore and in coastal regions have declined drastically as a consequence of toxicant contamination in the aquatic ecosystem and disease manifestation caused by pathogenic microorganisms [13.144]. These problems may be alleviated by introducing genetic traits that will enable seaweed to remove water-borne toxicants

Table 13.4 Parameters for electroporating Porphyra archespores

Electroporator	Conditions
Gene pulser II	
kV/cm	2.0; 1.75; 1.50
% Modulation	100
RF (kHz)	50
Burst #	10
Duration (μs)	6.4
Interval (s)	0.4
Baekonizer 2000	
kV/cm	3.0
# Pulses	2^6
Burst (s)	0.4
Cycle #	10
Pulse time (ms)	100

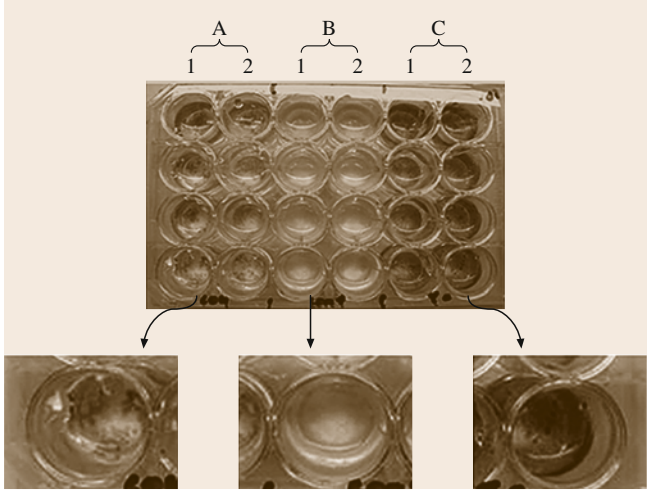

Fig. 13.14 Selection of transformants by BSD. A – Wild type *Porphyra* cultured in a growth medium without BSD; B – wild type *Porphyra* cultured in a growth medium with BSD (50 μg mL^{-1}); C – transformant *Prophyra* cultured in a growth medium with BSD (50 μg mL^{-1})

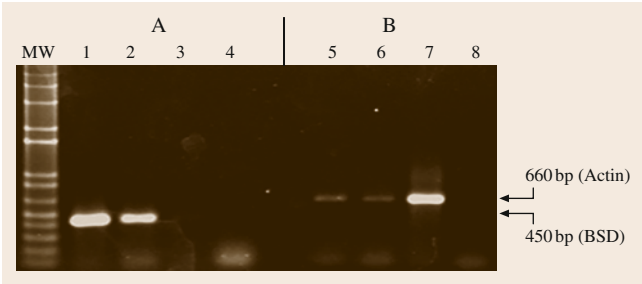

Fig. 13.15 Detection of transgene expression in transgenic *Porphyra* by RT-PCR. Total RNA isolated from transgenic and non-transgenic *Porphyra*, reversed transcribed, and PCR amplified using BSD specific and actin specific oligonucleotides as amplication primers. The products were analyzed by agrose electrophoresis. MW, molecular weight marker; A – Amplification of the BSD gene; B – amplification of the actin gene. Lanes 1 end 5, transformant clone 1; lanes 2 and 6, transformant clone 2; lanes 3 and 7, non-transgenic; lanes 4 and 8, PCR negative control

or confer the seaweed resistant to infection by disease-causing pathogens. Although numerous attempts have been made by scientists in the past to transfer genes into kelp or *Porphyra*, many years, very little success has been achieved in establishing stable transformants. Transient expression of the reporter GUS gene in the protoplasts of *Porphyra* has been reported by several investigators [13.145–148]. Although *Wang* et al. [13.149] reported the expression of the lacZ reporter gene in conchospores transformed through glass bead agitation, there are no data documenting the establishment of stable lines of transgenic *Porphyra*.

Since the genetic make-up of the archaeospore is haploid, it is suitable for conducting gene transfer studies. Our laboratory had initiated a project to establish stable transgenic *Porphyra* by electroporating a transgene carrying blasticidin (BSD) resistance marker driven by the promoter of Cytomegalovirus Mosaic Virus (CMV) early gene into achaeospores by Gene Pulser II with RF module (BioRad Company) or Beckonizer 2000 square wave generator. The conditions of electroporation are listed in Table 13.4. The results of our preliminary studies showed that an antibiotic, blasticidin, at a concentration of 50 μg/mL, is very effective to inhibit the growth of *Prophyra* archaeospores. Thus, it is used as a selection marker for transgenic *Porphyra*. Following electroporation, aschaeospores were incubated in the growth medium without BSD for 24 h to recover, and the transformants were selected by addition of BSD (50 μg/mL) to the growth medium. As is shown in Fig. 13.14, both electroporation conditions were able to deliver pCMV-BSD transgenes into *Prophyra*, and the expression of the BSD transgene was detected (Fig 13.15). Stable integration of pCMV-BSD

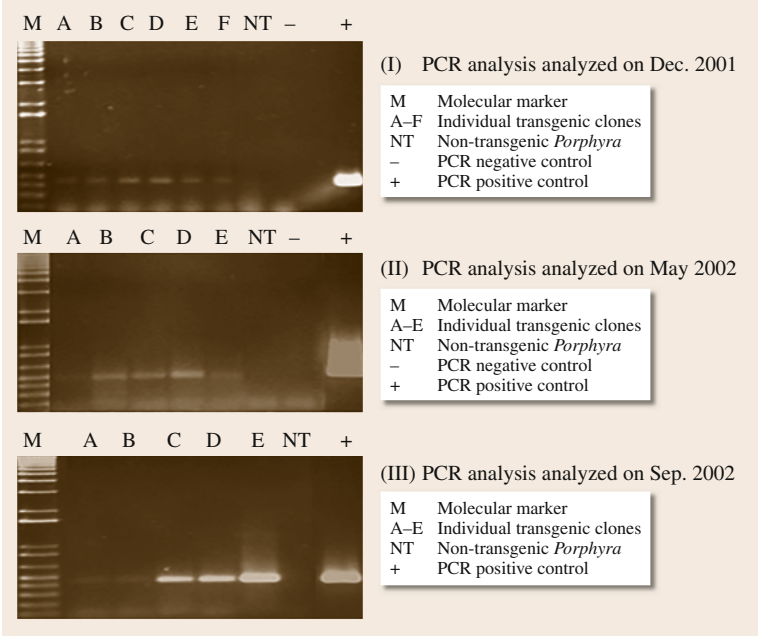

Fig. 13.16 Detection of the pCMV-BSD transgene in transgenic *Porphyra* at different passages. (I) Thallus analyzed in December 2001, (II) thallus analyzed in May 2002, (III) thallus analyzed in September 2002. DNA isolated from the transgenic thallus of different passages, analyzed by PCR amplification. Results show that the BSD transgene was present in the transgenic thallus of different passages, indicating permanent transformation

transgenes was detected in genomic DNA isolated from a *Porphyra* thallus after multiple passages (Fig 13.16). These results confirm the development of a protocol for transferring foreign genes into *Porphyra*.

13.5 Concerns and Future Perspectives

Application of transgenic technology to produce genetically modified (GM) food fish and ornamental fish with beneficial traits such as enhanced somatic growth and resistance to diseases, or diverse body color is on the rise. There have been ground-breaking discoveries made in the past few years based on transgenic fish models, and such applications are expected to continue to thrive in the future. Techniques to produce transgenic fish have improved tremendously in the last two decades; nonetheless, there are still concerns about low efficiency, mosaic expression, non-fish components of the transgenes, and the long generation time for certain fish species. The following advances would thus be essential in developing the next generation of transgenic fish:

i) The development of more efficient mass gene transfer technologies
ii) The development of targeted gene transfer technology such as the embryonic stem cell gene transfer method
iii) Identification of more suitable fish-origin promoters to direct the expression of transgenes at optimal levels and desired time
iv) The development of methods to shorten the time required to generate homozygous offspring.

Despite the promising future of the application of transgenic technology, there are great concerns about the environmental impacts of GM food fish or pets. Based on mathematical modeling, *Howard* et al. [13.150] proposed a *Trojan gene effect* hypothesis using male transgenic medaka expressing the salmon growth hormone gene. They proposed that the release of such fish into the environment could ultimately lead to the extinction of the wild population, owing to their advantage in mating with the wild females. The study provides a sound analysis of the potential impact of the accidental release of transgenic fish into the environment, reminding us of the delicate interaction between a newly introduced species and the native population and environment. However, we would reason that the potential environmental impact by transgenic fish is controllable and even avoidable. First of all, the Trojan gene effect might not necessarily exist for other types of transgenes or in other fish species. In practice, transgenic fish should be maintained in confined areas to avoid their escape into nearby waters. Moreover, as the desirable genetic traits can be introduced into fish, the undesirable behavior traits could be possibly removed from the transgenic population by screening the behavior pattern of each transgenic individual. Ultimately, the establishment of a sterile transgenic population can be applied to avoid the spreading of transgenes into the wild population. Ideally, such sterility should be reversible, otherwise it would mean the loss of the goose that lays the golden egg. Finally, from a historical perspective, the concerns in the early days that arose from the environmental impacts of recombinant DNA technology have not been realized and many lessons can be learned from this experience.

The acceptance of GM fish and other transgenic marine organisms as food by society is a key factor for the development of transgenic marine organisms as a new source of affordable and high-quality food. Largely, the concern is due to the public perception that GM food is unsafe to human health. Safety is the ultimate goal of GM food development and it relies on a thorough characterization of the transgenes used and the safety assessment of the transgenic products, resembling the development of new drugs. Unfortunately, it is a fact that there is always a subpopulation of people who are allergic, to different degrees, to many of the existing natural food sources, such as peanuts and shellfish. Therefore, the challenge to develop GM food with absolutely no adverse effect on every single individual in the human population is great. A thorough evaluation process to fully analyze any health risk of GM food to humans should be established, and the information should be clearly delivered to the public and well marked on the food products. The development of *gene-inactivated* transgenic marine organisms may be more acceptable by people who are concerned about the safety of the *new* genes that are introduced into the marine organisms. Take GM fish, for instance, one candidate GM fish is transgenic fish strains with their myostatin gene being inactivated. Inactivation of

the myostatin gene causes double muscling in animals, resulting in a significant increase in the mass of skeletal muscle. Natural mutation of the myostatin gene has been found in the meaty Belgian Blue and Piedmontese cattle, and the double muscling effect has been observed in mice whose myostatin gene was artificially inactivated [13.151, 152]. Such a *gene-inactivation* approach would be very valuable in producing transgenic fish with more flesh and of a higher food conversion rate, particularly for the economically important aquaculture species.

In summary, the application of transgenic technology in marine organisms has resulted in many valuable discoveries in basic biological science, many of which could lead to the next breakthrough for new cures for human diseases. On the other hand, the potential of transgenic marine organisms as bioreactors or as new renewable energy sources should not be deterred by environmental and safety concerns, but instead requires more research effort in the future to develop new GM species with beneficial traits that are safe to human health and also carry no harm to the environment.

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14. Marine Enzymes – Production and Applications

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The oceans provide an almost untapped reservoir of novel enzymes which might have a potential as biocatalysts for academic research and for industrial processes. With regard to the broad variety of environmental conditions, interesting enzymes with characteristic traits can be isolated. Especially enzymes from extremophiles, which are classified as thermophilic or psychrophilic, are of particular interest for industrial processes in terms of mass transfer or energy savings. Additionally, enzymes which are adapted towards high salt concentrations may be beneficial for industrial biotechnology applications, because the catalytic reactions can be performed in non-diluted solutions. Up to now only a minor amount of this treasure has found an application in the laboratory and industry. However, regarding the ongoing progress and developments in molecular biology it seems that the portfolio of enzymes that can be applied for the production of bulk and fine chemicals will be broadened in the near future.

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In this chapter an exemplary overview about the research work done on the production of enzymes from marine origin, as well as their potential application in industrial processes is given.

14.1 Overview

The oceans provide a broad variety of marine microorganisms whose products such as enzymes and bioactive metabolites are of interest for applications in the food and pharmaceutical industries, as well as for the processing of renewable resources to provide raw material, for example, for the production of biofuels. Considering the size of the marine habitats, which range from tropical to cold polar regions and from coastal to hydrothermal deep-sea zones, they harbor a pool of (micro)organisms that are able to produce enzymes with interesting traits like salt and barotolerance and/or adaptations to high or low temperatures, which are sometimes superior in comparison to their counterparts produced by terrestrial microorganisms.

During its initial phase, research in marine biotechnology was mainly driven by identifying novel bioactive compounds from the micro- and macro-organisms. The screening for novel metabolites was succeeded within the so-called two waves. The first wave began with the identification of novel nucleosides from the sponge *Cryptotethya crypta* by Bergmann more than 60 years ago [14.1] and resulted in the production of artificial nucleosides, e.g., ara-A (vidarabine) or ara-C (cytarabine), which are currently in use in many antiviral formulations. The second wave was initiated in the 1970s by isolation and identification of secondary metabolites featuring antitumoral, antiviral, anti-inflammatory, and/or analgesic activity. The first

drug from the sea was the peptide ziconotide, originally derived from a tropical marine cone snail. The compound was approved in the United States in 2004 under the trade name Prialt for the treatment of chronic pain in spinal cord injury [14.2]. A second drug (ET-743) from sea squirt origin was approved by the European Union in October 2007 for the treatment of soft-tissue sarcoma [14.2]. However, further promising candidates are still in several stages of clinical trials.

Today, natural product research also focuses on the enzyme systems that are responsible for the production of bioactive secondary metabolites, such as, for example, polyketide synthases [14.3, 4], which catalyzes the linkage of acyl-coenzyme A subunits via the generation of cyclic structures (e.g., bryostatin 1). Aside from the natural product research some (novel) marine enzymes, catalyzing a multitude of processes relevant for biocatalytic processes in industrial biotechnology, showing desired properties like high salt tolerance, hyperthermostability, barophilicity and cold adaptivity as mentioned before. Their performance results from the adaptation of their host organisms during the evolutional development to the environmental surroundings. However, the rising demand for environmentally-friendly and beneficial economical manufacturing of bulk and fine chemicals requires the exploitation of these enzymes for the specific and selective production of value-added products like chiral amines, alcohols, halogenated amino acids, as well as the enzymes themselves in the case of, for example, thermostable proteases or polymerases. Thus, marine microorganisms may contribute to expanding the number of (even more) stable and selective biocatalysts for industrial biotransformations. One major concern with establishing these enzymes in industrial-scale applications is their sustainable access. With respect to the difficulties of enzyme expression or cultivation of the natural expression system when transferred from its originally habitat to laboratory conditions, the responsible gene or even the gene cluster must be heterologously expressed. This approach requires identification, isolation, characterization, and cloning of the responsible genes in adequate host organisms (considering the codon usage of marine microorganisms), as well as optimization of enzyme stability and enzyme expression. In addition to microorganisms such as bacteria or fungi, higher organisms such as fish, prawns, crabs, snakes, plants, and algae can also be used for tapping of marine enzymes [14.5]. In particular the latter one represents a mentionable source for a number of halogenating enzymes.

The following chapters give an overview about handling and cultivation of the marine enzymes' hosts as well as expression of the biocatalysts. Moreover, some examples of enzymes from marine origin that are or may be of relevance for biotransformation processes are presented. In the main focus are saccharolytic enzymes such as amylases, cellulases, chitinases, agarases, as well as proteolytic enzymes, DNA polymerases, and halogenating enzymes. Since the supply of the biocatalyst depends on the expression and the applied downstream process, the final section of this review covers current purification techniques for intracellularly and extracellularly expressed proteins.

14.2 Cultivation Techniques of Marine Bacteria

The first step in the supply of an enzyme is the cultivation of the naturally occurring marine producing organism, except for using a metagenome approach, which is not addressed in this overview. Within the marine ecosystem microorganisms such as bacteria or fungi can be found in the free water column, in marine sediments, or in symbiotic associations (endosymbiontic and surface associated) with higher marine organisms even in the light and dark zones of the sea. Phototrophic microorganisms like cyanobacteria and microalgae are, however, primarily found within the zones affected by the sunlight.

The transfer of marine organisms from their natural ecosystems and their reproduction under laboratory conditions often fails, because a potential adaptation of the organism to its natural habitat and its participation in several symbiotic interactions with other organisms is commonly not considered. By establishing as far as possible similar environmental conditions to the marine strain these problems may be overcome in laboratory cultivations. Attention must be paid to the nutrient conditions for cultivation of the desired microorganism. Since bacteria or fungi deriving from the free water column exhibit different nutrient requirements than such organisms deriving from sediments or from the inner tissue of invertebrates, no standard nutrient broth that might be applied to support growth and production of all microorganisms exists.

Seawater contains approximately 10^4–10^6 cells ml^{-1}, whereas on surfaces exposed to seawater much higher cell densities, of six orders of magnitude, can be detected [14.6]. Thus, the applied nutrient media should reflect the nutrient conditions of the natural habitat. However, commonly used nutrient media feature a high carbon content, which is contradictory for the fermentation of marine strains. A special cultivation technique applicable for those strains that are classified as non-reproducible under laboratory conditions is the so-called dilution technique [14.7], whereas the populations in a sample are measured, diluted to a small and known number of cells, inoculated into unamended sterilized seawater, and afterwards examined for a defined period of time.

Another nutrient component which is often available to a less degree in seawater and in artificial nutrient broth is iron; therefore growth may be limited with respect to iron deficiency [14.8, 9]. Because of the limited bioavailability of iron, several organisms have adapted themselves and are able to release particular iron-complexing agents, so-called siderophores, enabling the organism to increase iron uptake [14.10].

Although marine microorganisms have specific nutrient requirements, many complex media have been developed for the cultivation of marine microorganisms under lab-scale conditions. The most prominent is the so-called Marine Broth 2216, which was developed in its primary formulation by *Zobell* in 1941 [14.11]. This complex medium can be regarded as the main reference medium in the field of marine microbiology. To reflect the special nutrient needs of distinct marine microorganisms it is sometimes utilized in diluted concentrations. Moreover, to satisfy the need for iron, it contains a well-accessible iron salt (0.1% iron-citrate).

Besides the cultivation of freely suspended cells, enrichment of organisms deriving from surfaces exposed to seawater or from tissues of higher marine organisms can be a challenging task. The organisms commonly grow as conglomerates of bacteria, fungi, algae, and protozoans and represent a biofilm. Within the biofilm the organisms benefit from the accompanying organisms in the extracellular matrix (commensalism and mutualism). Furthermore, the organisms are sheltered within the matrix thus they are often more tolerant towards unphysiological conditions than their suspended counterparts, e.g., towards high/low pH, pressure, and antibiotics. Microorganisms are exposed to varying conditions in their natural habitats with regard to pH, temperature, osmolality, or nutrients and they have evolved specific adaptation systems. Thus, they are able to respond to such fluctuations by activation or repression of gene expression [14.12]. Apart from these mechanisms alternative stimuli–response regulation patterns can be found in bacterial cell–cell communication by means of small signal molecules, called autoinducers. So far, a multitude of signal molecules and the induced signal cascades have been identified; this form of molecular regulation of signal transduction was defined as quorum sensing (QS). This term was given according to its special mechanism and was inaugurated by *Fuqua* et al. [14.13]. Hence, many interactions within such a microenvironment are mediated via different (chemical) QS communication patterns. During the phase of cell growth a specific autoinducer molecule is produced as a function of increasing cell-population density. The bacteria are able to detect and subsequently respond to the accumulation of these compounds, thus, they are capable of distinguishing between high and low cell population density and control gene expression in variation of cell population. By using such QS circuits a population of microorganisms can control the gene expression of the entire community [14.14]. Such a cell–cell communication was first described by *Hastings* and *Nealson* [14.15, 16], who elucidated the cell-density depending expression of luciferase of *Vibrio fischeri*. The microorganism emits light when growing inside the specialized light organs of the host organism but not when living freely in the ocean. So far, many communication strategies have been discovered, whereas distinct signal compounds have been found, depending on the species. Whereas Gram-positive bacteria commonly utilize amino acids or small peptides for QS [14.17, 18], derivatives of fatty acids (acyl-homoserine lactones) serve as autoinducers for Gram-negative bacteria [14.19]. Alternative communication strategies like hierarchical systems for cross-species communication have been summarized by *Whitehead* et al. and can be found elsewhere [14.19]. The discovery of the QS communication system is also of interest for the cultivation processes of marine microorganisms. For example, *Yan* et al. used a biofilm approach, where two epiphytic isolates were cultivated in a modified roller-bottle bioreactor to produce antimicrobial compounds [14.20]. Due to mimicking of the intertidal environment by application of this type of bioreactor, an enhanced production of antimicrobial compounds was found. When the organisms were cultivated in a planktonic state no antimicrobial activity was observed in the supernatant. Since QS is facilitated in the biofilm, it is assumed that production of the compounds is related to the communi-

cation of microorganisms. Moreover, *Yan* et al. have shown that secondary metabolite production of marine microorganisms can be induced by cross-species interactions [14.21]. They used a novel air-membrane surface bioreactor, allowing the bacteria to grow attached to a surface as a biofilm in contact with air. By exposure of the isolates to *Bacillus* sp. production of antimicrobial substances was induced. It can be assumed that this approach might also be transferable to the production of enzymes by raising the enzyme expression due to QS stimuli. Other reactor types that have shown their potential for cultivation of the biofilm state of marine microorganisms are the rotating disk contactor (rotating disk bioreactor, see Fig. 14.1) and a recently reported CCF (Fig. 14.2) approach. The latter one features an inner arrangement within a conical flask consisting of eight equidistantly spaced rectangular strips mounted radially on a circular disk to provide additional surface area for microbial attachment and ports to allow air supply [14.22]. So far, the CCF has proven its potential for the production of cellulase/xylanase and protease, whereas the biofilm state of the investigated microorganisms results in higher enzyme activities than the planktonic counterparts [14.23, 24]. By application of a novel rotating disk biofilm bioreactor *Sarkar* et al. faithfully mimicked the intertidal estuarine habitat of three marine isolates (salt-tolerant estuarine actinobacteria from the Sundarbans region), and supported biofilm formation, resulting in an increase of secondary metabolite production of the actinomycetes in comparison to stirred-tank cultivation [14.25].

Apart from using biofilms to enable the production of secondary metabolites, manipulating QS circuits by addition of signaling molecules may also be an approach to support the cultivation of bacteria. For instance, *Schink* et al. observed an improved growth of lake water bacteria, if acylated homoserine lactones are used as medium supplements [14.26]. Moreover, *Guan* and *Kamino* reported a three to eightfold increase of colony forming units when cultivation of marine-derived samples is accomplished in the presence of acyl-homoserine lactones and commercial siderophore desferrioxamine [14.27]. In addition to acylated homoserine lactones, other signaling molecules such as cyclic adenosine monophosphate (cAMP) might also influence the growth of marine bacteria. *Bruns* et al. reported a pronounced increase of the number of cultivated bacteria if cAMP, *N*-butyryl homoserine lactone, or N-oxohexanoyl-dl-homoserine lactone are supplemented even at a low concentration of 10 μM to the cultivation medium [14.29]. Interestingly enough, cAMP was identified as the most efficient inducer because the total bacterial counts were increased by 100%.

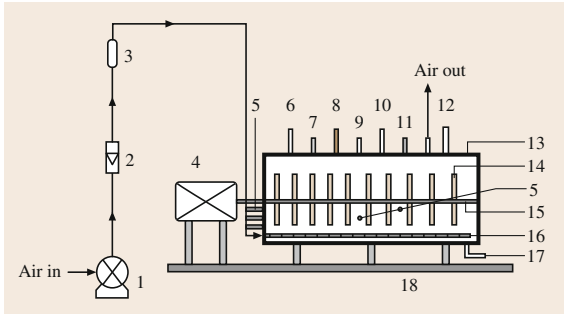

Fig. 14.1 Schematic of the ultra low-speed rotating disk bioreactor as described by *Sarkar* et al. after [14.28]. 1–Air pump, 2–rotameter, 3–air filter, 4–electrical motor and reducing gear train, 5–sampling port, 6–temperature sensor, 7–antifoam port, 8–inoculation and medium addition port, 9–acid port, 10–pH sensor, 11–alkali port, 12–DO sensor, 13–reactor vessel, 14–rotating coaxial disks, 15–shaft, 16–sparger, 17–drain, 18–base plate (courtesy of Springer)

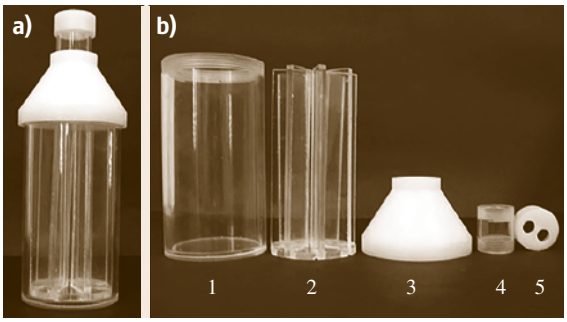

Fig. 14.2 **(a)** Conico-cylindrical flask (CCF) described by *Sarkar* et al. after [14.24] and US patent application number 20120295293A1 and **(b)** components of the CCF, 1–lower cylindrical portion, 2–inner arrangement, 3–upper funnel portion, 4–neck for joining top lid, 5–top lid for provision for aeration (courtesy of Elsevier)

14.3 Examples of Marine Enzymes

An important factor concerning the application of marine enzymes in industry and academic research is their amenability. Thus, cultivation of the enzyme-producing microorganism as well as identification (by molecular phylogenetic methods) and optimization of the enzymes' expression are crucial factors which must be addressed concerning the supply of the respected enzyme. However, as mentioned before molecular biology in terms of heterologous expression can be beneficial for reproducible (over)expression and production of enzymes on the requested scale. However, the symbiotic nature of the marine strains should be also taken into account, since marine microorganisms are often found in close association with various sponges, corals, and other species). Therefore mixed-cultures such as biofilms can be helpful to produce and identify the desired enzyme or, for example, bioactive secondary metabolites.

In the following sections some enzymes of marine origin, which are applicable in academic and industrial research/production, respectively, are highlighted.

14.3.1 Polysaccharide-Degrading Enzymes

Polysaccharolytic enzymes have well-known applications in the food processing, detergent, paper and textile industries. Furthermore, they have potential applications in the biofuel industry and in waste management. Cellulase, xylanase, and amylase are used in baking, brewing, and the production of natural sweeteners. Xylanases are also useful in the prebleaching of paper pulp and amylases are reported to be components of several laundry detergents. Carrageenolytic enzymes are used for liquefaction of carrageenan for applications in the food, pharmaceutical, and cosmetic industries. In addition to food, pharmaceutical, and cosmetic applications agarases have potential applications in biotechnological research. Cellulolytic and lignolytic enzymes are beneficial in the production of biofuels, since degradation of polysaccharide biomasses is the major challenge in the development of plant-derived biofuels. Plant biomasses often contain complex polysaccharides that need to be converted into simple fermentable sugars for bioethanol production. Cellulose, xylan, and lignocellulose are major groups of polysaccharides that are abundant in nature and they are major components of almost all biowaste and some industrial waste. Degradation of these materials is essential for pollution control. Various polysaccharolytic enzymes may be used for their degradation and conversion to value-added products.

Amylases

Amylase is an enzyme that catalyzes starch hydrolysis and is broadly used for the production of simple sugars. Versatile amylases have been reported from marine organisms. *Legin* et al. reported 70 amylase producing microorganisms while screening 269 thermophilic isolates from deep-sea hydrothermal vents [14.30]. Most thermostable enzymes were produced by several hyperthermophilic archaea belonging to the genus *Thermococcus*. Four of the archaeal strains showed pullulanase and α-glucosidase activity in addition to α-amylase activity. In another screening, several amylase producing psychrophilic bacteria were reported from deep-sea sediments of Antarctica [14.31]. Molecular phylogeny identified the amylase producing strains as members of the genera *Pseudomonas*, *Rhodococcus*, and *Nocardiopsis*. The two most active amylases were purified from two *Pseudomonas* strains and the enzymes were characterized. Both enzymes were optimally produced at low temperatures (15–20 °C), but their optimum activity was reported at a remarkably high temperature (40 °C), which is contradictory to their natural habitat. Both the enzymes retained almost 50% activity at 5 °C. However, the enzymes were unstable at a temperature above 30 °C and retained only half of their maximum activity after 5 min incubation at 60 °C [14.32]. An amylase encoding gene was isolated from a marine metagenomic library and overexpressed in *E. coli* [14.33]. The enzyme exhibited 72% identity of amino acid sequence to a putative glycosidase of the deep-sea bacterium *Photobacterium profundum*, followed by 67% and 53% identity to putative glycosidases of marine *Vibrio splendidus* and *Hahella chejuensis*. It showed about 50% sequence similarity to the putative glycoside hydrolase from the marine fish pathogen *Aeromonas salmonicida*. All these putative glycosidases were identified by genome sequences of marine bacteria and formed a new subfamily of glycoside hydrolase GH13, which is closely related to the bacterial α-amylases. The authors proposed that the subfamily may be an independent clade of ancestral marine bacterial α-amylases. Instead of low similarity of the full length sequence of any known α-amylases this enzyme showed a catalytic domain correlated with the α-amylase superfamily. A similar observation was reported in novel α-amylases isolated from *Thermo-*

toga maririma [14.34]. The putative enzyme had no amino acid sequence similarity to the glycoside hydrolase family (GHF) 13 α-amylases, yet the range of substrate hydrolysis and the product profile suggest the protein to be an α-amylase. A marine *Bacillus* was reported to produce several α-amylases that can degrade various types of plant-based raw starch to produce simple sugars [14.35, 36]. Two statistical analyses were applied to optimize the media composition and fermentation parameters for cold adapted amylase production by the marine isolate *Wangia* sp. C52 in a shake flask culture [14.33]. The study showed starch, tryptone, and initial pH had significant effects on production of this enzyme. The maximum amylase production was optimized at 20 °C and pH 7.18 with a shaking speed of 180 rpm. The experimental yield of the amylase in the optimized media closely matched the yield predicted by the statistical model when the fermentation was performed at theoretically optimized fermentations conditions. Tenfold higher amylase production was observed after this optimization. *Chakraborty* et al. isolated two thermostable alkaliphilic amylases from marine microorganisms that showed excellent stability towards surfactants and commercial detergents – the enzymes, therefore, have potential applications in the detergent industry [14.37, 38].

Cellulases and Lignocellulases

Cellulolytic enzymes were reported from a range of marine microorganisms, including bacteria and fungi. A *Saccharophagus degradans* strain was reported to degrade large numbers of complex polysaccharides from numbers of algae, plant, and invertebrate sources [14.39]. Complete genome sequencing of the bacteria revealed more than 180 open reading frames encoding carbohydrate degrading enzymes. It appears that this bacterium consists of a full array of polysaccharide degrading enzymes for complete degradation of plant cell walls. 13 cellulose depolymerases and seven accessory enzymes including two cellodextrinases, three cellobiases, a cellodextrin phosphorylase, and a cellobiose phosphorylase were predicted by genomic and proteomic analyses. A solvent stable alkaline cellulase was reported from a solvent tolerant marine strain of *Bacillus aquimaris* [14.40]. Enzyme stability increased with the presence of 20 v/v % benzene and it was significantly stable in the same concentration of methanol, acetone, and toluene. The enzyme activity was increased to 150% and 155%, respectively, by pre-incubation in two ionic liquids 1-ethyl-3-methylimidazolium methanesulfonate and 1-ethyl-3-methylimidazolium bromide. Cellulase productions by a group of marine microorganisms were optimized using industrial and agricultural wastes. Rice bran was reported to be a preferential nutrient for optimum production of three cellulases by a *Psychrobacter aquimaris* strain [14.41]. The microbe has optimum growth at 25 °C but maximum production of avicelase, CMCase, and filter paperase was achieved at 30 °C. Aeration was more important than agitation for cell growth and fermentation of all three cellulases. Enzyme production was scaled up to a 100 l pilot plant bioreactor. *El-Sersy* et al. screened six marine strains of actinomycetes for CMCase production and a *Streptomyces ruber* strain showed the best productivity [14.42]. Rice straw was used as the carbon source. The Plackett–Burman design was applied to optimize the media composition and fermentation parameters for maximum enzyme production. Two different cellulases were partially purified and their specific activities were determined as 4239.697 U mg^{-}1 and 846.752 U mg^{-1}, respectively. Three alkaline cellulase producing fungal strains were isolated from mangrove leaves and wood litters [14.43]. The production of β-endoglucanase, β-exoglucanase, and β-glucosidase by these isolates was optimized in submerged fermentation and solid-state fermentation using cheap agricultural and industrial waste as substrates. Maximum enzyme production was achieved by solid-state fermentation at alkaline pH using media containing cotton seed. It is interesting to note that the enzymes produced under alkaline conditions showed higher stability and activity than those produced by the same organisms under non-alkaline conditions. *Pointing* et al. studied 15 marine fungi for cellulolytic and lignolytic activity [14.44]. The lignolytic activity of these microorganisms was dependent on the primary carbon source, pH, and salinity of the medium. In another study, 29 marine fungi were screened for lignocellulose-degrading enzyme activities in agar media [14.45]. The isolates were identified as 27 ascomycetes, one basidiomycete (*Calathella mangrovei*), and a mitosporic fungus (*Cirrenalia tropicalis*). Cellulolytic, xylanolytic, and ligninolytic activities were studied in all 29 isolates. 21 isolates produced three enzymes: endoglucanase, xylanase, and laccase, while 2 isolates showed a lack of xylanase activity and 6 isolates had no laccase activity.

Chitinases

Chitin is a major component of fungal cell walls, the exoskeleton of arthropods including insects and crustaceans (mollusks, crabs, lobsters, and shrimps), and the

internal shell of cephalopods (squids and octopuses). Most of these organisms live in the habitat of the marine ecosystem, and it stands to reason that the chitin hydrolytic enzyme (chitinase) is abundant in the marine environment. Chitinolytic activity was reported in several marine bacteria (pathogens) that attack living arthropods or degrades the chitin-rich biomass derived from their exoskeleton. Chitinase is used for the production of single cell proteins for animal and aquaculture feed, for isolation of fungal protoplasts, and for the preparation of chitooligosaccharides. Chitinases are not widely used at commercial scale due to their high cost but they have interesting potential applications in pest control and preparation of medicines. Chitinase genes can be used to develop chitinase-producing transgenic plants that are resistant to insects and fungi. The cell walls of some human pathogens including fungi, protozoa, and helminths are composed of chitin, which could be hydrolyzed by microbial chitinases and thus, chitinase may be used for the treatment of these microbial infections.

A chitinase C encoding gene from the marine isolate *Salinivibrio costicola* 5SM-1 strain was cloned in the pGEM7Zf(+) vector and overexpressed in *E. coli* [14.46]. The protein was identified as an exochitinase that hydrolyzes colloidal chitin and produces chitobiose as the major hydrolysis product. One catalytic domain and two chitin binding domains were identified by amino acid sequence alignment. The complete loss of chitinase activity by mutation of three conserved amino acid residues at the putative catalytic site confirmed the location of the catalytic domain. Another chitinase C enzyme was reported from the marine *Alteromonas* sp. strain O-7, and the catalytic domain was predicted by a similar approach [14.47]. The same research group also isolated the novel cold-adapted chitinase B from this microorganism [14.48]. *Gohel* et al. used the Plackett–Burman design to optimize media composition for chitinase production by the marine strain of *Pantoea dispersa* [14.49]. In this optimized media chitinase production increases by 4.21 fold in comparison to the basal medium; also a 3.95-fold increase of endochitinase production and a 2.31-fold increase of chitobiase production was reported in this medium. In another study, the impact of process parameters was studied on extracellular chitinase production by a *Beauveria bassiana* strain in solid-state fermentation using wheat bran [14.50]. The chitinolytic marine fungus was isolated from marine sediments from the south coast of India and characterized as a spore-forming alkaliphilic fungus. The initial moisture content significantly affected the microbial growth rate and enzyme production. Maximum chitinase production was achieved after the third day of fermentation using the media containing wheat bran to a seawater ratio of 5 : 3 (w/v). Chitinase production by this fungus was also optimized in solid-state fermentation using chitin-rich prawn waste as nutrient [14.51]. The antifungal activity of chitinase was reported from the marine *Streptomyces* sp. DA11 isolated from a sponge from the South China Sea [14.52]. Amino acid sequence alignment identified the 34 kDa protein as chitinase C. The antifungal activity was confirmed against *Aspergillus niger* and *Candida albicans* with a zone of inhibition of 10.98 ± 0.49 mm and 10.48 ± 0.45 mm, respectively.

Agarases

Agarase is an important enzyme distributed in a wide range of marine organisms. This enzyme has potential application in the food, pharmaceutical, and cosmetics industries. It can also be used in microbiology and molecular biology research. Two β-agarase genes (*agaA* and *agaB*) were isolated from the marine *Pseudoalteromonas* sp.CY24 [14.53, 54]. The *agaB* gene was cloned and overexpressed in *E. coli* and the 50.8 kDa protein was characterized. This enzyme degrades agarose to generate neoagarooctaose and neoagarodecaose as the major products but cannot hydrolyze carrageenan, alginate, or chitosan. The substrate binding site and the mechanism of hydrolysis of this enzyme were predicted by analysis of the enzyme kinetics, the degradation pattern of different oligosaccharides, and ^{1}H-NMR analysis. The enzyme has a large substrate binding cleft and it appears to be structurally and functionally different from other known agarases. It hydrolyzes glycosidic bonds with inversion of anomeric configuration, in contrast to other known agarases that retain the anomeric configuration. On the other hand, the *agaA* gene encodes a 48.4 kDa protein corresponding to a catalytic domain of the glycosyl hydrolase family 16 and a carbohydrate-binding module type 13. It is an endo-type agarase that hydrolyzes β-1,4-linkages of agarose to yield neoagarotetraose and neoagarohexaose as the end products. Another β-agarase producing gene (*agrP*) was isolated from the marine *Pseudoalteromonas* sp. AG4 and was cloned and overexpressed in *E. coli* [14.55]. Sequence similarity suggests that this enzyme is a member of the glycosyl hydrolase family 16. It produces neoagarotetraose and neoagarohexaose from agar and additional neoagarobiose by hydrolysis of agarose. A β-agarase gene from the agarolytic marine bacterium *Agarivorans* sp. HZ105 showed 98.6%

sequence identity with a β-agarase (agaB) from *Vibrio* sp. JT0107 [14.56]. The enzyme was overexpressed in *E. coli* and identified as an endo-type β-agarase. It can hydrolyze neoagarooligosaccharides with a degree of oligomerization above four and produce neoagarotetraose as a dominant end product. Another endo-type β-agarase was reported from marine *Agarivorans* sp. LQ48 [14.57]. This enzyme showed 73% sequence similarity with the β-agarase (AgaB) isolated from the marine *Pseudoalteromonas* sp. CY24. Like AgaB this enzyme also hydrolyzes β-1,4-linkages of agarose and produces neoagarotetraose and neoagarohexaose as the main products. Notably, this agarase retains 95% of its initial activity after 1 h incubation at any pH between 3.0 and 11.0 – such a wide range of pH-stability has rarely been observed in any other agarase.

14.3.2 Proteases

Proteolytic enzymes account for above 60% of the global enzyme market [14.58], and there is no doubt that proteases are one of the most widely studied marine enzymes. They are vastly used in the food processing, detergent,leather, and pharmaceutical industries, and in biotechnological research. They have potential applications in bioremediation and waste management. The increased demand for industrial proteases over its supply from plant and animal sources led to an increased interest in microbial proteases. 70% of industrial proteases come from microbial sources. Alkaline and neutral proteases are obtained mainly from bacterial sources, mostly from different species of *Bacillus*, while fungi are a well-known source of acid proteases. Marine microbial proteases gained attention for their extremophilic properties and stability in the presence of a broad range of chemicals. They possess almost all the characteristics desired for various biotechnological applications of proteases.

A novel alkaline serine protease was reported from the marine fungus *Engyodontium album* BTMFS10 strain isolated from marine sediment from the west coast of India [14.59, 60]. The extracellular enzyme was produced by solid-state fermentation and the process parameters of protease production were optimized. The enzyme was active over a broad range of pH values (6–12) and temperature ranges (15–65 °C) and retained its activity in the presence of hydrocarbons, natural oils, surfactants, and organic solvents. The optimum activity of the purified enzyme at a high temperature (60 °C) and pH value (pH 11) suggest its potential application in detergent industry. *Sana* et al. reported another alkaline serine protease from a marine γ-proteobacterium isolated from the intertidal zone of the Bay of Bengal [14.61]. The enzyme was tolerant to salt, solvent, detergent, and bleaching agents, and showed potential for the detergent industry and dry washing. A protease encoding gene was isolated from *Engyodontium album*, which encodes a protein with 96% sequence similarity to proteinase R of *Tritirarchium album* [14.62]. A homology comparison of the amino acid sequence revealed that this protease belongs to the subtilase family of serine protease. A comparative homology modeling suggested a broad substrate specificity of the enzyme. The enzyme structure was reported to be stabilized by two disulfide bonds and more than two Ca^{2+} binding sites, which are assumed to contribute to the thermostability of the enzyme.

Surface-displaying recombinant alkaline proteases were produced on a *Yarrowia lipolytica* cell surface by overexpressing a marine-derived alkaline protease gene [14.63]. This surface-displaying protease can hydrolyze various proteins for the production of bioactive peptides. The peptides have potential pharmaceutical applications due to their angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant activity. Peptides with high ACE inhibitory activity have well-known potential application in the treatment of hypertension and other bioactive peptides can be used as clinical nutrient supplements.

The production of a thermostable alkaline protease by a marine *Bacillus* was optimized and scaled up in a stirred tank bioreactor using cheap and readily available substrates [14.64]. The authors studied the effects of environmental factors, carbon sources, nitrogen sources, and fermentation parameters on the protease production kinetics. Enzyme production increased with prolonged fermentation time and faster agitation rate. Maximum protease production was achieved after 40h fermentation at 42 °C with an aeration rate of 1.5 vvm and constant agitation at 400 rpm in a soybean–casein medium (pH 9.6). Protease production by the marine bacterium *Teredinobacter turnirae* was optimized for solid-state fermentation as well by immobilization on solid matrixes [14.65–67]. Media composition, initial pH, inoculum concentration, and the effect of different salts and carbon sources were studied to optimize protease production by solid-state fermentation in a soybean-based medium. The maximum protease production was obtained using 1 w/v % soybean concentration, initial pH 7.34, and a 2.5 v/v % inoculum level. The material and particle size of the immobilizing medium, and the method and conditions for

cell immobilization were also optimized for repeated batch fermentation of the alkaline protease by *T. turnirae*. Ceramic support, the different sizes of broken pumice stone, and silicone foam enhanced enzyme production by more than 200%, when compared to free cells. Electron micrograph evident *T. turnirae* colonization in porous support matrices and five repeated batch fermentation was achieved without significant decrease in the enzyme production. When the same strain was immobilized in calcium alginate beads maximum protease activity was achieved at 3 w/v % sodium alginate and 3% $CaCl_2$ concentrations with a 1 : 2 ratio of cell and alginate. Protease production was independent of bead size but decreased significantly when the beads were treated with glutaraldehyde. The immobilized cells were used for 8 cycles, each lasting 72h. Protease production increased with an increased number of fermentation cycles and was maximized at the fourth cycle when the enzyme activity reached about 3.5 times more than that of the first cycle.

14.3.3 Halogenating Enzymes

Halogenated molecules are widely distributed in the environment and their existence is not only related to human industrial processes but also to natural biotic as well as abiotic processes. To date almost 5000 organohalogens are known. Most of them contain chlorine or bromine rather than fluorine or iodine [14.68]. With respect to the relatively high concentration of bromide found in the marine environment, brominated compounds are found quite often and occur in a larger number than in freshwater samples [14.69, 70].

While establishing a halogen–carbon bond, several properties of the compound are changed. The following two effects are the most important: a) the introduction of halogen increases the molecule's thermal and oxidative activity, and b) increases the biological membrane permeability [14.71]. The halogenated compounds currently manufactured by the industry can be discriminated into bulk chemicals and higher value-added pharmaceutical compounds, where it is estimated that 20% of all pharmaceutical small drugs and approximately 30% of all active compounds in agrochemistry are halogenated [14.71]. Considering the problems of chemical halogenation, such as low yield, lack of sensitivity, and the use of harmful/toxic chemicals, there is an increasing demand for less toxic and highly specific halogenation processes. The latter is of particular interest for the production of halogenated pharmaceuticals. However, halogenated compounds are also of interest as intermediates in organic synthesis. Since they can be used as reactant within palladium-catalyzed coupling reactions, novel synthetic routes are accessible for manufacturing of organic fine chemicals.

The formulation of natural halogenated products occurs via abiotic and biotic processes. Particular enzymes are responsible for the biogenous halogenation process. However, halogenation can be accomplished by several types of enzymes featuring distinct substrate specificity. One can roughly distinguish the enzymes as haloperoxidases, flavin-dependent halogenases, non-heme iron halogenases, and the fluorinase/chlorinase system.

Haloperoxidases are versatile enzymes for transformation of many substrates. Most prominent is the chloroperoxidase from *Caldariomyces fumago*. Whereas the enzyme is more or less even unspecific with regard to the substrate, it can be beneficially applied to numerous biotranformations. Reactions such as epoxidations [14.72, 73], enatioselective sulfoxidations [14.74], *N*-oxidation of arylamines [14.75], hydroxylation of side chains of aromatic systems [14.76], oxidation of indole to oxyindole [14.77], or oxidation of primary alcohols to their corresponding aldehydes [14.78] have been reported. Since the expression of the enzyme is accompanied by the production of a black pigment, the efficient supply of the biocatalyst, and thus biotechnological application, is hampered. However, *Yazbik and Ansorge-Schumacher* et al. recently developed a novel purification strategy enabling an efficient supply of the enzyme for technical applications [14.79].

Regioselective halogenation can be accomplished by the application of flavin-dependent halogenases. However, halogenation of positions 4, 5, 6, and 7 of tryptophan-derived rings of natural products, as well as halogenation of tyrosines at the ortho position,and mono and di-chlorination of pyrroles are the preferred reactions catalyzed by such enzymes [14.80, 81]. Interestingly, similarly to haloperoxidases, the flavin-dependent halogenase uses hypochlorite for halogenation. However, unlike haloperoxidases, the generated hypohalous acid cannot leave the active site and can only react with substrates at the active site, resulting in selective halogenation reactions. Halogenation of inactivated carbon atoms can be accomplished by non-heme iron, α-ketoglutarate and O_2-dependent halogenases, which use a radical mechanism [14.82]. Up until now, the application of such enzymes for biocatalytic processes (in vitro) is still hampered due to the restricted substrate spectrum, as well as the required cofactor recycling.

A kinetic study of a *Streptomyces* sp. derived and recombinantly expressed flavin-dependent tryptophan-5-halogenase (including a cofactor regeneration) was also reported by *Muffler* et al. [14.83]. The study was accomplished due to the fact that halogenated tryptophan may serve as a potential serotonin precursor. By application of the FDH/formiate-system for cofactor regeneration, the space–time yield of the halogenation was improved by a factor of 1.6. Furthermore, *Muffler* et al. applied a genetic algorithm approach to optimize the enzyme's performance and the conditions for the biocatalytic process, whereby the yield of the halogenated product improved significantly from 3.5 to 65% [14.84].

Fluorinase and chlorinase have a commonality in that they mediate nucleophilic reactions of their respective halide ions to the C-5′ carbon of S-adenosyl-L-methionine (SAM) [14.85]. Biological fluorination is of tremendous interest, since it might help to overcome the problems associated with chemical fluorination. However, more basic research is needed to characterize the potential of these types of enzymes.

14.4 Molecular Biology

Marine-derived enzymes are inevitable in modern molecular biology research. Polymerase chain reaction (PCR) is a technique for continuous in vitro DNA synthesis by use of DNA polymerases. It is widely used for gene amplification, site directed mutagenesis, DNA sequencing, and diagnostic purposes. The reaction mixture needs to be treated at high temperature, which causes inactivation of the DNA polymerase [14.86]. It is essential to add fresh enzymes at each reaction cycle, and it was not possible to use the technique widely before discovery of the thermostable DNA polymerase. Today, two marine-derived thermostable DNA polymerases are at the center of this technique. Vent polymerase (New England Biolab) from *Thermococcus litoralis* and *Pfu* polymerase (Stratagene) from *Pyrococcus furiosus* are widely used DNA polymerases for this purpose. These enzymes show a better performance than the previously used *Taq* polymerase; they have a lower error rate than the *Taq* polymerase and also have a proofreading ability due to 3′–5′ exonuclease activity [14.87–89]. DNA ligase from *Thermal thermophillus* is another important marine enzyme used in molecular biology research [14.90, 91]. Marine microorganisms are also an important source of restriction endonucleases. *Asp*MD1 is an isoschizomer of *Sau*3AI that was purified from the marine isolate *Alcaligenes* sp. MD1 [14.92]. A novel restriction enzyme *Dma*I was isolated from the marine bacterium *Deleya marina* IAM 14 114 [14.93]. This enzyme is moderately thermostable and recognizes the hexanucleotide sequence 5′-CAGCTG-3′ to produce the blunt-ended digestion product. Several other restriction endonucleases, including *Dpa*I, *Age*I, *Hja*I, *Hac*1, *Hsa*1, and *Hag*1 have been isolated from marine bacteria. All these enzymes are potential tools for double-stranded DNA digestion. DNA topoisomerases play important roles in replication, transcription and gene expression by controlling DNA topology. Several DNA topoisomerases have been isolated from marine bacteria, including *Sulfolobus acidocalcarius*, *Desulfurococcus amylolyticus*, *Thermus acidophilum, Fervidobacterium islandicum*, *Thermus maritima*, and *Methanopyrus kandleri*. [14.94]. Some of these are characterized for their potential application in biotechnological research.

14.5 Downstream Processing of Marine Enzymes

Capturing and purification of a marine enzyme is a very challenging task, which is commonly addressed to a minor extent in comparison to the fermentation process. But both steps are directly linked together, since efficient downstream processing requires a minimum of the target compound otherwise every isolation strategy fails. Furthermore, the fermentation medium should contain only small amounts of supplements which might interfere with the purification strategy of the target enzyme. Thus, optimization of the cultivation process with regard to the yield of the product should be accomplished prior to establishing a downstream technique. This optimization should also focus on a minimization of by-product formation, as these must be separated from the product. Moreover, the production of (unwanted) side products also affects the

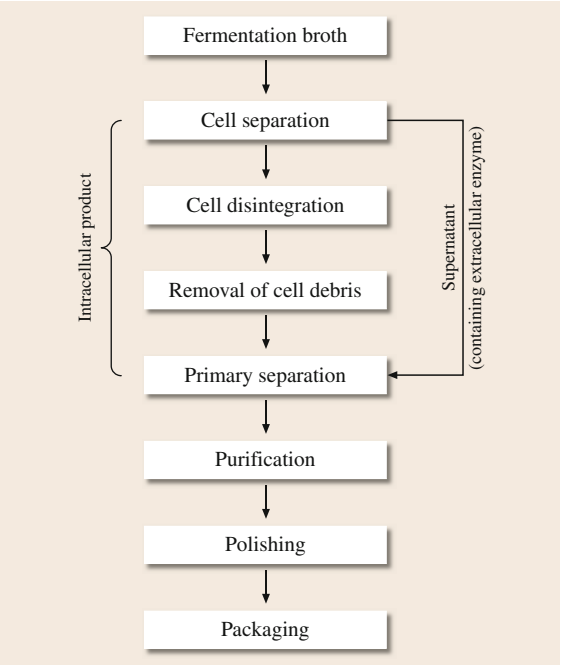

Fig. 14.3 Flow chart of a common downstream process according to *Muffler* and *Ulber* [14.95].

process costs, because the medium is used to a lesser extent for production of the relevant enzyme. In the following section a brief overview about the related steps of downstream processing is given. The interested reader is referred to a review article by *Muffler* and *Ulber* [14.95], which is exclusively dedicated to this topic.

Commonly, a diluted input stream is handled within downstream processing and is subsequently treated by several techniques to obtain a highly concentrated, purified, and storable product (liquid or solid). Initially, the kind of expression determines the first steps of the downstream process. If the enzyme is expressed in an extracellular fashion, it is available among other soluble compounds (e.g., medium components, side products) and insoluble matter (e.g., particles, intact cells). In the case of an intracellular occurrence of the enzyme, the biomass must be disrupted prior to its isolation. In principle, all downstream processes try to remove the insoluble particles and afterwards the product is isolated. A flow chart of the relevant steps of a downstream process is shown in Fig. 14.3.

Fermentation of the enzyme producing strain is followed by separation of the soluble and insoluble components of the fermentation broth. To avoid thermal deactivation of the product the broth is subsequently chilled after termination of the fermentation. The separation of cells can be achieved by application of filters (static filtration or cross-flow filtration), as well as by application of centrifuges or decanters. Selection of the separating unit depends, for example, on the cell concentration, as well as volume and viscosity of the fermentation broth. Extracellular enzymes can subsequently be obtained from the resulting supernatant, whereas disintegration of cells is required for recovery of intracellular enzymes.

Liberation of intracellularly expressed enzymes can be achieved by several procedures; the most frequently applied techniques are mechanical/physical and enzymatic treatment. Since it is difficult to predict the performance of the method of disintegration, some techniques are tested in parallel. The optimal procedure features a maximum of enzyme release and a minimum of structure alteration and deactivation of the target enzyme, respectively. Apparatuses such as homogenizers or bead mills are often applied to arrange mechanical treatment [14.96, 97]. Such techniques are even applicable for (continuous) disruption of biomass within pilot or industrial scale applications. A gentle method allowing cell disruption at lab-scale uses ultrasound. Enzyme release is accomplished via disruption of the cells by shear forces and cavitation. To prevent heat denaturation of the enzyme the cell suspension is cooled during ultrasonic treatment. An approach used to release the intracellular protein under mild conditions utilizes hydrolytic enzymes. Such enzymes are capable of degrading the cell wall of the relevant organism, and disintegration occurs as a result of osmotic pressure; thus the generated protoplasts burst and release the target enzyme into the surrounding medium. An enzyme that is often used for enzymatic degradation of bacterial cell walls is lysozyme, of hens' egg origin. Although lysozyme is most effective for the lysis of Gram-positive bacteria, it also facilitates the lysis of Gram-negative bacteria. However, ethylenediaminetetracetic acid (EDTA) is often added to the enzyme solution if the cell wall of Gram-negative bacteria should be attacked to trigger the hydrolysis via complexation of Ca^{2+} of the outer (protecting) membrane. Lysozyme is commercially available at moderate cost and the process requires only simple equipment. Therefore, enzymatic lysis can be considered as an option for large-scale processes [14.98]. Apart from the hens' egg lysozyme, cell wall degrading enzymes are also available from marine microorganisms, but up until now they have not been provided by commercial suppliers. These marine-derived lysozymes can feature

prospective traits such as high activity even at low temperatures, as well as activity in a broad range of pH values (e.g., [14.99, 100]). Hence the hydrolysis may be performed at low temperatures and thermal enzyme deactivation is minimized. Another approach is the so-called electroextraction, a technique which so far has not been applied for recovery of intracellular enzymes from marine microorganisms, although its potential has been proven, mainly for yeasts (e.g., [14.101]). Electroextraction is based on electro-induced changes in the cell envelope, which result in a release of intracellular proteins without formation of debris.

The diluted enzyme is subsequently concentrated by means of precipitation (e.g., with ammonium sulfate) or ultrafiltration. The unspecific concentration of proteins is followed by chromatographic steps, whereby the enzyme is separated from the impurities according to its charge, size, or hydrophobicity, or due to specific groups of the target enzyme. Analog separation principles are provided by membrane adsorbers. Such devices feature some advantages in comparison to conventional column chromatography. These can be summarized as follows: lower manufacturing costs, no diffusion-controlled exchange kinetics, easier handling, and up-scaling [14.102]. A membrane adsorber is based on a stationary, modified microporous matrix allowing high fluxes and maximization of the mass transfer, thus limiting diffusive transport phenomena as they occur in column chromatography can be minimized. Additionally, the compressibility can be neglected if the module is applied under moderate pressure. Membrane adsorbers are commercially available and can be purchased as ion-exchangers with acidic (e.g., sulfonic acid, or carboxylic acid), basic (e.g., quaternary ammonium or diethylamine), or chelating membranes, which are applicable for immobilized metal affinity chromatography. One example for a successful application of a cation-exchange membrane adsorber for recovery of a marine enzyme was reported by *Muffler* and *Ulber* [14.103]. They established a fed-batch process for optimizing the enzyme expression of the intracellular produced sulfite oxidase by *Sulfitobacter pontiacus*. The supernatant obtained after ultrasonication of the suspended biomass and centrifugation of the cell debris was directly applied to a cation-exchange membrane adsorber. Thus the specific activity within the pooled eluate fraction was increased about a factor of 8 compared to the crude extract. A further increase about a factor of 2.6 of the specific activity was finally achieved by ultrafiltration.

If applicable, affinity chromatography is the method of choice, because this technique relies on the highly specific binding interactions of the target enzyme and the stationary matrix. However, the target enzyme must feature a characteristic subunit or subgroup which can be used for binding to an affinity matrix. A successful application of this technique necessitates that such binding-groups are absent in the co-expressed proteins. Therefore, affinity chromatography is usually applied with recombinant enzymes rather than native enzymes, because the relevant groups, such as, for example, a histidine tag, a chitin binding tag, or maltose binding tag are linked to the native sequence of the protein, thus allowing a subsequent gentle purification of the recombinant protein. For instance, *Kang* et al. expressed an epoxide hydrolase of *Sphingophyxis alaskensis* origin in *E. coli* and purified the heterologously expressed protein by metal affinity chromatography via his-tag [14.104]. Another binding method was applied by *Han* et al., who expressed a chitinase with antifungal activity from marine *Streptomyces* sp.DA11 by *E. coli* and purified the chitinase, showing an antifungal activity by 80% ammonium sulfate, affinity binding to chitin, and diethylaminoethyl-cellulose anion-exchange chromatography [14.52]. However, affinity chromatography had already been successfully used for purification of native marine proteins. *Bertau* et al., for example, reported the purification and characterization of an α-L-fucosidase by application of the glycosidase inhibitor analogue 6-amino-deoxymannojirimycin as ligand for the saccharolytic enzyme [14.105].

14.6 Conclusion

Marine microorganisms can be considered as a powerful resource of enzymes with special traits which are often different from their terrestrial counterparts. In particular, enzymes from extremophiles can be valuable in a broad variety of processes. Enzymes from cryophilic microorganisms are often active at low temperatures and may contribute to, for example, energy savings in laundry processes (cryophilic lipases or proteases). Such enzymes may also contribute to increase the quality of food because processing can be

achieved at low temperatures. Moreover, cryophilic enzymes can simply be deactivated by a small shift in temperature. With respect to mass transfer and viscosity, elevated temperatures for biocatalytic processes can be desirable for several applications, thus thermo- or even hyperthermophilic marine enzymes are of great interest for this type of industrially relevant enzymes.

However, cultivation and expression of natural producing strains often represent a bottleneck for enzyme supply. Therefore, novel cultivation techniques must be introduced at lab scale but also examined and transferred to pilot scale, respectively. The cultivation of (single-species and mixed-species) biofilms is still in its infancy, but the examples presented here give an idea of the potential of this approach. One should bear in mind that up until now only a small fraction of marine bacterial diversity is accessible. Molecular biology and intensification of metagenome approaches may support the identification of many novel genes and enzymes, which will expand the pool of enzymes applicable for industrial processes in the near future.

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15. Biofouling Control by Quorum Quenching

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Bacteria above a threshold cell density regulate the expression of a specific group of their genes in response to small signal molecules called autoinducers. This *twitter* communication phenomenon is called quorum sensing (QS). Among the various genetic expressions mediated by QS, biofilm formation and expression of virulence factors are the most prominent. These phenotypes are a major cause of concern for health departments and a wide range of industries dealing especially with drinking water, waste water reclamation, and desalination. This phenomenon leads to heavy economic losses. Efforts to disrupt biofilms have met with little success. In fact, bacteria within the biofilms are 1000 times more resistant to antibiotics than their planktonic counterparts. Biosensors or reporter strains have been developed to screen QS inhibitors. A potential approach to inhibit the process of biofouling is to interfere with bacterial QS signals. QS inhibiting enzymes and molecules have been isolated from a wide range of organisms such as bacteria, fungi, algae, and marine organisms. Efforts to disrupt biofilm formation have been carried out through designing antifouling agents that primarily reduce surface adhesion of the organisms. These include fouling release coatings such as nanocomposites. In contrast to removing biofilms, there are certain areas where it is desirable to regenerate biofilm for a fresh round of biotransformation.

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15.1 Overview

Biofouling (BF) in aquacultures, water treatment, the pulp and paper industry, and marine systems is a major cause of concern for water purification industries, fisheries, merchant and defense naval departments, and manmade structures (monuments/paintings). Worldwide it is a problem of high practical and economic relevance. Micro and macrofoulers can cause severe industrial problems by increasing drag (leading to increased fuel consumption), promoting metal corrosion, and reducing heat transfer efficacy of heat exchangers. A major problem encountered during the production of drinking water, waste water reclamation, and desalination is the biofilm formation on membrane filters. Efforts to control this through the use of antifouling (AF) coating (incorporating biocides such as tributyrin) have imposed environmental burdens, especially since their usage has been banned [15.1–4].

The most evident form of BF is the formation of biofilms, which invariably harbor more than one type of microorganism. Bacterial biofilms play both negative and positive roles. During processing for drinking water, biofilms cause fouling of filtration membranes but act positively as biocatalysts in biologically active filters, riverbank filtration, and membrane biofilm reactors. In the wastewater treatment biofilms also act as biocatalysts in fixed film activated sludge, moving bed biofilm reactors, tertiary filters, and granular sludge, but cause BF of membrane bioreactors. The underlying phenomenon of the occurrence of bacterial communities in this specific niche depends largely on their ability

to communicate among themselves [15.5]. Among the various means of communication, the most widely studied and recognized is that occurring largely under high cell densities and exceptionally at low cell densities, such as in *Vibrio cholerae*. This phenomenon of cell-density-dependent communication is termed quorum-sensing (QS). Here, the release of QS signal molecules is proportional to the cell density and above a threshold level these are retrieved into the cell to trigger the expression of genes responsible for a large number of phenotypic features such as bioluminescence, biofilms, virulence, antibiotic production, and rhizospheric activities, etc. [15.6].

Bacteria have the ability to *sense* and adhere to surfaces to form biofilms. In aquacultures, bacterial pathogens such as *Aeromonas hydrophila, Vibrio anguillarum, V. alginolyticus, V. ichthyoenteri* form biofilms which are mediated by QS signal molecules, such as acyl-homoserine lactones (AHL), furanosyl borate diester, and peptides. Extremophiles – haloalkaliphilic archaeon – *Natronococcus occultus* living in alkaline biotope and acidophilic γ-proteobacterium, *Acidithiobacillus ferroxidans* cause BF through AHL-mediated QS. In water purification systems, *Legionella, Escherichia*, *Pseudomonas*, and *Rhizobium* are responsible for biofilm formation. Biofilms formed on stainless steel surfaces and other metal and alloys are typically dominated by diatoms [15.7–10]. For archaeological departments, the issue of deterioration of buildings and monuments caused by biofilm forming organisms such as *Gloethece*, is a matter of great worry [15.11].

15.2 Bacterial Biosensors

In order to detect the active principle underlying the biofilm formation by bacteria which produce QS signals to communicate among themselves, biosensor bacterial strains have been developed. These biosensors respond to AHLs with distinct acyl chains lengths. One of the most widely exploited bacterial biosensors which responds to C_6HSL is *Chromobacterium violaceum* strain CV026. McClean and his team developed a violacein pigment and AHL-negative double mini Tn5 mutant by transposons inserted into the *cviI* (AHL synthase) gene and in a violacein repressor locus. This biosensor strain produces purple violacein pigment in response to exogenously supplied AHLs. Biosensors for small acyl chains, C_4AHL, have been developed in *Escherichia coli* (pSB536), *E. coli* (pAL101). The plasmid pSB1075-based sensor responds to long acyl chain AHLs such as $3OC_{12}$HSL and pKDT17 responds to C_{12}-HSL and C_{10}-HSL. The biosensor strain *Agrobacterium tumefaciens* (TraI/R) can detect a wide range of AHL signals with acyl chain lengths ranging from C_4 to C_{14} [15.12].

15.3 Quorum Quenching (QQ)

QQ can be inhibited at different stages:

i) Biosynthesis of AHLs by inhibiting enzymes such as the acyl–acyl carrier protein (ACP) and S-adenosyl methionine synthase
ii) Destroying QS signal molecules through the activities of AHL-lactonases and AHL-acylases
iii) The inhibition of AHL efflux proteins
iv) The inhibition of transcriptional activators (LuxR homologs)
v) The use of QS analogs.

15.3.1 Enzymes

Among the various possibilities of down-regulating QS systems, enzymes produced by a range of organisms have been widely studied. These enzymes are of bacterial origin and are categorized as:

1. Lactonases
2. Decarboxylases
3. Acylases
4. Deaminases
5. Oxidoreductases.

Lactonases are produced by *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Bosea, Chryseobacterium*, *Geobacillus, Bacillus, Mycobacterium*, *Rhodococcus*, *Klebsiella*, *Ochrobactrum*, *Microbacterium*, *Solibacillus*, *Rhodococcus*, and *Nocardioides, Sphingopyxis*. Acylases having the ability to degrade long-chain AHLs ranging from C_6HSL to C_{12}HSL have been found to

be produced by *Pseudomonas aeruginosa, P. syringae* strain B728a, *Shewanella* sp. strain MIB615, *Streptomyces* sp. strain M64, *Anabaena* sp. PCC7120, and *Ralstonia solanacearum* GM1000. Oxidoreductases from *Bacillus megaterium* CYP102A1, *Burkholderia* sp. strain GG4, and *Rhodococcus erythropolis* W2 act by substituting an oxo group with a hydroxyl group of AHL [15.5, 6, 10, 13–17].

15.3.2 Quorum Sensing Inhibitors (QSIs)

A potential approach for inhibiting the process of BF is to interfere with bacterial QS signals [15.5]. AHL degraders have been isolated from guts of shrimp, *Penaeus vennamei*, European seabass, *Dicentrarchus labrax* L., and Asian seabass, *Lates calcarifer*. Antibiotic malyngolide produced by the cyanobacterium, *Lyngbya majuscula* inhibited QS at concentrations between 3.57–57 μM. Malyngamide C and 8-epi-malyngamide C inhibited luminescence of LuxR-based *E. coli* reporter strains. Malyngamides A and B, which are structurally different from malyngamide C, were effective in inhibiting QS-dependent violacein production by *C. violaceum* CV017.

Bacterial QSIs

The peptide antibiotic – siamycin I, produced by *Streptomyces* sp. strain Y33-1, inhibits the gelatinase and gelatin biosynthesis activating pheromone, which results in disruption of the biofilm formed by *Enterococcus faecalis*. The unique feature of siamycin is the ability to selectively inhibit the growth of Gram-positive bacteria but show no effect on the growth of Gram-negative bacteria. Butyrolactones (2(3H)-furanones) from *Streptomyces* sp. act as analogs of AHL. Naturally occurring intermediates of the butanolide (2(5H)-furanones) biosynthetic pathway in *Hortonia* sp. and *Streptomyces antibioticus* have also been found to be effective. In *Pseudomonas* sp., cyclopeptides and their diketopiperazines act on the QS system. There are few but certain secondary metabolites produced by bacteria: (i) *N*-(2'-phenylethyl)-isobutyramide and 3-methyl-*N*-(2'-phenylethyl)-butyramide produced by *Halobacillus alinus* C42, and (ii) 2-*N*-pentyl-4-quinolinol by *Alteromonas* sp. affect antibiotic (Andrimid) production, bioluminescence (HAI-1 pathway), and prevent surface colonization in different *Vibrio* spp. Based on the molecular size and structure of the inhibitors, phenylethylamides are AHL structural mimics that compete for receptor binding sites.

QSIs from Plants

The traditional of use of plant products for the treatment of BF and ailments has been attributed to the phytochemicals present in them. Plant extracts have been found to act as QSI because of the similarity in their chemical structure to that of QS signals (AHL) and also because of their ability to degrade signal receptors (transcriptional activators, LuxR/LasR). γ-aminobutyric acid produced by plants acts as promoter for the degradation of the AHL signal OHC_8HSL by AttM, lactonase of *A. tumefaciens*, attenuating the QS-dependent infection process. Pyrogallol, extracted from medicinal plants such as *Emblica officinalis* and its analogs exhibit antagonism against AI-2. L-canavanine from seed exudate of *Medicago sativa* affected QS-mediated exopolysaccharide production. *Curcuma longa*, a well-known spice plant produces curcumin, which inhibits the expression of virulence genes of *P. aeruginosa* PA01. QS-regulated activities such as biofilm formation in *P. aeruginosa* and AI-2-mediated QS in different *Vibrio* spp. have been reported to be affected by cinnamaldehyde and its derivatives. Extracts from leaves, flowers, fruit, and bark of *Combretum albiflorum*, *Laurus nobilis*, and *Sonchus oleraceus* were also found to possess anti-QS activities. Flavan-3-ol catechin, a flavonoid from the bark of *C. albiflorum* reduces the production of QS-mediated virulence factors – pyocyanin, elastase, and biofilm formation by *P. aeruginosa* PA01. QSIs have also been reported from garlic. Phenolic plant secondary metabolites such as salicylic acid stimulate AHL-lactonase enzyme expression. Aqueous extracts of edible plants and fruits such *as Ananas comosus, Musa paradiciaca, Manilkara zapota*, and *Ocimum sanctum* have been tested as QSI against violacein production by *C. violaceum* and pyocyanin pigment, staphylolytic protease, elastase production, and biofilm formation abilities of *P. aeruginosa* PA01. The QS system in *Staphylococcus* spp. consists of the AI RNA III activating protein (RAP) and its target molecule (TRAP). Inhibition of RAP by RNA III-inhibiting peptides (RIP) results in attenuation of virulence. Hamamelitanin extracted from the bark of *Hamamelis virginiana* (witch hazel), like RIP, did not affect the growth of *Staphylococcus* spp., but inhibited the QS regulator RNA III, and prevented biofilm formation and cell attachment in vitro. Evidence has also been provided by implantation of grafts soaked in Hamamelitanin into animals, where it decreased the bacterial load in comparison to control.

Extracts of the plants *Moringa oliefera* and *Acacia nilotica*, which contain gallic and ellagic acids,

have anti-QS potential. Epigallocatechin gallate (salt of gallic acid) and ellagic acid inhibited LasR and LuxR-based QS at the concentrations 15–30 μM. Gallic, but not ellagic, acid inhibited QS of *C. violaceum* CVO17 at 64.7 μM. Ellagic acid derivatives from *Terminalia chebula* Retz. fruit downregulate the expression of lasI/R and rhlI/R QS genes and attenuate *P. aeruginosa* PAO1 virulence. They also enhance the sensitivity of its biofilm towards the tobramycin antibiotic. Biomolecules like Hymenialdisine, demethoxyencecalin, microcolins A and B, and kojic acid have been shown to inhibit LuxR-based reporters (*E. coli* pSB401) induced by $3OC_6HSL$ at concentrations ranging from 0.2 μM to 36 μM. The ability to prevent microfouling by kojic acid at 330 μM and 1 mM was observed by a decrease in the cell densities of bacteria and diatoms. Inhibitory concentration of hymenialdisine, demethoxyencecalin, kojic acid, and microcolins A and B were comparable with ones of natural furanones, ellagic acid, malyngolide and manoalide. Other antibiotics such as – azithromycin, ceftazidime, and ciprofloxacin inhibited the QS of LuxR-based reporters based on the QS circuit of *Vibrio fischeri* at sub-lethal concentrations ranging between 0.1–11 μM [15.3, 9]. Even fruit and spices consumed as food in our day to day life have molecules with the ability to inhibit the QS of bacterial pathogens [15.18–21].

Marine Derivatives as QSIs

Extracts of Great Barrier Reef marine invertebrates and cyanobacteria act as QSIs against bacteria. Biochemical defenses against fouling are widely deployed by algae. Metabolites that affect microbial communities have been demonstrated in the red algal family *Bonnesmaisoniae* for *Delisea pulchra, Asparagopsis armata* and *Bonnesmaisonoa hamifera*. The most well-investigated example of this defensive strategy is that of the red alga, *D. pulchra*, which produces halogenated furanones at the surface of the thallus. A high concentration of metabolites at the surface strongly inhibits QS by targeting AHL-dependent signals, and thus the settlement of various fouling propagules, and deters the settlement and growth of both micro and macrofouling agents. Natural chemical defenses are not restricted to the red algal models, as the secondary metabolites from *Dictyota menstraulis* and phlorotannins from *Fucus evanescens* also have AF activity. Ursine triterpenes from *Diospyros dendo* also interfere with QS. Oxidized halogen (HOBr) from *Laminaria digitata*, a brown alga, reacts specifically with 3-oxo-acyl HSLs. Marine bacteria produce QSIs that interfere with the formation of biofilms. For example, the bacterium *Aeromonas veronii* inhibits QS through competition for AHL production. TAGE (*trans*-bromo ageliferin analog) and CAGE (*cis*-bromo ageliferin analog) are two derivatives of the marine natural product bromo ageliferin. Both compounds have been shown to be effective inhibitors of *P. aeruginosa, Staphylococcus aureus, Acinetobacter baumannii*, and *Bordetella bronchiseptica* biofilm formation. There are only a few studies on marine microbes and their metabolites with respect to their antibiofilm activities. This is because the majority (< 95%) of phylogenetic clades of marine bacteria cannot be cultivated in laboratories. AF metabolites from non-cultivable marine microbes can be obtained through a metagenomic approach [15.22].

Biomolecules produced by bacteria associated with sponges have been a very good source of QSIs. Extracts of bacteria such as *Bacillus horikoshi, B. pumilus*, and *Vibrio natrigens*, associated with the coral *Acropora digitifera*, have been found to display efficient activities against biofilm-forming *Streptococcus pyogenes*. Among these bacteria, *B. horikoshi* proved more efficient at a concentration of 50 $\mu g\,mL^{-1}$, in contrast to the other two strains, which were effective at 100 $\mu g\,mL^{-1}$. In a subsequent study, *Bacillus indicus, B. pumilus*, and *Bacillus* sp. SS4 from Palk Bay (Bay of Bengal) were shown to cause significant inhibition of QS-based activities in Gram-negative bacteria such as *P. aeruginosa* PAO1, *Serratia marcescens*, and *Vibrio* species. Marine bacterium *B. pumilus* S6-15 was found to have a wider application in its ability to inhibit biofilm formation in both Gram-positive and Gram-negative bacteria. The active principles were the enzyme acylase and other secondary metabolites like carboxylic acid derivatives (4-phenylbutanoic acid). These QSIs were also found to inhibit the intestinal colonization of *S. aureus* in nematode *Caenorhabditis elegans* and its subsequent killing [15.23–30].

Bacteria in a biofilm can also affect the growth of other bacteria in the same biofilm. The marine bacterium *Alteromonas* sp. produces antibiotic 2-*n*-phenyl-4-quinolinol, which alters the composition of the bacterial community developed on particles. To date, only two classes of marine sponge metabolites house non-bactericidal biofilm modulators, viz. terpenoids and pyrrole-imidazoles. Terpenes are structurally diverse molecules, and this diversity is contributed by their isoprene subunits. The biological activities of terpenes are also diverse, ranging from AF effects to antiproliferative cancer therapeutics [15.31].

Within sponge terpenoids, only ageloxime-D, manoalide, and two manoalide congeners have been reported to have the ability to interfere with bacterial biofilm formation without disrupting cellular growth. Besides terpenoids, marine sponges synthesize pyrrole-imidazole alkaloids (PIA), another class of potent AF molecules that is unique to their phylum only [15.31]. Many families of marine sponges are the source of PIAs, but most of the work has been done on the bromopyrrole derivatives extracted from the *Agelasidae* family [15.31].

Oroidin, sceptrin, and bromo ageliferin are natural PIA products that act as potent toxic AF compounds against microorganisms, as well as higher organisms. *Ageloxime-D*, a diterpenoid metabolite, (−)ageloxime-D 4, from a marine sponge, was shown to inhibit biofilm formation by *Staphylococcus epidermidis* without inhibiting its growth, whereas its parent molecule, (−)agelasine-D 5, showed the opposite effect. *Luffariella variabilis* synthesizes as ester terpenoid–Manoalide – which exhibits both antibiotic and anti-inflammatory activities. There are three additional derivatives of Manoalide, viz. seco-manoalide, (*E*)-neomanoalide, and (*Z*)-neomanoalide [15.31]. These derivative compounds and their parent molecule were tested for antibiotic activity and found to be effective against Gram-positive bacteria only. In 2007, Skindersoe and co-workers found that extracts obtained from *L. variabilis* act as potent QSI agents against both Gram-negative and Gram-positive bacteria. This QSI activity was attributed to the conserved 2(5*H*)-furanone substituent [15.31].

Manoalides work as bactericides against Gram-positive bacteria. Against Gram-negative bacteria, they are not bactericidal but exhibit significant QSI activity. This shows that marine sponges are potential sources for future biofilm modulator molecules, particularly against life-threatening infections caused by Gram-negative pathogens like *P. aeruginosa*, *A. baumannii*, and *Klebsiella pneumoniae* [15.31].

15.3.3 Designing Antifouling Agents

Surface Adhesion

Efforts to modify the surface energy of substrates to reduce adhesion (ca. 25 dynes cm^{-1} as the least hospitable) of organisms could not prevent the adhesion of all fouling organisms. The question arises as to which properties of the surface could be altered to discourage/reduce cellular adhesion. Amphiphilic coating systems having both hydrophobic and hydrophilic domains on the surface show excellent AF properties for both diatoms and macroalgae. Cells adhere more strongly to hydrophobic surfaces, presumably for better survival. Under stressed conditions, the cellular level of nitric oxide (NO)is a good indicator. Levels of NO were fourfold higher in cells on a hydrophilic surface than those on a hydrophobic surface (silicon), indicating that hydrophobic surfaces were less stressful. Incorporation of such molecules that induce stress in fouling organisms can make them potential candidates as AF agents. *Trans*,*trans*-2,4-decadienal (DD), implicated as a chemical defense molecule, inhibits the invertebrate grazing of phytoplankton. Since diatoms can produce DD, it can be exploited to disperse clumping of diatoms [15.4, 8]. Novel antibacterial agents have been developed as NO-releasing silica nanoparticles. With enhanced inhibitory activity and reduced toxicity against *P. aeruginosa* and *E. coli*, the strategy proved better than NO alone in removing biofilms [15.32, 33].

Marine mammal skin is a clean surface, free of all fouling organisms (epibionts). In the pilot whale *Globicephala melas*, such a phenomenon is attributed to nanobridge pores on the surface of the skin. The nanobridge pores ($0.1-1.2\,\mu m^2$) are smaller than most fouling organisms, and it is proposed that they provide few micro niches or contact points for adhesion. These skins thus reduce fouling by the common bacterium *P. aeruginosa* to the extent of 93% in comparison with unprotected surfaces. In the dogfish egg case and sea stars (echinoderms) are a unique group of organisms, whose surface is free of any fouling organisms. Glycoproteins from some species blocked adhesion of bacteria whilst adhesion was enhanced by glycoproteins from other species. Production of free radicals has also been proposed as an AF strategy for marine algae and barnacles. Shark skin is another case that demonstrates that fouling can be controlled based on texture and dimension [15.34].

Nanocomposites

Coatings made of amphiphilic diblock copolymers, consisting of a poly(dimethyl-siloxane) block and a PEGlyated (poly(ethylene glycolated)) fluoroalkyl modified polystyrene block, have proved to possess excellent fouling release properties against the macroalga *Ulva linza*. Enhancement in polymer performance can be achieved through dispersal of nanosized fillers into the polymeric matrix to create a nanocomposite. The use of fluorosilicones, i. e., polymers containing both siloxane and fluorinated moieties in diverse macro-

molecular architectures, e.g., siloxane-fluorinated linear, graft, block, or hyperbranched (co)polymers can add to these AF agents. Coatings incorporating low surface energy fluorinated moieties with hydrophilic poly(ethylene glycol) (PEG) also have strong AF properties against marine organisms. The ability of amphiphilic coatings to lower the adhesion strength of organisms is probably due to certain unique features: (i) the low interfacial tension with water of the hydrophilic PEGylated domains, and (ii) the non-sticky and slippery behavior of the hydrophobic perfluoroalkyl domains [15.35].

Urethane polymers alone have little fouling resistance but provide mechanical strength, whereas siloxanes are not mechanically strong but have fouling-release properties. The AF properties of polysiloxane polymers were also improved by the inclusion of biocides (quaternary ammonium compounds (quats)). Coatings with 18C length quats were effective in inhibiting bacterial biofilm formation. In contrast, 14C quats were effective as growth inhibitors of diatom *Navicula* sp. [15.8].

15.3.4 Fouling – Release Coatings

Commercially available fouling release coatings are based on silicone elastomers, such as polydimethylsiloxane. Here, foulants adhere weakly to the substratum and are released under the hydrodynamic forces. The use of biocides to control BF has not been effective against all microorganisms causing BF. Kojic acid incorporated into a non-toxic paint matrix at a concentration of 0.5% significantly reduced the densities of bacteria and diatoms growing in paint. In addition, it also decreased macrofouling over a period of 30 days. Kojic acid at non-toxic concentration (330 μM) inhibited bacterial density up to 2.5–3.2-fold and diatom density up to 4.7–3.6-fold in biofilms on glass slides. It was suggested that kojic acid reduced the formation of microbial communities by inhibiting non-QS regulatory cascades that affect biofilm formation. Alternative AF coatings employing copper and co-biocides, e.g., Irgarol 1051, zinc pyrithione, and Sea Nine 2011 are the principal replacement for tributyl tin coatings [15.3, 4, 35].

15.4 Applications

Microbes isolated from the gut of marine organisms such as shrimp, European seabass and Asian seabass have helped to improve the survival of larvae of *Macrobrachium rosenbergii*, which can also tolerate a higher concentration of ammonia. Microflora from the intestine of Ayu fish, *Plecoglossus altivel*, especially *Shewanella* sp. strain MIB010, proved to be an AHL degrader. It was effective in dispersing biofilms formed by fish pathogens, *Aeromonas* and *V. anguillarum*. The marine actinomycete, *Streptomyces albus* strain A66, was also effective in disrupting the biofilm. Seabass are inhabited by Gram-positive, *Halobacillus salinus* C42, which release phenethyliamide compounds, especially 2,3-methyl-*N*-(2'-phenylethyl)-butyramide, to inhibit bioluminescence of *Vibrio harveyi*. The marine cyanobacterium *Blennothrix cantharidosmum* exudes a variety of Tumonoic acids with the ability to inhibit bioluminescence, although not very strongly. *D. pulchra* with the ability to produce furanones, inhibits swarming motility in *Proteus mirabilis* and other phenotypes such as inhibition of luminescence and toxin production. *Ahnfeltiopsis flabelliformis*, a macroalgae, produces Floridoside, betonicine, and isethionic acid, which act as QS signal quenchers. *Bryozoa, Flustra foliacea*, isolated from the North sea, reduce a large variety of AHL signals through the action of bioactive molecules such as bromo-tryptamine-based alkaloids. A wide variety of sponges have been found to be very effective as quorum quenchers – *L. variabilis* produces secomanoalide and manoalide to degrade AHL signals. A nanofiltration membrane immobilized with porcine kidney acylase I suppresses exopolysaccharide production and, consequently, inhibits biofilm formation. It has thus proven to be a very good AF agent.

15.4.1 Clinical Settings

Biofilms are made up of proteins, polysaccharides, and DNA These polymers can be targeted by enzymes, e.g., glycosylases [15.36]. Proteases can be very efficient in breaking down the matrix, as has been observed for *Pseudoalteromonas*. Dispersin B (DspB) is a hexosaminidase, produced by a species of *Actinobacilli*, and it attacks polysaccharide intercellular adhesin (PIA)–poly-*N*-acetyl glucosamine polysaccharide (PNAG) [15.37]. Its effectiveness was shown through the prevention of biofilm formation by *S. epidermidis* on implanted devices, precoating plastic and polyurethane surfaces. DspB from the periodontal

pathogen *Aggregatibacter actinomycetemcomitans* is a b-hexosaminidase that exhibits a biofilm detachment ability. A combination of DspB with antiseptics (triclosan or chlorhexidine) has given a synergistic broad-spectrum antibiofilm and shown antimicrobial activity against *S. aureus*, *S. epidermidis*, and *E. coli* [15.38].

15.4.2 The Pulp and Paper Industry

Eight commercial enzymatic preparations were evaluated for biofilm reduction in the paper industry. These preparations contained proteases (pepsin, trypsin, Savinase1, and Alcalase1), glycosidases (dextranase and pectinase), and lipases (from *Candida rugosa* and Lipolase1). Savinase1 was the most efficient protease when used at concentrations of 40.5%. Cellulase is another enzyme that was successful in degrading mature biofilm formed by *P. aeruginosa*. It is possible to use this enzyme in combination with other treatments to increase the effectiveness to attack the mixture of polysaccharides in the EPS [15.39].

15.4.3 Deterrents/Biocides

Naturally produced AF biocide compounds have been divided into two categories: (i) non-polar metabolites which remain on the surface of an organism and may repel larvae exploring its surface, and (ii) polar metabolites, which are liberated into overlying water and may be detected by larval receptors to trigger avoidance behavior. Such AF compounds are classified as deterrents rather than toxins, since their mode of action may or may not be related to toxic effects. Glucose oxidase, hexose oxidase, and haloperoxidase are examples of oxidoreductases that can generate deterrents [15.36]. The oxidases and haloperoxidases catalyze the production of H_2O_2 and hypohalogenic acids, respectively. H_2O_2, like other reactive oxygen species (ROS), can cause oxidative damage in living cells. Hypohalogenic acids, e.g., HOBr or HOCl, being highly reactive, are thus used as oxidants for the treatment of water as disinfecting agents [15.36]. The oxidative damage of cells by H_2O_2 finds many applications. H_2O_2 has been used to reduce macrofouling in marine cooling water systems, particularly as Fenton's reagent when combined with ferrous ions [15.36].

15.4.4 Antifoulants

Hydrolases have been shown to abolish adhesion and biofilm formation by *Pseudoalteromonas* sp. D41 in natural seawater at 20 °C. This antibiofilm effect is due to the hydrolysis of extracellular polysaccharide (EPS), QS molecules, and adhesions as the substrates involved in bacterial adhesion. For example, hydrolysis of mutan and dextran EPS by mutanase and dextranase enzymes inhibits the formation of dental biofilms by *Streptococcus mutans* and *S. sobrinicus*, respectively [15.40]. Proteases, for example, Savinase, are very effective enzymes that prevent adhesion and biofilm formation by marine organisms. Savinase was effective and stable against biofilm formation, even when bacteria were allowed to adhere for 24 h [15.40]. Commercial protease (subtilisin) enzymes like Savinase or Alcalase are very effective when applied during the initial stages of bacterial adhesion. This can be done by conditioning the surface, e.g., by immobilizing the enzyme directly onto the surface or by applying the enzyme through an AF paint [15.40].

Lysostaphin, a *Staphylococcus simulans* metalloendopeptidase, degrades pentaglycine cross links in the cell wall and is effective against *S. aureus*, *S. epidermidis*. This enzyme can also detach biofilms, which suggests a potential contribution of cell wall-associated components to the matrix. Subtilisins, the serine proteases, cleave proteins in which serine serves as the nucleophilic amino acid. Such proteases are easily available as they are produced and secreted in large measures by many *Bacillus* species [15.41]. Subtilisins work on different biofilms, including those produced by industrially significant problematic species such as *Pseudomonas fluorescens* and *Pseudoalteromonas*.

DNA plays an important function in the stability of biofilms. This has been shown by the addition of deoxyribonuclease (DNaseI) leading to the dissolution of biofilms. Addition of bovine DNaseI to established biofilms can disrupt *S. aureus* biofilms formed on glass, plastic, and titanium surfaces. Studies have demonstrated that inducing expression of *S. aureus* endogenous nuclease prevents biofilm formation on abiotic surfaces. The use of DNase as a treatment for staphylococcal biofilm infections has not been attempted, although recombinant human DNase (also called Pulmozyme Dornase alfa) is approved as a treatment to reduce the viscosity of mucous in the lungs of cystic fibrosis (CF) patients. It is postulated that Dornase alfa plays a role in clearing bacterial infections that develop in the CF lung and *P. aeruginosa* is known to form a biofilm in this state. DNaseI also sensitizes biofilm bacteria to killing by various biocides and also to detachment by anionic detergents.

15.4.5 Engineered Bacteriophages

Phages that kill bacteria in a species-specific manner are engineered to express the most effective EPS-degrading enzymes specific to the target biofilm. This strategy permits the development of a diverse library of biofilm-dispersing phages rather than necessitating attempts to isolate such phages from the environment. These recombinant phages not only lyse the bacterial cells rapidly but express and produce the biofilm-degrading enzymes as well. This autocatalytic mechanism leads to efficient removal of bacterial biofilms in a wide range of scenarios, such as industrial, environmental, and clinical settings [15.42]. This strategy also eliminates the need to produce, purify, and deliver large doses of enzymes to target sites of infection that are otherwise difficult to access and would lead to improved and efficient phage therapy for removal of biofilms. For example, the engineered T7 phage expresses the K1-5 endosialidase enzyme, which allows it to replicate in the K1 polysaccharide capsule –producing *E. coli* [15.42].

DspB hydrolyzes adhesions which are important in the biofilm formation by several bacterial species, including clinical strains. Therefore, recombinant phages producing DspB can be applied to a large number of bacterial infections [15.42]. Also, several different biofilm-forming bacterial species, including *E. coli*, produce a group of biofilm promoting factors, required to produce a mature biofilm. These factors are shared among different strains and are, therefore, potential targets for an enzymatically engineered phage [15.42].

In contrast to most works which target only the removal of biofilms and its associated bacteria, a recent approach was aimed at reusing the platform for a fresh round of biofilms in biorefineries. Here, the intention was to reuse the platform for chemical transformations. In the initial step, proteins, global regulator (Hha) and cyclic diguanylate-binding (BdcA), were engineered to disperse biofilms. The dispersal cells were then removed through a chemical switch, isopropyl-β-*D*-thiogalactopyranoside. This technique can be applied to develop biosensors, biocorrosion, BF, and bioremediation [15.43].

Recently, QQ has been applied as a practical solution to control BF of membrane bioreactors (MBR) in water treatment systems. Cells of *Rhodococcus* sp. BH4, a QQ bacterium, were entrapped in alginate beads (cell entrapping beads, CEB), and designed with a porous surface microstructure. When these CEBs were used in MBRs, a transmembrane pressure of 70 kPa was reached in a time period that was ten times longer than MBRs without CEBs. The role of CEBs was dual in controlling the biofilm formation: biological, as quorum quenchers of AHLs produced by BF bacteria, thus inhibiting the synthesis of extracellular polymeric substances involved in biofilm formation, and physical as the source of friction between CEBs colliding with biofilm on the membrane surface. Sloughing off of biofilm was more pronounced with the QQ effect than friction. QQ is thus seen as an efficient and economical alternative to control BF of MBRs [15.44].

We should take inspiration from natural sources, especially skins of marine animals and microbes associated with them to develop the most sustainable marine technologies to control BF [15.9, 10].

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16. Detection of Invasive Species

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Marine invasive species incursions can cause significant ongoing damage to marine environments, aquaculture, biodiversity, infrastructure, and social amenity. They represent a significant and ongoing economic burden. Marine pests can be introduced by several vectors, including aquaculture, aquarium trading, commercial shipping, fishing, floating debris, mining activities, and recreational boating. Despite the inherent risks, there is currently relatively little routine surveillance of marine pest species conducted in the majority of countries worldwide. Accurate and rapid identification of marine pest species is central to early detection and management. Traditional techniques (e.g., physical sampling and sorting) have limitations, which has motivated recent progress towards the development of molecular surveillance tools. This chapter highlights the importance of effective sampling and Deoxyribonucleic acid (DNA) extraction strategies and describes developments in polymerase chain reaction (PCR)-based and ecogenomic methods for the detection and surveillance of marine invasive species. Recent advances provide a platform for the development of practical, specific, sensitive, and rapid diagnosis and surveillance tools for marine invasive species for use in effective prevention and control strategies.

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16.1 Background

Marine invasive species have the potential to cause significant harm to endemic biodiversity and habitats [16.1, 2]. Marine pests can be translocated and introduced by numerous vectors including ship ballast, hull fouling, floating debris, and man-made structures such as drilling platforms and canals [16.3]. Marine invasive species introductions continue to occur and threaten the marine environment and associated industries [16.4]. With increasing globalization comes faster and more frequent shipping and air transport of live seafood. Propagule pressure is only likely to increase unless effective strategies are employed for early detection, prevention, and control. Central to such strategies is the ability to rapidly identify invasive species from the environment.

In San Francisco Bay there are at least 230 established exotic species that account for 40–100% of the common species at many sites in the estuary [16.5]. Even if suitable eradication technologies were available, in some cases it may be un-economical to them, given the logistical difficulties associated with working in a marine environment. The main economic and social impacts of marine pests are decreases in the economic output of marine-based activities from aqua-

culture, fisheries, and tourism, and negative impacts on human health [16.3]. The total economic cost of a marine pest incursion is difficult to accurately define but in Australia, a major marine pest eradication has been estimated to cost up to AUD$263M [16.6]. The few examples of successful eradication of marine invasive species worldwide have been when the invasion has been detected in its very early stages, and/or it is restricted to an area that can be isolated from the rest of the marine environment (e.g., *Caulerpa taxifolia* eradication in Southern California [16.7]; black-striped mussels, *Mytilopsis sallei*, in Darwin, Australia [16.8, 9]).

Marine invasive species include a wide range of plant and animal taxa. These include macro and microalgae, molluscs, crustaceans, annelids, echinoderms, cnidarians, ascidians, and teleosts. The development and implementation of rapid, sensitive, and accurate diagnostic techniques for the identification and surveillance of marine pests from environmental samples (e.g., sea water, sediments, and ships' ballast), particularly in areas that are currently pest free, is an essential step in early detection and control of marine pests [16.10–12]. In this chapter, we:

i) Briefly describe techniques traditionally used for the identification and/or surveillance of marine pests.
ii) Review DNA technical approaches for the identification of species and definition of genetic markers.
iii) Describe recent PCR-based and ecogenomic methods for the detection of marine invasive species.
iv) Propose some options for the development of improved molecular diagnostic techniques.

16.2 Traditional Techniques

Baseline surveys and repeated monitoring conducted by experienced biologists are the prime requirement for early detection of marine pest species using standard methods. *Campbell* et al. [16.13] reviewed the five survey methods commonly employed for the detection of marine pest species: Hewitt and Martin protocols, rapid assessment surveys, Bishop Museum protocols, Chilean aquaculture surveys, and passive sampling protocols. These methods typically involve physical sampling, sorting, and identification to understand the resident biota and identify marine pests.

Traditional survey methods typically require specialized taxonomic experts, trained in identifying organisms across a range of life-cycle stages. The sorting and identification of plants and animals is time consuming, sometimes taking years before species are accurately identified, but the sampling data provided by surveys of this kind provide an important baseline understanding of biodiversity for future pest surveillance and ecological research. There is a global decline in taxonomic expertise [16.14, 15]. Given the wide range of taxa required to be identified in marine pest surveys and the inherent difficulties in identification, there is a need to develop methods that work in conjunction with, and are complementary to, available taxonomic expertise.

16.3 Sample Collection

The development of sensitive and specific molecular tools is highly important and has been the focus of most recent published studies on the development of marine invasive species surveillance. Without effective environmental sampling strategies and DNA extraction techniques, these molecular tools will prove largely ineffective.

16.3.1 Extraction of Genomic DNA from Environmental Samples

Mackie and *Geller* [16.16] developed a qPCR (quantitative PCR) for the detection of brine shrimp. The principle behind this was to use brine shrimp (*Artemia* spp.) as an additive to plankton samples. *Artemia* spp. inhabit hypersaline environments, so they are perfectly suited to being utilized as a quality control spike in marine plankton samples. Recently, the approach used by [16.16] was modified and used a sample quality control for the marine invasive species monitoring of Port plankton samples in Australia (personal observations). Freeze-dried *Artemia* sp. are added to each individual plankton sample at the time of collection and the *Artemia* qPCR assay is utilized to assess whether samples have been stored and preserved adequately for further analysis for marine invasive species.

Effective routine detection and surveillance of marine pests from environmental samples requires effective sample collection and genomic DNA (gDNA) isolation methodology. *Bott* et al. [16.11] highlighted the need to use samples that will be biologically informative for surveillance purposes. Organisms can be collected from water by filtering known amounts and extracting DNA from the filtrate. Larger sample sizes increase the likelihood of containing invasive species but pose issues surrounding DNA extraction.

Inhibition of PCR due to contaminants in environmental samples can be a limiting factor to accurate detection of invasive species. PCR inhibitors include organic and phenolic compounds, fats, glycogen, Ca^{2+}, humic acids, heavy metals, constituents of bacterial cells, over-abundance of nontarget DNA, and other contaminants [16.17]. There is a need, therefore, to rigorously assess DNA extractions for the presence of PCR inhibitors to reduce the occurrence of false negatives during analysis. Screening DNA extracts with control PCR assays of a known, added indicator organism is a useful approach in samples containing numerous DNA *types*. Correct preparation of environmental samples for DNA extraction can also prove useful in preventing PCR inhibition. For example, anecdotal evidence of freeze-drying marine sediment samples prior to DNA extraction greatly reduces the effects of PCR inhibition [16.11], which may be due to increased DNA extraction efficiency in freeze-dried samples [16.18]. A simple option to limit the effects of PCR inhibition is through the dilution of DNA; provided that a reliable detection limit to detect an organism is known, samples can be diluted to reduce inhibitors to below critical concentrations.

16.4 Molecular Approach to the Identification of Marine Invasive Species

As the use of molecular biological techniques has become more widespread, so too has their use in the identification of marine invasive species. A range of DNA-based techniques have been used (Table 16.1) to discriminate between species because of the ability of these methods to detect subtle genetic differences. DNA is relatively stable and can be readily isolated from fresh, frozen, ethanol-preserved, or commercially available nucleic acid storage buffer (e.g., RNAlater©, Ambion) preserved specimens for subsequent analysis using cloning, hybridization and enzymatic amplification procedures.

PCR [16.58, 59] is a reliable method of enzymatic nucleic acid amplification which has significantly advanced a wide range of scientific areas. The majority of molecular assays for the detection and identification of marine invasive species and their allies involve PCR-based technologies. The key to a reliable and robust molecular method for the identification of a marine pest is the definition of one or more suitable DNA target regions (genetic marker or locus).

Marine invasive species encompass a range of taxonomic groupings and no single marker should be considered universally appropriate to all taxa. Nuclear ribosomal genes and spacers, mitochondrial genes, and chloroplast genes (for plants), have been utilized to identify marine invasive species (Table 16.1). A thorough knowledge of the sequence variation within the marker of choice (both intra and inter-specific variation) must be obtained from previously published data (e.g., GenBank- http://www.ncbi.nlm.nih.gov/Genbank/). DNA sequencing of the marker of choice of both target and related organisms must be carried out in order to design an assay which is specific to the target taxon.

Genes evolve at different rates and a suitable DNA region should vary in sequence sufficiently to allow the identification of an individual to the taxonomic level required. For specific identification, the DNA marker should exhibit little or no genetic variation within a species but differ sufficiently between species so as to allow unequivocal delineation. For the identification of population variants (strains or genotypes), the marker should vary considerably in sequence within a species but still offer enough specificity so as to not cross-react (false-positives) with heterologous species.

In nuclear genes and spacers, there is typically little variation amongst individuals within a population and between other populations [16.43, 45]. Nuclear ribosomal DNA (rDNA) of eukaryotes is repetitive and consists of hundreds of tandem repeats. These tandem repeats are often distributed on different chromosomes [16.60, 61] and like other repetitive DNA elements they show sequence homogeneity as a result of concerted evolution [16.61, 62]. The rDNA array consists of the intergenic nontranscribed spacer (IGS or NTS), the external transcribed spacer (ETS), and the transcription unit comprising three rRNA genes (short subunit (SSU), 5.8 S and long subunit (LSU)), which are separated by the first and second internal transcribed

Table 16.1 Examples of molecular methods and markers used for the detection of different taxonomic groups of marine pests

Taxonomic group	DNA loci	Methods	References
Dinophyceae	ITS 1 and 2, 5.8S rDNA	PCR coupled rDNA probe	[16.19]
		qPCR	[16.20]
	rDNA	PCR	[16.21]
	LSU rDNA	PCR-RFLP	[16.22]
		PCR	[16.23, 24]
		FISH	[16.25]
		SHA	[16.7]
	SSU rDNA	FISH	[16.26]
		PCR-FISH	[16.27]
	5.8S rDNA	qPCR	[16.28]
Macroalgae	ITS 1 and 2, 5.8S rDNA	PCR coupled dot-blot hybridization	[16.29]
	IGS	PCR	[16.30, 31]
	*rbc*L gene	PCR	[16.32, 33]
	Mitochondrial: *cox*1, 16S	PCR	[16.34]
	Mitochondrial: cytochrome b and *cox1*	PCR	[16.35]
Gastropods	Mitochondrial: *cox*1	PCR	[16.36]
Bivalves	Mitochondrial: *cox*1, *nad*4, 16S	Multiplex PCR	[16.37]
		Nested PCR	[16.38]
		PCR	[16.39–41]
	Microsatellite loci	PCR	[16.42]
	SSU rDNA	Nested multiplex	[16.43, 44]
		PCR-SSCP	[16.45]
		FISH	[16.46, 47]
		SHA	[16.48]
	ITS	PCR-RFLP	[16.49]
	IGS	PCR	[16.30]
Crustaceans	Mitochondrial: *cox*1	qPCR 64	
		PCR	[16.50, 51]
		PCR-dot blot	[16.29]
	ITS	PCR	[16.50, 51]
	SSU-rDNA	PCR	[16.51]
	IGS	PCR	[16.30]
	Not applicable	RAPD-PCR	[16.52]
Echinoderms	SSU rDNA	PCR	[16.53]
		FISH	[16.54]
Ascidians	SSU rDNA	PCR	[16.55]
	SSU rDNA/Mitochondrial: *cox*1	PCR	[16.56]
	Microsatellite loci	PCR	[16.57]

spacers (ITS-1 and ITS-2). The rDNA genes, ITS, and IGS/NTS regions have been shown to be particularly useful in defining species specific markers for marine pest assay development (Table 16.1).

Genes in the circular mitochondrial genome generally evolve at a quicker rate than nuclear genes; animal cells typically contain hundreds to thousands of mitochondria per cell [16.63]. A high copy number increases detection potential, making mitochondrial markers potentially suitable for sensitive molecular assays. Evolutionary rates of mitochondrial DNA may vary between taxonomic lineages, but are generally believed

to undergo relatively rapid rates of evolution [16.64, 65]. Mitochondria are generally inherited maternally, making them particularly useful as a species-specific marker for the delineation of closely related species e.g., [16.35, 39]. The identification of different strains (i. e., population variants) has important implications for understanding the geographical origin and patterns of spread of marine pest species, potentially providing a better understanding of patterns and means of translocation. Mitochondrial DNA has proven particularly useful in understanding the genetic structure of populations due to their maternal inheritance which provides tracers to patterns of colonization and its smaller effective population size (when compared to the nuclear genome [16.66–68]).

Like mitochondria, chloroplasts have a circular genome that is smaller than the nuclear genome. Typically, chloroplast genomes display lower levels of heterogeneity than mitochondrial genomes; this is in part due to mitochondria having larger genomes which encode for less genes than chloroplasts [16.69, 70]. The ribulose-bisphosphate carboxylase (*rbc*L) gene is of interest for use as a diagnostic marker of algae. *Hanyuda* et al. [16.71] reported that the intron in the *rbc*L gene was highly variable amongst different species and strains of *Caluerpa*, and [16.32] used a *rbc*L PCR-based assay to identify invasive strains of *Caulerpa taxifolia*. *Freshwater* et al. [16.33] also used a *rbc*L gene PCR-based assay to discriminate invasive *Gracilaria* from nearly morphologically identical native species.

16.5 PCR-Based Methods Utilized for Routine Identification and/or Surveillance

A wide range of molecular-based techniques have been employed for the identification of species at adult and/or larval stages of marine invasive species or related species (Table 16.1). The advantages, limitations, and uses of these techniques for marine pest research and detection are summarized in Table 16.2, and the limitations of these approaches are further discussed in the following section. PCR has revolutionized many areas of biological research, including species and strain delineation. PCR can amplify minute amounts of template DNA, and its high specificity makes it highly effective for species and strain identification for a wide range of organisms. PCR with direct sequencing is a gold standard technique; however, it takes several days to obtain results, and a relevant question is whether other PCR-based procedures can be used as reliably in the same amount of time. The relatively low cost of equipment and reagents makes PCR accessible to small laboratories.

16.5.1 PCR-Based Methods

Species Detection Through Design of Specific Primers

PCR utilizing genus or species specific primers for marine pests has been utilized using a wide range of techniques: one-step reactions e.g. [16.23, 24, 30, 35, 38], nested PCR [16.37], and multiplex [16.44] and nested multiplex PCR [16.43, 75].

Random Amplified Polymorphic DNA (RAPD)

RAPD (RAPD) is based on the amplification of random fragments of genomic DNA using single primers of arbitrary sequence. RAPD offers rapid molecular assessment in that it does not require the design of specific primers that bind exclusively to one sequence type. It essentially scans the entire genome without prior knowledge of sequence information. RAPD will produce a specific banding pattern when amplicons are subjected to electrophoresis. It can be particularly useful in discriminating between closely related species or strains and understanding sequence variation between individuals [16.52]. *Zhou* and *Gao* [16.22] utilized RAPD for the identification of mitten crab (*Eriocheir sinensis*) populations, but it has not otherwise been widely utilized for marine pest identification.

Restriction Fragment Length Polymorphism (RFLP)

Restriction (RFLP) endonucleases are used to cut DNA at precise sequences producing specific bands which are visualized via electrophoresis. When RFLP is combined with PCR, a fragment will be amplified via PCR before being digested, using the restriction endonucleases to give a characteristic pattern based on the amplified fragment. RFLP has not been widely used for the identification and/or surveillance of marine pests. *Scholin* and *Anderson* [16.49] used PCR-RFLP to distinguish between

Table 16.2 Molecular diagnostic techniques suitable for different sample types for the identification and detection of marine pest species

Technique	Suitable sample types	Advantages	Limitations	Example references
PCR (end-point one step, nested and multiplex, agarose gel electrophoresis and/or sequencing)	Whole animals, larval and egg/spore stages, environmental samples	Can be highly specific, relatively inexpensive, able to amplify minute amounts of DNA.	Post-PCR handling time consuming, potential exposure to toxic reagents. Nested PCR has potential for PCR contamination. Multiplex PCR can be difficult to develop effectively.	[16.21, 30, 31, 55, 57]
PCR-dot-blot hybridization	Whole animals, larval and egg/spore stages, environmental samples	Can be highly specific, inexpensive, amplify minute amount of DNA, obtain results quickly.	Post-PCR handling time, if hybridization step requires specificity. Slower than qPCR applications.	[16.29]
PCR-RAPD	Whole animals, larval and egg/spore stages	Inexpensive	Not suited to specific amplification, requires post-PCR analysis	[16.52]
PCR-RFLP	Whole animals, larval and egg/spore stages	Inexpensive, can be useful in discriminating closely related species/strains	Not suited to specific amplification, requires post-PCR analysis	[16.22, 49]
PCR-SSCP	Whole animals, larval and egg/spore stages	Cost-effective, a powerful method of distinguishing closely related species/strains	SSCP electrophoresis is time consuming, not suited to widespread testing on environmental samples	[16.45]
Quantitative PCR	Whole animals, larval and egg/spore stages, environmental samples	Highly specific, rapid analysis, allows quantification of amount of target species in a sample, and potential for high-throughput application	Assay development can be expensive and time consuming. Incorrectly developed assays may produce false-positives.	[16.20, 28, 72]
Next generation sequencing	Whole animals, larval and egg/spore stages, environmental samples	Large amounts of data, provide comprehensive analysis. Allows examination for wide variety of species in environmental samples	Expensive per sample, not high-throughput like qPCR. Data processing time consuming	[16.73, 74]

six species of the dinoflagellate genus, *Alexandrium*. *Fernandez-Tajes*, *Medez* [16.76] utilized PCR-RFLP of ITS for the discrimination of razor clams (*Ensis* spp.), and *Yamasaki* et al. [16.77] utilized RFLP of the mitochondrial gene cytochrome oxidase subunit 1 (*cox1*) to identify Japanese mitten crab (*Eriocheir japonica*).

Single Strand Conformation Polymorphism (SSCP)

Single stranded conformation polymorphism (SSCP) is a mutation scanning approach that analyzes amplicons of 100–450 nucleotides by denaturing double-stranded DNA into single-stranded DNA and electrophoretically analyzes the single-stranded conformational profiles (conformers) in a nondenaturing gel matrix. SSCP has been shown to distinguish between sequences that differ by a single base [16.29]. It can be a highly effective cost saving tool. Once sequences (i. e., species or strains) are known for conformational profiles, the extra cost of direct DNA sequencing is eliminated. SSCP generally does not require optimization of different sequences or amplicons [16.29]. *Livi* et al. [16.45] utilized PCR-coupled SSCP of the short subunit (SSU)-rDNA for the discrimination of bivalve larvae.

PCR Coupled Dot-Blot Hybridization

PCR coupled dot-blot hybridization involves firstly PCR amplification and secondly detection of that amplicon using staining or radioassay (e.g., ^{32}P, DIG) of the complementary target bound to a support. The method is useful for differentiating between closely related species when species-specific probes are designed [16.19] and is a quick and relatively inexpensive means of identification and discrimination. *Blomster* et al. [16.19] used an ITS-based dot-blot hybridization to distinguish between species of the Chlorophyte genus, *Enteromorpha*, while [16.78] employed a ^{32}P-labeled probe to identify *Alexandrium* spp.

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) allows the amplification of a target PCR amplicon to be monitored in real-time as amplification occurs. qPCR utilizes two main detection systems: fluorescent intercalating dyes and probes. There are a number of intercalating dyes utilized for qPCR. The original method used ethidium bromide and measured the change in fluorescence after each cycle using a digital camera and a fluorometer [16.79]. Subsequent modifications incorporated intercalating dyes such as SYBR Green I [16.80] LCGreen [16.81], SYTO9 [16.82], and EvaGreen [16.83].

Probe-based detection systems include *Taq*Man probes [16.84], molecular beacons [16.85], fluorescence resonance energy transfer (FRET) [16.86], and minor groove binder (MGB) probes [16.87]. These probes are more expensive than intercalating dyes but ensure specificity of the assay through ensuring exclusive binding to the target sequence [16.82].

qPCR is typically analyzed in two ways. Relative quantification will provide a cycle threshold (C_t) for the specific amplification of the target sequence based on comparison with amplification of a *normalizer* gene from the same DNA template. It should be present with the same copy number of the locus of interest. Absolute quantification is performed by determining the C_t of test samples by amplifying target DNA standards (of known concentrations) at the same time as test samples; the C_t is calculated based on a standard curve generated from the DNA standards.

Quantitative PCR (qPCR) (offers a relatively rapid analysis (> 2 h)); the potential for high-throughput applications, allows linear quantification over a wide dynamic range (> 6 orders of magnitude) and the benefit of not requiring post-PCR handling (*closed-tube* format). It is now routinely used in a wide range of clinical applications for the detection of a wide range of bacterial, fungal, parasitic, and viral diseases of humans [16.28]. Recent advances have seen a number of studies utilizing qPCR-based techniques for the identification of marine pests [16.72, 88].

Melting Curve Analyses. When using intercalating dyes some qPCR thermocyclers can also perform melting analysis or high resolution melting (HRM) analysis mutation scanning techniques. Melting analyses allows for rapid post-PCR analysis of sequence variants or strains/species, or a secondary confirmation of the presence of the target amplicon in the sample based on consistency of the melt curve with the defined DNA standards. This approach has been employed for a number of studies concerned with human and livestock pathogens [16.89, 90]. Intercalating dyes typically bind and allow detection of any double-stranded DNA. This is advantageous but there is also the potential for binding to nonspecific products and primer dimers; careful PCR optimization is required to determine the optimum intercalating dye concentrations. The use of post-PCR applications such as melting analysis can be utilized to distinguish between a nonspecific product/primer dimer and a PCR amplicon.

Potential Issues with PCR-Based Approaches

The issue of PCR inhibition, due to DNA contaminants, potentially causing false negatives needs to be taken into consideration for all PCR-based procedures. Incorrect pipetting techniques or aerosol contamination of DNA and/or PCR products can contribute to false positives in all PCR-based applications. No template controls (NTC) should be used in every experiment to monitor PCR contamination, and laboratories should employ various decontamination procedures to avoid this (e.g., Ultraviolet sterilization, decontamination of bench tops).

16.6 Ecogenomic Techniques

Ecogenomics are methods that allow the examination of genetic material from complex environmental samples containing many different organisms. It provides a step-change approach to environmental surveillance methods through its ability to identify a wider range of taxonomic groupings [16.91]. Ecogenomic methods

have been effectively used for a wide range of taxa, localities, and environs (i. e., sediments and water) such as Sydney Harbour sediments [16.91], marine anoxic water [16.92], ecological characteristics of viruses [16.93], and biotic assemblages of soil DNA [16.94]. The development of molecular tools for ecological monitoring potentially allows for more complete, rapid, and cost effective assessment of biodiversity and ecological shifts to be conducted [16.73]. These methods, once developed and validated, will be applicable to a range of marine habitats that are currently poorly understood. These methods show great potential for the ongoing monitoring and early detection of marine invasive species. Ecogenomic methods can be used to develop baseline information of species assemblages, making subsequent monitoring exercises of ports and harbors more informative, allowing for the loss of endemic biodiversity to be monitored alongside invasive species monitoring.

Recent studies [16.74, 95] have shown the effectiveness of 454 pyrosequencing for marine invasive species detection. *Pochon* et al. [16.74] were able to demonstrate that the detection limit for the Northern Pacific seastar, *Asterias amurensis* is equivalent to qPCR-based detection methods.

Ecogenomic techniques offer the ability to screen environmental samples for a broad range of organisms at once, process large numbers of samples quickly, and offer the ability to screen not only for invasive species (or pathogens) but also for endemic species to provide a broader indication of environmental health [16.91]. Next generation sequencing technology may also facilitate baseline screening of ports and harbors to obtain a rapid understanding of the presence and population densities of all species. This may aid in tailoring more focussed screening for marine invasive species specific to a geographical area and provide valuable data for modeling invasion biology.

16.7 Future Approaches

Commercial shipping can act as a major vector in marine pest translocation [16.95, 96]. Ballast water commonly used to control the trim and draft of a vessel can contain larval and adult stages of marine pest species [16.97]. The potential for these pests to be discharged into a new environment and flourish is high for some species [16.4]. Hull-fouling is also a significant translocation vector, while solid ballast was a dominant vector in previous centuries. As a consequence, ports are the major entry points for the majority of new marine pest incursions. Thus port and ballast water surveys are targeted to areas where effective and efficient detection and management strategies can be implemented to aid in successful prevention and mitigation of marine invasive species invasions.

16.7.1 Routine Monitoring and Surveillance of Marine Invasive Species in Port Waters

Routine monitoring using molecular technologies can alleviate the demand for time-consuming and costly traditional port surveys. Understanding marine invasive species assemblages in ports is a vital tool for managing ballast water in a cost-effective manner as the management requirements are reduced or eliminated for transfers between ports with similar suites of pest species. Robust qPCR, particularly in laboratories with high-throughput capability, can be used to rapidly assess samples for a wide range of marine invasive species at a fraction of the cost of traditional surveys. The vast majority of existing molecular-based assays for marine pests (Table 16.1) are not suited to high-throughput applications, so conversion of tests to formats such as qPCR is important. Continued dedicated research is required to provide reliable, uniform testing procedures (e.g., qPCR) for pest species that display genetic differences across their range. Routine monitoring programs utilizing robust, sensitive, and specific assays for a wide range of marine pest species provide the ability for authorities to monitor and manage marine pest incursions in a cost-effective way.

16.7.2 Testing Ballast Water

Molecular methods could minimize delays and provide data on ballast water safety. There are technical and logistical hurdles to provide suitable testing including timeliness to avoid unnecessary delays. This would be best achieved by streamlining sample collection and DNA extraction.

An effective way to collect ballast water samples is to filter a known amount of water (and ballast sediment)

and collect the filtrate for subsequent DNA extraction and analysis. For this to occur routinely, laboratory facilities may need to be maintained at, or close to ports, and the DNA extraction method must not produce significant PCR inhibition. The use of microfluidic carbon nanotube platforms for the detection of invasive species from substrates such as ballast have been proposed by [16.98], and the use of laser transmission spectroscopy has also been proposed by [16.99]; technology such as this shows potential for on-board or portside applications. Well-defined quantitative testing systems could also be used to test the performance of ballast water treatment systems, particularly the concentrations of indicator microbes and invasive species of high economic importance.

16.7.3 Legal Requirements

It is possible that the result of detection of marine pests through molecular methods would be that legal action (e.g., seizures, quarantine) would be initiated by the relevant jurisdiction. It is, therefore, of utmost importance that positive results are verified through a number of methods: 1. manual discovery and identification of the pest; 2. obtaining DNA sequence data from the sample to further confirm identity; 3. using multiple detection methods (e.g., traditional identification with multiple molecular methods). It is also essential that the laboratory involved follow rigorous protocols and utilize a number of controls (including spikes) to provide quality control and assurance measures.

16.8 Concluding Remarks

This review summarizes studies about the molecular identification and/or detection of marine pest species. For the effective control of marine pest incursions, rapid specific identification is of utmost importance. An understanding and exploration of the genetic diversity of different pest species populations is also required, which will lead to more effective control and a better understanding of pest biology. The development of molecular-based assays for a wide range of marine pests provides a solid foundation for the continued development of robust, sensitive, and specific assays for screening environmental samples. It is important that the opportunity is grasped to develop robust surveillance methods of marine invasive species, as in some situations, detection may prevent release unless appropriate treatment is undertaken. Identification is vital in order for treatment and mitigation program to be implemented in a timely and effective manner.

Provided that they are robustly validated, high-throughput methods such as qPCR currently offers the most cost-effective and rapid means of analysis. The potential of ecogenomic applications is being realized, providing opportunities to not only understand what invasive species are present in a sample but also to analyze endemic species to assess overall environmental health.

An important component in the development of molecular surveillance strategies is an effective DNA isolation and purification technique that works effectively on a range of sample types and locations (e.g., spatial and temporal variation in samples). Methods need to be optimized relevant to the substrate to remove inhibitory substances to enzymatic amplification, and the development of an accepted quality control standard [16.16] for the effective purification of DNA from different sample types is essential.

With the worldwide decline in specialist taxonomic expertise there is an increasing need for accurate and rapid marine invasive species diagnostics. Continued development of marine invasive species molecular diagnostics must occur in conjunction with taxonomic specialists to ensure accuracy of assay development. Studies investigating the systematics and phylogenetic relationships of marine invasive species and related taxa will progress towards the development of robust diagnostics. These studies will help to further identify molecular markers with diagnostic potential and provide baseline genetic information necessary for the design of specific diagnostic assays.

Specific diagnosis is central to: (a) rapidly establishing the prevalence and distribution of marine invasive species in the environment in conjunction with traditional sampling techniques, (b) monitoring changes in marine invasive species distribution spatially and temporally, and (c) conducting targeted eradication and control programs if economics and logistics permit. Developing the capacity for detection and enumeration of marine invasive species seems achievable if appropriate support and funding is provided by relevant stakeholders.

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17. Marine Sponge Metagenomics

Valliappan Karuppiah, Zhiyong Li

The sponge is one of the oldest multicellular invertebrates in the world. Because of its special pore canal structure and characteristics of filter feeding, a large amount of microorganisms adhere to it. After hundreds of thousands of years' evolution, they form a kind of symbiosis relationship. A rather large amount of research shows the great diversity of microbes related to sponges using 16S rRNA (ribosomal ribonucleic acid) or 18S rRNA gene-based methods. Just in recent decades, many bioactive compounds have been separated from sponges. However, more and more studies confirm that those bioactive compounds do not derive from the sponges themselves but rather their symbionts. Considering that most of the microbes in sponges are unculturable, the culture-dependent approach is greatly limited. Using metagenomics, it may be possible to produce enzymes of special characteristics and secondary metabolites with biotechnological application from the sponge derived uncultured microbes. What is more, metagenomics can also serve as a way to understand the community structure, as well as the metabolism and function of a complex microbial community. This chapter focuses on techniques and advances in marine bacterial metagenomics, using sponge metagenomics as an example. Several aspects will be considered: The background and problems of sponge research (Sect. 17.1), the principle of metagenomics and related techniques (Sect. 17.2), the application and latest progress in sponge metagenomics (Sect. 17.3), and future perspectives (Sect. 17.4).

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17.1 Background and Problems of Sponge Research

Sponges are the simplest form of multicellular animals of the phylum *Porifera*, which literally means *pore-bearing*. Their morphology caused ancient scientists like Aristotle and Pliny to consider them as plants [17.1]. In 1765 John Ellis proved that they are actually animals [17.2]. Marine sponges represent one of the extant metazoans of 700–800 million years. They are classified into three classes according to skeletal type, calcareous sponges – *Calcarea*, demosponges – *Demospongiae*, and glass sponges – *Hexactinellida*. So

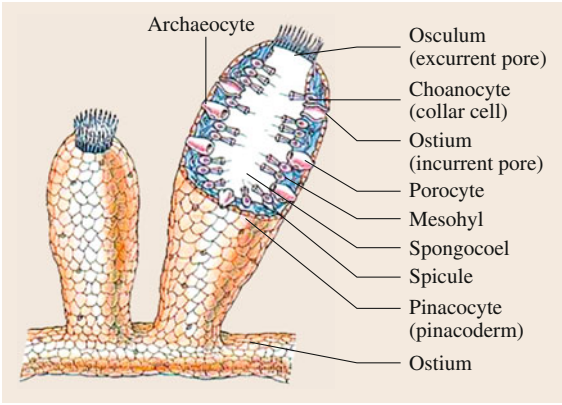

Fig. 17.1 Schematic representation of a sponge (adapted from http://universe-review.ca/R10-33-anatomy.htm)

far, 15 000 species have been described, but the true diversity is probably much higher. Among them, more than 6000 species are found in marine and freshwater systems throughout tropical, temperate, and polar regions [17.3]. An enormous diversity of sponges occurs on coral reefs, where they exhibit a wide range of shapes and colors.

The structure of sponges is different from that of other taxons (Fig. 17.1); the body of sponge is made up of extensive jelly-like material (mesohyl), armored by a network of collagen fibers. Sponges have special collar cells (choanocytes) that are unique in the animal kingdom. They have flagella, whip-like structures that form the external *skin*, breathing pores, and tubes to set up water currents to sieve food particles from the water. The seawater passes through an inhalant pore and leaves the sponge via an exhalant pore. The food particles, including bacteria and microalgae filtered by the choanocytes, are transferred to an extensive layer of connective tissue (mesohyl). In the mesohyl, the special cell archaeocytes will digest the food particles by phagocytosis. Sponges are also unique because nearly all their cells can change function as required (*totipotency*). The mesohyl of marine sponges represents dense communities of microorganisms [17.4].

Sponges are a most important component of benthic fauna and represent the second largest biomass on tropical reefs due to the mechanism of obtaining nutrients. Sponges feed on particles as small as bacteria as well as larger particles in food poor environments. Sponge symbioses were initially documented by electron microscopy. *Webster* et al. [17.5] identified the high density of bacterial cells within the mesohyl region and also choanocyte chambers surrounding the region by fluorescence in situ hybridization (FISH). As filter feeder it pumps large volumes of sea water containing bacteria and effectively removes 60 to 99% of the bacteria [17.6]. Most symbiotic bacteria occupy up to 60% of the sponge volume in the intercellular matrix or mesohyl region. Sometimes, this exceeds the total volume of sponge cells. These bacteria contribute up to 40% of the sponge biomass and play a role in nutrient balance, health, and diseases of sponges. These bacteria are permanently associated with the sponges until disturbed by external factors [17.7]. In addition to fitter feeding, some coral reef sponges also obtain nutrients by photosynthesis. In corals, dinoflagellates (zooxanthellae) perform the photosynthesis. In contrast, sponges contain a wide range of microbial symbionts, unicellular or filamentous cyanobacteria are the photosynthetic symbionts, providing nutrients to the sponges by carbon and nitrogen fixation under normal environmental conditions [17.8–10].

Microbial communities associated with marine sponges include bacteria, archaea, microalgae, and fungi. Initial studies found that bacteria associated with sponges are same as those found in ambient seawater [17.11]. Later, it was concluded that sponge-associated bacteria are exact symbionts and are different from ambient seawater bacteria [17.12, 13]. Radio labeled substrate feeding studies clarified the difference between symbiotic bacteria and ambient seawater bacteria [17.14]. The bacterial count in sponges exceeds by two to three times that in ambient seawater. The relationship between the sponge and microbes could be classified as obligatory mutualism (symbionts have a crucial role in the host metabolism), facultative mutualism (they have a valuable effect on their host, but the host will also exist without the symbiont), or commensalism (they live without providing any valuable effects to their host). In all cases, it is clear that the sponge provides shelter for their symbionts [17.15].

Many diverse studies such as light and electron microscopy, FISH, and cultivation methods have proved the presence of a wide range of microbial associations and their adaptation in a sponge specific host environment. More recently, classical cultivable and uncultivable studies based on the 16S ribosomal RNA gene sequence revealed a high microbial diversity within the sponges; for example, bacterial phyla include *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, *Proteobacteria* (*Alpha*, *Beta*,

Delta, and *Gamma Proteobacteria*), *Spirochaetes*, and *Verrucomicrobia*. Furthermore, a new phylum, *Poribacteria*, has also been found in a number of microbially rich sponges. Sequences from the *Chlorobi* (green sulfur bacteria) have not been obtained from sponges, although positive FISH signals were obtained from *Rhopaloeides odorabile*. Two archaea have also been reported from marine sponges, which belong to the members of the phyla *Crenarchaeota* and *Euryarchaeota* [17.4].

Sponges also host eukaryotic microbes such as dinoflagellates and diatoms, with the latter seemingly most prevalent in polar regions. Zoochlorella, an endosymbiotic microalgae, often reported from freshwater sponges. Two previous reports of cryptomonads in sponges were made by Wilkinson [17.16]. Marine sponges' associated fungi are receiving increasing attention due to their biotechnological potential. Fungal strains isolated from marine sponges, representing three phyla of *Ascomycota*, *Zygomycota*, and *Basidiomycota* fungi. In total 22 orders (*Boliniales*, *Botryosphaeriales*, *Capnodiales*, *Chaetosphaeriales*, *Claromycetales*, *Diaporthales*, *Dothideales*, *Eurotiales*, *Helotiales*, *Hypocreales*, *Microascales*, *Mitosporic Agricomycotina*, *Moniliales*, *Mucorales*, *Onygenales*, *Phyllachorales*, *Pleosporales*, *Polyporales*, *Saccharomycetales*, *Sordariales*, *Trichosphaeriales*, and *Xylariales*) within *Ascomycota*, 8 orders (*Agaricales*, *Agaricostilbales*, *Corticiales*, *Malasseziales*, *Polyporales*, *Sporidiobolales*, *Tremellales*, and *Wallemiales*) within *Basidiomycota* and 4 orders (*Rhizopus*, *Mucor*, *Rhizomucor*, and *Syncephalastrum*) within *Zygomycota* have been recorded in sponges [17.17]. Compelling evidence for symbiosis of yeast with sponges of the genus *Chondrilla* was obtained by extensive microscopy studies of both adult sponge tissue and reproductive structures [17.18]. It thus remains unclear in most cases whether such fungi are consistently associated with the source sponge, or even whether they are obligate marine species.

Viruses and virus-like particles (VLP) are abundant in coral reef environments and have been found in the water columns adjacent to sponges in coral reef environments. Virus-like particles have been observed in cell nuclei in *Aplysina* (Verongia) *cavernicola* [17.19]. It was suggested that VLP could be involved in sponge cell pathology. In another study, infection of an *Ircinia strobilina* derived *alphaproteobacterium* by a seawater bacteriophage was also demonstrated in laboratory conditions [17.20], but infection in nature is not known.

It has been assumed that the bacteria associated with the sponge synthesize several bioactive compounds [17.21]. However, cultivation of invertebrates for these bioactive compounds is too expensive and impossible, and hence cultivation of symbiotic microbes may provide an inexpensive supply of the natural compound. Yet, many sponge-associated bacteria have not been cultivable. Thus, metagenomic approaches are intended to isolate the bioactive genes responsible for bioactive compounds and express them in surrogate hosts. Usually, metagenomics begins with the construction and screening of metagenomic libraries for the targeted genes. This approach has resulted in the detection of numerous short biosynthetic pathways and novel bioactive compounds [17.21]. However, to date multidomain modular nonribosomal peptide synthetases (NRPS), type-I polyketide synthases (PKS-I), and type-II polyketide synthases (PKS-II) involved in the biosynthesis of secondary metabolites have been screened based on polymerase chain reaction (PCR) in the sponge metagenome for the novel bioactive compounds.

Marine sponges contain a wide range of microbial communities of significant ecological importance [17.4]. Although the perceptive of sponge microbial diversity is rapidly improving, the role of these microbes are mostly remains unknown [17.4]. Specific microbial mediated processes such as photosynthesis, sulphate reduction, nitrogen fixation, and nitrification have been quantified and identified by isotope enrichments, metagenomics and functional gene analyses [17.22]. These studies have extended our knowledge of the microbial symbiont function in sponges, yet they remain focused on specific processes or particular functional groups of organisms. Further, community wide assessment of microbial symbionts would be useful to elucidate their ecological role and contribution to the host.

Several methods have been developed and applied to evaluate sponge microbial symbiosis and diversity. Early studies in microbial ecology and the biosynthesis of natural products relied heavily on culturing bacteria on laboratory growth media. Unfortunately, most symbiotic microbes cannot be cultivated in laboratory conditions. The presence of abundant unculturable microbes in sponges makes it difficult to understand the functional traits. Therefore, in the last decades, microbial diversity studies have focused on molecular, culture-independent techniques based on the 16S rRNA gene. Cloning and sequencing of 16S rRNA genes are useful to infer phylogenetic relationships of the bacteria in a specific habitat. Cloning and characterization

of the environmental DNA (deoxyribonucleic acid) enables the renovation of metabolic and functional traits. This approach is generally termed *environmental genomics* or *metagenomics*. It has been effectively applied on several environmental samples, including marine sponges.

17.2 The Principle of Metagenomics and Related Techniques

Microbial research radically changed after 1985 due to the pioneering work on rRNA genes by *Carl Woese* [17.23]. This was followed by the work of *Pace* and colleagues, who directly analyzed the 5S and 16S rRNA genes from environmental samples without culturing [17.24]. The next technical step was the development of PCR and primers lead to amplifying the entire gene. This advancement in technology provided a larger microbial diversity that was not distorted by culturing bias.

Each organism in an environment has a unique set of genes in its genome; the combined genomes of all the community members make up the *metagenome*. Metagenome technology (metagenomics) has led to the accumulation of DNA sequences, and these sequences are exploited for novel biotechnological applications [17.25]. Metagenomics and the associated strategies have arrived at the forefront of biology, primarily because of the exploitation of next generation DNA sequencing technologies. This has significantly improved the potential for sequencing large environmental sets. Technological advances have created new opportunities for large-scale sequencing projects that would have been difficult to imagine several years ago. The next key development is an emerging appreciation for the importance of complex microbial communities in the marine environment. Due to the overwhelming majority of unculturable microbes in the marine environment, metagenome searches will always result in the identification of unknown genes and proteins. Currently, single-cell genomics is well suited for symbiosis research including bacterial symbionts of marine sponges. Thus, the probability of uncovering unknown sequences makes this approach more favorable than searches in cultured microbes (Fig. 17.2).

17.2.1 Isolation of Metagenomic DNA

Metagenomics has been confirmed to be a potent tool for studying the diverse environmental microorganism resources. Currently, there are two approaches in research on sponge metagenomics. In the first approach, a massive sequencing facility is employed to decode the metagenomic library with short sequences of fragments for assembly [17.26]. In the second one, scientists focus on the potential of functional expression of metagenomic library clones with large insert DNA [17.27]. In all cases, it starts with isolating DNA from the sponge sample.

Several methods are available for extracting DNA from sponge samples. Commercial kits are also available for extraction and purification of metagenomic DNA, which are easy to use with significant reproducibility. Isolation of metagenomic DNA from sponge samples can be classified into direct, indirect, and enrichment methods. The ultimate methodology should include cell lysis and DNA extraction. The direct method is based on cell lysis within the sample matrix and subsequent separation of DNA from the matrix and cell debris. Indirect DNA isolation is based on the separation of cells from the matrix, followed by cell lysis and DNA extraction. The enrichment method involves the separation of cells from the matrix, followed by cell enrichment, lysis, and DNA extraction [17.28].

17.2.2 Library Construction

Sponge metagenomic DNA is composed of a promising source of novel metabolites. The discovery of these novel metabolites requires the construction of a metagenomic DNA library and its efficient screening. During the construction of the metagenomic library two parameters have been widely considered. The first one is the large size and this should be the mixture of all bacterial genomes colonizing in a given environment. Such diversity improves cloning efficiency, so that the gene libraries provide sufficient functional genes of the entire metagenome. The second parameter is the size and cluster organization of genes involved in the secondary metabolite synthesis. Since the genes encoding the secondary metabolite biosynthesis are normally clustered, clones in the metagenomic libraries should contain larger DNA. Isolation of high molecular weight DNA facilitates the cloning of DNA into bacterial artificial chromosomes (BACs) and allows the characterization

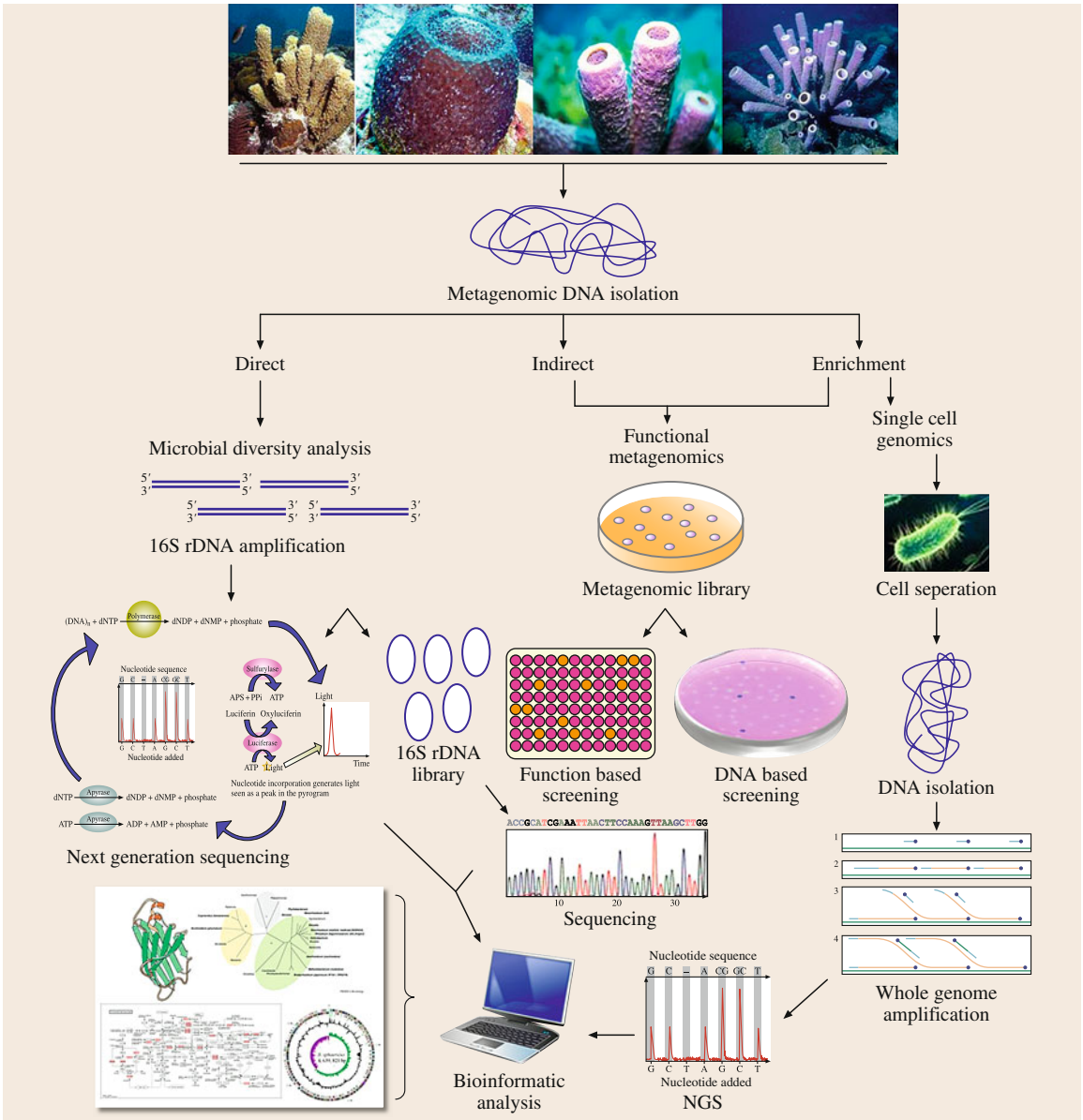

Fig. 17.2 Strategy of sponge metagenomics

of large regions of the genomes [17.28]. In general, the indirect and enrichment methods yield higher molecular weight DNA with high purity than direct methods. However, direct DNA isolation method from sponges is disadvantaged by the difficulty of mechanical shearing that occurs by the physical forces imposed on the sample during isolation. Further, nucleases released during cell lysis may possibly denature the released DNA.

The eukaryotic DNA content is also an important parameter effecting the construction of metagenomic library. Eukaryotic DNA will increase the cloning efficiency, but the expression of eukaryotic genes in bacterial hosts is restricted. In contrast, DNA obtained by the indirect and enrichment methods was suitable for library construction and gene expression due to the separation of bacterial DNA from the eukaryotic genome.

Therefore, it is emphasized that the direct method should be avoided when constructing the metagenomic library for natural product synthesis.

Metagenomic library construction methods have been extensively optimized for cloning in BAC and FOSMID (F1 origin-based cosmid vector); both have been successfully used in sponge metagenomics. Compared with fosmid libraries only capable of screening up to 40 kb inserts, environmental BAC libraries enable the screening of larger DNA inserts up to 200 kb [17.29], which can facilitate the reconstruction and functional analyses of environmental genomes [17.30].

17.2.3 Function-Based Screening of the Metagenomic Library

One of the simplest strategies used to detect antibiotic producing environmental DNA (eDNA) clones is top agar overlay assays. Metagenomic enzymes are usually screened on agar plates supplemented with substrates. Positive clones from the metagenomic library can be identified by visual screening for the appearance of a clear zone or color. Functional screening of a metagenomic library requires no special devices and high throughput can be performed, but the signals are often faint due to low hit rates.

Alternative approaches of cell lysates are applied to improve the sensitivity of agar plate-based screening. By growing the clones of a metagenomic library in 96-well plates, followed by preparing the lysates by either a chemical or a physical procedure, and mixing them with substrates, the sensitivity is improved. This method is frequently applied for screening gene resistance to toxic compounds such as antibiotics and heavy metals. This method can also be applied to screen essential genes using hosts that lack the target genes. A third approach is based on the reporter assay. By linking the target gene with the reporter gene, such as green fluorescent protein, β-galactosidase, and tetracycline resistance genes, screening can be conducted based on the expression of reporter genes [17.31].

17.2.4 Sequence-Based Screening of the Metagenomic Library

Type II PKS Biosynthetic Genes

ThePKSs are responsible for the production of bacterial polyketides, a diverse group of pharmacologically important natural products which include antibiotics, antitumor, immunosuppressive, and cholesterol lowering agents. Bacterial type II PKS biosynthetic gene clusters encode three enzymes, including two ketoacyl synthases, KS a (catalyzes the Claisen type condensation between the growing polyketide and incoming acyl-CoA subunits) and KSb (anchors the growing polyketide chain during chain elongation). The minimal PKS gene cassette is adequate to produce the polyketide in type II PKS systems. Additional enzymes such as ketoreductases (KR), cyclases, and aromatases are required to fold and cyclize the poly b-ketoacyl intermediate into the final structure. The structural diversity of the natural products originates from type II PKS, from divergence within the PKS domains, and their arrangement with assorted modification enzymes. With this knowledge of the functions of all three constituents of the minimal PKS (ketosynthase alpha subunit-ketosyntahase beta subunit-acyl carrier protein, KSa-KSb-ACP), a scheme was designed to determine overall polyketide chain length and structure. Degenerate PCR primers were designed based on conserved regions within KSa and ACP genes in type II PKS were used to amplify KSb sequences directly from metagenomic DNA. The discovery of novel small molecules from metagenomic DNA reveals the enormous genetic diversity encoded within the uncultured bacterial genomes for the discovery of natural products [17.32].

Type I PKS

Type I PKS generally consist of a set of multifunctional modules. Each module is responsible for one cycle of polyketide chain elongation. The module contains ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains that together catalyze a two-carbon extension of the chain. After such an extension, the resulting ketone can either be left as is or converted to a β-hydroxyl, a double bond, or an alkane group by stepwise processing of ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains. The substrate specificities of the ATs, the order and number of the modules, and the composition of catalytic domains within each module provide a *code* for the structure of the synthesized polyketide. Furthermore, the PKS genes for a particular polyketide are usually clustered and colinear with the order of its biosynthesis. Consequently, the structure of the polyketide product of an unknown PKS can be approximated from the sequence of its genes [17.33].

Nonribosomal Peptide Synthetases (NRPS)

NRPS gene clusters encode for a wide range of nonribosomal peptides, ranging from antibiotics, tox-

ins, siderophores, and anti-inflammatorials to immunosuppressants. These pharmacologically relevant bioactivities have motivated extensive searches for novel NRPS genes in microbial isolates and in environmental samples. NRPS are large, multimodular enzymes that are organized in modules containing specific domains that sequentially incorporate amino acid building blocks into a growing peptide chain. A typical NRPS module contains an adenylation (A) domain, a peptidyl carrier protein domain, and a condensation domain [17.32].

17.2.5 Next Generation Sequencing Technologies

Next generation sequencing (NGS) technologies are having a considerable impact on metagenomics. NGS technologies include three commercial platforms, namely the 454 pyrosequencer (Roche Diagnostic Corporation), GAII/HiSeq sequencer (Illumina), and SOLiD instrument (Life Technologies Corporation). The technologies behind each platform were reviewed in detail by *Metzker* [17.34].

Although a huge amount of phylogenetic data has been gathered for microbial identification using Sanger sequencing, new sequencing technologies are having a considerable impact on metagenomics. DNA pyrosequencing was developed to sequence the DNA with a fundamentally different approach in the mid 1990s [17.35]. Sequencing occurs by a DNA polymerase driven production of inorganic pyrophosphate with the formation of ATP and ATP-dependent conversion of luciferin to oxyluciferin (Fig. 17.3). The oxyluciferin generate the emission of light pulses and the amplitude of each signal is directly related to the presence of one or more nucleosides. The main drawback of pyrosequencing is its inability to sequence longer stretches of DNA. Hence, the hypervariable regions within bacterial 16S rRNA genes were amplified by the PCR and subjected to DNA pyrosequencing.

DNA pyrosequencing has been effectively applied in various fields such as genotyping, signle-nucleotide polymorphism, and microbial identification [17.36]. Pyrosequencing has been effectively applied to identify microbes based on the hypervariable regions within the 16S rRNA gene and signature sequence matching [17.37]. After DNA sequencing, sequences should be analyzed carefully to facilitate accurate bacterial identification.

Illumina performs solid surface PCR amplification by immobilizing random DNA fragments on a surface. The resulting cluster DNA fragments are sequenced with reversible terminators by synthesis process. The cluster density is enormous with hundreds of millions sequence reads for each surface channel. In total, 16 channels are run at the same time in the HiSeq2000 instrument. The sequence read length is about 150 bp and it can be sequenced from both ends. By overlapping both sequences, base pair reads can reach up to 300 bp. Approximately 60 Gbp can be expected in a single channel. As compared with 454, Illumina requires only a few nanogram of starting material and provides a superior amount of unassembled sequence reads. The limitation of Illumina is the run time; it takes approximately 10 days but 454 take only 1 day [17.38].

Apart from this, some other sequencing technologies are also available, but they need to prove themselves for metagenomic application. Among them, the Applied Biosystems SOLiD sequencer has been extensively used in genome re-sequencing. SOLiD possibly provides the lowest error rate compared to other NGS sequencing technologies, but the reliable read length is much beyond 50 nucleotides. This will limit its applicability for direct gene annotation of unassembled reads or assembly to large contigs. However, recent research work on the porphyrins from a metagenomic library of the marine sponge *Discodermia calyx* has showed N encouraging outcome [17.39]. Ion Torrent is another emerging technology and is based on the principle that nucleotide incorporation can be detected by proton release during DNA polymerization. This system promises read lengths of more than 100 bp. Most recently, the draft genome sequence of *Gillisia* sp., strain CBA3202 was announced based on the Ion Torrent Personal Genome Machine (PGM) sequencer [17.40].

17.2.6 Metagenomics and Informatics

The challenge for metagenomics is after getting hundreds or thousands of sequences, that is, how to identify the microbes in reasonable time. Existing bioinformatics methods are too slow and not adequate for large-scale projects. Currently, high-throughput metagenomics ribosomal DNA (rDNA) analysis tools are in development. Obviously, enough computational command, distributed computing networks, and robust server technology may ultimately solve the demands of current metagenomics data analysis. Starting with sequence collection and verification, various strategies have been followed to examine the quality of each read [17.41]. Before sequence assembly, the sequences are trimmed by using various algorithms to

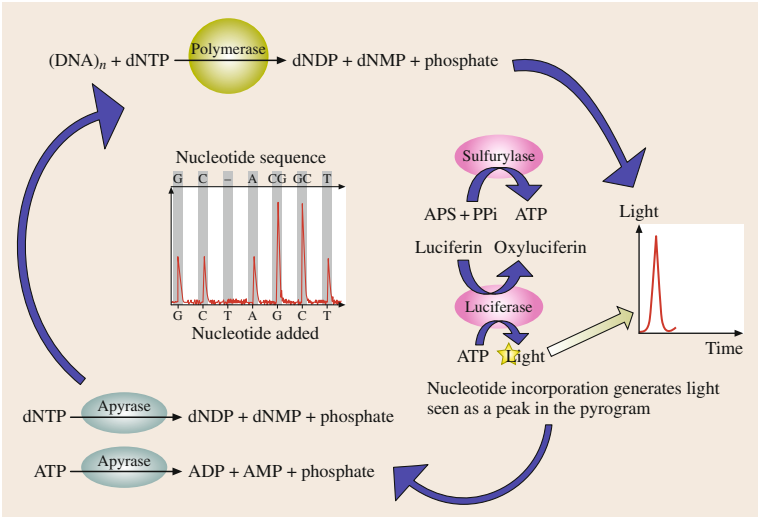

Fig. 17.3 The principle of DNA pyrosequencing [17.36]

remove primer and low quality sequence data. *Huse* et al. analyzed variable regions of 16S rRNA gene sequences generated by 454 sequencing for its error and explained a method for filtering low quality sequence data to produce robust data sets for 16S rDNA analyses [17.41]. Once the primer and low quality sequences have been trimmed, sequences can be aligned using sequence alignment programs such as NAST [17.42] or MUSCLE [17.43]. The next problem is the chimera sequences generated during the amplification process. They can be removed by chimera checking tools such as Greengenes [17.44], Ribosomal Database Project (RDP) [17.45], Bellerophon [17.46], or Pintail [17.47]. After obtaining the high quality sequences, they must be identified accurately in parallel. Sequences can be classified using classifiers such as the Bayesian classifier in the RDP system and can be compared with multiple sequence alignment programs such as RDP, Greengenes, or ARB-SILVA. Aligned sequences may also be classified against databases such asprok MSA (prokaryotic multiple sequence alignment) in Greengenes, and finally phyla may be identified and displayed as relative abundance histograms to compare different bacterial groups.

Novel bioinformatic tools such as CARMA (characterizing short read metagenomes) enable sequences encoding protein segments as small as 27 amino acid residues for microbial identification and comparative metagenomics [17.48]. A high throughput informatics approach, automated simultaneous analysis phylogenetics (ASAP) [17.49], recommend an automated approach for phylogenomics that may facilitate the analysis of large sequence data, especially whole genome-based microbiome explorations. Phylogenetic distance matrices may be constructed in programs such as DNAML (DNA maximum likelihood) [17.50]. Distance matrices may be transferred to DOTUR (Defining Operational Taxonomic Units and Estimating Species Richness) [17.51] for construction of collector's curves, rarefaction curves, calculations of Chao and ACE richness estimates, and computations of Simpson and Shannon indices of diversity.

17.2.7 Single-Cell Genomics

A recent addition to metagenomic research is single-cell genomics. It relies on genomic sequence information of a single culture-independent microbial cell. Comprehensive genomic information of single microbial cells was obtained by using the enzyme Phi29 polymerase. Single-cell genomics is especially well suited for symbiosis research in which the vast majority of symbionts has not been cultivated. Single-cell genomics is especially well suited for symbiosis research, including bacterial symbionts of marine sponges, insects (grasshoppers, termites), and vertebrates (mice, humans). In any case, new insights have been obtained into the repertoire of the bacterial partners; these findings have led to an improved understanding of the related host [17.52].

Single-cell genomics involves the separation of individual microbial cells from environmental samples to access their complete genomic DNA and to generate an adequate quantity of DNA by whole genome amplifi-

cation for sequencing (Fig. 17.2). Cell singularization mainly depends on the characteristics of the sample. If possible, for pure cultures obtained from symbionts, serial dilution is a promising method. Commonly used methods for cell singularization is fluorescence activated cell sorting (FACS). Target cells can be detected by FISH or labeled with a fluorescent dye.

Microfluidic chambers have also proven to be successful for obtaining single amplified genomes (SAG) [17.53]. This method uses reaction volumes of only 60 nl, which reduces the likelihood of contaminating the sample. Microfluidic devices have also been developed recently for FISH and tyramide signal amplification FISH (tsa-FISH) followed by cell sorting via flow cytometry directly on the device [17.54, 55]. This approach holds great promise for 16S rRNA gene-based identification of single cells, while bearing low risks of contamination. Micromanipulation techniques are useful to target morphologically distinct microorganisms. Micromanipulation using microcapillaries has been used successfully in combination with FISH to target the single cell [17.56]. The next step is cell lysis to provide the genomic material for consequent multiple displacement amplification (MDA).

MDA randomly amplifies primed template DNA using the Phi29 polymerase enzyme in an isothermal reaction. DNA synthesized by Phi29 polymerase becomes directly accessible for the next polymerase molecule, thus resulting in continuous DNA amplification. The next step is the whole genome sequencing of MDA products. Various sequencing methods have been employed for whole genome sequencing.

Most studies have relied on shotgun or paired end libraries using 454 pyrosequencing. However, mixture of 454 and Illumina have been also used to enhance the genome coverage. Combinations of 454 and Sanger sequencing have also been used. The first single-cell genome was obtained using Sanger, 454, and Illumina techniques [17.57]. With the evolution of new sequencing techniques, novel approaches such as the PacBio or IonTorrent techniques might facilitate obtaining better genomic data from single cells.

17.3 Application and Latest Progress in Sponge Metagenomics

17.3.1 Diversity of Bacterial Symbionts in Sponges

The use of culture-independent tools has greatly accelerated the understanding of the phylogeny of sponge-associated microbes. Underpinning this development is the application of next generation sequencing technologies. A recent study using 454 amplicon pyrosequencing revealed extraordinary species richness in Australia's Great Barrier Reef sponges (*Ianthella basta*, *Ircina ramose*, and *R. odorabile*) [17.58]. An enormous bacterial diversity was demonstrated in those sponges. This includes the dominant bacterial taxa *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* (*Alpha, Delta, Gamma*), which are the same phyla that featured in conventional 16S rRNA gene libraries [17.4]. Newly discovered phyla by 454 sequencing (e.g., *Deferribacters, Tenericutes*) were also present, but only in low abundance [17.58].

Next generation 16S rRNA gene tag sequencing suggests that the sponge microbiome may be classified into three main groups (species specific, variable, and core) [17.59, 60]. Species specific microbes comprise 72% of the taxa detected in sponges, whereas only 2% of the detected taxa correspond to the core. The five Mediterranean sponges *Aplysina aerophoba*, *Aplysina cavernicola*, *Ircinia variabilis*, *Petrosia ficiformis*, and *Pseudocorticium jarrei* share a core microbiome, which includes the phyla *Acidobacteria*, *Chloroflexi*, *Nitrospira*, *Poribacteria*, and *Proteobacteria*. The phylum *Chlamydiae* and *Lenthisphaerae* appears to be found only in association with *A. cavernicola* and *I. variabilis*, respectively [17.59]. The core communities are represented as globally distributed microbes that are horizontally acquired from the environment by a sponge, whereas the species specific community could consist of microbes that are vertically acquired from the progenitor [17.60]. Investigation of *Cymbastela concentrica* microbiome helped to further elucidate possible genetic mechanisms involved in the establishment of species specific microbiomes [17.61]. The authors sequenced about 190 623 shotgun sequences and 3545 16S rRNA gene sequences. *C. concentric* contains the vast majority of *Gammaproteobacteria*, *Phyllobacteriaceae*, *Sphingomondales*, *Neisseriales*, and *Nitrospiraceae*. Based on 16S rRNA gene identification (97% identity cutoff), only 34 different OTUs were common between *C. concentrica* and the surrounding seawater, which supported the idea of selection for a specific microbiome, possibly

consisting of several (although nonculturable) bacteria. A recent 16S rRNA gene sequence-based study in the southeastern Atlantic region generated 133 bacterial sequences from the sponges *Hymeniacidon heliophila* and *Polymastia janeirensis* [17.62]. These two sponge species appear to share several bacterial taxa affiliated with *Cyanobacteria* and *Proteobacteria*. An analysis of 254 archaeal 16S rRNA gene partial sequences from the sponges *Hymeniacidon heliophila*, *Polymastia janeirensis*, *Paraleucilla magna*, and *Petromica citrina* suggested that *Crenarchaeota* is well represented in sequence databases [17.63]. *Silva* et al. provided the first large-scale analysis of the taxonomic and metabolic diversity of the microbiome of the sponge *A. brasiliensis*. These results demonstrated that a complex microbiome exists within this sponge, which presents a particular metabolic profile [17.64]. It also indicates the *A. brasiliensis* microbiome is unique, differing from the microbiomes present in the water column surrounding these sponges and those associated with other marine organisms. Within the taxonomic signature of the *A. brasiliensis* microbiome, an enrichment of *Betaproteobacteria* (*Burkholderia*) and *Gammaproteobacteria* (*Pseudomonas* and *Alteromonas*) were detected, which indicates the specificity of the species.

17.3.2 Functional Genes Related to Natural Products of Bacterial Symbionts in Sponges

Natural products isolated from sponges are a key source of new biologically active compounds. However, the development of these compounds into drugs has been held back by the difficulties to achieve a sustainable supply of these, often complex, molecules for preclinical and clinical development. Increasing evidence implicates microbial symbionts as the source of many of these biologically active compounds, but the vast majority of the sponge microbial community remains uncultured [17.65]. Metagenomics offers a biotechnological solution to this problem. Metagenomes of sponge microbial communities have been shown to contain genes and gene clusters typical for the biosynthesis of biologically active natural products. Heterologous expression has also led to the isolation of secondary metabolism gene clusters from uncultured microbial symbionts. Combining a metagenomic approach with heterologous expression holds much promise for the sustainable exploitation of the chemical diversity present in the sponge microbial community.

Polyketides are considered as an important class of bacterial bioactive secondary metabolites. Efforts have been focused on isolating PKS gene clusters from sponge-associated bacterial metagenomic libraries [17.66]. *Piel* et al. [17.67] successfully employed this approach and identified the putative onnamide PKS gene cluster from a marine sponge *T. swinhoei* metagenomic library. Onnamide A (Fig. 17.4(1)), found in marine sponges of the genus *Theonella*, is very similar in structure to pederin (Fig. 17.4(2)) from Paederus beetles. A major difference is the addition of a terminal arginine, potentially added by NRPS domains, in the sponge compounds. The very clear homology between pederin and onnamide genes was used to identify the candidate onnamide gene cluster. The trans-AT feature of this gene cluster was used in the cloning strategy. Interestingly, PCR primers were designed for the PKS genes involved in pederin biosynthesis to identify the closely related onnamide gene cluster.

The dysidenin (Fig. 17.4(3)) group of nonribosomal peptide natural products was isolated from marine sponges of the genera *Dysidea* and *Lamellodysidea*, which harbor photosynthetic symbiotic cyanobacteria of the genus *Oscillatoria*. Synthesis of these nonribosomal peptides was not successfully determined [17.68]. The identification of the barbamide (Fig. 17.4(4)) biosynthetic gene cluster from *L. majuscula* facilitated identification of candidate dysidenin biosynthetic genes within marine sponges [17.69]. This family of compounds contains a very rare chemical motif, a multiply chlorinated leucine residue. The PCR primers were designed for this chemical motif, targeting the halogenase gene. This feature was used to isolate candidate halogenases from marine sponges containing the dysidenin family of compounds.

Fisch et al. [17.70] found that general KS primers amplify too many diverse PKS gene sequences, making it very difficult to identify the correct gene cluster. Hence, they applied a nested PCR strategy to amplify the specific gene cluster. First, the degenerate KS primers were used to amplify the KS region followed by the application of a trans-AT specific PCR primer pair. The primers were designed such that one primer was specific to 84% of all trans-AT KS, while the second primer was specific for certain chemical motifs in the resulting polyketide product. Using this strategy, probable gene clusters were identified for the pederin relatives mycalamide (Fig. 17.4(5)) and psymberin (Fig. 17.4(6)) from marine sponge metagenomes.

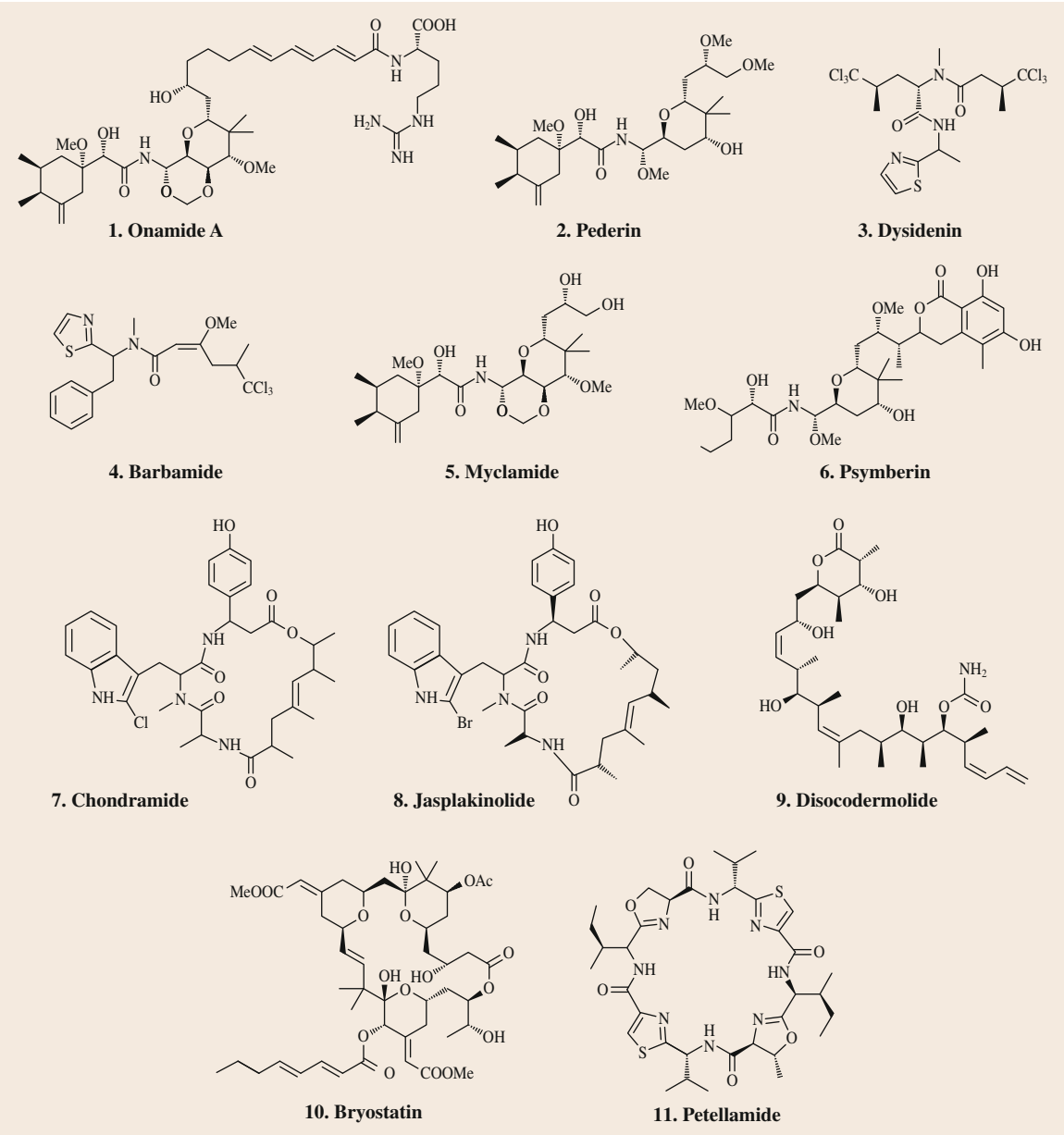

Fig. 17.4 Bioactive metabolites from marine sponges

Similarly, the cloning of the chondramide (Fig. 17.4(7)) biosynthesis cluster from *C. crocatus* [17.71] was used to isolate the biosynthesis genes for the closely related compound, Jasplakinolide (Fig. 17.4(8)) from the sponge *Jaspis* sp. *Kim* and *Fuerst* [17.72] investigated the secondary metabolic potential of the sponge *P. clavata* using PKS probes for isolating the biosynthetic gene cluster of discodermolide (Fig. 17.4(9)). Although this study did not identify the discodermolide gene cluster, it did demonstrate that many PKS genes were present in the sponge metagenome and that it was possible to clone large bacterial PKS gene clusters from the sponge metagenomic DNA.

Later, the investigation of PKS genes in cultivable and uncultivable bacteria proved that some of the PKS genes originating from the sponge metagenome appear to form a sponge specific bacteria and it is phylogenetically distinct from other PKSs. The large number of bacterial PKS genes that has been found using these metagenomic approaches provides compelling evidence in support of the symbiont hypothesis and also demonstrates the complexity of the *bacterio sponge*. Studies with other marine invertebrates have clearly demonstrated that microbial symbionts are the likely producers of the metabolites bryostatin (Fig. 17.4(10)) and patellamide (Fig. 17.4(11)) [17.73–75].

The discovery of bryostatin eventually led to the detection of a huge number of polyketide synthases of biotechnological interest. Metagenomics strategies have led to the identification of the Sup-PKS group, which is dominant in marine sponges and encodes synthases that are present exclusively in these animals. These synthases produce methyl branched fatty acids and they are assumed to be of bacterial origin [17.76].

Sponges exhibit a high level of fatty acid diversity, and it is generally assumed that the methyl branched fatty acids are of microbial origin [17.77]. This finding is supported by the observation that methylated fatty acids in bacterio sponges are more abundant than in bacteria free sponges. Similarly, methyl branched alkanes that are thought to originate from microbial methylated fatty acids are commonly present in fossil sediments and fuels [17.78]. A challenging hypothesis has been put forward that sponges as the most ancient metazoan animals might be extant reservoirs for ancient microbes and their corresponding methylated fatty acid encoding PKS genes.

The biological function of methyl branched fatty acids in sponges is unclear. Interestingly, structurally similar fatty acids are major constituents of the protective lipid cell layer of *Mycobacterium tuberculosis* [17.79]. Deletion mutants lacking intact mycolic acids are significantly attenuated in the mouse model and in macrophages [17.80]. Therefore, it is tempting to speculate the importance of methyl branched fatty acids. Marine sponges are also known to contain large amounts of halogenated organic compounds such as fatty acids and alkaloids and are thus a potential useful source of both the halogenases and the dehalogenases group of biotechnologically important enzymes, which can be used in the production of pharmaceuticals, herbicides, and pesticides [17.32]. A novel halo-tolerant lipase was isolated by following a functional screen of a marine sponge fosmid metagenomic library. The activity and stability profile of the recombinant enzyme over a wide range of salinities, pH, and temperatures, and in the presence of organic solvent and metal ions suggests that there would be a utility for this enzyme in a variety of industrial applications [17.81].

17.3.3 Functions of the Bacterial Community in Sponges

To date, the function of sponge-associated microorganisms lags behind the understanding of taxonomic affiliation. However, recent research on marine sponges has focused on the symbiont function, in particular for nitrogen metabolism. Initially, stable isotope experiments demonstrated denitrification in sponges *Geodia barretti* [17.82], *Dysidea avara*, and *Chondrosia reniformis* [17.83]. Further metagenomic analysis recovered gene encoding cytochrome cd1-type nitrite reductases (nirS) from sponge-associated *Betaproteobacteria* and *Gammaproteobacteria*, which are responsible for denitrification in sponges [17.82]. Gene encoding enzymes such as nitrite reductase and nitric oxide reductase were also identified in the metagenome of *Poribacteria* from the sponge *Aplysina aerophoba* [17.84]. Anammox activity was also demonstrated in the sponge *G. barretti* [17.83]. *Mohamed* et al. [17.85] provided the first molecular evidence for the presence of potential anammox bacteria in sponges. *Liu* et al. [17.86] detected the expression of the ammonia mono-oxygenase membrane bound subunits β and Υ (AmoB and C) and an ammonia transporter (AmtB) in the microbial community of *C. concentric*. The genes encoding AmoB and C were adjacent and orientated in opposite transcriptional directions on a contig of the *C. concentrica* metagenome. The contig also contains a gene for the α-subunit (amoA) and has overall striking synteny with a genomic region of *N. maritimus*.

Sponges are suggested to be capable of differentiating food bacteria from symbionts. *Wilkinson* et al. [17.12] suggest that the chemical composition of the bacterial outer layer may play a role in sponge symbiont recognition. *Wehrl* et al. [17.87] demonstrated that the retention rates of different food bacteria were similar, despite the difference in cell surface properties. However, the question of how sponges discriminate between food and symbionts remains unsolved. Metagenomic analysis of *C. concentrica* has led to the discovery of a number of important genomic factors, including tetratricopeptide repeat domain encoding proteins (TPR) and ankyrin repeat proteins (ARP). The presence of the TPR and ARP proteins in the *C. con-*

centrica metagenome indicate that it interacts with surrounding cells and proteins [17.88]. Ankyrins are especially likely to be of relevance, as they might be involved in the recognition of and protection from host phagocytosis. This theory is further strengthened by the fact that proteins with ankyrin domains are found in other obligate intracellular pathogenic and symbiotic systems, where they interfere with host cell function. Thus, these proteins could represent potential mechanisms that allow the host sponge to discriminate between food and symbiont bacteria [17.88]. Functional analysis performed via shotgun sequencing using the COG (Clusters of Orthologous Group) database suggested that the majority of the detected genes belong to bacteria. The authors also found a large number of sequences identified as transposable insertion elements. The sponge metagenomes contained a greater number of sequences identified as COG0610 (restriction enzymes, type I helicase) and COG1715 (restriction endonuclease) than the surrounding seawater. Both COG groups include specific DNA modification and restriction systems in bacteria, and therefore, the authors hypothesized that this would facilitate horizontal DNA exchange between sponge microorganisms and protect against DNA exchange with planktonic organisms in the surrounding seawater [17.61].

The symbiotic function of the microbial community in the sponge *A. aerophoba* was revealed by whole genome amplification (WGA). The functional data revealed a sponge specific PKS and an NRPS from the *Poribacteria* from the *Chloroflexi* clade, respectively [17.89]. The carbon monoxide dehydrogenase detected in the metagenome of *C. concentrica* [17.88] demonstrated that the *Poribacteria* are mixotrophs that can undertake autotrophic carbon fixation [17.84]. A large number of insertion elements was observed for the first time within the *C. concentrica* metagenome. Large numbers of mobile elements were previously seen within intracellular symbionts in other hosts [17.90] and were proposed to have important roles in the evolution of bacterial genomes for symbiotic relationship with their hosts.

Second, a high frequency of clustered regularly interspaced short palindromic repeats CRISPR and CRISPR-associated proteins (CAS) were observed in the metagenome data set. The elevated abundance of these CRISPRs and CAS were proposed to form a viral specific defense system [17.91]. The defense system may provide proteins that are effective against bacteriolytic viral infection for the sponge symbionts, as sponge bacteria are expected to be exposed to as many as 1000 viral particles on a single day. Therefore, the system would be essential for the survival of high cell density, nonmobile sponge microbial communities and to maintain a barrier against phage-mediated gene transfer from the surrounding planktonic community, where a high phage load could be expected. Third, several shared metabolic interactions between bacteria and host, including vitamin production, nutrient transport and utilization, and redox sensing and response were found. These new previously unrecognized genomic signatures and functions of sponge bacteria provide an insight into the potential metabolic interdependency between host and microorganisms, and into the specific mechanisms used by symbionts to remain in sponge tissue. Also, these novel markers could be used for monitoring the status of the symbiotic relationships between sponge and bacteria by assessing the abundance, diversity, and expression of these functional genes [17.88].

Liu et al. [17.86] unravel some of the interactions between microbial community and the sponge *Cymbastela concentrica* through a combined metagenomic and metaproteomic approach. They provided new data for the first time on specific transport functions for typical sponge metabolites (for example, halogenated aromatics and dipeptides). This study also indicated that there is a requirement for the microbial community to respond to variable environmental conditions and hence express an array of stress protection proteins. Finally, molecular interactions between symbionts and their host might also be mediated by a set of expressed eukaryotic-like proteins and cell-cell mediators, and some sponge-associated bacteria (for example, the *Phyllobacteriaceae* phylotype) may be undergoing evolutionary adaptation process to the sponge environment, as evidenced by active mobile genetic elements.

17.4 Future Perspectives

The application of metagenomics provides access to sponge microbiota as a rich resource for novel enzymes and metabolites, and opens new avenues for the sustainable production of biotechnologically rele-

vant compounds. However, as DNA sequencing and synthesis costs rapidly decrease, eDNA methods are becoming the mainstays of natural product discovery and research. Coupling metagenomics with expression holds much promise for the sustainable exploitation of the chemical diversity present in the sponge microbial community and also enables us to identify the cryptic pathways of natural product biosynthesis in individual microbes. The broadest application of next generation sequencing has enhanced our understanding of sponge microbial diversity and its symbiotic association. Further, the metagenomics has revealed the role of sponge microbes in nitrogen cycling by the analysis of functional genes involved in ammonia oxidization and denitrification. Thus, this method provides a bright future for the discovery of novel pharmaceuticals from marine sponges and for a better understanding of the microbial symbiotic roles in sponges.

However, the standard metagenomic approach of accessing the entire genetic material associated within sponges could not explore the active gene. To overcome this problem, metatranscriptomic-based approaches have been employed to study marine microbial populations, in which only transcriptionally active genes are accessed. Applying this approach to the study of sponges has resulted in the detection of genes known to be involved in key aspects of their metabolism, and in addition many unique transcripts, presumably also of biochemical importance have been detected. Another alternative strategy to accessing the metagenome for proteins within sponges is metaproteomics. This has proven to be useful in providing new physiological data such as the identification of proteins that are essential for sponge symbiosis. It is clear that novel screening tools would discover novel metabolites from sponges.

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18. Proteomics: Applications and Advances

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Proteomics provides a snapshot of gene expression in a particular cell, tissue, or organ at the time of sampling. Since the proteome is the link between the genotype and phenotype of an organism, characterization of the proteome provides information regarding the adaptability and physiology of an organism in relation to its environment. Proteomics is proving invaluable as a tool in marine biotechnology, where it has been employed to identify and characterize unique bioactive gene products, characterize the function and regulation of metabolic processes, elucidate marine biodiversity, and identify biomarkers of pollution and disease. Aquaculture of commercially important marine fish and shellfish has benefitted through a proteomics approach to address issues relating to disease, larviculture, and animal production in order to improve the profitability and sustainability of the industry. This chapter describes a wide variety of studies that illustrate the application of proteomics in marine biotechnology. The rapid improvements in proteomics technology expected over the next few years are guaranteed to result in dramatic advances in our understanding and use of the valuable resources provided by the marine environment.

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18.1 Omics

In 1998, the nematode *Caenorhabditis elegans* was the first multicellular organism to have its genome sequenced [18.1]. Since then, the *omics era* has essentially transformed molecular biology in terms of the vast volumes of data generated in our quest to better understand the cellular processes responsible for the evolutionary success and survival of living organisms. Omics is a collective term for genomics, transcriptomics and proteomics, which focus on the three basic levels of genetic expression in the cell, and is inclusive of additional technologies such as metabolomics, metagenomics, epigenomics, lipidomics, etc.

While genomics characterizes all the genes of a particular organism in an attempt to elucidate their function and regulation, transcriptomics and proteomics provide a more dynamic approach to studying gene expression and regulation at the cellular level. Whereas transcriptomics allows messenger ribonucleic acid (mRNA) levels to be assessed as a measure of gene expression, proteomics provides more detail since it detects the products of alternative mRNA splicing and post-translational modification of proteins [18.2, 3]. In addition to providing a *qualitative snap shot* of gene expression in a particular cell, tissue or organism, proteomics

can quantify protein expression at a particular time point. Thus, the proteome is the link between genotypic and phenotypic variation, and consequently, reflects the adaptability and physiology of an organism in relation to its environment [18.4].

While it is not the purpose of this chapter to provide an in-depth explanation and evaluation of the techniques used in proteomics, a general overview of the tools used in proteomic studies will be advantageous.

18.2 Overview of Proteomics Techniques

Polyacrylamide gel electrophoresis (PAGE) is the most common and simplest technique used in proteomic research [18.5]. A protein extract is suspended in a sample buffer incorporating the anionic detergent sodium dodecyl sulphate (SDS), which linearizes and binds to proteins, causing them to become negatively charged. Since there is an even distribution of charge per unit mass, the proteins are separated by approximate size during electrophoresis through a polyacrylamide gel. The technique can be improved by expanding protein fractionation by PAGE to two dimensions (2D-PAGE). The technique involves fractionation of a protein mixture according to the isoelectric point (pI) of each protein (first dimension) by electrophoresis through an immobilized pH gradient (IPG), followed by separation according to size by standard SDS-PAGE where the electric current is applied in a direction that is perpendicular to the first dimension [18.6].

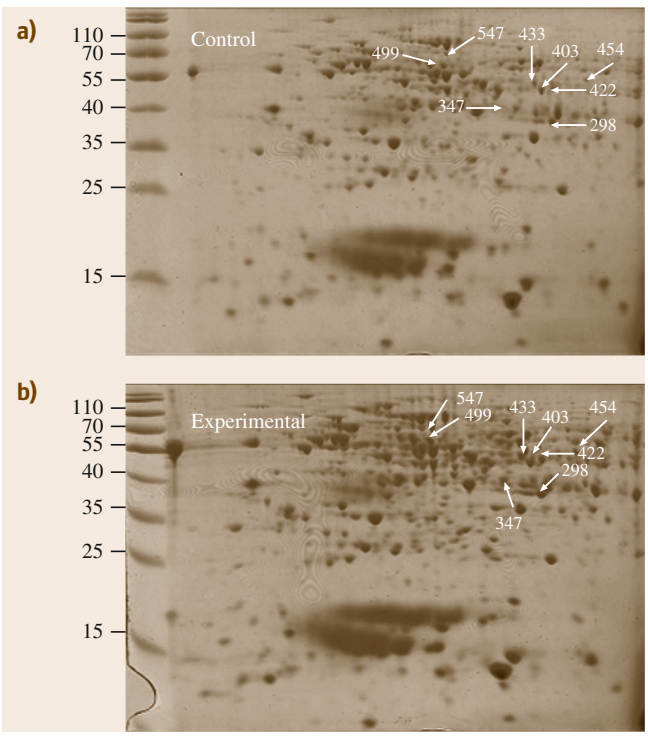

Fig. 18.1a,b Two-dimensional polyacrylamide gel electrophoresis was used to detect changes in the abundance of cellular proteins isolated from the red seaweed *Gracilaria gracilis* cultured in either nitrogen-replete (control) **(a)** or nitrogen-limited (experimental) **(b)** media for 6 h. Nitrogen-responsive proteins identified by tandem mass spectrometry (MS/MS) analysis are indicated by *arrows*. The levels of these proteins had increased in the nitrogen-limited samples relative to the control. A pre-stained molecular weight marker (Fermentas) on the *left side* of each gel image represents approximate molecular weights in kDa to enable estimation of the size of each protein spot (photograph by R.K. Naidoo)

Two-dimensional electrophoresis can routinely detect approximately 2000 proteins, which is an order of magnitude greater than what is possible with 1D-PAGE. Consequently, 2D-PAGE has been extensively employed in numerous proteomic studies in conjunction with mass spectroscopy (MS) [18.2, 6]. In addition to providing a qualitative analysis of the proteome in a particular sample, 2D-PAGE can also quantitate the relative expression of proteins sampled from organisms or cells exposed to different treatments through the use of various image analysis software packages (Fig. 18.1). Two-dimensional difference in-gel electrophoresis (2D-DIGE) is a refinement of 2D-PAGE. Since proteomics studies typically analyze multiple protein samples from various treatments, 2D-DIGE involves differentially labeling the proteins in various treatment groups with fluorescent cyanine dyes (Cy-Dyes) and then mixing equal quantities of the labeled proteins prior to fractionation by standard 2D-PAGE on the same gel, thus eliminating inter-gel variation [18.2, 3, 6]. Detection of proteins fractionated by 2D-PAGE is accomplished by staining with either colloidal Coomassie blue or silver nitrate, which detect 10 and 0.5 ng of protein per spot, respectively [18.7]. The sensitivity of protein spot detection following 2D-DIGE is a function of the method employed to

label the proteins with the fluorescent CyDyes, ranging from 1 to < 0.1 ng protein per spot with minimal or saturation labeling, respectively [18.8]. Once differentially expressed protein spots have been identified, either manual spot picking or a robotic spot picker is used to obtain samples of the spots for identification by MS.

MS is widely used for the identification and quantitation of proteins in biological samples [18.9, 10]. The majority of protocols involve trypsin digestion of the protein in each of the sampled spots to generate peptides with a mass range that is more conducive to MS, since they can be effectively ionized by electrospray [18.4, 9]. The resulting peptide mixture is fractionated by reverse phase liquid chromatography and analyzed by tandem mass spectrometry (MS/MS) [18.10]. Briefly, MS/MS involves ionization of the peptides followed by separation of the peptide ions according to their mass to charge ratio and further fragmentation for sequence analysis [18.2].

Data obtained by MS/MS includes information concerning the peptide masses, fragment ion masses, and intensities, which is transformed into a peak list that is searched against a protein database of in silico-digested proteins using open-source or proprietary software packages [18.2]. If insufficient sequence data is available regarding the organism under investigation, de novo peptide sequences can be used to interrogate protein sequence databases. Besides identification of the proteins present in a particular biological sample, MS can also be used to quantitate the proteins present in individual samples. Isobaric tags for relative and absolute quantification (iTRAQ) is a widely used chemical labeling technique that allows quantitative comparison of protein samples from up to eight treatments simultaneously [18.11, 12]. Since the eight isobaric mass tags result in different mass spectra following peptide fragmentation, iTRAQ allows simultaneous identification and quantitation of the peptides in the eight samples [18.2].

18.3 Proteomics and Marine Biotechnology

The marine environment covers 70% of the Earth's surface and is inhabited by a broad diversity of organisms [18.13, 14]. The latter reflects the fact that life originated in the sea approximately 3.8 million years ago and has adapted to the wide range of habitats that comprise the marine environment, many of which are considered extreme [18.14].

Given the extent of the marine environment and the biodiversity it contains, marine biotechnology promises to deliver a vast array of commercially important products that include novel bioactive compounds, enzymes, and biopolymers. In addition, technology has been developed that has allowed aquaculture of a number of fish and shellfish species to provide an increasingly important source of protein for human consumption.

Indeed, marine aquaculture production is increasing steadily, achieving a 20% growth in production from 2004 (16.7 mt) to 2009 (20.1 mt), while wild fisheries harvest decreased by almost 5% from 83.8 to 79.9 mt over the same period [18.15]. Despite the many potential benefits that may be derived from marine organisms, the health of the marine environment is being damaged enormously due to rapidly elevating levels of anthropogenic pollution, over-fishing, and the effects of climate change. In fact, a significant proportion of the world's population lives within 75 mi of an ocean coast and the number is expected to continue to increase together with a substantial impact on the marine environment [18.16].

In order for us to derive the benefits that the marine environment offers humanity through marine biotechnology and mitigate the damage done to the marine environment, it is necessary for us to utilize modern technologies to better understand the functioning of the organisms that inhabit the marine environment in terms of their ecology and biology. As discussed earlier, the omics era is generating new insights into the molecular biology of living organisms at a rate never before achieved. Proteomics provides a unique opportunity to characterize the phenotype of marine organisms, which dynamically responds to changing environmental conditions and associated stresses. Indeed, proteomics has a range of applications in marine biotechnology, such as identification of unique bioactive gene products, characterization of the function and regulation of metabolic processes, determination of biodiversity, and identification of biomarkers of pollution and disease. The following sections of this chapter will discuss many areas of marine biotechnology which have been investigated using a proteomic approach.

18.4 Aquaculture

In order to continue to meet the worldwide demand for seafood, it is estimated that fish production will have to increase sevenfold by 2020 and that this will need to be accomplished through aquaculture [18.13]. Although production levels of farmed shellfish and fish are steadily increasing, the industry faces many challenges that must be met in order to be sustainable. Some of the issues that the industry faces include improved larviculture, prevention and treatment of disease, feed quality and increased growth rates, nutritional quality of the product, etc. Perhaps the greatest threat to the future growth of aquaculture is the escalating rate of climate change that will have a significant impact on aquaculture in the same way that it will affect terrestrial farming [18.17, 18].

18.4.1 Disease

Aquaculture of fish and shellfish is subject to the same constant threat of infectious disease as farmed terrestrial animals and plants [18.19–23]. For example, even though Scotland generates 97% of farmed Atlantic salmon (*Salmosalar*) in the United Kingdom, it is estimated that a third of production is lost due to disease [18.24]. Similarly, although 2.7 million metric tons of the white leg shrimp *Penaeus vannamei* was produced in 2010, with a value of US$ 11.3 billion [18.25], the cumulative loss in global production since the emergence of white spot disease in the early 1990s is estimated at nearly US$ 15 billion [18.26]. Although it is difficult to find definitive figures, it is estimated that the global loss of aquacultured animals to disease amounts to approximately several billion US$ per annum [18.27]. Marine organisms are subject to infection by viruses, bacteria, and parasites, many of which are uncharacterized and thus designated as *emerging diseases* [18.28]. Consequently, the means to either prevent or mitigate disease in aquacultured marine organisms is currently quite limited. Antibiotics are no longer an acceptable treatment against bacterial pathogens due to the potential of resistance arising in target bacteria, as well as the fact that antibiotic residues in seafood destined for human consumption are not acceptable [18.27]. Consequently, the development of vaccines for treatment and disease prevention is receiving a great deal of attention [18.29, 30], while probiotics are being investigated as an alternative to antibiotics [18.21, 31].

Proteomics has been employed in a number of studies investigating disease in aquacultured marine animals. It has become increasingly evident that stress is intimately linked to the onset of disease in aquacultured organisms [18.17, 32–34]. Hypoxic conditions in shrimp ponds has been shown to lead to stunted growth rates and an increased susceptibility to infectious disease [18.35]. Consequently, the proteome of the hepatopancreas of adult Chinese shrimp *Fenneropenaeus chinensis* was characterized to obtain a better understanding of the hypoxia stress response in this commercially important crustacean.

Two-dimensional electrophoresis identified 67 protein spots that were differentially expressed in response to anoxic conditions [18.35]; 51 of these spots were identified as 33 proteins by LC-ESI-MS/MS (LC: liquid chromatography; ESI: electrospray ionization), including preamylase, arginine kinase, phosphopyruvate hydratase, citrate synthase, adenosine triphosphate (ATP) synthase alpha subunit, chymotrypsin B1, chitinase, ferritin, C-type lectin receptors, transketolase, formylglutathione hydrolase, formyltetrahydrofolate dehydrogenase, aldehyde dehydrogenase, glutathione peroxidase, cytosolic manganese superoxide dismutase, protein disulphide isomerase, β-actin, oncoprotein 23 nm, and crustacyanin-C1. Many of the proteins could be classified into functional groups such as energy production, immunity, chaperones, and antioxidants, thus providing insight into the type of metabolic processes and stress response systems evoked in response to hypoxia [18.35].

The effect of hypoxia on humoral immunity in the Eurasian perch *Perca fluviatilis*, currently undergoing domestication for aquaculture, was investigated by exposing captive fish from two generations (F1 and F5) to either single or multiple 4 h hypoxic episodes over a period of 46 days and examining the serum proteome using 2D-DIGE [18.36]. C3 complement proteins were found to be up-regulated following a single exposure to hypoxia, while exposure to multiple hypoxic events resulted in up-regulation of transferrin and differential expression (some up and others down-regulated) of a number of C3 complement proteins. The study concluded that the serum proteome responded to single and multiple hypoxic events in the same way and that repeated exposure resulted in acclimation to the stress, suggesting that *Perca fluviatilis* can adapt to hypoxic conditions that may arise during aquaculture [18.36].

In another study conducted to identify biomarkers of chronic stress, the liver proteome from gilthead sea bream (*Sparus aurata*, L.) that had been stressed by repetitive handling or stocked at high density was compared to that of control fish using comparative proteomics [18.37]. Approximately 299 differentially expressed proteins were detected by 2D-PAGE, with 235 spots differentially expressed in response to handling stress, 223 spots regulated in response to overcrowding, and 70 spots that responded to both treatments. LC-MS/MS analysis allowed identification of 12 differentially expressed proteins: fatty acid binding protein and haemoglobin were up-regulated, while calmodulin, triose-phosphate isomerase, β-tubulin, glutamine synthetase, pyruvate dehydrogenase, heat shock cognate protein 70, glyceraldehyde 3-phosphate dehydrogenase, α-enolase, voltage-dependent anion channel 1, and cofilin were all down-regulated [18.37]. The proteins could be assigned to key functional groups, reflecting their role in lipid transport, antioxidation, chaperoning, Ca^{2+} signaling, lipid oxidation, ammonia metabolism, cytoskeleton, and carbohydrate metabolism. *Alves* et al. [18.37] list fatty acid binding protein, calmodulin, pyruvate dehydrogenase, heat shock cognate protein 70, and voltage-dependent anion channel 1 as the most promising molecular markers identified in their study since their role in the stress response is better understood. However, the authors caution that additional proteomic studies need to be conducted using different tissues in order to ensure that biomarkers used to monitor chronic stress in farmed fish are robust. Furthermore, expression of these potential biomarkers during activities that affect metabolism, such as feeding and exercise, needs to be assessed to ensure their specificity with regard to chronic stress.

Although *Moraxella* sp. colonizing the internal organs of farmed fish has not been directly associated with disease, farmers have reported slow growth rates, poor feed conversion, and decreased weight gain in sea cage-farmed fish [18.38]. A proteomic investigation of kidney samples obtained from cage-farmed *Sparus aurata* that were positive or negative for *Moraxella* sp. showed that several mitochondrial enzymes were significantly up-regulated in the *Moraxella* sp. infected kidneys, reflecting what has been reported in lipopolysaccharide (LPS) challenge experiments [18.38]. A number of nonmitochondrial proteins were found to be up-regulated, including peroxiredoxins, *S*-adenosyl-homocysteine hydrolase, antiquitin, warm acclimation-related protein 65, transferrin, glutathione *S*-transferase, carbonic anhydrase, and Cu/Zn superoxide dismutase, many of which function in oxidative stress, infection, inflammation, or apoptosis. The study suggested that metabolic alterations do occur in *Moraxella* sp. infected fish and identified various proteins that could potentially be used as biomarkers of *Moraxella* sp. infection in farmed fish.

The protozoan parasite *Marteilia sydneyi* is responsible for QX disease in Sydney rock oysters (*Saccostrea glomerata*), which can result in up to 95% mortality [18.39]. A proteomic approach to identify markers of QX disease resistance among Sydney rock oysters was undertaken by developing proteome maps of total haemolymph proteins from the fifth generation of a QX resistant oyster line derived from successive generations of selective breeding in which parental broodstock had survived disease outbreaks (QXR5), and comparing these to proteome maps from uninfected and infected wild-type oysters. Six proteins, found to be uniquely associated with resistance, were characterized by ESIMS/MS, which showed that two proteins were homologous to cavortin from the Pacific oyster *Crassostrea agigas* and domin in from the Eastern oyster *Crassostrea avirginica* [18.39]. Although the remaining four proteins could not be identified due to the absence of homologous sequences in the National Center for Biotechnology Information (NCBI) database, the two superoxide dismutase-like molecules are being investigated further as potential markers for selective breeding of QX disease-resistant oysters.

As previously mentioned, probiotic treatment of aquacultured animals is being developed as an alternative to antibiotics (Fig. 18.2). In a study aimed at investigating the effects of probiotic treatment on lar-

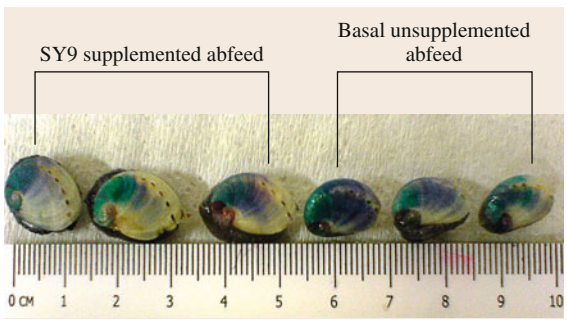

Fig. 18.2 Juvenile South African abalone *Haliotis midae* ($\approx$ 7.5 mm shell length) fed a diet supplemented with the probiotic bacterium *Vibrio midae* SY9 for 60 days had a 55% increased growth rate (shell length) in comparison to abalone that had been fed an unsupplemented diet (Coyne, unpublished)

val Atlantic cod (*Gadus morhua*), 2D-PAGE and MS were used to identify changes in the expression of larval proteins [18.40]. Interestingly, the study found that the five identified cod larval proteins that were downregulated in the probiotic-treated larvae function in the immune system of the fish, while two of the four upregulated proteins identified in the probiotic-treated larvae were α-actins from skeletal muscle. *Sveinsdóttir* et al. [18.40] concluded that probiotic treatment may have decreased the effect of environmental stress on the larvae, resulting in increased growth and development as indicated by decreased expression of immune system proteins and increased α-actin expression, respectively.

Proteomics has also proved to be invaluable for investigating the virulence response of bacterial pathogens of marine organisms. Although members of the genus *Vibrio* are ubiquitous in the ocean, a number of species are pathogenic to sea urchins, oysters, abalone, and fish, and thus are a major threat to aquaculture operations farming these animals. *Vibrio splendidus* is known to infect and cause disease in fish and shellfish, and is responsible for major mortalities of farmed animals [18.41]. The virulence mechanism of this bacterium towards marine invertebrates is poorly characterized due to the absence of molluscan cell lines, making it necessary to employ an ex vivo approach to investigate *Vibrio splendidus* virulence. Consequently, *Binesse* et al. [18.42] conducted a study of the extracellular products (ECPs) of the oyster pathogen *Vibrio splendidus* LGP32 which induce a strong cytopathic effect in infected oysters. Since the ECPs of a *Δvsm* strain, a metalloprotease deletion mutant of LGP32, were significantly less toxic, a proteomic analysis of the ECPs of both LGP32 and the *Δvsm* mutant was performed to identify additional cytotoxic proteins [18.42]. One of the differentially expressed proteins (VSA1062), found to have 41% identity to the *Bacillus thuringiensis* virulence factor immune inhibitor A precursor (InHA), was identified as a metalloprotease. The observation that the cytotoxicity of the ECPs of a *Δvsm*-1062 double mutant was similar to that of the *Δvsm* mutant even though metalloprotease activity could not be detected in the *Δvsm*-1062 mutant, led *Binesse* et al. [18.42] to discard the hypothesis that the VSA1062 metalloprotease compensated for the loss of VSM activity in the *Δvsm* mutant. Instead, they proposed that VSA1062 plays a role in bacterial resistance to the oyster immune response in a similar manner to InHA which functions in antibacterial peptide resistance.

In another study, the cellular and extracellular protein profiles of two *Vibrio anguillarum* strains, *Vibrio anguillarum* MN and 3010, were compared by 2D-PAGE and MALDI-TOF/TOF analysis since *Vibrio anguillarum* MN is 50-fold more virulent than 3010 towards the Japanese flounder *Paralichthys olivaceus* [18.43]. Two of the nine spots found to be differentially expressed between the two strains were identified as the outer membrane protein OmpU and the metalloprotease PrtV, both of which were highly upregulated in *Vibrio anguillarum* MN. Since OmpU increases the bacterium's resistance to bile [18.44], allowing it to colonize the fish digestive system, while PrtV from closely related *Vibrio cholera* is cytotoxic and can degrade extracellular matrix components such as fibronectin, fibrinogen, and plasminogen [18.45], *Zhao* et al. [18.43] concluded that these proteins most likely participate in the virulence response of *V. anguillarum*.

18.4.2 Reproduction

Aquaculture of commercially important marine animals relies on breeding healthy, fast-growing, high-quality animals. Consequently, a great deal of research and development has been invested in larviculture and hatchery design. As the aquaculture industry expands, there is a growing reliance on hatchery-produced seed for restocking farms. Many hatcheries maintain broodstock animals that are artificially induced to spawn in relation to a schedule based on the routine of the farm. Up to now, broodstock animals have either been obtained from the wild and cared for under optimal conditions or from what are perceived as being fast-growing animals produced on the farm and retained for breeding. However, it is becoming increasingly evident that a more directed approach to selecting broodstock for seed production is required in order to ensure production of robust, fast-growing spat.

Some studies have been published that employed a proteomics approach to address various challenges associated with breeding marine animals for commercial production. For example, it is necessary to determine that the gonads of broodstock animals have reached the appropriate developmental stage in order to induce spawning. The most common approach to this is visual inspection, which is acceptable for gastropods such as abalone, but inappropriate for bivalves such as oysters. *Li* et al. [18.46] employed surface enhanced laser desorption/ionization (SELDI) technology (ProteinChip Array System, Ciphergen Biosystems, Fremont, CA, USA) to obtain haemolymph protein profiles

during reproductive development of the Eastern oyster *Crassostrea virginica*. SELDI-TOF-MS combines array technology, which allows high resolution separation of proteins from complex mixtures on a chip, and mass spectrometry [18.47]. SELDI-TOF-MS is more sensitive than traditional MS and allows rapid sample throughput [18.46, 47]. The study identified 62 peptides associated with oyster reproduction and, consequently, was able to categorize individual broodstock animals into one of five groups: undifferentiated, female developing, female ripe, male developing, and male ripe with an accuracy of 98.8% [18.46].

Besides ensuring that broodstock animals are ripe for spawning, the quality of the eggs produced is also important in terms of ensuring that viable progeny are consistently produced in the hatchery. *Corporeau* et al. [18.48] employed a proteomic approach to identify abundant proteins linked to oocyte quality in the Pacific oyster *Crassostrea gigas*, rather than measuring oocyte diameter or total lipid content. Ten proteins were found to be up-regulated in low quality oocytes, which included vitellogenin-breakdown products and metabolic enzymes, while six up-regulated proteins from high quality oocytes included the chaperone heat-shock cognate protein 70 (HSC70) and cell-cycle control proteins [18.48]. The authors of the study postulated that increased levels of HSC70 may ensure improved protein folding in two-cell embryos and enhanced protection of oocytes and early embryos directly exposed to environmental stresses such as oxidative stress and temperature fluctuations. Likewise, up-regulation of the putative cell-cycle control protein 14-3-3 ε may be important in oocyte cell-cycle control and enhanced protection of embryos, while proliferative cell nuclear antigen (PCNA) has been associated with processivity of deoxyribonucleic acid (DNA) synthesis during early development [18.48].

18.4.3 Animal Production

To ensure that an aquaculture enterprise remains profitable, it is important that the growth rate of the animals is optimized so that the time they spend on the farm is minimized in order to keep production costs relatively low and reduce the risk of loss to stress and disease. Maslinic acid (MA, 2α,3β-dihydroxyolean-12-en-28-oic acid) has been found to improve the growth rate of rainbow trout by increasing hepatic protein-turnover rates, elevating the hyperplasia level and augmenting liver glycogen content [18.49]. Indeed, *Fernández-Navarro* et al. [18.50] found that trout fed a diet supplemented with 250 mg MA per kg feed for 225 days exhibited a 29% increase in weight in comparison to control animals.

Since *Sparus aurata* is an economically valuable marine species for fish farming, the effect of MA supplementation on the liver proteome of this fish was tested under farm conditions [18.49]. Two-dimensional electrophoresis of total protein samples from the experimental and control groups showed that 49 protein spots were differentially regulated in fish fed the MA-supplemented diet for 210 days. MALDI-TOF/TOF analysis allowed unambiguous identification of 19 different proteins that play a role in metabolism of glucose (phosphoglucomutase 1, phosphoglucose isomerase, and *S*-adenosyl methionine-dependent methyltransferase class 1), lipids (17β-hydroxysteroid dehydrogenase type 4 (HSDH4)), amino acids (fumarylacetoacetate hydrolase, 4-hydroxy-phenylpyruvic dioxygenase, methylmalonate-semialdehyde dehydrogenase), and purines (urate oxidase), as well as detoxification and metabolism of xenobiotics (aldehyde dehydrogenase), oxidative stress (catalase and 6-phosphogluconate dehydrogenase), immune system (lysozyme chain A), protein synthesis (elongation factor 2 of eukaryotic protein synthesis), protein folding (Hsp60, 58 kDa glucose-regulated protein), signalling (kinase suppressor of Ras1 (KSR1)), and cytoskeleton formation (cytokeratin E7, intermediate filament proteins, KERII).

Fernández-Navarro et al. [18.50] proposed a signaling mechanism to explain how MA affected the liver of gilthead sea bream at the molecular and cellular level. The interaction between MA and its receptors, modulated by increased HDSH4, inhibits KSR1 expression and consequently activates the signal-transduction pathway mediated by Ras. This affects cellular functions such as glucose and other metabolism, detoxification and xenobiotic metabolism, oxidative stress, amino acid catabolism, nonspecific immune system, purine catabolism, protein synthesis and folding, and cytoskeleton proteins, leading to stimulation of growth, protein-turnover rates, total content of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins, nicotinamide adenine dinucleotide phosphate (NADPH)-production systems, and antioxidant defence.

A similar approach was used to assess the effect of the glutamine precursor α-ketoglutarate (AKG), used as a dietary supplement, on the pituitary proteome of gilt-head sea bream [18.51]. The rationale for the study was based on the fact that pituitary hor-

mones regulate metabolism and somatic growth, which in turn, require a sufficient nutrient supply. Thus the aim of the study was to determine whether a feedback loop exists between nutritional status and pituitary hormone synthesis and secretion, since there is very little information available regarding the effect of dietary supplementation with AKG on the fish endocrine system. *Ibarz* et al. [18.51] found that AKG supplementation resulted in up-regulation of proteins such as fructose-bis-phosphate aldolase, glyceraldehyde-phosphate dehydrogenase and malate dehydrogenase that function in glucose metabolism. Proteins responsible for protein folding, such as two heat shock protein isoforms, cyclophylin and chaperonin, were also up-regulated, as well as cofilin and Vat-protein, which are involved in regeneration of neural function. On the other hand, an unusual apolipoprotein-A-1 and the hormone somatolactin were down-regulated in fish fed the AKG-supplemented diet. These observations led the authors to suggest that an endocrine/metabolic regulatory loop is, indeed, activated by AKG supplementation [18.51].

Since the muscle tissue of the fish is the portion that is consumed, it is important to safeguard the quality of farmed fish muscle tissue. Muscle quality is based on its composition, which is a function of the number and integrity of muscular fiber [18.52]. Since muscle tissue is central to many aspects of fish aquaculture, such as physiology, growth, food safety, seafood authentication and quality, traceability, and shelf-life, *Addis* et al. [18.52] conducted a study of the muscle proteome of *Sparus aurata* in which fish from four offshore floating cages and two re-population lagoons, located at remote sites from each other, were sampled on a two-monthly basis over a period of 22 months in order to investigate muscle proteome variability in relation to fish size and environmental conditions. Although the expression pattern of the muscle proteome was found to be relatively stable, the study revealed significant variation in the abundance of parvalbumins, troponins, and warm temperature acclimation-related protein 65 isoforms (Wap65) in relation to fish length and water temperature [18.52]. The authors also noted that structural proteins were more abundant in longer wild fish, while shorter farmed fish had an abundance of proteins involved in glycolysis. However, the muscle proteome was found to be comparable in wild and farmed fish of the same size, indicating that cage farming does generate high-quality fish for commercial production [18.52].

Similarly, since sea urchin gonads are a high-value seafood product, farmed sea urchins must be provided a quality diet to ensure gonad quality and quantity. Consequently, *Sewell* et al. [18.53] employed a proteomic approach to define the proteome of mature ovaries of the New Zealand sea urchin *Evechinus chloroticus*. The study successfully identified 138 proteins, of which only 12 were linked to ovarian reproductive function, and established a foundation for quantification of changes in expression of the ovary proteome which could be correlated with gonad quality and diet formulation [18.53].

18.5 Environment

As mentioned previously, the world's oceans occupy two-thirds of the earth's surface and harbor a vast diversity of microbes, plants, and animals. The marine environment plays an important role in regulating the earth's climate and in turn, the ocean's currents and temperatures are significantly influenced by climate [18.18]. Climate change is not only a serious threat to terrestrial environments, but is also impacting the marine environment and its inhabitants. Carbon dioxide emissions are directly responsible for ocean acidification and increased water temperatures, which in turn, leads to oxygen limitation [18.17, 54]. In addition, the large proportion of the human population living in close proximity to the coast has had a detrimental impact on the marine environment due to over-exploitation of biological resources and significant microbial and chemical contamination of coastal and near-shore regions [18.18, 55].

18.5.1 Climate Change

Ocean acidification (OA) affects marine organisms by disrupting acid–base regulation, respiration, energy turnover, and metabolism, as well as reducing growth rates, reproductive success, calcification, and sensory mechanisms [18.17]. Since climate change is responsible for increased temperatures and hypercapnia (elevated CO_2 levels), the negative effects on marine biota are likely to be interactive and cumulative [18.56].

Most marine invertebrates have a biphasic life cycle, where the fitness of the pelagic larval stage is responsible for the success of the benthic adult stage in that they must locate a suitable substrate for attachment and subsequent metamorphosis to the adult form, while simultaneously dealing with the cumulative effects of environmental stress imposed by climate change. Consequently, larvae that undergo settlement and metamorphosis may exhibit a more detectable proteome response to environmental stressors compared to the adult form due to their rapid development, greater susceptibility, and the complex developmental reprogramming that occurs in the proteome during the larval–juvenile transition [18.57]. Since barnacles are model organisms used in studies of larval development in intertidal benthic invertebrates, *Wong* et al. [18.54] investigated the effect of OA on protein expression in metamorphosing larvae of the economically and ecologically important barnacle *Balanus amphitrite*, which were cultured from nauplius to the cyprid stage under current and projected CO_2 concentrations for the year 2100. Two-dimensional polyacrylamide gel electrophoresis revealed proteins that were differentially up or down-regulated in response to OA which could be placed in three major functional groups: energy-metabolism, respiration, and molecular chaperones, possibly reflecting the strategy employed by the barnacle larvae to tolerate OA stress.

In a study investigating the effect of OA on *C. virginica*, the proteomes of mantle tissue samples obtained from animals exposed to normal ($\approx$39 Pa pCO_2) and hypercapnic ($\approx$357 Pa pCO_2) conditions were analyzed for changes induced by elevated CO_2 [18.58]. Proteomic analysis revealed 54 of the 456 proteins (12%) detected by 2D-PAGE were differentially expressed in response to hypercapnic conditions; 17 of the proteins responding to elevated CO_2 were identified and found to either be associated with the cytoskeleton or the oxidative stress response. *Tomanek* et al. [18.58] suggest that the mantel cytoskeleton is a major target of oxidative stress caused by elevated CO_2 levels which increase reactive oxygen species (ROS) either indirectly by lowering the pH of the organism and/or directly by interactions between CO_2 and other ROS to form more free radicals.

One consequence of climate change will be altered distribution patterns of fish and shellfish as a function of increased water temperatures [18.18, 55]. The Mediterranean blue mussel *Mytilus galloprovincialis* is an invasive species along the southern Californian coast, which has displaced the more heat-sensitive native species *Mytilus trossulus* [18.59]. *Tomanek* and *Zuzow* [18.59] investigated changes in the proteome of gill tissue sampled from the two congeners in response to acute heat stress (exposure to 24, 28, and 32 °C for 1 h, followed by a 24 h recovery at 13 °C) in order to detect responses common to both species as well as differences that account for the greater heat tolerance of the invasive mussel. The study found that the expression of proteins involved in molecular chaperoning, proteolysis, energy metabolism, oxidative damage, cytoskeleton, and deacetylation reflected a common heat stress response in both mussels, but also showed a lower sensitivity to high-temperature damage in the warm-adapted Mediterranean mussel, which may explain its increased range in warmer waters. For example, increased expression of a number of molecular chaperones occurred at a lower temperature in the cold-adapted *Mytilus trossulus*, which also seemed to be incapable of responding to heat-induced oxidative stress beyond 28 °C when nicotinamide adenine dinucleotide phosphate (NADPH)-producing pathways were found to be down-regulated in order to decrease the production of ROS via the electron transport chain [18.59].

The *Tomanek* and *Zuzow* study [18.59] was the first comparative investigation which identified systems-level differences in the proteome's response to acute thermal stress in a pair of congeners whose biogeographic ranges are in flux. In a follow-up study that investigated temperature acclimation (7, 13, and 19 °C for 4 weeks) in the two mussel species, warm acclimation was found to affect proteins that comprise the cytoskeleton and function in energy metabolism, which is consistent with increased filtration and respiration rates associated with increased culinary activity [18.60]. Increased levels of molecular chaperones were detected in *Mytilus trossulus* at 19 °C that were not evident in *Mytilus galloprovincialis*, reflecting differences in the long-term upper thermal limits of the congeners and suggesting that 19 °C is close to the chronic thermal limit of *Mytilus trossulus*. Although their data indicate that *Mytilus trossulus* may have a competitive advantage at colder temperatures, *Fields* et al. [18.60] suggest that their study shows why *Mytilus galloprovincialis* displaced *Mytilus trossulus* in southern California and indicates that climate change could increase this competitive advantage.

18.5.2 Pollution

Coastal and near-shore regions receive a steady influx of anthropogenic pollutants, which accumulate in

sediments and are a constant threat to the health of marine organisms [18.61]. Pollutants accumulating in coastal and near-shore zones affect marine animals by altering metabolic and developmental processes and, consequently, disrupting reproduction, modifying behavior, and decreasing disease resistance [18.55, 62]. Biomonitoring of marine pollution has been extensively used as a means of determining the extent of pollution. The rationale is that xenobiotic pollutants are biomagnified and accumulate in marine organisms, especially filter feeding invertebrates, in direct correlation to the concentration in which they occur in the environment [18.63]. Thus, pollutants can be quantified in tissue samples from key marine animals, referred to as sentinel organisms, living in the monitored environment [18.64, 65]. Bivalves are favored as sentinel organisms, since they are sessile filter feeders capable of accumulating high levels of xenobiotics, and consequently, they provide temporal and spatial information regarding the extent of contamination [18.64]. Since many biological and abiotic factors have been found to affect xenobiotic bioaccumulation, biomonitoring programs now include the use of biomarkers, such as metallothioneins, cytochrome P450, glutathione transferase, and heat-shock proteins, which specifically respond to xenobiotics encountered by sentinel organisms [18.63–66]. Even so, effective use of biomolecules as a means of monitoring marine pollution has its limitations, since their activity, which fluctuates naturally in response to biotic and abiotic factors, is governed by the route of chemical exposure (dietary or waterborne), the extent of bioaccumulation of the xenobiotic and detoxification mechanisms of the sentinel organism [18.64–66]. Consequently, *environmental proteomics* or *ecotoxicoproteomics* is increasingly becoming the biomonitoring tool of choice [18.63, 66].

The advantage of using proteomics for biomonitoring is that it provides information regarding the response of the entire proteome to the pollutant, thus identifying groups of proteins that respond strongly and specifically to particular pollutants [18.64]. In addition to establishing a protein expression signature (PES) specific to a particular xenobiotic, proteomics provides additional information that may elucidate the molecular processes and pathways that detoxify and catabolize particular pollutants [18.63, 65–67].

Ecotoxicoproteomics is already proving invaluable as a biomonitoring tool. *Liu* et al. [18.63] used a proteomic approach to investigate the effect of cadmium (Cd) on bioaccumulation of other heavy metals in four bivalves: the scallop *Chlamys nobilis*, the clam *Ruditapes philippinarum*, the mussel *Perna viridis*, and the oyster *Saccostrea cucullata*. Cd, which pollutes the marine environment through activities such as mining, mineral ore refining, electro-plating and fertilizers, is highly toxic to marine organisms as it results in oxidative damage by ROS and disrupts physiological processes involved with essential metal ions such as Ca^{2+} [18.66]. Although they found that exposure to cadmium influenced the bioaccumulation of other trace metals, Cd had little effect on the bivalve proteomes, and the identified proteins were insufficient to explain the observed disruption of trace element metabolism. Nevertheless, the PES could distinguish between clams and mussels that had accumulated different concentrations of Cd, and even though a PES for oysters and scallops was not obtained, the strong up-regulation of galectin in Cd-exposed oysters suggests that this protein has potential as a novel biomarker in environmental monitoring [18.63].

In another study, *Leung* et al. [18.66] investigated the proteomic response in hepatopancreas and adductor muscle tissue sampled from the green lipped mussel *Perna viridis* after a two-week exposure to Cd and hydrogen peroxide (H_2O_2), a persistent ROS linked to anthropogenic pollution such as dissolved organic matter and sewage disposal. Cd and H_2O_2 resulted in two unique sets of tissue-specific PESs and revealed that the former xenobiotic was responsible for endoplasmic reticulum and oxidative stress in the hepatopancreas and disrupted Ca^{2+} homeostasis in adductor muscle [18.66].

A study investigating changes in the proteome of the hepatopancreas of the blue mussel *Mytilus edulis* sampled from four sites in the vicinity of Gothenburg harbor, Sweden, generated a PES composed of 13 proteins that allowed the pollution-free site (Fjällbacka) to be distinguished from the 3 polluted sites [18.65]. Principal component analysis (PCA) and hierarchical clustering validated the discriminatory power of the PES and provided a spatial gradient from the polluted source.

Not only has proteomics proved invaluable for the discovery ofbiomarkers for monitoring environmental pollution, the technology has also been successfully used to investigate the effects of environmental pollutants on marine organisms, both in terms of their health and the health of seafood consumers. Mercury is a heavy metal pollutant that poses a danger worldwide as a consequence of its neurotoxic effect on fish and human brain tissue [18.68]. A report recently released by the Biodiversity Research Institute's Center

for Mercury Research stated that commercially important seafood such as lobster, tuna, and swordfish have muscle mercury concentrations (0.22–0.95 ppm, wet weight (ww)) that exceed the consumption guideline of one meal per month [18.69]. Since information regarding the molecular mechanisms of mercury neurotoxicity in fish is scarce, *Berg* et al. [18.68] conducted a proteomic study of Atlantic cod (*Gadus morhua*) brain tissue from fish exposed to methylmercury (MeHg) for 2 weeks after being injected intraperitoneally with MeHg at a concentration of 0.5 or 2 mg/kg. Although many of the proteins that were significantly expressed in response to MeHg had already been shown to respond to MeHg in mammals, confirming the validity of the data, the study also identified a number of proteins that have critically important, nervous system-specific functions that had never been associated with MeHg. These proteins included pyridoxal kinase (essential for the synthesis of several neurotransmitters), G protein (coupled to neurotransmitter receptors), nicotinamide phosphoribosyl-transferase (protects against axonal degeneration), dihydropyrimidinase-like 5 (axon guidance and regeneration), septin (dendrite development), phosphatidylethanolamine binding protein (precursor for hippocampal cholinergic neurostimulating peptide) and protein phosphatase 1 (control of brain recovery by synaptic plasticity) [18.68]. Not only has the data obtained from this study proved invaluable in confirming the involvement of a number of molecular processes associated with MeHg exposure and toxicity, but it also identified proteins that had not been investigated in the context of MeHg accumulation, which may prove invaluable as biomarkers of MeHg contaminated fish.

Endocrine disrupting compounds (EDC) are a diverse group of anthropogenic chemicals that are occurring in ever increasing concentrations in the marine environment. EDCs, thought to be responsible for the significant decline in many populations of aquatic species in a number of geographical locations worldwide [18.70], can be defined as [18.71]:

> *an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub)populations.*

EDCs interfere with hormonal function by binding to cytoplasmic oestrogen receptors and consequently, have been shown to affect sexual development, growth, osmoregulation, stress, and immune responses in fish [18.70, 72, 73]. Although the concentrations of nonylphenol mixtures (NPs) and bisphenol A (BPA) detected in polluted environments are much lower (<10 µg/L) than the median effective concentrations (EC_{50}) of these compounds for marine invertebrates such as adult *Mytilus edulis* and the adult cnidarian *Hydra vulgaris* (30 mg NPs/L [18.74] and 6.9 mg BPA/L [18.75], respectively), environmental concentrations of EDCs have been reported to affect the more susceptible larval stages of marine organisms [18.76]. Indeed, many invertebrate larvae settle on benthic diatoms that have been shown to biomagnify EDCs to levels greater than those in the environment, making them more likely to be harmful to the larval stage of marine organisms [18.77].

Since not much information is available regarding the effect of EDCs on marine invertebrate larvae, and since many of the life cycles of these animals include larval settlement and ingestion of benthic diatoms, *Liu* et al. [18.76] used a proteomic approach to investigate the effect of NPs and BPA on larval *Haliotis diversicolor supertexta* (a commercially important abalone from Taiwan). The study showed that toxicity of the EDCs towards the abalone larvae increased due to EDC accumulation by the diatom *Navicula incerta* that was used as a settlement substrate in the experimental setup. Larval proteins affected by EDC exposure function in ATP synthesis (down-regulated), the stress and defence response (up-regulated), metabolism, signal transduction (up-regulated), cytoskeleton (mostly down-regulated), and cilia formation (down-regulated) [18.76]. Consequently, Liu et al. [18.76] suggested that inhibition of ATP synthesis, actin, β-tubulin, and cilia formation protein could explain the malformations observed during embryogenesis and consequent failure of metamorphosis in *Haliotis diversicolor supertexta* larvae settled on *Navicula incerta* exposed to EDCs.

18.5.3 Population Proteomics

Marine fish populations are under severe pressure due to over-exploitation in conjunction with the effects of climate change and pollution, and consequently, it is becoming increasingly important that fish populations be accurately defined and characterized if wild fisheries and fish biodiversity are to be sustained and protected. Indeed, a United Nations report states that more than two-thirds of the world's fisheries have been overfished or fully harvested, and more than one third is in a state of decline due to lost habitats, increasing pollution levels, and climate change [18.78]. Although traditional population studies of marine fish have been conducted

using a genetics approach, proteomics may be invaluable to population biology and structure studies, since the proteome more accurately represents physiology and phenotypic plasticity [18.55, 79].

Gonzalez et al. [18.79] investigated the potential of population proteomics as a tool for detecting differences between populations of commercially important fish. Two distinct stocks of the European hake (*Merluccius merluccius*, Linnaeus 1758), namely an Atlantic Ocean and a Mediterranean Sea group, have been defined on the basis of genetic population studies. Two-dimensional DIGE and MS analysis of protein expression in liver and brain tissue samples from hake captured in the Mediterranean Sea, Cantabrian Sea, and Atlantic Ocean identified 84 and 145 protein spots in the liver and brain, respectively, that had significantly different expression levels between the three groups [18.79]. Using a variety of statistical tools, *Gonzalez* et al. [18.79] were able to reduce the number of proteins required to assign fish samples to a particular population group to just three, achieving a discriminant score of 90–100% accuracy. The study found that differences in the expression of two brain proteins, involved in cell signaling and metabolism/energy, discriminated between the three fish populations significantly better than differences in expression of the liver proteins, suggesting that brain protein profiles may more accurately reflect the functional adaptation of populations [18.79]. This study clearly demonstrates the value of population proteomics as a tool for defining and monitoring fish populations which are at a critical point in terms of ensuring the sustainability of the fishing industry and preserving the biodiversity of the oceans.

18.6 Natural Products

Antibiotic discovery in the twentieth century enabled treatment of diseases caused by previously untreatable infectious bacteria. However, the situation has changed dramatically over the past few decades with rapidly emerging bacterial resistance to antibiotics as a consequence of over-prescription and inappropriate use of antibiotics as prophylactics in farmed animals, including aquacultured fish and shellfish [18.80–82]. The problem has been exacerbated by a steady decline in the discovery of novel antibiotics, with just one new antibiotic class, the oxazolidinones, added to the approved list during the 1990s [18.80]. Traditionally, drug discovery from natural products has depended on high-throughput screening of microbial, plant, and animal extracts. The majority of bioactive compounds with medical applications such as antibiotics, anticancer therapeutics, anti-inflammatories, etc., have been obtained from microorganisms, particularly the filamentous bacteria belonging to the order Actinomycetales and filamentous fungi [18.83]. However, as stated earlier, the discovery of novel pharmaceuticals has significantly decreased due to frequent re-isolation of the same compounds. Since marine organisms comprise approximately half of the total biodiversity on earth, the marine ecosystem has the greatest potential as a source of novel bioactive compounds [18.82–84].

Since sessile marine invertebrates such as sponges, bryozoans, soft corals, seaweeds, and tunicates generally lack defence structures, they have developed a broad range of secondary-metabolites with novel molecular structures, and thus, are proving to be an invaluable source of novel pharmaceuticals [18.4, 84–87]. Interestingly, many of the bioactive compounds thought to be produced by marine invertebrates are, indeed, synthesized by endosymbiotic bacteria associated with these animals [18.85, 88]. The latter has broadened the potential of the marine environment as a source of novel therapeutics since the biodiversity of marine microorganisms is immense, especially when one considers that only 1–5% are culturable using standard laboratory techniques [18.89]. Consequently, metagenomics is a technique that has been adopted for screening for novel bioactive compounds from the marine environment, since it involves generating gene libraries from DNA isolated directly from environmental samples and screening the gene sequences in the libraries for those that may code for proteins with novel functions or structures [18.88, 90]. These candidates would be expressed in a heterologous host in order to produce sufficient quantities of the gene product for further analysis. Unfortunately, this approach is not simple, since many bioactive compounds are produced via multigene pathways, all of which would need to be cloned and expressed correctly [18.88].

A further complicating factor is that very low yields of cloned proteins are obtained due to poor levels of gene expression in heterologous hosts [18.89]. Although proteomics has great potential as a tool that

could assist in identifying novel bioactive proteins, proteomic techniques have not been utilized to any great extent in marine bioprospecting [18.4]. Nevertheless, there are a few studies which have employed a proteomic approach to identify or investigate bioactive products from marine organisms.

Evans et al. [18.91] employed iTRAQ as a means of characterizing and quantifying expression of the secretome of the marine bacterium *Pseudoalteromonas tunicate*. The bacterium, which is associated with marine eukaryotes, produces a broad spectrum of extracellular compounds that are active against invertebrate larvae, algal spores, diatoms, fungi, heterotrophic flagellates, and bacteria, and are thought to provide the bacterium with a competitive advantage in mixed communities [18.91]. The study compared the wild-type secretome to that of a transposon mutant that was no longer pigmented and did not produce the bioactive compounds as a consequence of a disrupted type-II secretion pathway. *Evans* et al. [18.91] found a range of cytosolic proteins and an ABC-transporter (ABC: ATP-binding cassette) that were significantly differentially expressed between the two strains. They were unable to determine the functions of ≈23% of the proteins comprising the *Pseudoalteromonas tunicate* secretome, and suggested that the type-II secretion pathway may play a role in iron uptake. The authors make the point that the data obtained from this study will lead to a better understanding of the regulation of pigmentation and production of the bioactive compounds in *Pseudoalteromonas tunicate*, as well as elucidate the strategies the bacterium uses to eliminate competitors and survive as a surface-associated bacterium in the marine environment [18.91].

It is essential that macromolecular targets of small bioactive molecules are identified in order to understand their mechanism of action and develop new pharmaceuticals. *Margarucci* et al. [18.92] used chemical proteomics [18.93] to investigate the target of petrosaspongiolide M (PM), a γ-hydroxy butenolide marine terpenoid isolated from the sponge *Petrosaspongia nigra* that has anti-inflammatory properties. The protocol involved chemically immobilizing PM by covalently linking the molecule to solid beads which are subsequently incubated with cell lysates. Proteins anchored to the beads are released, resolved by SDS-PAGE, and finally identified by LC MS/MS analysis [18.92]. The protein mixture eluted from the beads included a number of proteins known to comprise the ubiquitin–proteasome system responsible for degrading proteins involved in the cell cycle, apoptosis, signal transduction, and the NF-$\kappa\beta$ (nuclear factor) pathway. Since abnormal expression of the ubiquitin–proteasome system is responsible for many human diseases such as cancer, inflammation, cardiovascular diseases, and neurodegenerative disorders, *Margarucci* et al. [18.92] concluded that the ability to block proteasome activity using PM suggests that the molecule has great potential as a new lead therapeutic agent. Chemical proteomics was also used in two independent studies that showed that the eukaryotic translation initiation factor eIF4A is the target of pateamine A from the marine sponge *Mycale* sp., which is a potent cytotoxin against rapidly growing cells (such as P388 leukaemia cells; IC50 0.15 ng/ml [18.94]) [18.95, 96].

The genus *Conus* is comprised of ≈700 species of cone snails, which are carnivorous marine gastropods that use venom for defence, competition, and predation [18.97–100]. Their venoms, termed conotoxins or conopeptides, have been extensively studied due to the number and diversity of the neuropharmacologically active peptides they contain (50 to >1000 per species), which specifically target a broad range of therapeutically relevant receptors and ion channels [18.97, 99, 100]. Indeed, the drug Ziconotide (Prialt), derived from the venom of *Conus magus*, has been approved for treating severe chronic pain [18.101, 102]. A number of proteomic studies have been conducted to characterize the venom gland peptidome and proteome of cone snails in order to increase our understanding of conotoxin biosynthesis, maturation, secretion, and interspecies variation. A total of 161 and 157 proteins and protein isoforms were identified in the venom glands of *Conus novaehollandiae* and *Conus victoriae*, respectively, with a large fraction of the proteins found to function in protein/peptide translation, folding, and protection events [18.99].

In another study, *Tayo* et al. [18.97] used an LTQ-Orbitrap tandem mass spectrometer to investigate the venom proteome in the proximal, central, and distal portions of the tubular venom duct of *Conus textile* in order to elucidate the overall envenomation strategy employed by *Conus* species. 31 conotoxin sequences and 25 post-translational modification variants were identified following searches using SEQUEST (tandem mass spectrometry data analysis program used for protein identification) and ProLuCID (name of a tandem mass spectrabased protein identification program) for unmodified and post-translationally modified peptides, respectively. The study found both qualitative and quantitative differences in the conotoxin components sampled from the different sections of the

Conus textile venom duct and that post-translational modification variants significantly enrich the peptide pool for the combinatorial mechanism of envenomation, which may contribute to the production of toxin groups suitable to a particular predatory or defence requirement [18.97].

Since there is a paucity of information regarding the global protein content of injected venom, *Violette* et al. [18.100] used liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) to analyze native injectable venom of *Conus consors* and coupled the data to extensive venom duct transcriptomic data to match the results and deduce nucleic and amino acid sequences. This approach resulted in identification of 105 components out of ≈400 molecular masses detected in the venom, which included new conotoxins belonging to the A, M, and O1-superfamilies, as well as a novel super family of disulphide-free conopeptides. The study demonstrated that it is possible to identify more than 100 peptides and proteins in injectable cone snail venom using a combined proteomic/transcriptomic approach and established a promising strategy for the discovery of novel therapeutics from cone snail venom [18.100].

18.7 Algal Toxins

Marine algal toxins are produced by unicellular algae that proliferate and/or aggregate to form dense concentrations of cells or *blooms* in response to favorable environmental conditions often associated with upwelling systems of the eastern boundaries of the world's oceans (Fig. 18.3) [18.103–105]. Only 2% (60–90 species, belonging to the dinoflagellate and diatom groups) of the 3400–4000 known phytoplankton taxa are toxic and responsible for more than 60 000 intoxication incidents per year worldwide, with an overall mortality rate of 1.5% [18.103, 105]. The incidence, geographic ranges, and variety of harmful algal blooms (HABs) and their toxins appears to be increasing, possibly due to increased anthropogenic nutrient loading and/or climate change [18.16, 103, 105]. HABs impact human health as a consequence of consuming contaminated seafood (such as filter-feeding shellfish), causing diseases which include paralytic shellfish poisoning (PSP), diarrhoeic shellfish poisoning (DSP), ciguatera fish poisoning, neurotoxic shellfish poisoning, amnesic shellfish poisoning, and azaspiracid shellfish poisoning [18.16, 103, 105]. Since vaccines to protect against dinoflagellate toxins have not yet been developed, prevention of HAB-related diseases relies on monitoring algal blooms and seafood, as well as surveillance of human populations for the incidence of disease [18.16, 105].

PSP is caused by consumption of shellfish contaminated with paralytic shell-fish poisoning toxin (PST), which is a mixture of saxitoxin analogs of approximately two dozen naturally occurring cyclic perhydropurine compounds that differ in their specific toxicities [18.106]. PSTs are produced by several dinoflagellates, particularly species belonging to the genera *Alexandrium, Gymnodinium* and *Pyrodinium* [18.106]. Although rapid identification of dinoflagellates present in a particular bloom would allow an accurate assessment of the potential health risk of the event, allowing appropriate contingency measures to be set in place, dinoflagellate identification is currently accomplished by light and scanning electron microscopy, which are both time consuming and complicated due to the difficulty associated with employing morphology as a sole criterion of unicellular algal taxonomic identity [18.107]. Consequently, *Chan* et al. [18.107] employed 2D-PAGE to generate proteome reference maps for nine species responsible for HABs off Hong Kong on the basis that the proteome of unicellular algae is simple and unique to each species. Species-specific

Fig. 18.3 Dinoflagellate bloom at Elands Bay, South Africa on 26th March, 2012. The bloom, clearly recognized by its red-brown color, consisted of *Alexandrium catenella* and a *Ceratium* species (photograph by C.J. Foord)

2D-PAGE protein profiles were obtained for all species and could be used to distinguish between closely related species belonging to the same family, confirming 2D-PAGE as an excellent monitoring tool for distinguishing dinoflagellate species comprising a HAB [18.107].

Since some dinoflagellate species are morphologically similar, and/or toxic and nontoxic varieties of the same species exist and sometimes even co-occur, the ability to use 2D-PAGE to differentiate between toxic and nontoxic variants of a species was investigated [18.106, 108]. Although the protein profiles of a toxic and nontoxic strain of *Alexandrium minutum* were essentially similar, reflecting the absence of significant morphological variation between the strains, pronounced differences were detected [18.106]. Thus, four proteins (designated NT1, NT2, NT3, and NT4) could be consistently detected in all nontoxic strains, while proteins T1 and T2 were associated with the toxic strains regardless of the culture conditions.

A similar study, employing a combination of 2-PAGE and MS was conducted on several strains of *Alexandrium tamarense* with different toxin compositions and originating from different geographical locations, identified several proteins that were always present in all toxic *Alexandrium tamarense* strains [18.108]. Interestingly, MS analysis indicated that the proteins associated with toxic *Alexandrium tamarense* are isoforms of the same protein, which shares amino acid similarity to that of T1 from toxic *Alexandriunm minutum*. Since the peptide mass fingerprints of these toxicity biomarkers were highly strain- and species-specific, differentiating between toxic and nontoxic strains as well as *Alexandrium tamarense* and *Alexandrium minutum*, the study has made a significant contribution to the complex task of monitoring HABs for toxic species. Indeed, the use of these toxicity biomarkers was validated by western blot experiments using a murine AT-T1-polycolonal antibody that detected all the toxic *Alexandrium tamarense* and *Alexandrium minutum* strains, but not the nontoxic *Alexandrium tamarense* strain [18.108].

Besides monitoring HABs in the ocean, it is also of utmost importance to monitor seafood destined for human consumption for toxin contamination. Although this may be accomplished using analytical techniques, the diversity of toxins and their analogs that may contaminate seafood makes this approach problematic due to the lack of specific standards and reference compounds required to ensure the accuracy and specificity of the protocol. Consequently, *Ronzitti* et al. [18.109] investigated a proteomics approach that would provide a functional assay for toxin-contaminated seafood by screening for toxin biomarkers in situ. They used 2D-PAGE to analyze digestive gland samples obtained from mussels harvested from four sites in the Adriatic Sea for biomarkers of contamination with okadaic acid and related dinophysis toxins, responsible for diarrhoeic shellfish poisoning in humans. Two proteins that correlated with okadaic acid contamination of the digestive gland samples were identified as a component of photosystem II and a subunit of NADH dehydrogenase, the former being identical to its counterpart in *Dinophysis* species that produce this group of toxins [18.109].

Ronzitti et al. [18.109] concluded that the photosystem II component originated from *Dinophysis* dinoflagellates ingested by the filter feeding mussels, and the amount ingested is reflected by the quantity of this protein which correlated with the degree of okadaic acid contamination of the mussel digestive gland samples (as determined by chemical analysis). Thus the study demonstrated the feasibility of biomarkers for monitoring seafood, where the biomarkers can either reflect the origin of the toxin (photosystem II component) or the animal's response to the toxin (NADH dehydrogenase subunit – possibly functions as a component of a protective response to the presence of a strong apoptogenic compound in the mussel digestive gland) [18.109].

Besides inducing diarrhoeic shellfish poisoning in humans after consuming bivalves contaminated with *Dinophysis* dinoflagellates, okadaic acid has been recognized as a general tumor promoter in various murine organs, including the skin, glandular stomach, and liver [18.110, 111]. Although the genes and signaling pathways affected by okadaic acid toxicity have been elucidated using a mouse toxicological microarray that identified a total of 177 differentially expressed genes [18.111], almost all the available knowledge concerning the toxicological mechanisms of okadaic acid has been obtained from in vitro assays. Furthermore, because the effects of okadaic acid on cell transformation, cell proliferation, and apoptosis vary considerably, the molecular events underlying the effects of okadaic acid are not well understood [18.110, 111]. Consequently, *Wang* et al. [18.110] employed 2D-DIGE and MALDI-TOF-TOF MS to investigate the acute toxicity of okadaic acid in mice at the proteomic level to further elucidate the molecular mechanism of okadaic acid-induced diarrhoea in mammals. Male Imprinting Control Region (ICR) mice were treated with 750 μg/kg okadaic acid, administered in a single dose by gavage, and protein profiles of the small intestines were de-

termined at four time points (0, 3, 6, and 24 h post-treatment). The study identified 58 proteins that were differentially expressed over the 24 h period which functioned in macromolecular metabolism, cytoskeleton reorganization, signal transduction, molecular chaperoning, and oxidative stress [18.110]. Although it has been known for some time that okadaic acid acts as an inhibitor of types 1, 2a, and 3 serine/threonine protein phosphatases (PPs), which results in the diarrhoeic effects as a consequence of the accumulation of phosphorylated proteins that regulate metabolism, transport, and secretion, the data obtained from the in vivo proteomic study suggests that okadaic acid toxicity in mouse intestines is complex and diverse, involving multiple proteins other than PP in the diarrheic process [18.110]. Indeed, the proteomic analysis revealed that okadaic acid toxicity interrupted cytoskeleton reorganization of intestinal epithelial cells, destroyed the digestive enzyme system by affecting lipid, amino acid, and sugar metabolism, induced oxidative stress, and simultaneously interfered with cell signal transduction in intestinal cells [18.110]. The authors suggested that villin 1 (cytoskeletal protein; down-regulated by okadaic acid) and hnRNP F (RNA binding protein that may function in apoptotic pathways; up-regulated by okadaic acid 3 h post-administration) may be key triggers that induce diarrhoea by interrupting cytoskeleton reorganization and cell apoptosis in the mouse small intestine, and thus, may be candidate biomarkers for acute okadaic acid toxicity [18.110].

18.8 Conclusion

It is clear that proteomics is a tool that will strongly influence all aspects of marine biotechnology, since it provides information on a scale that reflects the new omics era that is a hallmark of twenty-first century science. The technology is constantly improving, and as such, our ability to better understand the biology of marine organisms, their interactions with each other, and the environment will be enhanced by new developments in proteomics that are expected to be realized in the near future.

Goals anticipated to be achieved over the next 5 years include a 100 to 1000-fold increase in the sensitivity of MS-based protein identification that will allow characterization of biologically relevant, low abundance proteins; increased sensitivity will reduce the amount of sample required for proteomic analysis from 10^6 to 10^3 cells and eventually to single cells, while enhanced throughput of proteomics assays will allow the analysis of hundreds of specimens per day, which would be beneficial to research areas that investigate large numbers of samples such as in marine natural product identification and characterization [18.112].

Nagaraj et al. [18.113] used a high-performance benchtop quadrupole Orbitrap mass spectrometer to identify the majority of proteins expressed in the yeast *Saccharomyces cerevisiae* W303 MATα (an average of 4000 proteins per run), which allowed almost complete coverage of the yeast proteome in a few hours using a minute amount of sample (4 µg of peptide sample). Indeed, the proteomics field is said to be moving rapidly from a *second generation* state to a *third generation* phase where it is expected that MS-based proteomics will be routinely used to determine absolute protein levels as opposed to relative quantification, as is currently the case, and to characterize the dynamic response of entire proteomes in both space and time [18.114].

The development of new software that will allow integration of proteomics data with that obtained from next-generation sequencing and metabolomics will allow a systems biological approach to the study of marine organisms. Thus, proteogenomics is expected to provide a more holistic understanding of marine organisms, since proteomics data can be used to validate and annotate genome sequences as well as identify novel genes and pseudogenes which may be missed during DNA sequence annotation [18.3, 115]. The study of marine organisms will be elevated to an unprecedented level through the detailed information that will be gained with regard to their functional genetics, the regulation of these processes and in turn their metabolism, physiology, and interaction with the environment. This knowledge will significantly influence all aspects of marine biotechnology in that the impact of climate change and anthropogenic pollution on marine organisms will be understood in greater depth, the discovery of novel bioactive compounds will be greatly facilitated, aquaculture will benefit through a deeper understanding of reproduction, metabolism and stress leading to improved quality, survival and growth rates in farmed species, and most importantly, a greatly improved balance between exploitation and conservation of resources from the marine environment.

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19. Marine Metagenome and Supporting Technology

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Bacteria are known to be highly diverse and unique to the various environments they reside in. Covering more than 70% of the earth's surface, marine bacterial ecosystems in particular have long been regarded as reservoirs for novel and unique genes important to industry and pharmaceutics. In the first part of the chapter, we reviewed the importance and potential of bacteria from marine environments as an important genetic resource and some of the recent efforts in the implementation of marine metagenomic research to screen for genes applicable in bioprocesses, bioremediation and bioethanol production. Nevertheless, metagenomic research has also provided new challenges that will need to be addressed in order to use these resources efficiently. Here, in the second part of the chapter, we introduced several supporting technologies that show great potential in assisting metagenomic research to overcome such challenges including high-throughput screening using microfluidics, single-cell analysis and in silico data mining of metagenomic data. The introduction of such technologies, with metagenomic research, does not only allowed us to exploit these genetic resources to the fullest but may also provide new perspectives and insights towards living organisms and natural ecosystems.

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19.1 Bacteria and Marine Ecosystems

Nature, as we know it, comprises various unique environments that have provided shelter and homes to diverse organisms. These unique environments have long been regarded as treasure boxes that have yet to be revealed and explored. It not only provides a unique environment to large vertebrates, invertebrates, plants, etc., but to microorganisms, including bacteria. Based on recent studies, it seems prominent that the bacteria present in each environment is highly diverse and their existence is not coincidental but instead they are comprised of microecosystems that are highly specific to the environment [19.1, 2]. It is, therefore, considered that bacteria living within these microecosystems harbor unique and novel characteristics that allow them to thrive and survive in their natural environment. The presence of these specific bacterial populations clearly indicates that they play a direct and crucial role either for the benefit of the bacteria themselves or for the environment they reside in [19.3]. In the exploration of the terrestrial environment, for example, mainly soil has led to the identification of numerous bacterial ecosystems comprised of diverse bacterial species [19.4–6]. The marine environment on the other hand, which covers more that 70% of the earth's surface, provides both mild and extreme conditions that allow the presence of bacterial species that harbor unique characteristics, which have evolved to thrive in such conditions. Although large collections of bacterial species are constantly being studied and identified, we are merely at the tip of

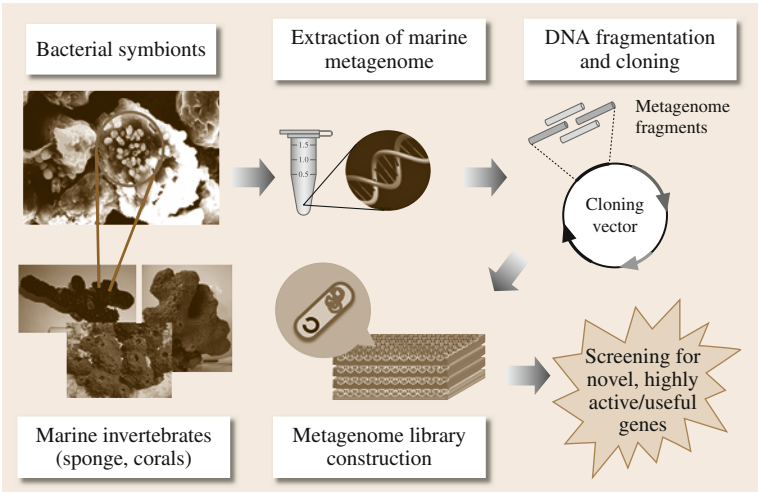

Fig. 19.1 Schematics of metagenome library construction from environmental samples

the iceberg and there is still much to be learned and discovered.

In the marine environment, bacteria in particular, are highly diverse and harbor unique and novel enzymes that are indispensable to research, pharmaceutics, and industry. Extracellular-polymer-degrading enzymes, DNA-modifying (DNA: deoxyribonucleic acid) enzymes and many others have been attained from bacteria in marine environments [19.7]. In addition, secondary metabolites or natural products that are of high pharmaceutical value have also been abundantly discovered from marine bacteria [19.8, 9]. Nevertheless, even with the availability of such unique resources, we still face the challenge of fully exploiting them due to a lack of technologies or techniques. Techniques for the bacterial culture have been and still are being introduced constantly, but it is regarded that culturable bacteria represent only $< 1\%$ of the currently identified bacteria, while the remaining $> 99\%$ are still considered as uncultivable. Thus, in this chapter we focus on metagenomic research, a tool introduced for the exploitation of novel and useful genes from bacteria, and the introduction of several tools that have been established to support metagenomic research.

19.1.1 Marine Metagenomic Research

As was briefly described above, the challenge for the extraction of novel and unique genes from bacteria surviving within the natural environment has been hindered due to the presence of uncultivable bacteria. Therefore, such a challenge has led to the introduction of a powerful tool that is currently known as metagenomics [19.10–12]. Metagenomics integrates molecular biology with genetics, enabling us to assess *nonassessable* genetic materials from the natural environment, thus, allowing the exploitation of such a resource to the fullest. The principle of this technique involves the extraction of genetic materials, such as DNA, directly from a pool of bacteria, regardless of their cultivability, and cloning them into cultivable hosts, namely *Escherichia coli*, resulting in the establishment of a *metagenome* library. The constructed metagenome library would then comprise millions of clones, individually harboring a random DNA fragment, which may potentially be comprised of useful and unique genes to be identified from subsequent large-scale screening of specific target genes via protein expression and functional analysis (Fig. 19.1).

Marine metagenomics, which describes the exploitation of samples from oceanic or marine samples, is supposed to have begun with initial work conducted by *Schmidt* et al., who proposed the idea to extract bulk DNA directly from picoplankton communities and to use the bacterial 16S small subunit ribosomal DNA (rDNA) as a reference to identify microorganisms in natural samples without the requirement of laboratory cultivation [19.13]. Subsequent work was followed by the exploration of bacterial diversity from larger pools of 16S rDNA sequences conducted by *Hugenholtz* et al. [19.1]. *Stein* et al. presented one of the first reports on the construction of a metagenome library using fosmids from marine picoplankton, but this research was directed at marine archaea [19.14]. In 2003, the Global Ocean Sampling Expedition (GOS) led by *Venter* conducted a global expedition to collect oceanic

metagenome samples among which one of their analysis conducted in the Sargasso Sea, resulted in the identification of at least 1800 different species, including 148 previously unknown bacterial phylotypes [19.15]. Based on these efforts, the application of marine metagenomic research has further expanded to other marine microorganisms, including viruses [19.16], archaea [19.17], and cyanobacteria [19.18].

In addition to the large amount of research conducted on marine ecosystems thus far, our research is mainly focused on bacteria associated with marine sponges collected from Ishigaki island, Okinawa, Japan. Bacterial symbionts were isolated from the marine sponges *Stylissa massa* and *Hyrtios erecta*, two species that are widely distributed on the ocean floor. Metagenome DNA was extracted from these sponges and 16S rRNA analyses of the diversity of bacterial symbionts and metagenome libraries were prepared. Based on the diversity of the analyses, it was observed that sponges show similarity in bacterial populations (phylum level) between individual sponges. As for the metagenome libraries, libraries comprised of DNA fragments in the range of 3–40 kbp were established, and subsequent gene annotation and in silico screening of useful genes via large-scale paired end sequencing also resulted in the establishment of a database. We would also like to introduce several unique and novel enzymes that have been discovered from marine metagenomes in the course of the work conducted by our research group.

Esterase Screening from a Sponge Metagenome Library

Esterases are enzymes that function in the breakdown of esters into alcohol and acid via hydrolysis and are regarded as potentially important enzymes of industrial value since they degrade natural materials, industrial pollutants, and toxic wastes. They can also be useful in the synthesis of drugs, cosmetics, and antioxidants [19.19]. Although numerous cultivable bacterial strains and species have been reported thus far, novel esterases showing high activity, thermostability, or unique characteristics are still in demand. In this study, we show the efforts to identify novel or unique esterases from a metagenome library constructed from marine sponge bacterial symbionts [19.20].

The sponge metagenome library MGSB3, comprised of 26 496 clones harboring DNA fragments of 3–5 kbp in size, was constructed from metagenomic DNA extracted from bacterial symbionts of the marine black sponge *Hyrtios erecta* collected off the coast of Ishigaki Island, Okinawa, Japan. Screening for esterase activity was carried out on agar plates containing Tween-20 and $CaCl_2$, in which screening of all the clones resulted in the identification of only one positive clone. Upon sequencing and further gene annotations, the clone harbored an approximate 3.4 kbp insert comprised of four putative open reading frames (ORFs). Among these ORFs, the third putative ORF had a 54% sequence identity with an arylesterase isolated from *Marinobacter aquaeolei* VT8 [19.21]. Designating the putative ORF as *EstHE1*, alignment of its encoded protein, EstHE1, amino acid sequences with several other esterases revealed that EstHE1 had the characteristic GDSLS amino acid consensus sequence (GDSLS: an amino acid residues considered as an important motif in the EstHE1 esterase) within its N-terminus and five distinct blocks of sequence homology to the GDSL family of esterases (GDSL: amino acid motif specific to the esterases) (Fig. 19.2a). In addition, the highly conserved catalytic residues Ser (S), Gly (G), Asn (N), and His (H) were identified in the conserved blocks, I, II, III, and V, respectively.

It is known that many esterases and lipases contain the pentapeptide motif GxSxG, which is well

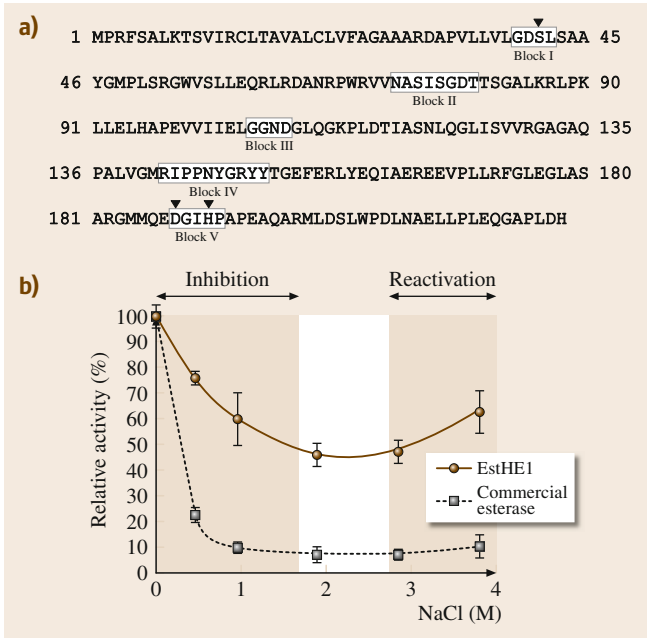

Fig. 19.2a,b The (a) structure and (b) relative activity of the esterase EstHE1

conserved among lipolytic enzymes [19.22], but enzymes conserved with the SGNH (SGNH: a motif representing amino acid residues) amino acid residues is seen in a new subfamily of hydrolytic/lipolytic enzymes [19.23–26]. These four residues are the namesake of the SGNH superfamily and utilize a different catalytic mechanism for hydrolase activity from that of common α/β-hydrolases [19.27]. While SGNH hydrolases are well represented in eukaryotic organisms, the isolation and characterization of SGNH hydrolases from bacteria is limited [19.27, 28]. This further suggests that EstHE1 may be novel, belonging to the SGNH hydrolases superfamily. Furthermore, a signal peptide (Met1-Ala29) was identified at the N-terminus of EstHE1, also suggesting that EstHE1 may function extracellularly.

For the biochemical characterization of EstHE1 esterase activity, the functional domain of EstHE1, excluding the signal peptide sequence was cloned, purified by nickel nitrilotriacetic acid (Ni-NTA) column chromatography, and identified by immunoblot analysis. EstHE1 was also proven to be an esterase rather than a lipase via a substrate specificity assay showing that the enzyme hydrolyzed chain-fatty acid substrates (C_6>) and not triglycerides or tributyrin (C_4). To determine the optimal temperature for esterase activity and to evaluate the thermostability of EstHE1, *p*NP-acetate was used as the substrate. The optimal temperature for EstHE1 activity was near 40 °C; however, the enzyme was active over a broad range of temperatures, retaining over 50% of its relative activity between 25−55 °C. The thermostability of EstHE1 was tested by incubation at 40 °C, whereby the retention of esterase activity was analyzed. EstHE1 retained more than 80% of its relative activity after 3 h at 40 °C, and 58% of its relative activity even after 12 h at 40 °C (Fig. 19.2b).

Since EstEH1 originated from marine bacteria, stability of the enzyme was also tested in various NaCl concentrations. Upon a gradual increase in NaCl concentration, enzyme activity showed a gradual reduction in esterase activity, eventually showing 55% of its activity at 1.9 M NaCl. However, as we further increased the salt concentration from 1.9 M to 3.8 M, esterase activity was recovered to 62% (Fig. 19.1). This result indicates that EstHE1 exhibits halotolerance, however, similar to the actinidain esterase found in kiwifruit, and increased salt appears to re-activate EstHE1 esterase activity [19.29].

As described above, the behavior of EstHE1 for halotolerance is very unique and its thermostability is desirable for application in the industry.

Cadmium Accumulation Protein from Sponge Metagenome

Heavy metals such as cadmium, lead, mercury, manganese, etc., are regarded as important industrial materials, if applied in the appropriate manner, due to their use in the energy and fuel production, the fertilizer and pesticide industry, and their applications in metallurgy, iron and steelmaking and electroplating. Nevertheless, the misuse or inappropriate handling of these metals can also result in generation of environmental pollutants that threaten human health and the ecosystem [19.30, 31].

In contrast to their organic counterparts, heavy metals are not degraded or destroyed. In developing countries, heavy metal pollution has become a problem since sanitary sewers are not equipped to treat toxic wastes [19.32]. Conventional techniques or technology to remove heavy metal ions such as chemical precipitation, filtration, ion exchange, electrochemical treatment, and membrane technologies have been introduced but the large-scale use of these technologies is hindered mainly because of problems due to high cost and low efficiency [19.33]. Therefore, such a challenge has been approached by the use of natural materials, termed biosorbents, including plants (mostly algae), fungi, yeast, and bacteria, resulting in the decrease of heavy metal ion concentrations in solution from ppm to ppb levels [19.34]. These biological systems, acknowledged for their low cost and high efficiency, have now resulted in the establishment of new technologies such as bioremediation and phytoremediation [19.35]. In addition, the use of useful genes for the expression of heavy metal accumulation proteins identified from various organisms or from metagenomic samples has also promoted this field [19.35, 36].

To contribute to the requirements of *environmental friendly* novel accumulation proteins or peptides, here, we focused our research on the screening of heavy metal accumulation genes from a metagenome library that was constructed from the marine sponge *Stylissa massa*. It is stated that 40% of the sponge body comprises of sponge-associated bacteria [19.37], and numerous antibiotics and important enzymes have been extracted from them [19.20, 38]. A total of approximately 3000 clones from the library were evaluated with the presence of cadmium (Cd), manganese (Mon), strontium (Sir), chromium (Cr), nickel (Ni), gadolinium (Gd), and selenium (Se). As a result, we were successful in identifying a novel Cd accumulation gene that showed unique protein structural features and Cd binding properties higher than the currently

known Cd accumulation proteins, metallothionein or phytochelatin. Currently we are in the process of analyzing the characteristics and properties of this novel protein.

Screening for Saline Tolerant Enzymes

Salt or saline tolerant enzymes have long been regarded as important enzymes required in industry, since many industrial processes are highly dependent on conventional salt removal steps that result in high-energy consumption. Thus, the isolation and use of salt-tolerant enzymes in such processes may greatly contribute to overcoming this problem. Several attempts have resulted in the isolation of salt-tolerant enzymes from microorganisms found in high saline marine environments. Serine hydroxymethyltransferase (SMTH), for example, commonly found in living organisms and is a key enzyme in the cellular one-carbon pathway. *Waditee-Sirisattha* et al. successfully identified this enzyme from the halotolerant cyanobacterium *Aphanothece halophytica*, expressed it in *Escherichia coli* and further showed that enzyme activity was promoted in the presence of high salinity [19.39]. *Mai* et al. on the other hand, successfully cloned a β-glucosidase from a marine streptomycete showing that the enzyme also showed remarkable activity enhancements under saline conditions [19.40]. In addition, salt-tolerant enzymes such as general stress proteins, enoyl-CoA hydratase [19.41] and bacterial lipolytic enzymes [19.42] have also been identified from metagenome library screening.

In our efforts, we have also been successful in identifying a gene encoding for the FtsH protein from our metagenome libraries. Upon comparison of the isolated FtsH protein with that of *Escherichia coli*, our isolate showed high saline resistant properties, suggesting the possibility of it being a novel enzyme originating from a marine bacterium. Although further functional analyses are in progress, the potential of this enzyme is promising, since FtsH proteins are known to function as crucial enzymes that play key roles in bacterial survival, and it has been suggested that it participates in several cellular processes, including protein assembly, export, and degradation [19.43, 44]. Further studies may provide important insights to the function of FtsH proteins in high salinity environments.

Potential of Marine Metagenomic in Bioethanol Production

Bioethanol is a renewable energy that has received much attention due to its potential in replacing current fossil fuel. Its production is dependent on biomass resources such as starch-based biomasses (corn, sugarcane), cellulose or lignocellulose biomasses (wheat grain), and marine biomasses (seaweed, microalgae). Although conversion of sugar to ethanol is relatively easy, starch-based biomasses conflict with the food industry. Thus, the latter two types of biomasses play a more important role in providing the resource for bioethanol production. Nevertheless, conversion of these biomasses to bioethanol is much more challenging. In countries like Japan, providing resources for land-cultivated biomasses is problematic due to limited land resources, but marine biomasses show greater potential. Marine biomasses grow relatively fast and produce a higher yield [19.45]. For example, *Laminaria japonica* (brown algae) yields six and a half times more than sugarcane. Nevertheless, although it has great potential, the conversion of marine biomasses to bioethanol is still challenging.

Marine biomasses, seaweed in particular, are composed of polysaccharides such as ulvans (green algae), agarans and carrageenans (red algae), and alginates, fucans, and laminarin (brown algae), which are required to be processed in order to be utilized [19.46]. Oligosaccharides from alginate, for instance, undergo saccharification by partial acid and alkali hydrolysis. These saccharification processes are complex and by-products can pose technical, environmental, and economic problems [19.47]. Thus, in order to overcome this problem, the use of polysaccharide degrading enzymes extracted from the environment has received much attention. Recent reports on the identification of novel genes encoding for enzymes such as porphyranases, agarases [19.48], ulvan lyases [19.49], cellulase, alginate lyase, laminarinase, and kelp-lyase [19.50] from various marine resources have shown that microorganisms may, in fact, provide an ideal source for the identification of highly active algae degrading enzymes to assist in the processing of marine biomasses.

Therefore, in order to attain highly active algae degrading enzymes, it is believed that the application of metagenomes from microorganisms can be very useful. As described above, by implementing metagenomic research, these enzymes can be fully exploited from both cultivable and uncultivable microorganisms. Furthermore, it is speculated that metagenomic research may play a major role and be of importance to this area of research.

19.2 Technologies Supporting Metagenomic Research

In the first part of this chapter, we described the advantages and new insights attained from metagenomic research, whereby the technology has opened doors to a new way to exploit environmental gene resources. Nevertheless, metagenomic research has also provided new challenges that will need to be addressed in order for us to use gene resources to their fullest. Two major challenges that have arisen from metagenomic research are 1) the techniques required for the screening of clones from large metagenomic libraries and 2) the approaches required to process or analyze the metadata attained from large-scale sequencing. Here, we introduce several supporting technologies that show great potential in assisting metagenomic research to overcome these challenges (Fig. 19.3).

19.2.1 High-Throughput Analysis/Screening of Metagenome Samples

Currently, metagenomic libraries can be constructed with the use of commercially available kits by introducing large fragmented DNA extracted from environmental samples into fosmids, cosmids, or bacterial artificial chromosome (BAC) systems. These libraries may comprise of tens to hundreds of thousands of clones that are required to be screened in order for a gene of interest to be obtained. Thus far, screening of target genes can be conducted mainly via polymerase chain reaction (PCR) analysis (genetic approach) or plate assays (functional approach). PCR analysis serves as a reliable tool if genetic information of the target gene is available, whereby degenerate primers can be specifically designed to amplify novel genes within a gene cluster or family. Plate assays, on the other hand, serve as a useful tool for the direct screening of genes based on the functional characteristics of the target gene. Although these conventional techniques have provided new information and novel insights on unique ecosystems for the discovery of novel proteins or enzymes of significant use, they are still incompatible for the exploitation of the potential of metagenomic samples. PCR analyses, for instance, can only be effective if sufficient information is available for the class of proteins that one is interested in. In the case of plate assays, it is rather challenging in the sense that in order to conduct screening for an unknown compound of enzymes, new assay development for the screening of these enzymes is required, which is very time consuming [19.51].

Therefore, in reference to these current techniques, it is clear that time and number is a very important aspect in the analysis of metagenome samples. In our group, in addition to metagenomic research, we have begun to include supporting technologies to assist us in optimizing throughput.

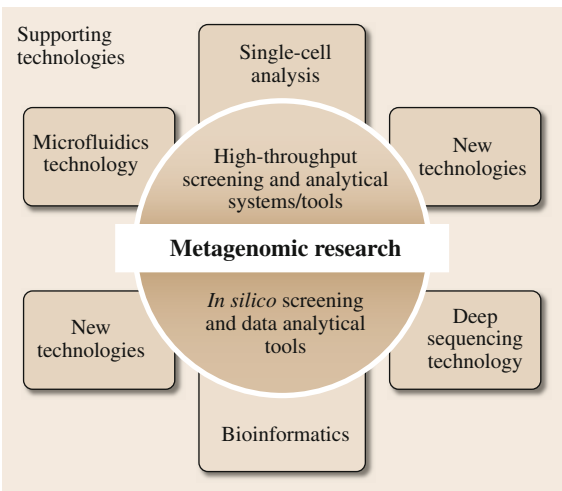

Fig. 19.3 Metagenomic research (core technology) and its supporting technologies

19.2.2 Increasing Throughput Using Microfluidics

Microfluidics is an area of research that has received much attention due to its ability to manipulate liquid flow at micrometer scale via devices that have been uniquely designed to include miniaturized pumps, mixers, valves, and channels [19.52, 53]. In recent years, this technology has niched itself to various fields ranging from synthetic chemistry and biology, right up to information technology. It also holds the promise of integrating an entire laboratory onto a single chip, termed lab-on-a-chip [19.54]. Among the various types of microfluidic systems, droplet-based microfluidics is a rapidly growing interdisciplinary field that has been shown to be compatible with many chemical and biological reagents, making it an ideal high-throughput platform for biomedical research, biotechnology applications, biochemistry, and many more [19.55]. Thus far, this technology has been applied in protein crystallization, biological assays against living cells, in single-cell

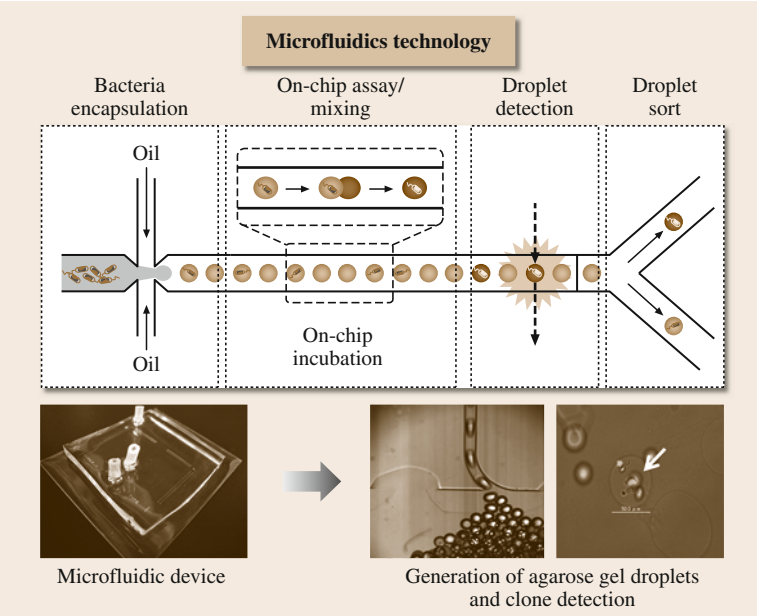

Fig. 19.4 Microfluidics technology and its application in metagenome library screening

analysis, and even in the cultivation of uncultivable bacteria [19.56–59].

Looking at the advantages of such a technology, we believe that metagenomic research can greatly benefit from it. In fact, some examples of microfluidics in the screening of enzymes and other related applications have recently been reported. *Aharoni* et al. showed the screening of paraoxonase (PON1) mutants for improved thiolactonase activity from a bacterial library by encapsulating each variant at the single-cell level using water-in-oil-in water emulsion [19.60]. Using this approach, they isolated a range of new PON1 variants that were catalytically active, approaching 100-fold higher than the wild type PON1. *Leung* et al. showed the development of a programmable droplet-based microfluidic device that can be tuned for various applications, including cell sorting, culture, PCR-based genotyping of bacteria, and whole genome amplification (WGA) [19.61]. They also showed the applicability of their system with environmental samples by showing taxonomic profiles from metagenomes of a marine enrichment culture, deep-sea sediments, and the human oral cavity. As such, our group is also focused on the establishment and development of droplet-based microfluidic systems, whereby we are currently establishing a system for the handling of single bacterial variants from metagenomic libraries directed towards the screening of intra and extracellular enzymes (Fig. 19.4).

19.2.3 Single-Cell Analysis

Metagenomic research has allowed us to identify unique and novel genes from pools of uncultivable and cultivable bacteria. However, the setback of this technology comes once a gene of interest has been identified; it is often the case where one may be curious to know more about the producer of the gene of interest. However, identification of the producers from metagenomic resources is not feasible particularly if one's interest involves novel genes where online database information is scarce. Therefore, with reference to metagenomic data or analysis, a new approach would be required to support this challenge.

Single-cell analysis is a field that was introduced to understand various biological phenomena such as cellular mechanisms, protein functionality, gene expression, etc., which occur within individual cells in a heterogeneous population [19.62]. Since its introduction, single-cell analysis has emerged as a technology that is constantly applied in genomics, transcriptomics, proteomics, and metabolomics [19.63–65]. Working in this field since its introduction, we have also assisted in the development of single-cell techniques for mammalian systems [19.66, 67]. Nevertheless, although the approaches introduced for single-cell analysis thus far have proven to be highly applicable and promising,

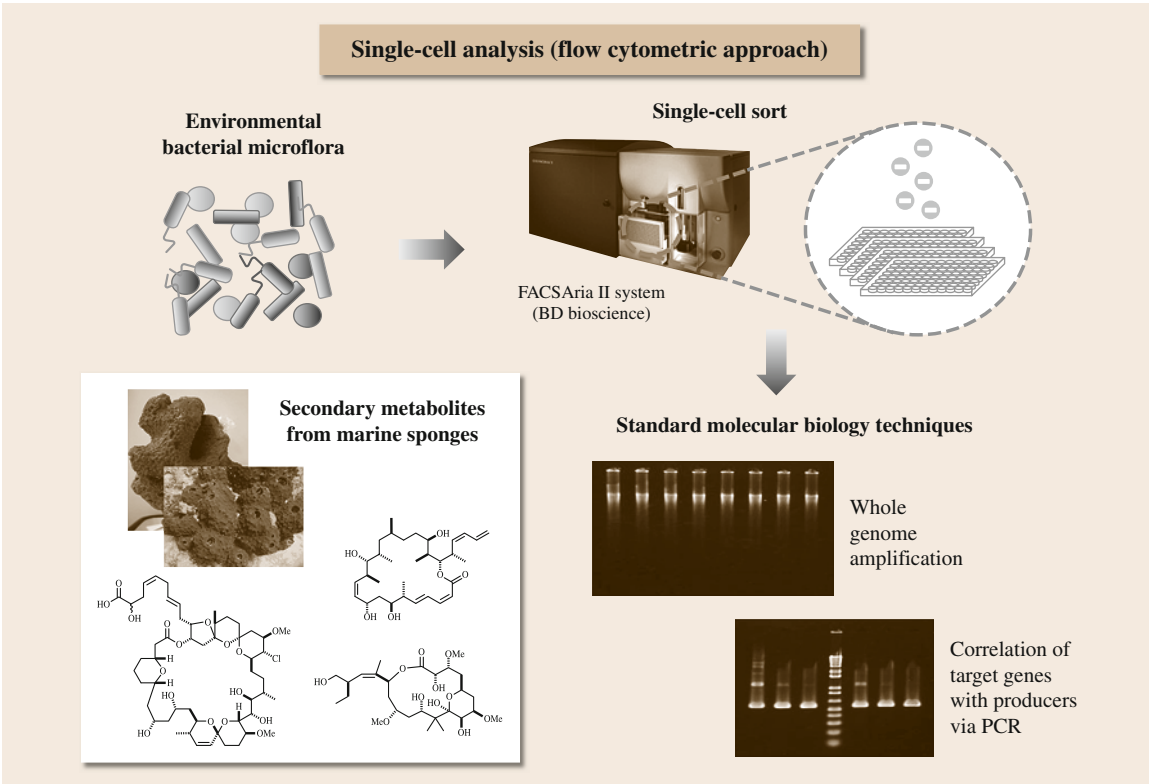

Fig. 19.5 Single-cell analysis in the identification of secondary metabolite producers

the majority has been directed at analytical studies on mammalian or eukaryotic cells. Currently, single-cell analysis has even expanded its application to the analysis of prokaryotes, using tools such as raman microscopy [19.68], flow cytometry [19.69], and microfluidic digital PCR [19.70].

In our group, we have established a single-cell analysis workflow using flow cytometry to investigate the producers of biosynthetic compounds from marine sponges (Fig. 19.5). Marine sponges are invertebrates that possess a chemical defense mechanism to survive in harsh ecosystems, resulting in them remaining the most important phylum, as they provide numerous novel biocompounds showing interesting biomedical potential, pharmaceutical relevance, and diverse biotechnological applications [19.71, 72]. Recent speculations however, suggest that such natural compounds may not originate from the sponge but instead from bacteria associated to them [19.73]. This theory is currently attracting considerable interest, since it shows that there may be interaction between host and bacteria in order for either to survive in such harsh environments. As a proof-of-concept in the establishment of our research, the marine sponge *Theonella swinhoei* was used as a model, with the research goal of identifying the bacteria symbiont producing the biocompound onnamide A as described by *Piel* et al. [19.73]. By utilizing flow cytometry, with the combination of molecular biology-based techniques such as multiple displacement amplification (MDA) and nested-PCR, we were successful in identifying the true producer of the biocompound. Our results suggest that the implementation of the single-cell approach was useful in allowing us to verify the producers of a desired enzyme or protein, thus allowing us to take the first step to combine such a method with metagenomic analysis to search for important or novel bacteria that could be of pharmaceutical or industrial value.

19.2.4 In silico Analysis of Metadata from Metagenome Samples

As was previously mentioned, in addition to the requirements for supportive technology in the screen-

ing of large metagenome libraries, technologies are also required for the analysis of the huge amounts of data generated by metagenomic studies. Already in the *post-genomic* era, in the past few years many of us have experienced the dramatic change from capillary sequencing approaches to the recent next-generation sequencing platforms. What was considered known already from capillary sequencing was the ability for one to identify sequences of new genes from unknown samples was completely changed with the introduction of deep sequencing platforms such as the 454 (Roche), Solexa (Illumina), SOLiD (Applied Biosystems), HeliScope (Helicos), PACBIO (Pacific Biosciences), and Ion torrent (Life technologies) [19.74, 75] systems. These platforms can generate giga or terabases of sequencing reads in an equal amount of time to capillary sequencing, allowing not only genes but genomes of organisms be sequenced in a matter of days or weeks. In addition, software and techniques have also been developed to support the vast amount of data generated by these sequencing platforms [19.76]. Even in metagenomics, several analytical and annotation tools such as the meta genome analyzer (MEGAN) [19.77] and the metagenomics RAST server (MG-RAST; RAST: rapid annotation using subsystem technology) [19.78], and databases such as the Genomes OnLine Database (GOLD) [19.79] and Genomic Encyclopedia of Bacteria and Archaea (GEBA, http://genome.jgi.doe.gov) project have been established to assist researchers in their respective metagenomic analyses and studies.

In our group, we have taken approaches to analyze the data attained from metagenome sequencing (approximately 110 Mbp) by conducting in silico screening for homologs to 45 enzymes that are of industrial value. Using analysis of homologs at $> 80\%$, $50–80\%$, and $< 50\%$ similarity against a total of 4075 genes, hits rates were 1%, 29%, and 70%, respectively.

19.3 Summary

To summarize this chapter, it is clear that since the very first introduction of metagenomic research for the analysis of various natural environments, not only have we gained new insights or discovered numerous new and novel genes that are of great use, we have also come to realize that new technology and techniques are required for the field to grow further. High-throughput analytical systems, such as microfluidics and single-cell analysis are just some examples of technology that have shown its potential in supporting the rapidly growing metagenomic research field. With the rate at which the amounts of data are being generated, it is almost certain that in the very near future, and with the support of bioinformatics, methods or software for data processing and analysis will also be required. Judging from the rate at which the field is expanding and evolving, our perspective towards living organisms or natural ecosystems will certainly change drastically.

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20. Microfluidic Systems for Marine Biotechnology

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During the past decade, rapid progress in physics, electronics, and material sciences has facilitated the development of miniaturized microfluidic systems, also known as Lab-on-a-Chip (LOC), that represent the next generation of analytical laboratories, miniaturized to be held in one's hand. Microfluidics has appeared as a concrete alternative than can address problems and overcome limitation in various biological and chemical domains, including stem cell research, drug discovery, and food sciences just to name a few, and has proven to be more than state-of-the-art techniques. Microfluidic techniques provide accurate and fast results with small amounts of reagents and can bring various analytical tools for in situ studies. In this chapter, we describe some of the systems developed to overcome the limitations of conventional marine biotechnology processes.

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Occupying three-fourths of our planet, oceans are vital to human beings [20.1], providing us in kelp, food, recreation, mining, natural resources (such as gas and oil), shipping and transportation, and biomedical and cosmetic products; more than 80% of the Earth's living organisms are found only in marine ecosystems [20.2] and about two-thirds of the world's population live within 100 km of the coastline [20.3]. Although the importance of seas on our lives is evident, marine ecosystems remain relatively unknown, underexploited and under-protected compared to those from terrestrial and freshwater environments. In this context, marine biotechnology aims to develop technologies to enable scientists to differentiate sea populations, to address emerging diseases to protect fishery and ecological resources, and to discover the life-enhancing and lifesaving properties these populations possess, while identifying sources of ecological stress to develop strategies to protect and restore coastal resources and ensuring the safety of the seafood we eat.

In this chapter, we will discuss how microfluidics can provide cheap and portable systems to help marine biotechnology to study marine ecosystems, to protect human health and safety, and to provide resources for research, using a reduced amount of samples in a shorter time when compared with conventional methodology.

20.1 Basic Principal of Microfluidics

Microfluidics encompasses many multidisciplinary areas, including physics, chemistry, engineering, and biotechnology. More precisely, it deals with the science and technology of systems that manipulate very small fluid volumes, 10^{-9}–10^{-18} L, through channels with dimensions in the range of tens to hundreds of micrometers [20.4]. Microfluidics emphasizes many advantages compared to conventional fluidic handling and may be summarized as follows: lower consumption of sample volumes, less reagent wastage, cost savings of samples, faster reaction times, faster diffusion, quick heating and cooling, better process control, and integration of reaction steps on a single chip [20.5, 6]. Integration is the key part of microfluidics, and integrated microfluidic systems manipulate very small fluid volumes in micro/nanometer scale channels for the automation and integration of many biological or chemical processes [20.4]. However, the integration aspect of microfluidics is still very young, and hence to fully utilize the above-mentioned advantages further engineering innovation of different strategies and optimization processes is required [20.4].

The seed of microfluidics started around three decades ago with the development of gas chromatography and the inkjet printer [20.5]. Since then a significantly large number of applications of microfluidics has been successfully demonstrated for chromatography, electrophoretic separation systems, electro-osmotic pumping systems, diffusive separation systems, micromixers, deoxyribonucleic acid (DNA) amplification, cytometers, and chemical microreactors, nearly in chronological order [20.6]. In recent years, integrated microfluidic systems have captured diverse application fields such as flow cytometry [20.7], protein crystallization [20.8], DNA extraction [20.9], digital polymerase chain reaction (PCR) amplification [20.10], stem cell culture [20.11], and for synthetic ecosystems consisting of *Escherichia coli* populations [20.12], determination of enzyme kinetic parameters with a single experiment [20.13], dose response analysis [20.14], catalytic competence analysis [20.15], and cytotoxicity analysis [20.16].

The field of microfluidics has many peculiar features from physics and chemistry, and because of this the above-mentioned advantages of microfluidics could be exploited successfully and few of these features are explained below.

20.1.1 Laminar Flow

Laminar flow consists of well-streamlined flow where particles move parallel to the direction of flow. The flow inside the microfluidic channels is characteristically laminar. The presence of laminar flow inside the microchannel is decided by the value of Reynolds number and by ensuring the parabolic velocity profile (Fig. 20.1a).

20.1.2 Surface Area to Volume Ratio

Surface area to volume ratio (SAV) could be defined as the ratio of surface area to the volume of fluid under consideration. In microfluidics, SAV increases significantly with a decrease in channel dimensions. A higher SAV value increases the rate of reaction and heat transfer. A higher SAV value creates adsorption of

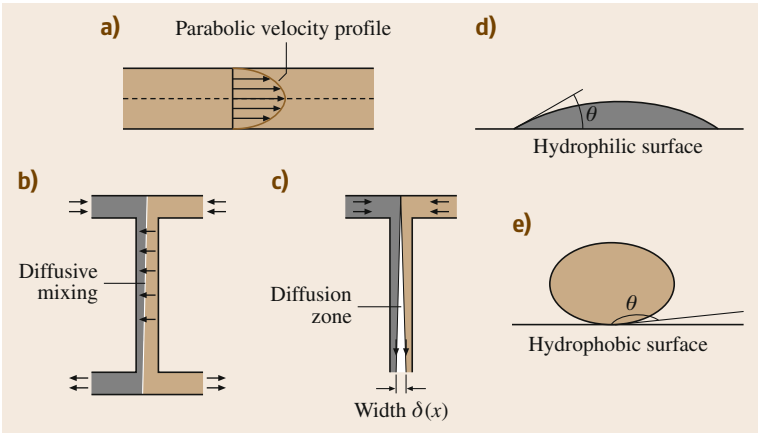


Fig. 20.1 Physical features involved in microfluidics. **(a)** Laminar flow in microchannel, **(b)** diffusion between two coflowing fluids, **(c)** diffusive mixing between two co-flowing laminar flows, **(d)** droplet on hydrophilic surface, **(e)** droplet on hydrophobic surface

macromolecules to the channel wall. A higher SAV of liquid in the microchannel helps in conducting efficient capillary electrophoreses.

20.1.3 Diffusion

Diffusion arises when two fluids with a concentration gradient come into contact with each other (Fig. 20.1b). In microfluidic systems, diffusion is an inefficient way of mixing two or more fluids because of the presence of a laminar regime. Mixing at microscale mainly occurs by the process of diffusion and due to the absence of turbulence.

20.1.4 Mixing

In microfluidics, quick mixing of fluids flowing through the microchannel is one of the key challenges. In the microchannel, the Reynolds number is very small due to the smaller channel dimensions. In the laminar flow regime, mixing of fluids occurs mainly by diffusion and, hence, happens very slowly (Fig. 20.1c). To solve this problem, various micromixers, such as chaotic flow micromixers, electrokinetic micromixers, peristaltic micromixers, etc., have been developed in recent years [20.17–21].

20.1.5 Wetting

Wetting is an interfacial phenomenon due to the balance of surface energies at gas-liquid-solid interfaces. The value of contact angle (θ) determines the type of wetting on the surfaces, viz. hydrophilicity and hydrophobicity. The behavior of fluid wetting on hydrophilic and hydrophobic surfaces is shown in Fig. 20.1d and e, respectively. The principle of wetting is exhaustively used in droplet-based microfluidic devices [20.4, 15].

20.2 Microfluidic Devices for Marine Biology and Ecosystem Studies

A major challenge in the field of marine biotechnology is to develop an efficient procedure for the discovery of novel biomolecules in the marine environment or marine bioprospecting. Because of the high level of biodiversity of marine organisms, an understanding of marine biology and ecology is an important first step to the discovery of new active molecules.

20.2.1 Microfluidics for Cell Analysis (RNA Amplification, Single-Cell Analysis, and Fish Embryology)

In this part, we will describe how microfluidic systems can be used to study marine organisms at different levels: molecular (genomics), cellular, and multicellular (tissues and the whole organism).

Genomics

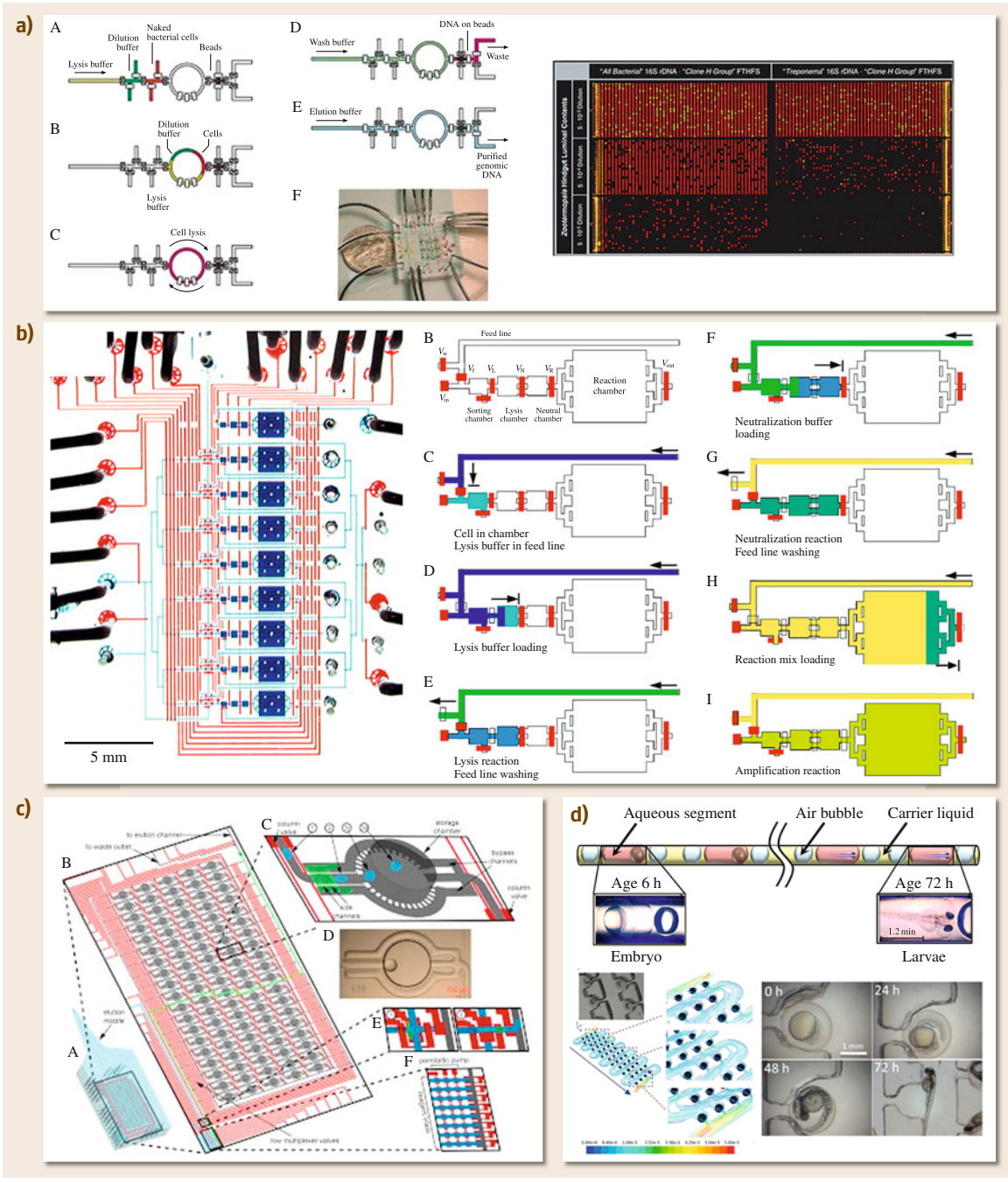
Genome sequencing is a powerful tool that can be applied to explore the genetic materials of organisms, such as microorganisms [20.22–24]. Recently, single-cell genomics (SCG) emerged both as a potent complement to cultivation and metagenomics [20.25] and as an independent analytical tool [20.26, 27], by providing genomic information and delivering unequivocal information about the organization of the genes discovered within genomes [20.28–34]. However, to obtain high-quality SCG, expensive and large instrumentation (e.g., cell sorters, robotic liquid handlers, DNA sequencers) and infrastructure (clean rooms, high-performance information technology (IT)) are required, which limit their use in most individual research groups. Microfluidic technology provides an alternative to conventional methods, offering numerous advantages including automation, enhanced sensitivity and reaction efficiency in small volumes [20.35, 36], and the potential for scalable and cost effective small volume assays [20.37] that can be used for genomics (Fig. 20.2a) [20.9, 38]. It effectively addresses the three classes of contamination with the potential to compromise single-cell amplification reactions: the laboratory environment, amplification reagents, and cells/DNA from the sample itself [20.39]. Microfluidic devices produced by *Blainey* et al. illustrate the capability to perform SCG analysis on a chip [20.39]. The device, shown in Fig. 20.2b, originally developed by *Marcy* et al. [20.26], was used to perform multiple displacement amplification (MDA) reactions with nanoliters of reagent. The microfluidic chip was composed of 32 microchambers implemented with a laser tweezer system [20.40, 41]. A mixture of ammonia-oxidizing archaea (AOA) microorganisms was introduced into the device. Single cells, smaller than 1 μm, were selected upon microscopic inspection and then guided with a laser trap

into separate chambers, where each sorted cell was lysed individually. The genome of each individual cell was then amplified by MDA in 50 nL volumes [20.29]. Amplified genomic DNA from each cell was then separately recovered from the chip and sequenced by high-throughput DNA pyrosequencing. Combining SCG genome amplification with metagenomics, *Blainey* et al. sequenced and identified the genome of *Candidatus Nitrosoarchaeum limnia* SFB1, an AOA from low-salinity sediments in San Francisco Bay, using single-cell and metagenomic genome sequence data [20.39]. Another interesting example demonstrates the possibility of combining different genome analysis techniques in the same chip for marine cell genome analysis. *Leung* et al. [20.42] developed a microfluidic device capable of running multiple single-cell applications, such as phenotypic sorting of bacteria followed by clonal analysis of growth rates, taxonomic identification of single bacteria by small subunit ribosomal ribonucleic acid (RNA) gene quantitative PCR (qPCR) and sequencing, and high-throughput single-cell whole genome amplification (WGA) and sequencing. Interestingly, this device, shown in Fig. 20.2c, combines a programmable droplet-based microfluidic device with the reconfigurable flow-routing capabilities of integrated microvalve technology. It is composed of 95 individual microchambers, a reagent-metering module, a cell-sorting module, and an integrated nozzle that allows for automated recovery of on-chip reaction products without cross-contamination. A three-valve peristaltic pump is used to deliver arbitrary picoliter droplets of eight separated reagents in discrete increments from eight separate inlets. Reagent droplets are dispensed directly into a flowing stream of carrier fluid; delivered to a selected storage chamber it merges with any previously dispensed droplets. When a cell of interest is identified, it is pumped into a droplet and delivered to a microchamber where it can be analyzed by qPCR and WGA. This device was used to analyze the genome of single cells and microbial consortia from diverse environmental samples, such as marine enrichment cultures and deep-sea sediments.

Cell Analysis

A variety of analytical methods have been deployed to identify and study populations of marine microorganisms with a view to understanding both the organisms and their behavior. Optical microscopy is generally used to study marine microorganisms but the large ratio between the size of the target cell and the size of the culture system (plate, petri dish, or tube) makes the study labor intensive and time consuming. In the early 1980s, flow cytometry was introduced as a complementary technique to analyze mixed microbial populations [20.30, 31, 45]. However, the high cost and the large size of the apparatus limited its use as a standard tool for laboratory-based research and in situ analysis. Toward the realization of a portable and affordable microfluidic cytometer, *Benazzi* et al. [20.46] have developed a microfluidic chip capable of high-speed analysis of phytoplankton. The chip was made from a glass substrate with planar microelectrodes patterned on the surface. A microfluidic channel made of polydimetylsiloxane (PDMS) was bonded on the surface of the patterned glass substrate, perpendicularly to the microelectrodes. The chip was placed above a microscope objective and the cells were pumped into the microchannel with the use of a syringe pump. The phytoplankton flowed through the detection zone where optical and electrical properties were measured simultaneously. When a mixture of different species of phytoplankton (here *Synechococcus* sp., *Rhodosorus m.*, and *I. galbana*) were introduced into the chip, measurement of the optical properties differentiated cells in function of their autofluorescence, while electrical

Fig. 20.2a–d Microfluidic system for genome, cell, and whole organism studies. **(a)** *Top*: DNA purification chip and schematic diagram of one instance of the DNA isolation process. *Bottom*: multiplex digital PCR platform for single-cell analysis in the environmental sample for amplification of *all bacteria* 16S rDNA and termite cluster 16S rDNA (red fluorescence) and clone H FTHFS gene (green fluorescence), (after [20.9] and [20.38]). **(b)** *Left*: picture of the single-cell genome amplification device. *Right*: step-by-step process (after [20.35]). **(c)** Programmable droplet-based microfluidic device applied to multiparameter analysis of single marine microbes and microbial communities. *Left*: schematic representation of the entire chip. *Right* (from top to bottom): storage element geometry for droplet immobilization and coalescence by flow-controlled wetting. Micrograph of a water droplet in a single chamber. Graphic representation of the cell-sorting module and of the reagent-metering module (after [20.42]). **(d)** Microfluidic chips for fish embryology study. *Top*: schematic representation of microfluidic segment technique for screening and development studies on fish embryos. *Bottom*: photo and 3-D streamline of flow obtained by computational fluid dynamic simulations across the fully loaded device. Time-lapse images of developing fish embryos collected every 24 h (after [20.43, 44]) ▶



impedance, measured at different frequencies, characterized the cells in function of their size and the capacitance of their membrane. The results obtained by this microfluidic flow cytometer were comparable with those obtained with a commercial flow cytometer, and the low-frequency electrical impedance signal used to size the particles was analogous to the forward scatter signal obtained in a flow cytometer. However, the mi-

croflow cytometer, with its present dimensions, was not able to measure particles with sizes $< 2\,\mu m$.

In addition to the distinction between the different cells in the same solution, microfluidic technology can be used to separate cells with different characteristics to study their functions. *Sasaki* et al. [20.47] developed a microfluidic device to separate bioluminescent bacteria in function of their motility. The device is made of PDMS and composed of two inlets connected to two separated channels that merged in one main channel. The end of the channels, which is 35 mm long, was split into two channels connected to two outlets. This configuration of the microchannel allows the formation in the main channel of two parallel flows, each coming from a different inlet, without mixing the two liquids. *Sasaki* et al. used this property to separate luminescent *Photobacterium kishitanii* bacteria according to their motility [20.47]. The cell suspension was introduced in one inlet and filtered liquid broth in the other. In the main channel, motile bacteria swim and crossed the flow to reach the liquid broth while nonmotile cells remain in the cell suspension flow. Suspensions collected from both outlets were analyzed. The luminescent intensity of the separated samples was measured using a luminometer. The luminescent intensity per cell was calculated. The result was that the value from the mobile (swimmers) bacteria was larger than the nonmobile cells (nonswimmers).

So far, very few systems have been used to study marine cells. However, other microfluidic reactors have been developed to analyze the cell proteins from multiple or single cells [20.37, 38, 48–51], which could be used in marine biotechnology.

Tissues and Whole Organisms

Fish embryogenesis-based biotechnologies have various promising applications in the field of marine biotechnology, from commercially valuable fish production to threatened marine species protection [20.52]. Conventionally, handling of embryos, treatment, and analysis are performed manually, in 96–384 microtiter plates, [20.53–56] and combined with laborious imaging methodology [20.57]. Under such conditions, analysis and imaging of fish embryos and juveniles in a high-throughput and high-content manner remains challenging [20.53, 55, 57, 58]. These limitations can, however, be overcome with miniaturized LOC technologies that can provide an automated platform for the manipulation and immobilization of micron-sized organisms [20.53, 59–62] and for high-throughput screening [20.63]. Recently, several microfluidic-based methodologies have been developed that involve droplet-based microfluidic systems [20.43, 64] or continuous-flow systems [20.44, 65–68]. Droplet-based microfluidic systems were the first technology to be used for controlled fish embryogenesis. In 2006, *Funfak* et al. studied the behavior of multicellular systems, in particular embryonic development in a microfluidic system [20.43]. The system, which is schematically represented in Fig. 20.2d, is composed of a Teflon microtube in which fish eggs in aqueous solution are introduced into the tube, perfused with immiscible perfluoromethyldecalin (PP9), resulting in droplets containing one single embryo each. The development processes of fish embryos were observed over a time period of 80 h. Finally, after 5 days, the fish larvae were collected from the droplets and transferred into breeding reservoirs for further experiments. More recently, *Akagi* et al. introduced a miniaturized embryo array that automatically traps, immobilizes, and perfuses fish embryos [20.44]. The device, which is shown in Fig. 20.2d, is composed of a serpentine channel that contains embryo traps (where embryos are trapped and immobilized), suction channels (to attract embryos in the embryo trap), and hydrodynamic deflectors (to enhance embryo trapping). Embryos were loaded in the chip one by one every 5 s. After entering the serpentine channel, embryos rolled on the bottom surface of the main channels under the influence of drag force. Embryos approaching an empty trap were affected by the flow passing through the suction channel and directed toward the trap, which was designed to assure single-embryo occupancy, and unobstructed passage of other embryos in the main channel. The following embryo rolled freely in the serpentine channel toward the next available trap. The process was repeated until all traps were occupied. After loading, the chip was perfused for long-term culture (up to 72 h), and normal and uniform development of all embryos was observed. Although most microfluidic technology techniques for fish embryological studies were performed on *Danio rerio* (a freshwater fish), one can easily transpose those techniques to marine fish embryogenesis studies and engineering.

20.2.2 Microfluidic Systems for Cell Behavior Studies

In the last decade, microfluidic systems have been widely used for the study of cell behavior, mainly with respect to how cells sense mechanical, electrical, and chemical signals, and for the measurement

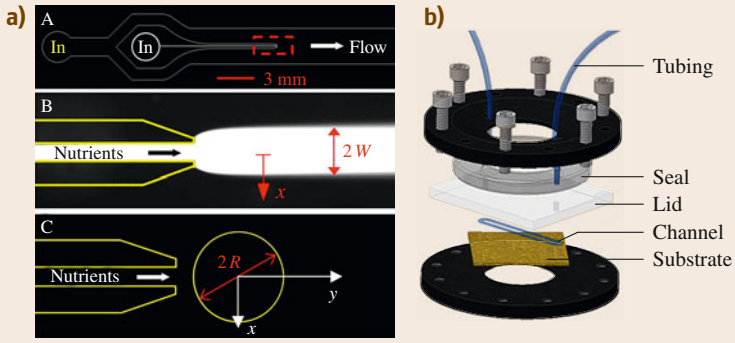


Fig. 20.3a,b Microfluidic system for cell behavior studies. **(a)** Microfluidic system for the study of marine bacteria motility response to microscale nutrient patches (after [20.69]). **(b)** Microfluidic setup for the study of marine bacteria biofouling on modified substrate (after [20.70])

of events and their biological responses surrounding and within cells. More specifically, the study of cell behavior in microfluidic systems includes: cell growth [20.11], differentiation [20.71], signal transduction [20.72], protein secretion and transport [20.73], gene expression [20.74], cell and extracellular matrix behaviors [20.75], and cell cytoskeleton dynamics [20.76]. Applied to marine microorganisms, microfluidic systems have been successfully developed for the study of cell behavior. For example, to investigate how marine bacteria are capable of exploiting ephemeral nutrient patches generated at environmentally realistic spatiotemporal scales, *Stocker* et al. [20.69] used a simple microfluidic system (shown in Fig. 20.3a) that creates two types of patches (a purely diffusive pulse and a plume governed by diffusion and advection) with spatial and temporal scales consistent with those expected in the ocean [20.77]. By simultaneously measuring the spatiotemporal distribution of nutrients and cells, they quantified the nutrient exposure experienced by chemotactic marine bacteria, indicating that the response time scale of opportunitrophs can be commensurate with patch evolution time scales, enabling the utilization of a wide range of patchily distributed nutrient resources in the ocean.

Arpa-Sancet et al. developed a microfluidic system to quantify the adhesion of marine bacteria [20.70]. The quantification of bacterial adhesion is of major importance for the understanding and support of the development of new materials with environmental or medical applications. For marine application, the colonization of submerged surfaces (artificial or natural) by undesired biological organisms is a major problem for many marine industries, which results in environmental and economic penalties [20.78–80]. With increasing restrictions in the application of biocidal antifouling, fouling-release (FR) coatings are currently considered as an alternative. Bacteria are among the first microorganisms to colonize submersed interfaces to form biofilms [20.78] that will influence subsequent colonization by invertebrates, algae [20.81], and tube worms [20.82, 83], resulting in fine in ship damage [20.84]. The microfluidic chip is composed of a single microchannel in PDMS sandwiched between a glass window and the coated surface, as shown in Fig. 20.3b. The window, the channel, and the sample of interest were mechanically held together by two disks connected by screws. Different shear stresses were generated by flowing liquid in the channel at different pressure (between 0.002−700 Pa, resulting in shear stresses between 0.02−7000 dyn/cm^2). Observation with an optical microscope showed that in these conditions, bacteria detached when a shear stress between 40−200 dyn/cm^2 was applied. To quantify bacteria adhesion, microfluidic assays have a number of advantages: the adhesion strength can be quantified on relatively small sample areas with only small amounts of bacteria in a very short time (few hours), and different substrates can easily be tested in parallel. The main advantage of a microfluidic assay lies in the possibility of analyzing the effect of the variation of the shear force across several orders of magnitude on the adhesion on the substrate of each single cell [20.85–87].

Microfluidic Systems for Water Analysis

Seawater analysis is essential in marine biology for the exploration and classification of underwater environments, particularly when investigating the distribution and diversity of microbial communities, underwater marine geology [20.89], and various other marine biotechnology-based industrial applications, including civil and environmental monitoring, soil and geotechnical engineering, plant and crop science, food, and bio-based and industrial chemistry.

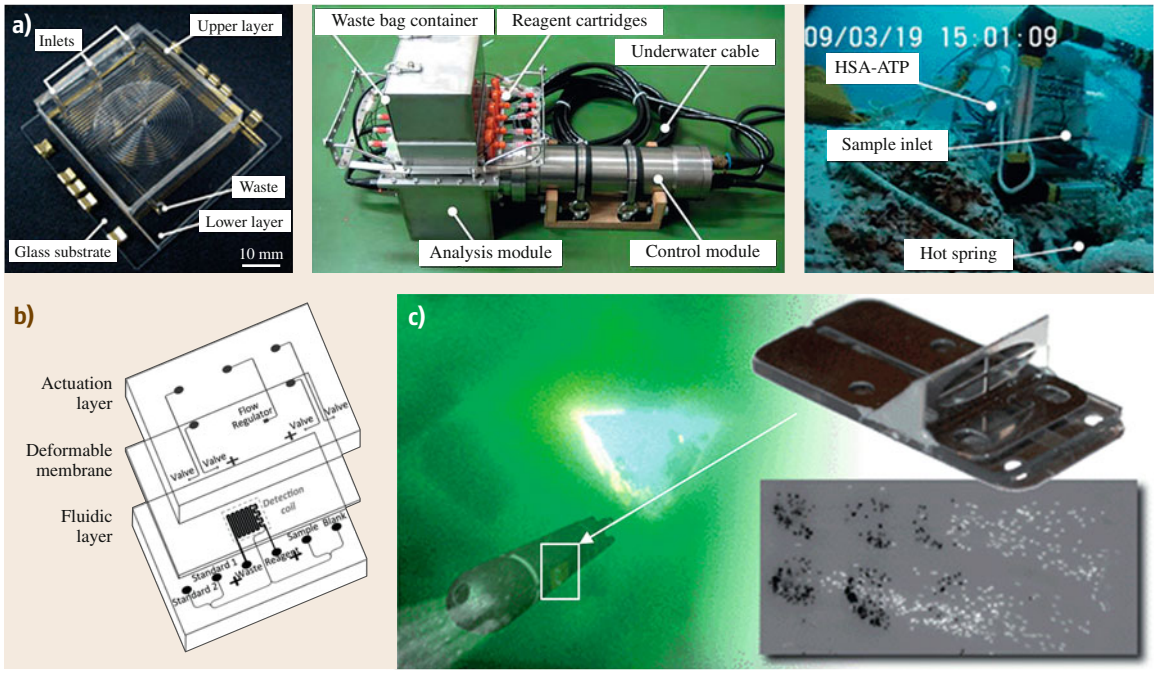


Fig. 20.4a–c Microfluidic systems for in situ water analysis. **(a)** Microfluidic in situ analyzer for ATP quantification in ocean environments. *Left*: picture of the microfluidic chip. *Middle*: photo of the completed IISA-ATP system for in situ operation. *Right*: photo of the IISA-ATP on the seafloor during the in situ operation at the bubbling site in the Taketomi submarine hot spring area (after [20.88]). **(b)** Schematic exploded view of the integrated microfluidic device for manganese anomaly detection composed of three layers: actuation, membrane, and fluidic layers (after [20.89]). **(c)** Representation of a microfluidic system for yeast cell detection at a depth of 1200 m attached to a submersible. *Right, top*: photo of the microchip. *Right, bottom*: images of the particles captured on the device (after [20.90])

Microfluidic systems have been developed to detect the presence of ion concentration in seawater, [20.89], which is essential for the maintenance and management of reinforced concrete structures in harsh saltwater environments [20.91, 92], and to detect and evaluate new hydrothermal sources in the sea [20.89]. However, the main advantage of using microfluidic systems for seawater analysis is the possibility of integrating fully functional LOC in submersible vehicles for in situ seawater analysis. The use of submersible vehicles as carriers for microfluidic LOC allows the investigation of hardly accessible environments, such as large deep-water or ice-covered bodies of water and can replace the large and powerful pump needed for sample collection and filtration. Such microfluidic systems have been used to analyze the composition of seawater in microbes by detecting the presence of adenosine triphosphate (ATP) in water (Fig. 20.4a) [20.88] or by trapping and collecting microorganisms, such as yeast, for further investigations (Fig. 20.4c) [20.90]. For example, *Fukuba* et al. [20.88] developed and tested a functional IISA (IISA) system for the detection of ATP, called IISA-ATP (shown in Fig. 20.4a), for microbial activity assays based on the total ATP quantitative determination in ocean environments. The device utilizes a PDMS-glass hybrid microfluidic device as its core functional element and can perform cell lysis and total ATP quantification by a luciferin–luciferase bioluminescence assay in situ. As a result of the evaluation using the microfluidic device with ATP standard solutions, the bioluminescence intensity was linearly correlated with 2×10^{12} to 2×10^{8} M of ATP, with a detection limit of 11.1×10^{11} M. The system was successfully operated in situ at a shallow submarine hot spring area in Okinawa, Japan, and the total ATP was determined to be 3.4×10^{10} M [20.88]. Another example was presented by *Provin* et al. [20.89], who created a miniaturized and integrated microfluidic system (Fig. 20.4b) for the detection of Mn in deep-sea environments, called ISSA for Mn^{2+} (IISA-Mn). This

high-sensitivity detection system is based on the chemiluminescence reaction of Mn contained in the seawater sample with a luminol-based reagent. It is composed of a microdevice for mixing and reaction, a pumping unit, several valving units, and a photomultiplier (PMT) detector. Mn concentrations above 280 nM in seawater were detected, and a quite linear response was obtained until 500 nM. In addition, this device worked continuously during the 8 h of an actual remotely operated vehicle dive and led to the discovery of a previously unknown hydrothermal site in the Okinawa Trough.

20.3 Microfluidic Devices for Sea-Related Health and Safety

Microfluidic-based marine biotechnology provides a promising approach for rapid and accurate in situ detection of toxic microorganisms, toxins, or pollutants that can threaten human and animal health and safety.

20.3.1 Microfluidics in Toxic Algae Detection

The detection of algae proliferation in drinking water and marine recreational water is an important environmental and public health issue. Traditional laboratory-based identification of algae in water requires sophisticated and expensive procedures that result in delays of effectively reacting to an outbreak. However, increased governmental and public concerns on algae-related health and safety issues has raised the demands for low-cost, portable, and real-time algae monitoring. The development of microfluidic devices provides a promising approach for toxic algae detection. To date, optical detection [20.93–95], flow cytometry [20.95, 96], electrical impedance [20.97–99], and nucleic acid amplification [20.38, 100] are successfully exploited on microfluidic platforms.

Optical detection has been use for algae detection based on shapes, structures, or pigments. *Schaap* et al. [20.101, 102] developed a femtosecond laser to construct a waveguide in a microfluidic device (Fig. 20.5a) to qualitatively distinguish various algae types in a water stream by detecting the distinctive wavelets that are associated with the particular algae geometry. This sensing principle relies on distorted transmitted wavelet-like signals induced by an alga crossing a monochromatic coherent light beam when moving along a microfluidic channel.

Flow cytometry is a common optical approach used to detect algae by measuring the a number of optical and side scatter properties. For example, the ratio of chlorophyll (CHL) to algae size (side scatter signals) and/or to phycoerythrin (PE) or phycocyanin (PC) has been used to identify different algae with conventional macroscale experiments [20.105]. In 2007, *Benazzi* et al. [20.46] developed a microfluidic flow cytometry that measures fluorescence, which was used to distinguish three mixed algae with similar results to those obtained from a commercial flow cytometry. More recently, *Hashemi* et al. [20.103, 106] developed an improved microfluidic flow cytometry for algae species evaluation using CHL, PE, and side scatter. The chip was composed of chevron grooves, as shown in Fig. 20.5b, combined with PDMS-embedded optical fibers, and external optical filters and photomultiplier tubes were used to collect fluoresced and scattered lights. The device generates a sheath stream that focuses the sample stream in the center of channel horizontally and reduces the impact of pulses from the peristaltic pump on the sample stream. The chevron grooves formed on the top and bottom of channels directs the sheath flow toward the top and bottom to compress the sample stream vertically.

Impedance spectroscopy is a noninvasive electrical method used for measuring size and dielectric characterization of cells when they pass over electrodes that have an alternating current (AC) potential. This method has been applied to LOC devices to differentiate cells [20.107, 108]. *Benazzi* et al. [20.46] developed a microfluidic device integrated with a impedance spectroscopy and fluorescence measurements to distinguish a mixture of three algae. A microfluidic channel made from PDMS was placed on a glass substrate where microelectrodes were patterned (Fig. 20.5c). Two frequencies (327 kHz and 6.030 MHz) were used to measure the electrical impedance; the lower frequency was chosen to measure the cell size, while the higher one was used to probe small changes in the membrane capacitance of the cells. The magnitude of the electrical impedance signal and fluorescent measurement were used to resolve three algae species (Fig. 20.5c).

The detection of harmful marine organisms can be achieved by using a benchtop isothermal nucleic acid sequence-based amplification (NASBA) method [20.109]. Recently, a hand-held system integrated

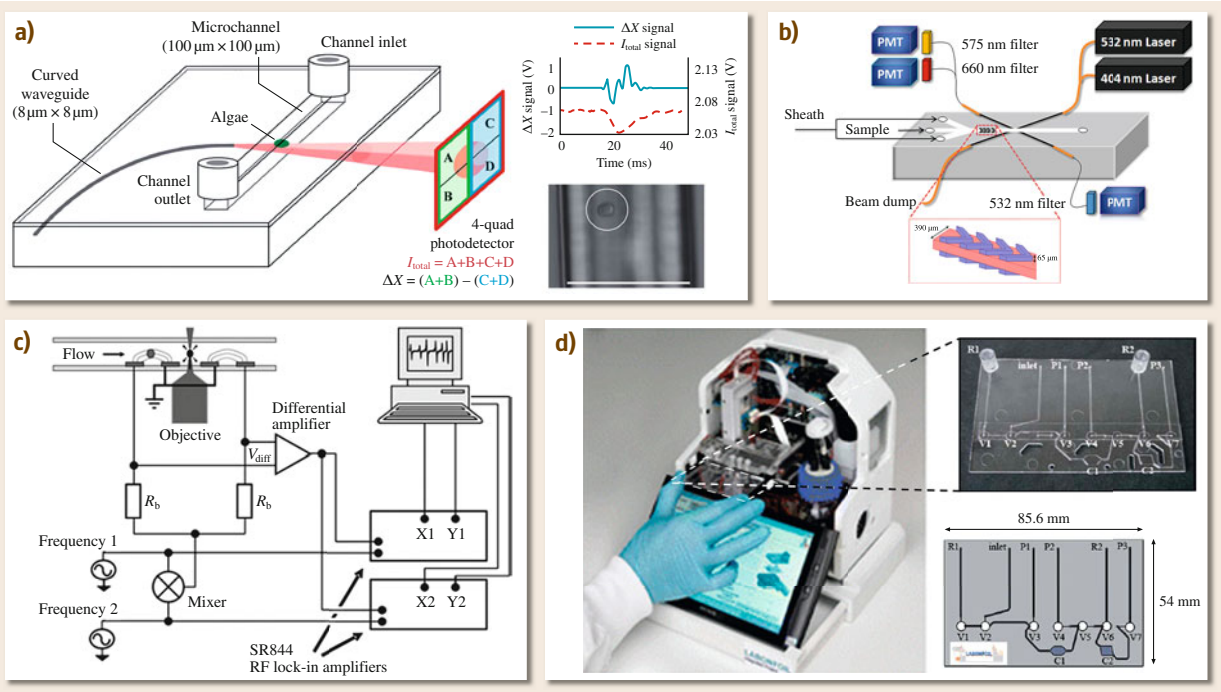


Fig. 20.5a–d Microfluidic devices for toxic algae detection. **(a)** Schematic representation of a microfluidic device for algae classification based on optical detection (after [20.102]); **(b)** microfluidic flow cytometer for algae detection (after [20.103]); **(c)** schematic of an microfluidic impedance spectroscopy flow cytometer; **(d)** toxic marine microalgae concentration and lysis, RNA extraction/purification and quantitative RNA detection in microfluidic device (close-up). Illustration of the microfluidic chip (called lab-card), (after [20.104])

disposable microfluidic cartridges for cell concentration, lysis, RNA extraction/purification, and quantitative RNA detection was developed to detect harmful algal blooms [20.110]. The microfluidic cartridges (lab-cards) were fabricated by injection molding into a cyclic olefin co-polymer (COC). Fluid control was achieved by using seven polymer valves integrated within the lab-card. The cell sample and reagent master-mix were prepared and preserved in the lab-card. The lab-card reader system is an assembly of fluid control, temperature control, real-time fluorescence detection, and tablet computer. This portable analytical system (shown in Fig. 20.5d) was demonstrated to address the significant demand for in situ phytoplankton analysis [20.104].

Microfluidics in Marine Toxin Detection

Of the 4000 marine planktonic microalgae, around 80 species (mainly dinoflagellates and diatoms) are known to produce marine toxins (phycotoxins) that are potentially harmful to shellfishes, fishes, and humans, causing direct or indirect impacts to fisheries, aquaculture, tourism, and even public health [20.112]. The toxins produced by algae accumulate in fish, crabs, or shellfish and can cause severe intoxication when substantial amounts of contaminated seafood are consumed by humans. In order to protect human health from seafood poisoning, legislation has been established worldwide to limit and monitor the toxin content of seafood. Currently, the most widely used methods are in vivo bioassays (mouse bioassays and rat bioassays) that are conducted by administrating seafood extracts to laboratory animals and monitoring symptoms and time to death. However, the lack of specificity and sensitivity, the high rate of generating false positive results, and the ethical concerns of using laboratory animal are making this method unacceptable [20.113–115]. In vitro cytotoxicity assays have been developed as an alternative to in vivo bioassays, using cell types and cytotoxicity markers to detect marine toxins. For example, immuno-based methods, such as enzyme-linked immunosorbent assay (ELISA),

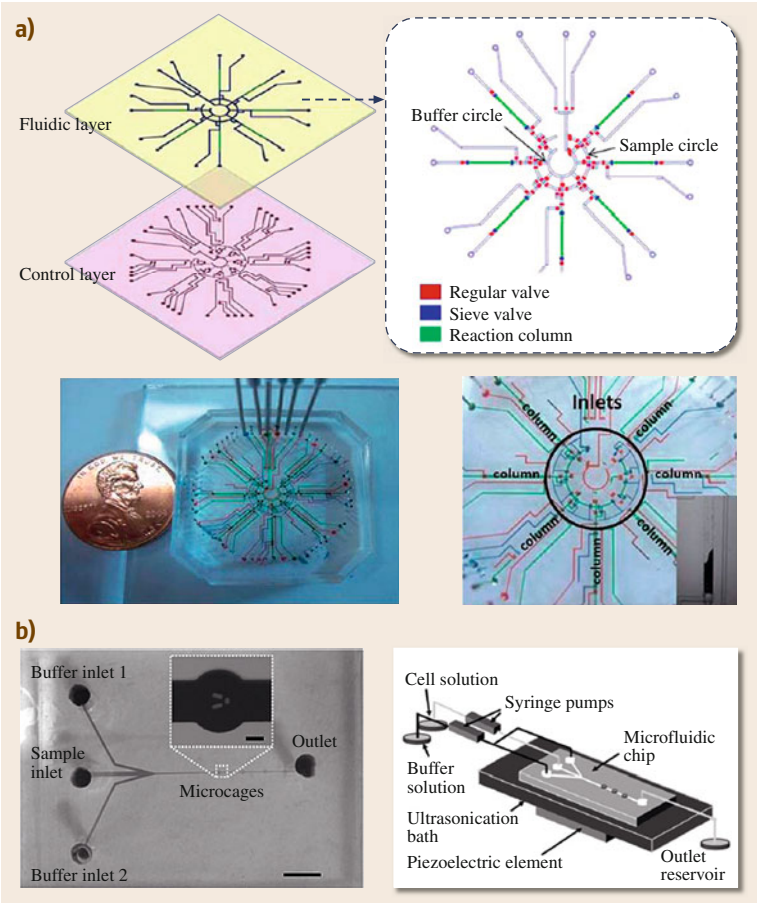


Fig. 20.6a,b Microfluidic device for algal toxin detection. **(a)** Immunoreaction chip used for rapid detection of algal toxin. *Top*: schematic representation. *Bottom*: optical micrography of the entire chip (*left*) and of the immune-reaction columns (after [20.103]) **(b)** Picture and schematic representation of a microfluidic device with cell trapping and lysis parts for toxin detection (after [20.111])

have been widely used to detect marine toxins. Analytical methods, such as liquid chromatographic and mass spectrometry techniques are used for the detection of toxins [20.113]. Recently, biosensors have been developed as fast, cheap, reliable, and more sensitive bioanalytical tools to screen toxicity of marine samples [20.116]. However, this methodology remains time consuming, which limits the capability to promptly provide an adequate answer to protect the population from food-borne intoxication.

Microfluidic-based analytical methodologies are a promising alternative as they provide rapid and accurate tools for marine toxin detection and analysis, and can bring the detection process on the field for in situ analysis. A microfluidic chip-integrated ELISA has been developed for rapid and automatic analysis of algal toxin [20.117]. The platform includes a core microfluidic chip, cartridges for reagents, a digital camera, the control system, digital inlet/outlet (I/O), and a laptop computer. The two-layered PDMS microfluidic device shown in Fig. 20.6a was fabricated to complete immune reactions based on a succession of specific steps. First, protein A coated microbeads were loaded into the chip. Then a secondary antibody was introduced and incubated with the beads, followed by the introduction of the toxin-specific antibody. Finally, the antigen and HRP-labeled (HRP: horseradish peroxidase) antigen were introduced, incubated, and revealed with load enzyme substrate solution. The fluorescence signal after enzyme catalysis reaction was measured. The use of this microfluidic-based detection system significantly reduced the time to detect toxins two to fourfold, with the limit of detection (LOD) much lower than that of common methods.

Recently advanced microdevices, such as microelectromechanical systems (MEMS), enable handling and processing of biological cells [20.121]. With one

of these devices, microfluidic methods can be used for single marine cell isolation to detect and study toxin [20.122]. *Wu* et al. [20.111] reported a microfluidic method to trap and lyse single marine algae prior to toxin detection. The system, shown in Fig. 20.6b, contained 3 microcages in a single channel for cell trapping and was coupled to an ultrasonic method for cell lysis to prevent chemical reagent contamination which can observed in conventional cell lysis methods. After lysis, toxin from the algae can be collected for further analysis. This method can be further incorporated into a biodetection system for in-field rapid toxin detection.

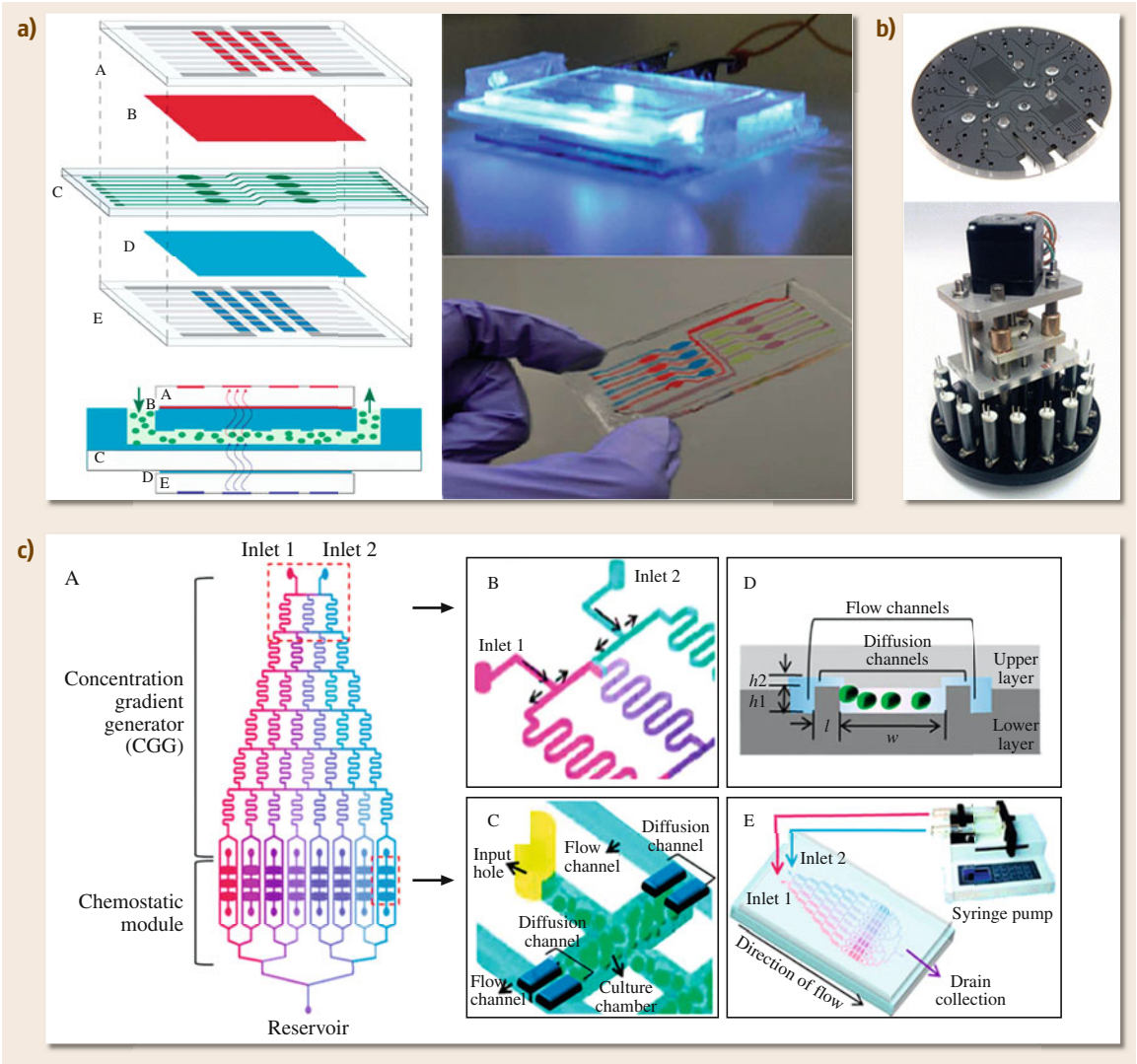


Fig. 20.7a–c Microfluidic devices for water pollutant monitoring. **(a)** Microfluidic chip integrated with a fluorescent sensor. *Left*: exploded and edge view. *Right*: assembled microfluidic chip illuminated with OLED (*top*) and colored with dyes (*bottom*) (after [20.118]). **(b)** PMMA microfluidic chip with micrometer scale fluidic channels (*top*) and the assembled analysis system (*bottom*) (after [20.119]). **(c)** Illustration of a microfluidic chip for toxicity assessment of heavy metals based on measurement of mobility of marine microalgae. A) Layout of the integrated microfluidic device, B) close-up of the mixing process of two solution (represented by colors) in the concentration gradient generator, C) close-up and D) cross-section of the chemotactic module, and E) representation of the experimental set-up (after [20.120])

20.3.2 Microfluidics in Sea Pollutant Detection

The rapid industrialization in chemical, biological, and medical fields has resulted in the release of toxic chemicals or potentially harmful pollutants into water sources, including sea, groundwater, and rivers. These pollutants are a great threat to human health. Therefore, a rapid and highly sensitive trace analysis of water pollutants and minerals in marine and different water sources needs to be investigated according to the stringent guidelines provided by different environmental protection agencies (EPA). Thus, the development of a highly sensitive detection system for monitoring trace amounts of various pollutants and minerals has become very important. A number of bulky and conventional systems based on fluorescence, chromatographic, capillary electrophoresis, chemiluminescence, and electrochemical detection methods were reported previously. However, these bulky methods have many limitations, such as a long sample preparation time, a low detection limit, a lengthy measurement time, and indirect measurement results.

Recently, there have been reports of various portable microfluidic systems with extreme sensitivity for pollutants and minerals, such as phosphate levels in treated wastewater [20.123] or fecal indicator in coastal recreational water [20.124]. Another interesting approach uses whole-cell biosensor technology to detect pollutants like herbicides and metal ions from agricultural run-offs [20.125]. This technology presents great advantages compared with conventional methodology, including portability, low cost, ease-to use, and real-time monitoring. Unicellular microalgae have been extensively exploited for evaluating toxicity of pollutants in aquatic ecosystems, due to their high sensitivity to a number of pollutants and reproducibility [20.126]. Algae-based biosensors for water pollutant detection have been reported by many groups [20.126–128]. For example, a microfluidic platform for water pollutant detection was developed to improve detection sensitivity by integrating an algal fluorescence sensor [20.118]. This device consists of an organic light emitting diode (OLED) and an organic photodetector (OPD) integrated into a microfluidic chip. OLED and OPD were used to detect algal fluorescence, as shown in Fig. 20.7a. The presence of herbicides or metal ions was detected with the diminution of the algae's chlorophyll fluorescence. This device demonstrated a 10 times higher sensitivity ($\approx 10^{-8}$ M) towards herbicide than values detected with a commercial fluorescence sensor, and 100 times higher compared to amperometric algal biosensors. Another device developed by *Beaton* et al. has demonstrated the possibility to measure with high resolution in situ nitrate and nitrite [20.119]. The circular poly (methyl methacrylate) (PMMA) microfluidic chip shown in Fig. 20.7b contains a fluidic manifold that allows selection of standards, sample, and blank solutions. The chip integrated a Griess assay and a spectrophotometric method for colorimetric nitrite analysis. This LOC system demonstrates the potential of nutrient analysis for inclusion in large-scale networks for ocean pollution monitoring.

Another microfluidic device developed by *Zheng* et al. (Fig. 20.7c) [20.120] has been reported for the assessment of heavy metals in water, based on the measurement of motility of marine microalgae. The device consists of a toxicant concentration gradient generator where serial concentration gradients are generated by diffusion, and a chemostatic module where cells are seeded and migrate under different concentrations of toxic heavy metals. It is known that marine microorganisms are often motile [20.129], and heavy metals, such as, Cd and Cu, can directly inhibit cellular respiration processes that provide energy for motility [20.130, 131]. Therefore, a dose-response manner of Cd and Cu on the motility of two marine bacteria was observed on their microfluidic device.

20.4 Microfluidic Systems for Other Marine Biotechnology Applications

20.4.1 Synthesis of Marine Chemicals

Marine life forms are an important source of structurally diverse molecules with various biological properties, including toxic, pharmacological, and cosmetic ones, as well as being important in biomedical research. However, although nature provides structurally diverse molecules with varying biological activities, it is not always possible to obtain the required quantities of the desired molecule for research or commercialization; either because the quantity of raw material is not sufficient to obtain the required amount of active product of interest, or because active chemicals can only be isolated from endangered species protected by the Washington Convention (Convention on International Trade in Endangered Species of Wild Fauna and Flora). This

lack of material has inspired innovative chemistry that develops new methodologies for the synthesis and modification of natural products [20.132–135]. Still, these methodologies, realized at conventional macroscale, are labor intensive, time consuming, and expensive. In addition, scale-up from research to mass production remains troublesome because of the volume to surface ratio that affects the thermal and mass transport properties of the reaction and requires readjustment in the process at each stage of the scale-up.

Microreaction technology provides the means of addressing those limitations. Single microfluidic devices can be used in a laboratory to reduce the cost, time, and labor of the experiments. In addition, microfluidic technologies render easier scale-up of a reaction process from research to mass production, as multiple devices can be used simultaneously. For example, a recent study reported the use of microfluidic systems for the synthesis of pristine [20.136] (or 2,6,10,14-tetramethylpentadecane), a saturated isoprenoid isolated from the basking shark, *Cetorhinus maximus* [20.139, 140]. The synthesis of pristane involves different steps, namely oxidation, alkylation, dehydratation, and hydrogenation. In this process, dehydration is the most challenging part. When the reaction is performed at a gram scale, purity of the final product does not exceed 20%, even with repeated distillation or silica gel chromatography, which cannot be used for kilogram-scale mass production. Using ten commercially available Comet X-01 micromixers arranged in parallel (Fig. 20.8a), *Tanaka* et al. successfully performed an 8 kg-scale dehydration over 3–4 days in one batch [20.136]. The solution eluted from the micromixing system was then quenched, extracted, and concentrated. The mixtures were quickly passed through a short silica gel pad to remove hydrophilic byproducts. Finally, hydrogenation in the presence of 10% Pd/C under hydrogen atmosphere provided pristane in 50–55% overall yields (≈ 5 kg) with $> 99\%$ purity, based on gas chromatographic analysis. In addition, the synthetic pristane obtained with the microfluidic system was confirmed to induce antibody production in mice twice as efficiently as natural pristane or other synthetic products, due to the non-negligible contamination of the other hydrophobic compounds [20.136].

20.4.2 Marine Bacteria–Based Microrobot Fabrication and Control

With the development of MEMS and robotics [20.141–145], microrobotics has become the focus of attention worldwide for its potential applications in medical diagnosis, targeted delivery, in vivo sensing, cell manipulation, and material removal in the human body [20.146–149]. However, because of the size limitation, energy supply is always one of the bottlenecks in the development of microrobots [20.145, 149–151]. Recently, *E. coli* [20.152] and marine bacteria [20.137] have been successfully integrated in the design of microrobots and has been used as a propulsion system [20.151]. Obviously, the use of conventional cell culture system, such as flasks or plates, is not the most suitable for studying the control of microrobot behavior, because of the high surface ratio between the substrate and the bacteria. The ability to easily control biological conditions and the dynamic fluidic environment within the system [20.150–155] makes microfluidics ideal tools for microrobot-based studies. *Quifeng* et al. recently developed a microfluidic device to control and track marine magnetotactic bacteria (MBT) ovoid MO-1 cell-based microrobot (MBT-microrobot) [20.137]. The system is shown in Fig. 20.7b. In this study, MO-1 bacteria were used as a carrier system on which microbeads were attached. The control and tracking system consisted of an inverted microscope tracking device, a set of electromagnetic coil devices embedded in the stage of the inverted microscope brackets, and a microfluidic chip at the center of the set of the electromagnetic coil device. The microfluidic chip was made of PDMS and composed of a single channel with two pools, as shown in Fig. 20.8b. The control and tracking device could manipulate the movement of the MTB-microrobots within the microfluidic channels and trap the swimming cells within the pools where circular magnetic fields were applied, showing the possibility to use the circular field as a *stop control* system.

20.4.3 Seawater Desalination

The shortage of fresh water is one of the most important challenges facing today's world. Population growth, enhanced living standards, and the expansion of industrial and agricultural activities have created unprecedented demands on clean water supplies all over the world. Nowadays, nearly half a billion people in 31 different countries, mainly in Africa and the Middle East, suffer from water shortage. In 2025, this will grow to four billion people in 48 countries [20.156, 157]. Converting seawater, which represents about 97% of the total water resources on Earth, into freshwater may provide the solution to the worldwide water-shortage problem. However, current desalination methods, such

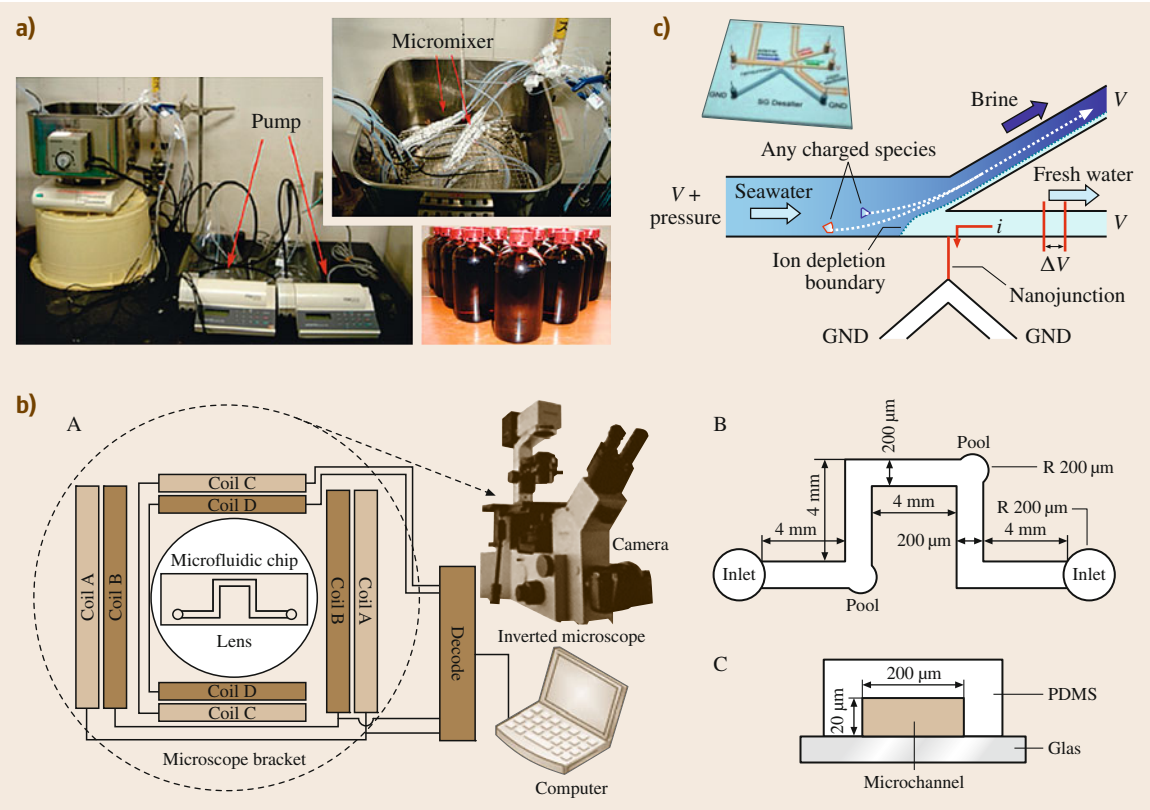


Fig. 20.8a–c **(a)** Microfluidics-based system for the in vitro synthesis of precious marine chemicals (here pristane) and photo of the quantity obtained in one single operation during 3–4 days (after [20.136]) **(b)** Schematic representation of microfluidic chip for the study of MO-1 magnetotactic bacteria-based microrobot (after [20.137]). **(c)** Schematic of micro/nanofluidic desalination system with embedded microelectrode for measuring the potential drop (*top*) and electrokinetic desalination operations associated with the external pressure field (*bottom*) (after [20.138])

as distillation, reverse osmosis (RO), and electrodialysis (ED), require high power consumption and operating costs or large-scale infrastructures, which make them difficult to implement in resource-limited areas, which are often in the poorest, most underdeveloped countries that lack the necessary and expensive power and water-delivery infrastructures to use these methods. Microfluidic technology that can be used to create small-scale and affordable seawater desalination systems with low power consumption and high throughput would be very useful in resource-limited areas. Toward the realization of such systems, *Kim* et al. [20.138] have reported an on-chip desalinization process that uses ion concentration polarization (ICP) for membraneless direct desalination of seawater. The device is made of PDMS with a perm-selective nanojunction [20.158]. It is composed of two microchannels connected by a nanojunction, as shown in Fig. 20.8c. The nanojunction preferentially conducts only cations (cation exchange membrane) that do not match the ion conductivities in the bulk electrolyte, which generate ion concentration gradients on both sides of the membrane. Once ICP is triggered near the cation exchange membrane, the concentrations of both cations and anions decrease on the anodic side of the junction (ion depletion) and increase on the cathodic side (ion enrichment) [20.158, 159]. The use of the nanojunction instead of classical membrane geometry, prevents the blocking of fluid flow by the membrane and rather flows in directions tangential to it. Gold microelectrodes, with titanium as an adhesion layer, were deposited on a glass slide. The device was then bonded with the patterned glass plate by means of plasma treatment in a way that the electrodes were located at the inlet, desalted

and salted microchannels for electric potential measurement, using an evaporation/lift-off process. To perform seawater desalinization, seawater from Crane Beach, Massachusetts, was physically prefiltered to remove any precipitation and debris larger than the channel dimension (dirt, sand, and seaweed) and then loaded (using an external pressure pump) into the reservoir of the chip. Once the ICP process was initiated, a depletion zone formed and diverted charged ions into the *salted* stream. In addition, the ICP layer acted as a virtual barrier for any charged particles found in the seawater (both negative and positive), including solid particles, microorganisms, and biomolecules (including proteins, bacteria, viruses, red blood cells, and white blood cells (WBCs)), because most water-borne microorganisms and microparticles have a nonzero zeta potential. Consequently, both small salt ions and large microorganisms could be removed simultaneously from the output desalted stream, making this process highly attractive for direct desalination of seawater from natural sources. However, this system cannot remove neutral particles and must be associated to other conventional system, such as charcoal absorption, in order to to obtain drinkable water. The present technology would not replace large-scale RO or ED plants for general seawater desalination (for agricultural and industrial purposes), but the technique could be used as a small portable unit with low power consumption. It is, therefore, suitable for seawater desalination in disaster and poverty-stricken areas where the necessary infrastructures for operating large-scale desalination systems are unavailable.

20.5 Conclusion

Microfluidic technologies are powerful tools that can revolutionize marine biotechnology. In this chapter, we described some of the systems and concepts that overcome a number of limitations imposed by conventional macroscale experimentations, including expensive and time-consuming methods, the supply of rare and large volumes of reagents, and the large space and apparatus required. With the miniaturization of the biological and chemical processes on a single chip, there is a new set of tools that dramatically changes the approach to marine biotechnology. These tools render marine biotechnology studies faster, cheaper, and easier. In addition, because of their small size, these tools are highly suitable for in situ studies and analysis, and consequently perfect to increase the response time during biological or chemical pollution of costal seawater or to prevent sample deterioration after harvesting. However, microfluidics is still an infant in marine biotechnology, and various applications, such as marine-based drug discovery, have not been investigated. In the field of drug discovery, microfluidics has demonstrated its potential. Marine biotechnology will benefit highly from the development of such tools, making the discovery of active marine biocompounds much easier and faster. Moreover, in order for microfluidic-based marine biotechnology to efficiently grow and substitute conventional methodologies, several challenges must be overcome. For example, in order to bring about lab-in-the-field, portability of the system should remain one of the main cornerstones of chip development and important efforts must be made to get rid of all external tubings, pumps, gas tanks, and other bulky components that anchor the chip into the lab. More importantly, mindsets need to be changed and researchers in marine biotechnology need to be convinced of the suitability of these systems by increasing the number of validation studies.

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21. Genome Mining for Bioactive Compounds

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Coral reef environments are a rich source of biologically active natural products. However, due to their vulnerable nature and stringent environmental laws, it is always difficult to explore such environments physically. With the advent of molecular biology, many new strategies have evolved for the discovery of novel natural bioproducts from sensitive marine environments. Analyses of plant and microbial genome sequences have revealed many genes and gene clusters encoding proteins similar to those known to be involved in the biosynthesis of structurally complex natural products. The concept of genome mining evolved from this concept, where large-scale deoxyribonucleic acid (DNA) sequencing approaches have been used. Basically, this strategy involves two approaches: sequence-based metagenomics and functional metagenomics. Although many breakthroughs have been achieved, presently, the global pharmaceutical pipeline consists of few approved drugs and a large number of marine chemicals in different stages of preclinical trials. It is anticipated that with the advent of new molecular techniques, the metagenomics approach will be more useful for the discovery of natural bioactive compounds.

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In the present chapter, an attempt has been made to gather detailed information on the present status of coral-derived bioactive compounds and on the reasons for momentary halt in biodiscovery of marine natural products, and to outline future prospects of research on marine/coral associated bioactive compounds.

21.1 Overview

Marine organisms contain many potential novel bioactive compounds because of the unique environmental conditions (high ionic strengths, low level of light, cold or warm temperatures, and pressure) prevailing in their habitats. Several groups of marine organisms, including soft-bodied sessile invertebrates, such as tunicates, soft corals, and certain sponges, appear defenceless yet they produce bioactive compounds to protect themselves from predators. These organisms are rich in nutritionally important substances and use an arsenal of chemical defences and chemical repellents to protect themselves when competing for space. The incidence of predation is low because of the production of toxic compounds and the possession of some form of calcareous sclerites. The abundance and diversity of natural products having potential biological activities have provided scientists with the opportunity to discover new drugs. In the recent past, bioprospecting of new marine natural products (NMNP) has resulted in the discovery of several thousand novel molecules. The rich biodiversity

of the ocean, which prevails over the diversity of land, shows a huge promise as a source of potential natural products in future. In a recent study, *Leal* et al. [21.1] compiled yearly reviews from 1990 to 1999 and 2000 to 2009 on marine natural products, which were published in the journal Natural Products Reports. The reviews pointed out that there was a noticeable increase in the discovery of new natural products from porifera in the first five years of the 1990s, which subsequently maintained a flat trend. However, the discovery of NMNP from Cnidaria increased over the decades with a noticeable increase in the last 20 years. The class Anthozoa generally comprise 99% of NMNP, which belongs to the phylum Cnidaria. Further, most scleractinian corals are representative of this class. The discovery of new natural products from the Anthozoa class increased by 72% from the 1990s to the 2000s.

The investigation of natural products as novel human therapeutics reached its peak in the western pharmaceutical industry during 1970–1980 . It was reported that between 1981 and 2002, a total of 877 small molecules, new chemical entities (NCEs), were introduced and interestingly, approximately half of these were derived from natural products, semisynthetic natural product analogs, or synthetic compounds based on natural product pharmacophores [21.2].

Recently, *Cheng* et al. [21.3] discovered three new diterpenoids designed as gyrosanols A–C from the soft coral *Sinularia gyrosa*. An in vitro cytotoxicity study with these compounds revealed activity against human lung carcinoma (A-459), human colon adenocarcinoma (HT-29), and mouse lymphocytic leukemia (P-388) cancer cell lines. It also showed antiviral activity against the human cytomegalovirus and antibacterial activity against five pathogenic bacterial strains, *Enterobacter aerogenes*, *Salmonella enteritidis*, *Serratia marcescens*, *Shigella sonnei*, and *Yersinia enterocolitica*.

Initially, studies on another species, *S. crassa*, yielded sphingosine and steroids possessing anti-inflammatory activity. A further study by *Cheo* et al. [21.4] confirmed isolation of eight new cembranoids showing bioactivity against selected human carcinoma cells.

In a recent study, five new polyoxygenated steroids were derived from the bamboo coral *Isis hippuris*, with anti-HCMV (HCMV) activity as well as cytotoxicity against selected cell lines. One of these compounds showed inhibitory activity against HCMV, with an EC_{50} value of 6.0 μg/ml [21.5].

Nematocyst venom of fire warm *Millepora platyphylla* (Mp-TX) was extracted and simultaneous biological and biochemical assays were performed to monitor the hemolytic, using washed human red blood cells (RBC) and phospholipase A2 (using radio labeled sn-2 C14-arachidonyl phosphatidylcholine as a substrate) active venom fractions. The magnitude of both hemolysis and phospholipase A2 activity was found in a fraction rich in proteins of molecular masses of approximately 30 000–34 000 Da. The former fraction was purified by ion exchange chromatography, and a major bioactive protein factor (approximately 32 500 Da, here named milleporin-1) was recovered. Milleporin-1 enzymatic activity showed a significant contribution to the overall hemolysis of human RBCs [21.6]. *Su* and *Wen* [21.7] recently isolated five membrane-based diterpenoids from the Taiwanese soft coral *Sinularia triangular*. Among them, triangulenes A and B were reported to be new metabolites and 3 and 5 exhibited moderate cytotoxicity to human tumor cell line (CCRF-CEM) and DLD-1 (dihydrolipoamide dehydrogenase). Metabolites 4 and 5 also effectively reduced the expression of the COX-2 protein of the macrophages.

Pharmaceutical research, especially the development of different antibiotics based on the screening of different microbes expanded rapidly following the discovery of penicillin just after the Second World War. By 1990, about 80% of drugs were either natural products or analogs inspired by them. Antibiotics (e.g., penicillin, tetracycline, erythromycin), antiparasitics (e.g., avermectin), antimalarials (e.g., quinine), lipid control agents (e.g., lovastatin and analogs), immune suppressants for organ transplants (e.g., cyclosporine, rapamycin), and anticancer drugs (e.g., taxol) revolutionized the pharmaceutical industry.

Bryozoans are an independent group of animals closely related to corals and are the source of many potent bioactive compounds. A common bryozoan species, *Bugula neritina*, is a potent source of the protein kinase C inhibitor called bryostatin. Bryostatin-1 has been granted orphan drug status by the Food and Drug Administration (FDA) and has been designated as an orphan medicinal product in Europe for oesophageal cancer in combination with paclitaxel [21.8].

21.2 Temporary Halt in the Discovery of Bioactive Compounds

Koehn and *Carter* [21.2] accumulated the numbers of natural products derived from small molecules for which patents were granted between 1984–2003. According to report, increasing patent activity was observed throughout the 1980s, a flattening or even slight decline between 1990–1999, and a pick up of activity between 2000–2003. The number of natural product-derived drugs declined further during the period of 2003 to 2007 (Fig. 21.1). The decreased emphasis in the pharmaceutical industry on the discovery of natural products during the last decade can be attributed to many factors, such as: the introduction of high throughput screening (HTS) against defined molecular targets, which prompted many pharmaceutical industries to move from the natural products library towards the synthetic chemical library. Further adoption of new strategies to offer the prospect of simpler, more drug-like screening libraries of wide diversified chemicals also reduced the thrust on the search for natural compounds. In the recent past, major pharmaceutical companies have reduced the emphasis on infectious disease therapy, a traditional area of strength for natural products [21.9]. Finally, stringent rules for collection of natural products from eco-sensitive environments has also prompted many companies to shift the focus from the discovery of natural products to other modes of discovery. This is mainly because infectious disease treatment with antibiotics is of short duration in relation to that with other drugs, such as cholesterol lowering or hypersensitive agents, which are consumed daily for prolonged periods and relieve symptoms rather than provide a cure. However, development costs, standards for safety, and requirements for limited side effects are similar for both antibiotics and long-term drugs [21.10].

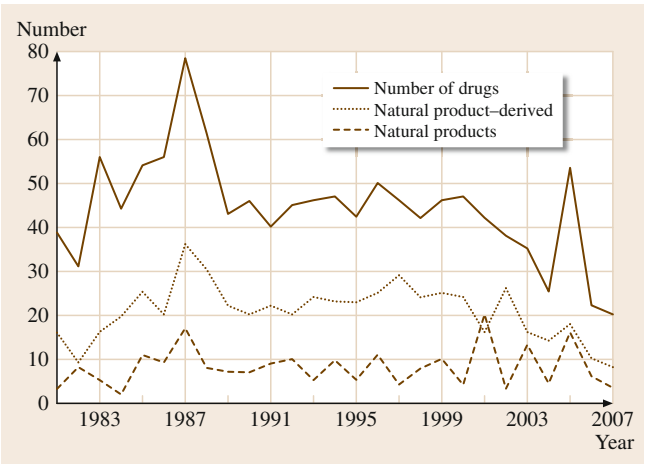

Fig. 21.1 Number of drugs approved in the US between 1981–2007

21.3 Future Directions

With the current strategies, the numbers of new natural product-derived drugs may be reduced; however, this is likely to be temporary. Access to rapid and inexpensive genome sequencing has enabled novel applications such as rapid sequencing of environmental DNA samples and has substantially widened the scope of discovery of new compounds from extreme environmental conditions. These technologies are not only changing our genome sequencing approaches and associated timelines and costs, but also accelerating and altering a wide variety of biological enquiries, including characterization of ecological diversity and identification of unknown natural compounds [21.11].

21.3.1 Genome Mining

Sequencing of huge set of data has opened up a new avenue in genomic research. The analysis of microbial genome sequences has revealed the potential to direct the production of novel, structurally complex natural products. A dramatic and sustained increase in the understanding of the genetics and enzymology of biosynthesis of microbial natural products throughout the 1990s has facilitated the identification and analysis of gene clusters likely to encode for biosynthetic pathways. At the onset of the last decade, publicly deposited genomic data began to accumulate rapidly and analysis of these data revealed that most sequenced microorganisms or plants contain a large number of genes that encodes proteins involved in the biosynthesis of secondary metabolites, the products of which are unknown at the metabolome level. To date, only a fraction of natural products has been analyzed, and the majority ia waiting to be discovered. The discrepancy between the small numbers of isolated compounds and the huge biosynthetic capacity and diversity demonstrated by various organisms on the genomic level highlights the huge potential of genomic mining for the discovery

of novel natural products and represents a strategy to access tremendous sources of new, biologically active metabolites [21.12]. The concept of genome mining for the discovery of novel natural products appears to be more suitable for vulnerable and sensitive natural environments like the coral reef ecosystem, the mangrove ecosystem, etc., which are difficult to access, although they have great potential as a repository of many novel bioactive compounds.

21.3.2 Functional Metagenomics

A functional metagenomics approach is the most effective and suitable for the screening novel gene products or genome mining from any sensitive ecosystem including the coral reef ecosystem. This is because this method mainly focuses on the screening of DNA library clones directly for a phenotypic trait, i. e., the genes are recognizable by their function rather than by their sequence.

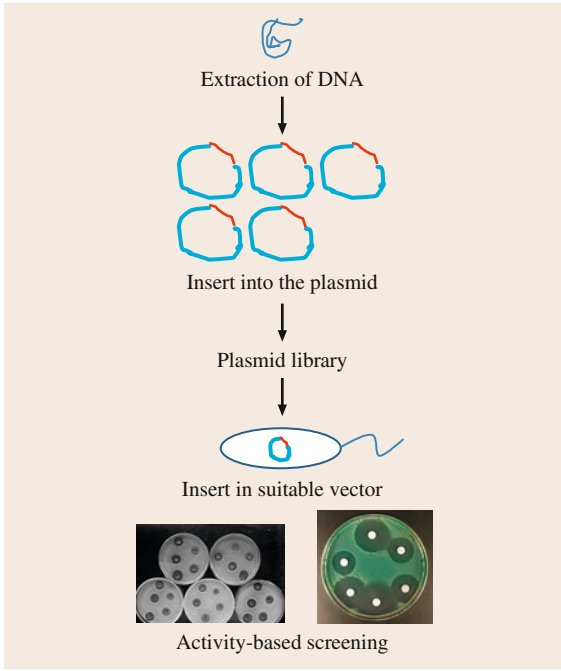

Fig. 21.2 Functional metagenomics

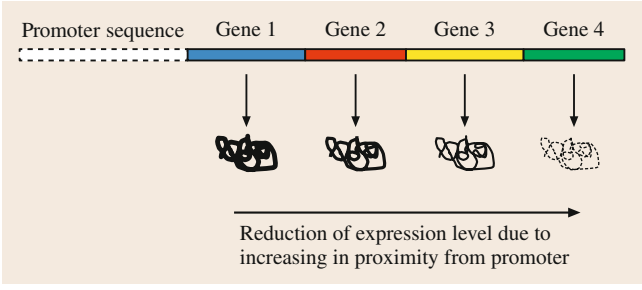

Fig. 21.3 Schematic diagram showing reduction of gene expression with the proximity of the promoter sequence ◄

The basic strategy for functional metagenomics is outlined in Fig. 21.2. In this method the total community DNA can be extracted from an environmental sample, e.g., seawater or marine invertebrate. The isolated DNA is then used to generate a metagenomics library using a suitable cloning vector. This library is then transferred to a suitable host strain, usually *Escherichia coli*, and individual clones can then be screened for the presence of enzymatic or other bioactivities encoded by the environmental DNA fragment. When coupled with a robust and high-throughput screen, this is an extremely effective method to isolate novel bioactivities from otherwise inaccessible microbes (Fig. 21.2) [21.13].

Most of the screens for the isolation of genes encoding novel biomolecules are based on the metabolic activities of metagenomic-library-containing clones. As sequence information is not required, this is the only strategy that bears the potential to identify novel target genes with encoding known functions [21.14–19]. Three different function driven approaches have been used to recover novel biomolecules: phenotypical detection of the desired activity [21.16, 19, 20], heterologous complementation of host strains or mutants [21.21–23], and induced gene expression [21.24–26].

21.3.3 Phenotypic Detection of the Desired Activity

In this type of screening, some chemical dyes are incorporated into the growth medium that showed phenotypic change in the presence of a specific enzyme. There are two distinct strategies taken in functional metagenomics according to the primary goal. Large insert libraries are constructed for archival and sequence homology screening purposes. The main advantage of this approach is the capacity to capture the largest amount of available genetic resources in the sample and to archive it for further studies/interrogation. Although some genes in such libraries are expressed and may be found in active screens, these will be biased towards genes from organisms related to the host, since

they generally lie far from vector expression signals and, therefore, are expressed from their own expression signals (Fig. 21.3). On the other hand, small insert expression libraries, especially those made in lambda phage vectors, are constructed for activity screening. The small size of the cloned fragments means that most genes present in the appropriate orientation will be under the influence of extremely strong vector expression signals and thus have a good chance of being expressed and detected by activity screens [21.15].

Another function-driven screen is based on the principle of heterologous complementation of host strains or their mutants, which requires the targeted genes for growth under selective conditions. This technique allows a simple and fast screening of complex metagenomic libraries comprising millions of clones [21.27]. Recently, *Donato* et al. [21.28] isolated genes that confer resistance to tetracycline, β-lactams or amino glycoside antibiotics from a complementation-based screen of 446 000 clones derived from specialized soil samples.

Resistance genes were identified by subcloning and/or in vitro transposon mutagenesis with the Genome Priming System, GPS-1TM (New England Biolabs). Subcloning was accomplished by partial digestion of plasmid DNA with *Sau*3AI, ligation into the *Bam*HI site of pUC19 (encoding ampicillin resistance) and selection for subclones on Luria-Bertani (LB) broth with the appropriate antibiotics. Subclones were sized by gel electrophoresis after digestion with *Pvu*II. In vitro transposon mutagenesis inserts transposons randomly into plasmid DNA, and antibiotic-susceptible mutants were identified by patching cells onto media containing the appropriate antibiotic. Sequencing reactions, using M13 forward and M13 reverse primers or primers specific for the transposon ends, were performed on both DNA strands [21.18].

It is well known that marine invertebrates, including corals, produce many secondary metabolites, including antimicrobials. Different functional metagenomic approaches can be very useful tools to explore novel bioactivities.

21.3.4 Induced Gene Expression

This is a novel method to screen small molecules. In this intracellular screen, the biosensor that detects active clones is inside the same cell along with the metagenomic DNA. The biosensor detects small diffusible signal molecules that generally induce quorum sensing. Generally, when the signal molecules reach a sufficient concentration, they start binding to the transcriptional activator (LuxR) which activates promoters of the *lux* gene and induces expression of target genes, commonly green fluorescent protein (GFP) [21.26]. This method is highly accurate and there is minimum chance of a false positive result. Coral environments

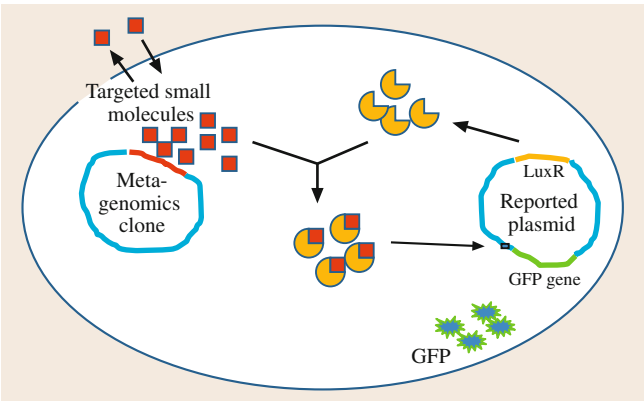

Fig. 21.4 Both biosensor and active clones present inside the same cell as the metagenomic DNA. When target molecules reach a sufficient concentration they bind the LuxR transcriptional activator, which activates Plux and induces expression of the signal molecule (GFP) (after [21.26])

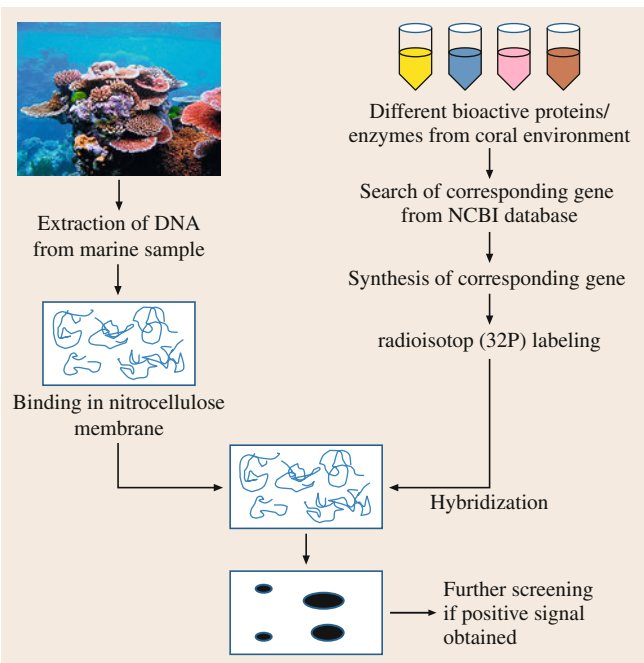

Fig. 21.5 Targeted metagenomics

generally harbor many such small molecules, which can be screened well with induced gene expression. However, the main hindrance in this method is that only specific types of molecules can be screened out (Fig. 21.4).

21.3.5 Sequence-Based Metagenomics

This method generally involves the design and use of polymerase chain reaction (PCR) primers or hybridization probes for the gene being targeted, which for enzymes is usually based on conserved regions of an already well-characterized protein family (Fig. 21.5).

In brief, an environmental sample is collected and then the total community DNA is extracted from the sample. The isolated DNA is then embedded into the suitable nitrocellulose membrane.

Preparation of the Probe

A list of the novel compounds reported to be present in this environment should be made. A detailed search for corresponding genes should be made through Basic Local Alignment Search Tool (BLAST) and the probe can be designed either by PCR amplification or by synthesis of part of these target genes. The targeted probe is labeled in vitro with [5' c¢32p]dCTP (375−3000 mCi mM^{-1}) with a Nick translation kit following the recommended protocol supplied with the kit. ^{32}P-labeled oligonucleotides are separated from the ^{32}P-labeled probe by elution through a 3 cm 3 Sephadex G-50 column with a column buffer that contains 0.25 M NaC1, 0.05 M Tris, 0.002 M EDTA, and 0.5 SDS (pH 8.0) [21.29].

Hybridization

Binding of the ^{32}P-labeled DNA probe with a complementary sequence of the DNA-bound nitrocellulose membrane is a crucial and tricky step in this method. In brief, the DNA-bound nitrocellulose membrane is deeped in a prehybridization buffer for initial washing of the membrane. Subsequently, the prehybridization buffer is replaced by a hybridization buffer with the addition of the required amount of DNA probe. After completion of hybridization, a final washing is performed in a temperature fixed on the basis of the required stringency condition.

21.4 The Hurdles of Drug Discovery

The prevention of different forms of cancer is the biggest challenge to the medical scientist at present. However, the development of new anticancer medicines is an arduous task that requires the coordinated efforts of a team of scientists from chemical and biological sciences. Any potential cancer chemotherapeutic candidate must undergo a rigorous series of tests prior to gaining FDA approval for commercialization. The process generally starts with in vitro tests against a panel of 60 different types of cancer cells. Additionally, xenograft models are also conducted on many occasions. Determination of the effective dose (ED_{50}) is the next step. A molecule becomes a candidate for testing in humans if it displays both toxicity against cancer cells and tolerance by healthy cells and in the later stage by whole animals. Human testing generally follows a three-phase protocol. Initially, Phase I involves treatment of a small number of healthy (paid) volunteers with the drug candidate in order to ascertain whether humans tolerate the compounds. If no adverse effects are detected, then the drug is generally used on a small number of patients with different cancers that do not respond to conventional treatment (Phase II). Drug candidates that continue to show therapeutic potential at this point then enter Phase III trials, in which they are administered to a broad range of cancer patients. Eventually, if the drug candidate survives these experimental challenges, FDA sanctions the approval after analyzing all the data. It is reported that generally more than 99.9% preclinical drug candidates fail this rigorous screening method [21.30]. Only a handful of compounds was discovered in the last 10 years. In fact, the National Cancer Institute (NCI) maintains an ever-expanding repository of over 60 000 plant and marine samples available for testing, and about 700 new marine samples are added each year.

21.5 Marine Pharmaceuticals Under Clinical Trials

The first reported structure of marine product was in 1880 by *Vosmaer* et al. [21.31]. The development of different chromatographic techniques increased the number of structurally-complex molecules obtained from

Table 21.1 Drugs in pharmaceutical clinical pipeline

Clinical status	Compound name	Trademark	Major organism	Clinical trial	Chemical class	Company	Disease area
Approved	Cytarabine, Ara-C	Cytosar-U	Sponge	>50/748	Nucleoside	Bedford, Enzon	Cancer
	Vibarabine, Ara-A	Vira-A	Sponge	0	Nucleoside	King Pharmaceuticals	Antiviral
	Ziconotide	Prialt	Cone snail	1/5	Peptide	Elan Corporation	Pain
	Eribulin Mesylate (E7389)	Halaven	Sponge	27/39	Macrolide	PharmaMar	Cancer
	Omega-3-acid ethyl esters	Lovaza	Fish	13/56	Omega-3-fatty acid	GlaxoSmithKline	Hypertriglyceridemia
	Trabectedin (ET-743)	Yondelis	Tunicate	13/37	Alkaloid	PharmaMar	Cancer
	(EU Registered only)						
	Brentuximab vedotin (SGN-35)	Adcetris	Mollusc	15/19	Antibody drug conjugate	Seattle Genetics	Cancer
Phase III	Plitidepsin	Aplidin	Tunicate	1/7	Depsipeptide	PharmaMar	Cancer
Phase II	DMXBA (GTS-21)	NA	Worm	2/2	Alkaloid	UCHSC	Cognition Schizophrenia
	Plinabulin (NPI2358)	NA	Fungus	1/2	Diketopiperazine	Nereus Pharmaceuticals, Inc.	Cancer
	PM00104	Zalypsis	Mollusc	1/3	Alkaloid	PharmaMar	Cancer
	Elisidepsin	Irvalec	Mollusc	1/2	Depsipeptide	PharmaMar	Cancer
	PMO1183	NA	Tunicate	2/3	Alkaloid	PharmaMar	Cancer
	CDX-011	NA	Mollusc	1/3	Antibody drug conjugate	Calldex Therapeutics	Cancer
Phase I	Marizomib	NA	Bacterium	4/4	Beta lactone-gammalactam	Nereus Pharmaceuticals, Inc.	Cancer
	PM060184	NA	Mollusc	1/1	Unavailable	PharmaMar	Cancer
	SGN-75	NA	Mollusc	1/1	Antibody drug	Seattle Genetics	Cancer

bioactive natural extracts. In the last 10 years, more than 1000 reports on bioactive molecules isolated from marine organisms have been published [21.32]. Several antitumor marine natural products, derived mainly from marine sponges or molluscs and also from bryozoans and cyanobacteria, have exhibited potent antimitotic properties. Presently, the global marine pharmaceutical pipeline consists of 3 FDA approved drugs, 1 EU (European Union) registered drug, 13 natural products (or derivatives thereof) in different phases of clinical trials, and a large number of marine chemicals in the preclinical pipeline [21.33]. Three FDA-approved drugs in US pharmacopeia are cytarabine (Cytosar-U, Depocyt), vidarabine (Vira-A), and ziconotide (Prialt). Currently, Trabectedin (Yondelis) has been approved by the European Agency for the Evaluation of Medicinal Products (EMEA) and is completing key Phase III studies in the US for approval. A list of marine-derived compounds that have been FDA approved or are in Phases III, II, or I of drug development (as determined at http://clinicaltrials.gov/, as of January 2012) are shown in Table 21.1 with slight modifications [21.34]. It is reported that the majority of compounds are derived from marine invertebrate such as sponges, molluscs, tunicates, etc. Significantly, the majority of compounds showed activity against cancer and some serious diseases. So far, corals do not figure prominently in the clinical pipeline. However, due to the enormous species diversity, the coral reef environment is always considered to be a repertoire of many potential bioactive compounds [21.35]. However, due to their highly endangered status and limited permissibility, coral reef ecosystems throughout the world are less explored.

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Marine Algae Part D

Part D Marine Algal Biotechnology

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22. Cell Wall Polysaccharides of Marine Algae

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Marine algae are interesting as a plentiful source of many bioactive compounds, including polysaccharides, which represent various structurally different polymers of high diversity in monosaccharide composition, absolute and anomeric configuration, glycosidic linkages, molecular mass, and the presence and distribution of various functional groups. These polysaccharides are used by algae as cell wall structural components or food reserve. Algal polysaccharides are highly indicative for main algal taxa. Sulfated galactans of periodical unit/linkage sequence (agars, carrageenans) are typical for red algae, alginates, and fucoidans for brown algae; sulfated glucuronoxylorhamnans (ulvans) and other sulphated glycans for green algae. Algal cell wall polysaccharides are extracted from the raw material and further purified by preparative chromatography and/or chemical treatment. Many algal polysaccharides are assigned as phycocolloids due to their good solubility in water and their ability to create colloid systems, including gels and films. They also demonstrate various biological activities (immunomodulation, antitumor, anticoagulant, antiviral, and many other activities), which are prerequisites of pharmaceutical and medicinal applications. Finally, initially inactive or weakly active natural algal polysaccharides can be partially degraded or structurally modified to be fitted to various medicinal applications.

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22.1 Overview

Marine algae are a plentiful source of many bioactive compounds. Among these chemical components, polysaccharides are of interest because of their physical and chemical properties, which are useful for food and medicinal applications. Algae synthesize various types of polysaccharides as cell wall structural components or energy reserves. Algal polysaccharides demonstrate a high specificity to raw algae, which has been used by phycologists to clarify the phylogenetic relationship between main algal taxa and higher (vascular) plants [22.1–3]. Several species of common marine algae that are the source for the isolation of polysaccharides for food and medicinal purposes are shown in Fig. 22.1.

Fig. 22.1a–i Marine algae producing polysaccharides of medicinal importance: **(a)** *Enteromorpha (Ulva) linza* (Ulvales, Chlorophyta); **(b)** *Capsosiphon fulvescens* (Ulotrichales, Chlorophyta); **(c)** *Sargassum muticum* (Fucales, Phaeophycea); **(d)** *Ecklonia cava* (Laminariales, Phaeophycea); **(e)** *Undaria pinnatifida* (Laminariales, Phaeophycea); **(f)** *Laminaria japonica* (Laminariales, Phaeophycea); **(g)** *Carpopeltis affinis* (Cryptonemiales, Rhodophyta); **(h)** *Chondrus ocellatus* (Gigartinales, Rhodophyta); **(i)** *Scinaia japonica* (Nemaliales, Rhodophyta). Algal photos were kindly provided by Dr. Myounglae Cho, East Sea Institute of the Korea Institute of Ocean Science and Technology (KIOSTI), Korea

22.1.1 Structure of Algal Cell Walls

Algal cell walls are composed of a highly integrated network of biopolymers, mainly polysaccharides, which interact with water, metal cations, and other molecules [22.2]. Structural specificities, including substitution patterns and degrees, are prerequisites of the physical and functional properties of cell wall polysaccharides in marine algae. Crystalline and fibrous parts (cellulose, xylans, hemicelluloses, etc.) are embedded in a gel-like matrix made of carboxylic and/or sulfated polysaccharides. Other biopolymers (proteins, proteoglycans, and polymeric phenolics) may also participate in cell wall formation.

22.1.2 Polysaccharides as Taxonomic Markers of Marine Algae

The great variability of cell wall polysaccharides in marine algae is determined by the specie/taxa [22.3–7], anatomical part of the alga [22.7], the developmental and life-cycle stage [22.7–11], and season and habitat [22.12, 13]. Algae represent a diverse group of photosynthetic eukaryotes with a wide range of cell wall types [22.2]. Besides pigmentation and food reserve specificity, matrix cell wall polysaccharides are useful as structural and taxonomical markers of marine algae. Cell wall polysaccharides from various marine algae of three main divisions, i. e., *Clorophyta* (green algae), *Rhodophyta* (red algae), and *Phaephyta* (brown algae), are summarized in Table 22.1.

Green algae produce various sulfated and/or carboxylic cell wall polysaccharides of specific structure. Red algae have complex composite cell walls made of cellulose, xylan, or mannan fibrils and sulfated galactans (agars, carrageenans) as the main matrix components [22.14]. Brown algae produce cell walls containing cellulose fibrils and two main types of matrix polysaccharides (alginates, fucoidans). Cell walls of all marine algae are rich with sulfated matrix polysaccharides. This is an adaptation to marine habitats that has also been observed for many other marine organisms, including angiosperms and invertebrates [22.15]. There are two groups of algal sulfated polysaccharides (SPS) [22.16], i. e., (i) uronic acid-rich (UA: uronic acid) and (ii) UA-limited ones. In the green algae of Ulvophyceae [22.3], the first group is represented by ulvans (genera *Ulva*, *Monostroma*, *Gayralia*, and *Acetabularia*) [22.5]; the second

Table 22.1 Major cell-wall polysaccharides in the main groups of algae [22.1–3]

Polysaccharides	Chlorophyta	Rhodophyta	Phaeophyta (Phaleophycea)
Crystalline	Cellulose – (1 → 4)-β-D-glucan (1 → 4)-β-D-mannan (1 → 4)-β-D-xylan (1 → 3)-β-D-xylan		
Hemicelluloses	Xyloglucan Mannans (1 → 4)-β-D-glucuronan (1 → 3)(1 → 4)-β-D-glucan	Glucomannan (1 → 3)(1 → 4)-β-D-xylan	Sulfated xylofucoglucan Sulfated xylofucoglucuronan (1 → 3)(1 → 4)-β-D-glucan
Matrix	Ulvans Sulfated glycans	Sulfated galactans (agars, carrageenans)	Alginates Fucoidans

one includes sulfated (1 → 3)-β-D-galactans [22.17–22], (1 → 3)-β-L-arabinopyranans [22.23], and β-(1 → 4)-D-mannans [22.24] (genera *Caulerpa*, *Codium*, and *Bryopsis*). In addition, alkali-soluble linear (1 → 4)-β-D-glucuronan found in some green algae plays the role of hemicellulose [22.25]. Red algae contain linear and branched sulfated galactans having no uronic acids. By contrast, brown algae have specific kinds of carboxylic and sulfated polysaccharides, namely alginates and fucoidans; some of the latter ones may also contain galactose or glucuronic acid residues. More complex highly branched sulfated heteropolysaccharides (xylofucoglucans, xylofucoglucuronans, etc.) of brown algae were assigned as hemicelluloses.

22.2 Structural Diversity of Algal Polysaccharides

Algal cell wall polysaccharides are structurally variable polymers showing significant differences in molecular mass, monosaccharide composition, absolute and anomeric configuration of sugar units, glycosidic linkages, presence and distribution of functional groups (carboxylates, methyl ethers, acetyl esters, sulfate semiesters, 3,6-anhydro and others). There are linear and branched polysaccharides, the latter may have stubs, longer side chains or even very complex branching with defined core, inner and peripheral parts. Basic structure of common algal polysaccharides is demonstrated in Fig. 22.2. Structural variability of specific algal polysaccharides is reviewed below.

22.2.1 Alginates

Alginates are matrix cell wall polysaccharides of brown algae (Phaeophyceae). They are linear co-polymers of (1 → 4)-linked units of β-D-Man*p*A and its C-5 epimer α-L-Gul*p*A. These uronic acids are distributed in three types of blocks containing (i) only β-D-Man*p*A (M-blocks, M: mannuronic acid), (ii) α-L-Gul*p*A (G-blocks, G: guluronic acid), or (iii) alternating sequences of both these residues (MG-blocks) [22.44]. In algal cells alginates are synthesized as homomannuronan polymeric chains and then β-D-Man*p*A units are partially converted to α-L-Gul*p*A ones by enzyme C-5 epimerase. This conversion is irreversible and both types of blocks are formed. The $^4C_1$ and $^1C_4$ conformations were confirmed for β-D-Man*p*A and α-L-Gul*p*A alginate units, respectively [22.30, 45]. In the homopolymeric blocks the units are bound with diequatorial (M-blocks) and diaxial (G-blocks) glycosidic linkages, while in the alternating sequence the units are bound by mixed equatorial-axial (MG) and axial-equatorial (GM/MG – alternating blocks in alginates) linkages (Fig. 22.2a). The homopolymeric blocks form helical structures stabilized by intra-molecular hydrogen bonds involving hydroxyls, ring oxygens, and/or carboxyls; the MG-blocks take up disorder conformations. These helices demonstrate different stiffnesses: G-blocks look like *buckled ribbons* and they are stiffer than *flat-ribbon* M-blocks [22.41, 46, 47]. The M/G ratio (molar ratio between mannuronic and glucuronic acids in alginates) and distribution of co-monomer residues along an alginate chain depend on the source and geographic location of raw algal material, season of collection, the age of the parts of the plant, and extraction methodology used. The M/G ratio of alginates originating from Indian algae (the coast of Ki-

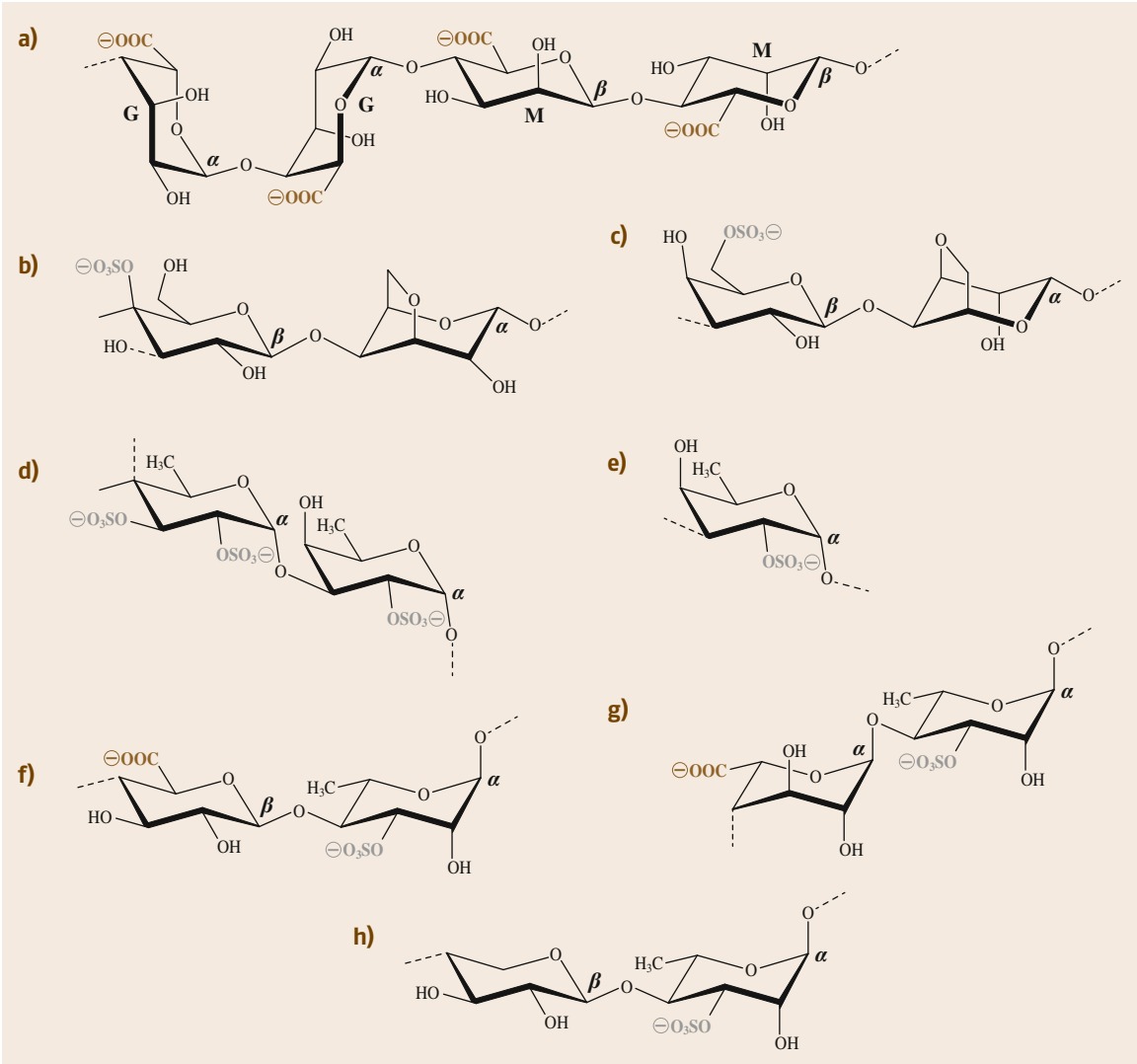

Fig. 22.2a–h Basic structures of marine algal polysaccharides: **(a)** alginate (G – guluronic acid, M – mannuronic acid); **(b)** κ-carrageenan; **(c)** agarose-6-sulfate; **(d,e)** fucoidans; **(f–h)** ulvans

lakarai, the Gulf of Mannar, South India) was reported to be maximal in summer and minimal in premonsoon seasons; this value also depends on the anatomical part of algal thalli used for isolation [22.12, 13]. The high variability of the M/G ratio for alginates from various brown algae is illustrated in Table 22.2. The block composition of alginates was investigated by a partial acidic hydrolysis (0.3 mol L^{-1} HCl, 100 °C, 2 h) followed by precipitation of the resistant fragments [22.11, 32]. Strong acidic hydrolysis of alginates from *Laminaria brasiliensis* gave rise mainly to M and G-oligosaccharide blocks, indicating preferential hydrolysis of glycosidic bonds in the MG-regions [22.42]. Two types of alginates were defined based on the distribution of monomers [22.32], i. e., those containing (i) many G-blocks, less MG-blocks, and even less M-blocks (GG > MM > MM), and (ii) a small proportion of GG and near equal proportions of MG and M-blocks (GG < MG≈MM). Alternatively, algal alginates could be classified as being high-M, low-M (or high-G), or intermediate type [22.27]. Well-defined MG-blocks were detected only for the intermediate alginates, whereas

Table 22.2 M/G values obtained for alginates from various algal sources

Order	Species	M/G value	References
Chordariales	*Myriogloeia intestinalis*	0.33	[22.26]
	Papenfusiella lutea	0.53	
	Splachnidium rugosum	0.56	
	Spermatochnus paradoxus	1.3	[22.11]
	Chordaria flagelliformis	0.9	
Desmarestiales	*Desmurestiu aculeatu*	0.85	
Dictyosiphonales	*Dictyosiphon foeniculaceus*	0.85	
Durvillaeales	*Durvillaea antarctica*	3.0–4.0	[22.26, 27]
	Durvillaea potatorum	3.17	[22.11]
	Durvillaea willana	2.57	
Dictyotales	*Zonaria* sp.	0.41	[22.28]
	Spatogossum sp.	0.75	
Ectocarpales	*Chnoospora* sp.	0.51	
	Stilophoru rhizodes	0.44–0.47	[22.29]
	Leathesia difformis	0.37	
Fucales	*Ascophyllum nodosum*	1.29–1.56	[22.11, 29–32]
	Cystophora retroflexa	1.08	[22.11]
	Cystophora torulosa	0.99	[22.26]
	Captophyllum maschalocarpum	0.94	
	Xiphpophore chondrophylla	1.36	
	Pelvetia canaliculata	1.50	[22.11]
	Sargassum sp.	0.44–1.09	[22.28]
	Sargassum fluitans	0.19–1.18	[22.33–35]
	Sargassum oligocystum	0.49–0.62	[22.34]
	Sargassum filipendula	0.19	[22.36]
	Sargassum muticum	0.31	
	Sargassum thunbergii	0.53	
	Sargassum dentifolium	0.52	[22.37]
	Sargassum asperiifolium	0.69	
	Sargassum latifolium	0.82	
	Sargassum tenerrimum	high-G	[22.38]
	Sargassum trichophyllum	1.88	[22.39]
	Sargassum siliquosum	0.72	[22.33]
	Sargassum vulgare	1.27–1.56	[22.40]
	Sargassum polycystum	0.54–0.74; 0.21	[22.13, 36]
	Turbinaria conoides	0.60–0.77	[22.12]
	Hormosira banksii	1.50; 1.33	[22.11, 26]
Laminariales	*Laminaria hyperborea*	0.62	[22.32]
		0.54–1.35 (fronds)	[22.11, 29, 31, 32, 41]
		0.40–0.65 (stipes)	[22.11, 30, 32]
	Undaria pinnatifida	1.45–2.65	[22.7]
	Laminaria japonica	2.26	
		2.34–3.18 (basal)	
		1.61–2.02 (apical)	
	Laminaria brasilliensis	1.2	[22.42]
	Kjellmaniella crassifolia	13	[22.43]

Table 22.2 (continued)

Order	Species	M/G value	References
Laminariales	*Laminaria pallida*	2.33 (stipes)	[22.48]
	Laminaria digitata	1.45	[22.11]
		1.35–2.08 (blade)	[22.29]
	Laminaria longicruris	1.44–2.17	
	Agarum cribosum	1.30 (blade)	
	Macrocystis pyriphera	1.38–1.70	[22.7, 11, 29, 32, 49]
		1.52 (frond)	
		1.02 (stipes)	
		1.41 (blade)	
	Macrocystis boryana	0.79	[22.11]
	Lessonia trabeculata	1.73	[22.50]
	Lessonia variegata	1.95	[22.26]
	Ecklonia radiata	1.60	
Scytosiphonales	*Scytosiphon lomentaria*	1.15; 0.67	

the high-M and low-M alginates consist mainly of homopolymeric blocks (GG, MM), which are almost directly connected and contain few or no heteropolymeric blocks. It was also found that young algal tissues are rich in M-blocks, and that the difference between the alginates from different species was mainly due to the contribution of the older parts of thalli [22.11].

22.2.2 Sulfated Galactans

Sulfated galactans are common cell wall matrix polysaccharides of green (*Clorophyta*) and red (*Rhodophyta*) algae. They are polymers of partially sulfated β-D- sand/or α-D(L)-galactopyranosyl (Gal*p*) units.

Sulfated galactans from cell walls of green algae (genera *Codium* and *Bryopsis*) were reported to be both homo and heteropolysaccharides, depending on the species [22.17, 19, 20, 22, 51]. Polysaccharides isolated from *Codium isthmocladum* [22.20] and *Codium yezoense* [22.17, 52] are sulfated homogalactans containing similar backbones, composed mainly of (1 → 3)-linked β-D-Gal*p* units and short oligosaccharide side chains bound at *O*-6. The units were sulfated mainly at *O*-4 and rarely at *O*-6, and nonreducing terminal residues were pyruvylated forming cyclic 3,4-ketals. Sulfated galactan isolated from *Bryopsis plumosa* (Bryopsidales, Chlorophyta) consist of a (1 → 3)-linked β-D-Gal*p* partially sulfated on *O*-6 and substituted with pyruvic acid, forming 4,6-ketals [22.19]. A branched sulfated (1 → 3), (1 → 6)-β-D-galactan was isolated from *Codium fragile*. This polysaccharide has a (1 → 3)-linked partially 4-*O*-sulfated β-D-Gal*p* backbone with some 3,4-pyruvated β-D-Gal*p* stubs at *O*-6 positions [22.21]. By contrast, galactans isolated from *Codium fragile* [22.51] and *Codium cylindricum* [22.22] contain other sugar units – arabinose and glucose, respectively. Nevertheless, the →3)-β-D-Gal*p*4S-(1→ units still predominate in these polysaccharides. Water-soluble highly sulfated arabinans, galactans, and/or arabinogalactans were isolated from *Codium fragile* and *Codium vermilara* [22.18]. These polysaccharides are composed of (1 → 3)-linked β-D-Gal*p* and β-L-Ara*p* residues; they are partially substituted with pyruvic acid ketals.

Red algal cell walls contain (1 → 3), (1 → 4) – mixed linkage α, β-D(L)-galactans. Most of these polysaccharides are nonbranched. Their backbone is made of disaccharide repeating fragments of alternating (1 → 3)-β- and (1 → 4)-α-linked Gal*p*. The former units (G) always have D-configuration, whereas the latter ones (D or L) are present as the D- (the carrageenan family) or L- (the agaran family) enantiomers (Fig. 22.2b,c). Sulfate hemiesters, methyl ethers, and pyruvic acid are commonly bound to these polysaccharides. Sulfate and/or occasionally methyl groups may occur at the *O*-2 and/or *O*-4 position(s) of the (1 → 3)-β-linked Gal*p* units and at *O*-2, *O*-3-, and/or *O*-6 position(s) of the (1 → 4)-α-linked Gal*p* units; pyruvic acid may be attached to (1 → 3)-β-D-Gal*p* residues as ketals at *O*-4 and *O*-6 positions [22.81, 82]. The 3,6-anhydro derivatives of D(L) units may be present in the backbone of carrageenose (agarose) after elimination of sulfate from *O*-6 by enzymatic or alkali treatment. Agarose, a simplified structure of agar, is a sequence of

Table 22.3 Disaccharide repeating structure and abbreviation [22.14] of some agarans and carrageenans

Agaran	Formulae (IUPAC[a])	Abbreviation
Agarose	[3)-β-D-Gal*p*-(1 → 4)-3,6a-α-L-Gal*p*-(1→]$_n$	**G**-LA[b,c]
Agarose precursor	[3)-β-D-Gal*p*-(1 → 4)-α-L-Gal*p*6S-(1→]$_n$	**G**-L6S[d;f]
Agarose sulfate	[3)-β-D-Gal*p*6S-(1 → 4)-3,6a-α-L-Gal*p*-(1→]$_n$	**G6S**-LA
Agarose sulfate precursor	[3)-β-D-Gal*p*6S-(1 → 4)-α-L-Gal*p*6S-(1→]$_n$	**G6S**-L6S
	[3)-β-D-Gal*p*-(1 → 4)-α-L-Gal*p*-(1→]$_n$	**G**-L
Carrageenan	**Formulae (IUPAC)**	**Abbreviation**
β-family	β [3-β-D-Gal*p*-(1 → 4)-3,6a-α-D-Gal*p*-(1→]$_n$	**G**-DA[c]
	γ [3-β-D-Gal*p*-(1 → 4)-α-D-Gal*p*6S-(1→]$_n$	**G**-D6S[e]
	ψ [3-β-D-Gal*p*6S-(1 → 4)-3,6a-α-D-Gal*p*2S-(1→]$_n$	**G6S**-DA**2S**
	ω [3-β-D-Gal*p*6S-(1 → 4)-α-D-Gal*p*2,6diS-(1→]$_n$	**G6S**-D**2S**6S
λ-family	α [3-β-D-Gal*p*-(1 → 4)-α-D-Galp2S-(1→]$_n$	**G**-DA**2S**
	δ [3)-β-D-Gal*p*-(1 → 4)-α-D-Gal*p*2,6diS-(1→]$_n$	**G**-D**2S**6S
	θ [3)-β-D-Gal*p*2S-(1 → 4)-3,6a-α-D-Gal*p*2S-(1→]$_n$	**G2S**-DA2S
	λ [3)-β-D-Gal*p*2S-(1 → 4)-α-D-Gal*p*2,6diS-(1→]$_n$	**G2S**-D2S6S
	ξ [3)-β-D-Gal*p*2S-(1 → 4)-α-D-Gal*p*2S-(1→]$_n$	**G2S**-D2S
	π [3)-β-D-Gal*p*Pyr2S-(1 → 4)-α-D-Gal*p*2S-(1→]$_n$ [g]	**GP2S**-D2S
κ-family	κ [3-β-D-Gal*p*4S-(1 → 4)-3,6a-α-D-Gal*p*-(1→]$_n$	**G4S**-DA
	ι [3-β-D-Gal*p*4S-(1 → 4)-3,6a-α-D-Gal*p*2S-(1→]$_n$	**G4S**-DA2S
	μ [3-β-D-Gal*p*4S-(1 → 4)-α-D-Gal*p*6S-(1→]$_n$	**G4S**-D6S
	ν [3-β-D-Gal*p*4S-(1 → 4)-α-D-Gal*p*2,6diS-(1→]$_n$	**G4S**-D2S6S
	o [3-β-D-Gal*p*4S-(1 → 4)-α-D-Gal*p*2S-(1→]$_n$	**G4S**-D2S

[a] International Union of Pure and Applied Chemistry (IUPAC); [b] first unit in agarans and carrageenans (G); [c] 3,6-anhydro units (LA, DA); [d] second unit in agarans (L); [e] second unit in carrageenans (D); [f] sulfation of hydroxyls (S); [g] pyruvation of hydroxyls (Pyr)

identical disaccharide (agarobiose) fragments without sulfates. By contrast, real agars have some α-L-Gal*p*6S (porphyran), pyruvic acid 4,6-ketals, *O*-methyl, or sulfate groups attached to β-D-Gal*p* residues [22.81, 83]. Similarly, carrageenans may have a structure described by one of the defined disaccharides (carrabioses) and thus are classified by Greek prefixes according to their structural specificity, i. e., sulfation patterns and the presence of 3,6-anhydro groups (Table 22.3). More often, however, they contain several types of disaccharide fragments, i. e., hybrid types (Table 22.4). Structural and conformational analysis of κ/ι hybrid carrageenans from *Sarcothalia crispala*, *Mazaella laminarioitles*, and *Chonrlrus crispus* showed that they are not a mix of pure carrageenans but have a mixed chain containing both κ and ι-disaccharide fragments [22.84]. Gametophyte and sporophyte generations of the algal biological cycle contain carrageenans of different structure, especially sulfate and 3,6-anhydride groups. Carrageenans of *Phyllophoraceae* and *Gigartinaceae* were described to be hybrids of κ, ι, and ν forms for gametophytes and rather λ subfamily forms (λ, ξ, and π) for tetrasporophytes [22.9, 10, 64, 68, 69, 85, 86]. Carrageenans usually contain more sulfates than agarans, while the latter often contain more methyl and glycosyl groups (Table 22.3). The structural variability of red algal galactans, especially of their substitution patterns, has been described for different species, harvesting location, or seasons. Sometimes the structure of red algal galactans (α/β anomers, D/L enantiomers, sequence of linkages, substitution patterns, etc.) deviates from the defined structural types of agars and carrageenans. Other sugars (Xyl, Glc, GlcA, Ara, or Man) are bound to some of them as backbone or side chain parts [22.87]. There are also some polysaccharides, named DL-galactan hybrids, having both D and L-units distributed within the same polysaccharide chain [22.88]. The real structure of red algae sulfated galactans is probably a result of the following stepwise biosynthesis: (i) formation of the regular polymer backbone, (ii) sulfation, methylation, and/or pyruvation of specific hydroxyls, and (iii) enzymatic

Table 22.4 Hybrid carrageenans and DL-galactans

Algal species	Structure	References
Mastocarpus stellatus	κ/ι [a]	[22.53]
Kappaphycus striatum	$\kappa/\iota/\kappa_{6Me}/\iota_{6Me}$ [b]	[22.54]
Furcellaria lumbricalis	κ/β	[22.55]
Tichocarpus crinitus	$\kappa/\beta/\mu$	[22.56]
Kappaphycus alvarezzi	κ/μ	[22.57]
Sarconema filiforme	$\iota/\alpha/\alpha_{Pyr}$ [c]	[22.58]
Stenogramme interrupta	$\iota/\alpha/\alpha_{Pyr}$ (cystocarpic) λ/ξ (tetrasporophyte)	[22.8]
Eucheuma denticulatum	ι/ν	[22.59, 60]
Coccotylus truncatus	$\iota/\nu/\alpha_{Pyr}$	[22.61]
Botryocladia occidentalis	Unusual	[22.62]
Meristiella gelidium	$\kappa/\iota/\nu$	[22.63]
Gigartina pistillata	$\kappa/\iota/\nu$ (gametophyte) $\lambda/\xi/\pi$ (tetrasporophyte)	[22.10, 64]
Gigartina alveata, Gigartina clavifera	κ/ι (gametophyte) ξ/θ (tetrasporophyte)	[22.9]
Gigartina chapmanii	$\xi/\lambda/\theta/\pi$ (tetrasporophyte)	[22.10]
Gigartina skottsbergii	$\lambda/\xi/\delta$, μ/ν, D/L κ/ι, μ/ν (gametophyte) λ/θ (tetrasporophyte)	[22.65] [22.66, 67] [22.68] [22.69]
Sarcothalia crispate	κ/ι, μ/ν (gametophyte) λ/θ (tetrasporophyte)	[22.69]
Gigartina atropurpurea	κ/ι, μ/ν (gametophyte) λ/θ (tetrasporophyte)	[22.70]
Schizymenia binderi	D/L	[22.71]
Gymnogongrus torulosus	D/L	[22.72, 73]
Cryptonemia seminervis	D/L	[22.74]
Cryptonemia crenulata	D/L	[22.75, 76]
Halymenia durvillei	D/L	[22.77]
Gloiopeltis furcata	D/L	[22.78]
Digenea simplex	D/L	[22.79]

[a] More κ/ι hybrid carrageenans are reviewed by [22.80]; [b] methylation of hydroxyls (Me); [c] pyruvation of hydroxyls (Pyr)

conversion of some (1 → 4)-α-linked Gal*p*6S residues to 3,6-anhydro derivatives. According to this sequence, porphyran is a precursor of agarose, and, similarly, it is true for the pairs of carrageenans. Formation of 3,6-anhydrogalactose, similar to the final step of biosynthesis, is possible by the treatment with heated dilute alkali on the corresponding precursor. The corallinans, which are agar-like branched sulfated and methylated galactans from red algae of the order *Corallinales*, have (a) an agaran backbone, (b) a β-D-Gal*p* unit almost completely substituted at *O*-6 mostly with β-D-xylosyl side chains and less by sulfate, and (c) α-L-Gal*p* units partly substituted by methoxyl and sulfate groups at *O*-2 [22.89]. In addition, the latter units can be substituted at *O*-3 or *O*-6 with partially *O*-methylated α-D- or α-L-Gal*p* side chains (Table 22.5). Polysaccharides from various algae may differ in the position of these branches. There is a balance between the hydrophobic (methoxyl) and the hydrophilic (sulfate) groups, so the algal enzymatic system equilibrates their proportions to fulfill their biological design [22.90]. Decalcified tissue from *Calliarthron cheilosporioides* contains sulfated galactan, highly methoxylated but lowly glycosylated with xylose at *O*-6 of the β-D-Gal*p* units [22.91]. By contrast, calcified segments of this alga produce xylogalactans with high levels of xylose and low levels of 6-*O*-methyl ethers. Therefore, decalcification of algal tissues led to replacing xylosyl branches by methyl groups. These structural changes are caused by specific methoxyl transferase blocking xylosylation in decalcified tissue.

22.2.3 Fucoidans

Fucoidans are sulfated matrix cell wall polysaccharides of brown algae (Phaeophyceae). Simple fucoidans are sulfated homofucans, i. e., polysaccharides consisting of sulfated fucose only. However, in some reports this term is used to describe complex fucose-containing heteropolysaccharides. The chemical composition and structure of fucoidans are complex and variable, reflecting the differences in biosynthesis [22.6, 152]. Very often the exact structure of these polysaccharides remains unclear even when modern methods of structural analysis like selective enzymatic cleavage or correlation NMR analyses are applied. This problem is caused by significant irregularities in the structure of algal fucoidans, including the presence of numerous minor sugar and nonsugar components, random sulfation, and/or acetylation [22.6, 153]. There are two main types of backbone structure

Table 22.5 Side chain positions in corallinans and other agar-like branched sulfated xylogalactans

Source	Side chain	Position	References
Bossiella cretacea	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S) [a]	[22.82, 92]
Gymnogongrus torulosus	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S)	[22.93]
Pachymenia lusoria	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S)	[22.94]
Calliarthron cheilosporioides	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S)	[22.91]
Polysiphonia nigrescens	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S)	[22.95]
Joculator maximus	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S)	[22.96]
Corallina pilulifera	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S)	[22.97, 98]
Cryptonemia crenulata	β-D-Xyl*p*	*O*-6 of β-D-Gal*p*(2S)	[22.75]
Bostrychia montagnei	β-D-Xyl*p*,β-D-Gal*p*	*O*-6 of β-D-Gal*p*(2S)	[22.99]
Kappaphycus alvarezii	β-D-Xyl*p*,β-D-Gal*p*	*O*-2 and/or *O*-4 of β-D-Gal*p* *O*-3 and/or di-*O*-2,3 of α-D(L)-Gal*p*	[22.100]
Lithothamnion heterocladum	β-D-Xyl*p* 2,3-di-*O*-Me-D-Gal*p* [b]	*O*-6 of β-D-Gal*p* *O*-3 and *O*-6 of α-L-Gal*p*	[22.89]
Jania rubens	β-D-Xyl*p* 2,3-di-*O*-Me-D-Gal*p* 3-*O*-Me-D-Gal*p* 3-*O*-Me-L-Gal*p*	*O*-6 of β-D-Gal*p* *O*-3 and/or *O*-6 of α-L-Gal*p*	[22.101]
Corallina officinalis	β-D-Xyl*p* 4-*O*-Me-D-Gal*p*	*O*-6 of β-D-Gal*p* *O*-6 of α-L-Gal*p*	[22.90, 102]
Gracilaria tikvahiae	4-*O*-Me-L-Gal*p*	*O*-6 of β-D-Gal*p*	[22.103]
Gracilaria verrucosa	4-*O*-Me-L-Gal*p*	*O*-6 of β-D-Gal*p*	[22.104]
Acanthophora spicifera	β-D-Xyl*p*	*O*-3 of α-L-Gal*p*2,6diS	[22.105]
Palisada flagellifera	β-D-Xyl*p*, β-D-Gal*p*, 2,3-di-*O*-Me-D-Gal*p*	*O*-3 of α-L-Gal*p*6S	[22.106]
Phacelocarpus peperocarpos	β-D-Xyl*p*	*O*-3 of α-L-Gal*p*6S	[22.107]
Laurencia nipponica	β-D-Xyl*p*	*O*-3 of α-L-Gal*p*6S	[22.108]
Nothogenia fastigiata	β-D-Xyl*p*,β-D-Gal*p*	*O*-3 of α-L-Gal*p*6S	[22.109]
Chondria macrocarpa	β-D-Xyl*p*	*O*-3 of α-L-Gal*p*6S	[22.110]
Ceramium rubrum	β-D-Xyl*p*	Not defined	[22.110]
Okamurina (Laingia) pacifica	β-D-Xyl*p*	*O*-2 of α-L-3,6aGal*p*	[22.111]
Pterocladiella capillacea	β-D-Xyl*p*	*O*-6 of β-D-Gal*p* *O*-3 of α-L(D)-Gal*p*	[22.112]

[a] Sulfation of hydroxyls (S); [b] methylation of hydroxyls (Me)

found in fucoidans [22.152–154] (Fig. 22.2d,e, Table 22.6). The first type of fucoidan backbone structure consisting of (1 → 3)-linked partially or completely sulfated α-L-Fuc*p* units revealed fucoidans from orders *Ectocarpales* and *Laminares*: *Adenocystis utricularis* [22.141], *Analipus japonicus* [22.114], *Chordaria flagelliformis* [22.140], *Chorda filum* [22.127], *Ecklonia kurome* [22.123], and *Saccharina latissima* [22.128]. The second type is characterized by alternating the (1 → 3) and the (1 → 4)-linked α-L-Fuc*p* sequence. This backbone structure is common for fucoidans from *Stoechospermum marginatum* (order Dictyotales) [22.116] and from algae of the order Fucales, i. e., *Ascophyllum nodosum* [22.117], genera *Fucus* [22.118–120] and *Sargassum* [22.121]. Single α-L-Fuc stubs or short fuco-oligosaccharide sequences are present in many fucoidans as side chains attached mainly at *O*-2 of the backbone units. Structurally different fucoidans are commonly produced by the same algal species. For example, fucoidan isolated from *Ascophyllum nodosum* has a linear structure of alternating (1 → 3) and (1 → 4)-linked sulfated α-L-Fuc*p* [22.116]. By contrast, another fucoidan isolated from this alga is defined as a highly-

Table 22.6 Basic structures of sulfated fucans (fucoidans) from brown algae

Order	Source	Backbone	Side chains	Position	Reference
Dictyotales	*Stoechospermum marginatum*	→ 3)-α-L-Fuc*p*(2S,4S)-(1 → 4)-α-L-Fuc*p*(2S)-(1→			[22.113]
Ectocarpales	*Analipus japonicus*	→ 3)-α-L-Fuc*p*(2S,4Ac,S)-(1→	α-L-Fuc*p*	*O*-4;*O*-2	[22.114]
Fucales	*Ascophyllum nodosum*	→ 3)-α-L-Fuc*p*2S(4S)-(1→ ; →4)-α-L-Fuc*p*2S-(1→	α-L-Fuc*p* FOS[a]	*O*-2	[22.115]
	Fucus vesiculosus	→ 3)-α-L-Fuc*p*2S-(1 → 4)-α-L-Fuc*p*2,3diS-(1→			[22.116, 117]
	Fucus distichus	→ 3)-α-L-Fuc*p*2,4diS-(1 → 4)-α-L-Fuc*p*2S-(1→			[22.118]
	Fucus evanescens	→ 3)-α-L-Fuc*p*2S(4S)-(1 → 4)-α-L-Fuc*p*2S-(1→			[22.119]
	Fucus serratus	→ 3)-α-L-Fuc*p*2S4Ac-(1 → 4)-α-L-Fuc*p*2S3Ac-(1→	FOS[a]	*O*-4	[22.120]
	Sargassum horneri	→ 3)-α-L-Fuc*p*2S(4S)-(1 → 4)-α-L-Fuc*p*(2S)3S-(1→	α-L-Fuc*p*3S	*O*-2	[22.121]
		→ 3)-α-L-Fuc*p*2S-(1→; →4)-α-L-Fuc*p*2S-(1→ → 3)-α-L-Fuc*p*-(1 → 4)-α-L-Fuc*p*-(1→ (three fractions)			[22.122]
Laminariales	*Ecklonia cava*	→ 3)-α-L-Fuc*p*2S-(1 → 4)-α-L-Fuc*p*2S-(1→			
	Ecklonia kurome	→ 3)-α-L-Fuc*p*4S-(1 → 3)-α-L-Fuc*p*-(1→ (major) →?)-α-L-Fuc*p*-(1→2)-Gal-(1 → 4)-α-L-Fuc*p*-(1→ (minor)	α-L-Fuc α-L-Fuc4S	*O*-4 *O*-2	[22.123]
	Laminaria cichorioides	→ 3)-α-L-Fuc*p*2S(4S)-(1→			[22.124]
		→ 4)-α-L-Fuc*p*2,3diS-(1→			[22.125]
		→ 3)-α-L-Fuc*p*(2S,4S)-(1→; →4)-α-L-Fuc*p*(4S)-(1→	α-L-Fuc	*O*-2;*O*-4	[22.126]
	Chorda filum	→ 3)-α-L-Fuc*p*(2S,Ac)4S-(1→	α-L-Fuc*p*	*O*-2	[22.127]
	Saccharina latissima	→ 3)-α-L-Fuc*p*(2S)4S-(1→	α-L-Fuc*p*	*O*-2	[22.128]

[a] FOS – fucooligosaccharides

branched polysaccharide with mainly (1 → 3) and a few α-(1 → 4)-linked sulfated α-L-Fuc in the core region; branch points are at *O*-2 position of the (1 → 3)-linked backbone units [22.115]. Three fucoidans from *Sargassum hornery* have a different backbone structure: (1 → 3), (1 → 4), and mixed (1 → 3),(1 → 4)-linked α-L-fucans; the latter polysaccharide was nonsulfated [22.122].

Some fucoidans were identified as not true fucans but rather complex heteropolysaccharides containing fucose as the major or minor component of the backbone (core), stubs, or longer side chains [22.152–154]. The structural features of these polysaccharides are summarized in Table 22.7. The core of such fucoidans is commonly built of alternating hexuronic acids–hexose sequences. This backbone is very sta-

Table 22.7 Basic structures of fucose containing heteroglycans from brown algae

Order	Source	Backbone	Side chains	Position	Reference
Laminariales	*Kjellmaniella crassifolia*	→ 4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*(6S)-(1→	α-L-Fuc*p*(2S,3S,4S)	*O*-3 of α-D-Man*p*	[22.129]
	Saccharina (Laminaria) japonica	→ 4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→	α-L-Fuc*p*(2S,4S) fucooligosaccharides		[22.130]
		→ 3)-β-D-Glc*p*A-(1→	α-L-Fuc*p*(2S,4S) fucooligosaccharides		
		→ 3)-α-L-Fuc*p*(2S,4S)-(1→ (major)	α-L-Fuc*p*4S	*O*-2 (major)	[22.131]
		→ 4)-α-L-Fuc*p*(2S)-(1→ (minor)	β-D-Gal*p*3,4diS-(1→6)-β-D-Gal*p*4S	*O*-4	
	Saccharina latissima	→ 6)-β-D-Gal*p*-(1→	α-L-Fuc*p*, β-D-Gal*p*	*O*-2	[22.128]
		→ 4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→	α-L-Fuc*p*	*O*-3 of α-D-Man*p*	
		→ 3)-β-D-Glc*p*A-(1→	α-L-Fuc*p*	*O*-3	
	Saccharina longicruris	→ 3)-α-L-Fuc*p*4S-(1→; →6)-β-D-Gal*p*3S-(1→			[22.132]
	Undaria pinnatifida (sporophyll)	→ 3)-α-L-Fuc*p*2S-(1→; →3)-β-D-Gal*p*6S-(1→ → 4)-β-D-Gal*p*(3S,6S)-(1→; →6)-β-D-Gal*p*3S-(1→			[22.133–137]
	Costaria costata	→ 3)-α-L-Fuc*p*(2S,4S)-(1 → 3)-β-D-Gal*p*(2S,6S)-(1→			[22.122, 138]
		→ 3)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→ (core)	Galacto- and fucooligosaccharides	*O*-4 of β-D-Glc*p*A	
Ectocarpales	*Cladosiphon okamuranus*	→ 3)-α-L-Fuc*p*(4S)-(1→	α-D-Glc*p*A	*O*-2	[22.129, 139]
	Chordaria flagelliformis	→ 3)-α-L-Fuc*p*(2S,4S)-(1→	α-D-Glc*p*A	*O*-2	[22.140]
			α-L-Fuc*p*, α-L-Fuc*f*-(1→2)-α-L-Fuc*f*	*O*-4	
	Adenocystis utricularis	→ 3)-α-L-Fuc*p*4S-(1→; →3)-β-D-Gal*p*4S-(1→ → 6)-β-D-Gal*p*4S-(1→	α-L-Fuc*f* , α-L-Fuc*p*(2S)	*O*-2	[22.141]
Dictyotales	*Spatoglossum schroederi*	→ 4)-β-D-Gal*p*3S-(1 → 4)-β-D-Gal*p*-(1→ (core)	Xylofuco-oligosaccharides	*O*-2 of β-D-Gal*p*	[22.142]
		→ 3)-β-D-Glc*p*A-(1→ (core)		*O*-4	[22.143]
	Padina gymnospora	→ 3)-β-D-Glc*p*A-(1→; →4)-β-D-Glc*p*A-(1→	α-L-Fuc*p*3S, β-D-Xyl*p*	*O*-2	[22.144]

ble and other sugars including sulfated or nonsulfated α-L-Fuc*p* can be attached to it as stubs or longer side chains. The complete highly branched molecule is thus structurally similar to rhamnogalacturonans of higher plants and may perform similar functions in algal cell walls. For example, highly branched fucoidan (92.7 kDa) isolated from *Hizikia fusiforme* contains a fucose-free core composed of the alternating sequence → 2)-α-D-Man*p*6S-(1 → 4)-β-D-Glc*p*A-(1 → with a little amount of → 4)-β-D-Gal*p*-(1 → [22.146]. The α-D-Man*p*6S units are sulfated or glycosylated (branching points) at the *O*-6 position. Side chains are formed by α-D-Xyl*p*, sulfated α-L-Fuc*p*, fuco and xylo-oligosaccharides, and longer → 6)-β-D-Gal*p*3S-(1→

Table 22.7 (continued)

Order	Source	Backbone	Side chains	Position	Reference
Fucales	*Ascophyllum nodosum* (ascophyllan)	→ 4)-β-D-Man*p*A-(1→; other UA	β-D-Xyl*p*-(1 → 3)-α-L-Fuc*p*4S		[22.145]
	Hizikia fusiforme	→ 4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*(4S)6S-(1→ → 4)-β-D-Gal*p*-(1→ (core)	→ 6)-β-D-Gal*p*3S-(1→ sulfated α-L-Fuc*p*; α-D-Xyl*p* fuco- and xylooligosaccharides	*O*-2 of β-D-Gal*p* *O*-3 of α-D-Man*p*	[22.146]
	Sargassum stenophyllum	→ 6)-β-D-Gal*p*-(1→2)-β-D-Man*p*-(1→ (core)	→ 3)-α-L-Fuc*p*4S-(1→ → 4)-α-L-Fuc*p*4S-(1→ → 4)-α-D-Glc*p*A-(1→ → 4)-α-D-Glc*p*-(1→; β-D-Xyl*p*		[22.147]
	Sargassum linifolium (sargassan)	→ 4)-β-D-Glc*p*A-(1 → 4)-β-D-Man*p*-(1→ (core)	→ 4)-β-D-Gal*p*(3S,6S)-(1→ → 2)-β-D-Xyl*p*-(1→ → 3)-α-L-Fuc*p*4S-(1→	internal peripheral	[22.148–151]
	Sargassum trichophyllum	→ 3)-α-L-Fuc*p*(2S,4S)-(1→; →6)-β-D-Gal*p*-(1→			[22.39]

Table 22.8 Basic structures of ulvans from *Ulva* sp. (after [22.5])

Name	Structure	Abbreviation	Species
Ulvanobiuronic acid 3-sulfate A	→ 4)-β-D-Glc*p*A-(1 → 4)-α-L-Rha*p*3S-(1→	$\mathbf{A_{3s}}$	*Ulva rigida*
Ulvanobiuronic acid 3-sulfate B	→ 4)-α-L-Ido*p*A-(1 → 4)-α-L-Rha*p*3S-(1→	$\mathbf{B_{3s}}$	*Ulva* sp.
Ulvanobiose 3-sulfate	→ 4)-β-D-Xyl*p*-(1 → 4)-α-L-Rha*p*3S-(1→	$\mathbf{U_{3s}}$	*Ulva rigida*
Ulvanobiose 2′, 3-sulfate	→ 4)-β-D-Xyl*p*2S-(1 → 4)-α-L-Rha*p*3S-(1→	$\mathbf{U_{2's,3s}}$	*Ulva rigida*
Ulvanobiuronic acid 2′-glucuronic acid 3-sulfate A	→ 4)-β-D-Glc*p*A[β-D-Glc*p*A-(1→2)]-(1 → 4)-α-L-Rha*p*3S-(1→	$\mathbf{A_{2'g,3s}}$	*Ulva rigida*

chains. Another variant of the core was found in fucoidan of *Padina gymnospora* [22.144]. The backbone of this polysaccharide is glucuronan, consisting mainly of (1 → 3)- or (1 → 4)-linked β-D-GlcA. Ascophyllan, a branched xylofucoglycuronan isolated from *Ascophyllum nodosum*. It has a backbone made of uronic acid, mainly (1 → 4)-linked β-D-Man*p*A, and branches containing sulfated α-L-Fuc*p* and β-D-Xyl*p* [22.145]. Sargassan, a complex polysaccharide from *Sargassum linifolium*, has a glucuronomannan core with internal side chains of (1 → 4)-linked sulfated β-D-GlcA and peripheral side chains made of sulfated α-L-Fuc*p* and β-D-Xyl*p* [22.148–151].

Complex highly branched polysaccharides have been isolated from *Sargassum stenophyllum* [22.147]. One of these had a linear galactomannan core formed by (1 → 6)-β-D-Gal*p* and/or (1 → 2)-β-D-Man*p* units with side chains containing β-D-Xyl*p*, (1 → 3)- and/or (1 → 4)-linked α-L-Fuc*p*, (1 → 4)-linked α-L-Glc*p*A, and α-D-Glc*p*. Two polysaccharides obtained from *Adenocytis utricularis* were identified as galactofucan and glycuronofucan [22.141].

22.2.4 Ulvans

Ulvans are principal matrix cell wall polysaccharides of green algae, genus *Ulva* [22.155]. The composition of these polysaccharides varied according to species, harvest season, growth conditions, and method of isolation [22.5, 156]. Four monosaccharides, i. e. α-L-Rha*p*, β-D-Xyl*p*, β-D-Glc*p*A and α-L-Ido*p*A, and sulfate are the main constituents of ulvans [22.155, 156]. Like in the case of alginates, these two uronic acids are C-5 epimers. All these types of sugar units are commonly arranged in the backbone, while single β-D-Glc*p*A can also constitute the side chains. The basic structure of ulvans is described by several repeating disaccharide fragments called ulvanobiuronic acids or ulvanobioses. Ulvanobiuronic acids (Fig. 22.2f,g) contain α-L-Rha*p* and uronic acid residues, while ulvanobioses contain

β-D-Xyl*p* instead of uronic acid (Fig. 22.2h). Ulvans isolated from several Ulvales species are composed of variable proportions of these disaccharides. Table 22.8 summarizes various oligosaccharides defined as structural fragments of ulvans.

22.2.5 Other Polysaccharides

Cell walls of many green algae contain alkali soluble linear (1 → 4)-β-D-glucuronans [22.25, 157–159]. Sulfated rhamnans have been isolated from *Monostroma nitidum* and *Monostroma latissinum* (Ulotrichales, Chlorophyta) [22.25, 160–165] and from *Gayralia oxysperma* (Ulvales, Chlorophyta) [22.25, 166]. Similar polysaccharides but in smaller amounts have been detected in *Bryopsis plumosa* (Bryopsidales, Chlorophyta) [22.19]. These polysaccharides have mainly (1 → 2)-linked, but also (1 → 3) and/or (1 → 4)-linked partially sulfated α-L-Rha*p* units. Moreover, they have single stubs of uronic acid residues as side chains. Rhamnan sulfate from *Monostroma nitidum* consists of (1 → 3)-linked α-L-Rha*p* residues, some of which are sulfated mainly at *O*-2 [22.162]. Minor amounts of internal (1 → 2)-linked and branched α-L-Rha*p* linkages have also been found. Branched sulfated glucorhamnan has been isolated from the same alga [22.162]. This polysaccharide is mainly composed of (1 → 2) and (1 → 3)-linked α-L-Rha*p* (1 : 2) partially sulfated at the *O*-3 and *O*-2 positions, respectively. In addition, (1 → 4)-linked β-D-Glc*p* fragments are bound at *O*-2 of some (1 → 3)-linked backbone units. By contrast, polysaccharide from *Monostroma latissinum* is defined as highly sulfated rhamnan composed mainly of (1 → 2)-linked α-L-Rha*p* residues with sulfate groups substituted at *O*-3 and/or *O*-4 [22.163]. Sulfated heterorhamnan isolated from *Gayralia oxysperma* has (1 → 3) and (1 → 2)-linked α-L-Rha*p* units in the backbone [22.166]. The latter units are partially substituted at C-3 by side chains containing single Glc*p*A, Gal*p*A or Xyl*p*. Both types of Rha*p* units are partially sulfated at *O*-2 and/or *O*-4; side chain uronic acids are also sulfated but only at *O*-2.

Sulfated xylomannans, linear or containing single stubs of β-D-Xyl*p*, or 3-*O*-methyl-β-D-Xyl*p* at *O*-2, have been described for many red algae. Branched sulfated xylomannans (33–222 kDa) have been isolated from two species of the genus *Chondrophycus* (Ceramiales, Rhodophyta) [22.167]. These polysaccharides consist of (1 → 4)-linked β-D-Man*p*2S backbone carrying stubs (15–25%) of single β-D-Xyl*p* (70–80%) and β-D-Man*p*2S (20–30%). Several fractions from *Nothogenia fastigiata* (Nemaliales, Rhodophyta) have been identified as xylomannans with a backbone of (1 → 3)-linked α-D-Man*p* sulfated at the *O*-2 and *O*-6 positions and single stubs of β-D-Xyl*p* at *O*-2 [22.168, 169]. Sulfated xylomannan (150 kDa) has been isolated from *Sebdenia polydactyla* (Sebdeniales, Rhodophyta) [22.170]. This polysaccharide contains a backbone of (1 → 3)-linked α-D-Man*p* units, partially substituted with a single stub of β-D-Xyl*p* at *O*-6. The main polysaccharide fraction isolated from *Nemalion helminthoides* (Nemaliales, Rhodophyta) has been identified as (1 → 3)-α-D-mannan sulfated at the *O*-4 and *O*-6 positions; other fractions are similar mannans but they contain single stubs of β-D-Xyl*p* [22.171, 172]. Sulfated arabinopyranan has been isolated from the green alga *Enteromorpha clathrata* [22.173]. The backbone of this polysaccharide is mainly composed of (1 → 4)-linked β-L-Ara*p* residues, partially sulfated at the *O*-3 position. Two sulfated polysaccharides, i. e., arabinan and arabinogalactan, have been isolated from *Codium dwarkense* (Bryopsidales, Chlorophyta) [22.174]. Two water-soluble sulfated polysaccharides from *Codium vermilara* (Bryopsidales, Chlorophyta) have been identified as (i) partially 2-*O*-sulfated (1 → 4)-β-D-mannan and (ii) (1 → 3)-β-L-arabinopyranan highly sulfated at the *O*-2 and *O*-4 positions [22.23, 24].

22.3 Isolation from Algal Raw Material

Crystalline, fibrilar, and matrix polysaccharides of algal cell walls can be successfully isolated from raw material by subsequent extractions. The isolation is based on the difference in solubility of these polysaccharides in various media at neutral, acidic, or alkali conditions. Usually matrix polysaccharides are well soluble in hot water, hemicellulose components are soluble in alkali solutions, while crystalline cellulose and, in some cases, xylans or mannans, remain insoluble at these conditions. Before subsequent extraction of polysaccharides, raw material is usually washed with organic solvents to remove lipids, pigments, and other small molecules, and then washed with distilled water to remove small hydrophilic molecules. Finally, raw material is dried and milled.

22.3.1 Extraction Procedures

The subsequent extraction steps vary depending on whether the polysaccharides contain sulfate and/or carboxylic groups. Routine procedures of common extraction of polysaccharides from various algae are outlined in Table 22.9. Extraction with cold water is followed by extractions with hot water and alkali solutions at different pH and temperatures [22.175]. This approach could be defined as universal because it is effective for the isolation of major polysaccharides from red algae (sulfated galactans, agars, and carrageenans), brown algae (sulfated fucoidans and xylans), and green algae (heteroglycuronans, ulvans). Neutral sugar (NS) and UA compositions vary significantly for crude water and alkali extracts, so it is possible to separate water and alkali soluble polysaccharides. For example, sulfated galactans predominate in water extracts, while floridean starch predominates in the alkali extracts. Heterofucan was assigned as the major polysaccharide in extracts from brown algae; however, alginates were not analyzed in this report. Alternatively, a combination of other media can be used for extractions.

In other work [22.176], brown algae (*Ascophyllum nodosum*, *Fucus vesiculosus*, and *Saccharina longicruris*) were subsequently treated with selective extraction media at 70 °C to obtain three fractions: fucoidan in a mixture with laminaran (2% aq. $CaCl_2$), fucoidan (0.01 mol L^{-1} aq. HCl) and alginate (3% aq. Na_2CO_3). Three different ways of sodium alginate isolation from brown algae *Macrocystis pyrifera* (Table 22.10) were compared in [22.49]. Acid pre-treatment was used in order to eliminate polyvalent cations prior to the extraction.Alginate was then solubilized under basic conditions and purified in different ways. Three methods of precipitation/purification (ethanol, HCl, and $CaCl_2$ routes) were used to study the influence of process conditions on the final products. Both HCl and $CaCl_2$ routes give low M_w alginates of poor mechanical properties owing to the partial acidic hydrolysis of the polymeric chain by the treatment with 1 mol L^{-1} HCl. By contrast, the ethanol route showed the highest yield and good rheological properties of the products. The diluted acid pre-treatment improved the yield of the ethanol route, avoiding the acidic hydrolysis of alginate.

Extraction of agars and carrageenans needs different conditions because these polysaccharides have a marked difference in sulfate contents [22.178]. Carrageenophytes (*Mastocarpus stellatus, Chondrus crispus, Calliblepharis jubata, Chondracanthus acicularis*, and *Chondracanthus teedei*) have been used for carrageenan extraction by alkali treatment (1 mol L^{-1} of NaOH, 80 °C, 3 h), whereas agars have been isolated from agarophyte (*Gracilaria gracilis*) by hot water extraction (90 °C, 4 h). Two major cell-wall polysaccharides have been extracted from the green seaweed *Ulva rigida* sequentially with oxalate and KOH containing media [22.157]. Sulfated glucuronorhamnoxylan (ulvan) has mainly been extracted with oxalate, while two hemicelluloses, i. e., (1 → 4)-β-D-glucuronan and glucoxylan, have been isolated at alkali conditions.

Kinetic modeling (KM) is often used for optimization of extraction procedures and to obtain good quality products. For example, a kinetic model has been proposed for the alkaline extraction of alginate from the fresh brown alga *Laminaria digitata* in order to predict the yield as a function of the stirring level and the size of algae pieces [22.179]. Such models may help to

Table 22.9 Extraction of marine algal polysaccharides

Extraction media/reagents	Main polysaccharides			References
	Red algae	Green algae	Brown algae	
Sodium oxalate		Ulvan		[22.157]
$CaCl_2$			Laminaran/fucoidan	[22.176]
Diluted HCl			Fucoidan	
Cold water	Sulfated galactans	Ulvan Sulfated glycans	Fucoidan	[22.175]
Hot water	Sulfated galactans Agar	Ulvan Sulfated glycans	Fucoidan	[22.175, 177]
Na_2CO_3			Alginate	[22.176]
	Floridean starch	Glucuronan	Fucoidan	[22.175]
NaOH	Floridean starch Carrageenan	Glucuronan	Fucoidan	[22.175] [22.177]
KOH		Glucuronan		[22.157]
$NaClO_2$/AcOH		Glucoxylan		[22.157]

Table 22.10 Three ways of sodium alginate isolation from algal material according to [22.49]

Steps	Conditions/routes		
	I	II	III
Soaking	**0.1 mol l^{-1} HCl**		
Extraction	**1 mol l^{-1} Na_2CO_3**		
Precipitation			**1 mol l^{-1} $CaCl_2$**
Washing			Water reflux
Precipitation		**1 mol l^{-1} HCl**	
Solubilization		**1 mol l^{-1} Na_2CO_3**	
Precipitation	**Ethanol (1 : 1)**		
Washing	Ethanol reflux		

reduce the extraction time of high-quality alginate production. The proposed model was defined as a first step in the more complete extraction kinetic model, taking into account the effect of temperature. The effects of various variables (alkali treatment, relative amount of water, soaking/extraction time, and temperature) have been investigated on agar extraction from the red alga *Gracilaria cliftonii* [22.180]. The agar yield was significantly affected by all these variables.

Response surface methodology (RSM) has been used to determine the optimum extraction conditions for yield and further parameters of agar extraction from *Hydropuntia cornea* [22.177] and carrageenan extraction from *Kappaphycus alvarezii* [22.181]. This method explores the relationships between several explanatory (reaction conditions of designed experiments – temperature, extraction time, pH, concentration of reagents, etc.) and response (yield, molecular weight, structural features, and physical properties of extracted polysaccharides) variables [22.182]. Among the parameters of reaction conditions, the extraction temperature is supposed to be the most important for increasing of the yield. Extraction procedures can be also optimized by the assistance of other additional factors like extrusion, microwaves (MW), or specific enzymes. Alkaline extraction of alginates from *Laminaria digitata* has been modified by reactive extrusion, which enables process time savings, yield increase, and improvement of the rheological properties of the products [22.183].

Microwave-assisted extractions (MAE) of algal polysaccharides have been tested and optimized [22.183]. A combination of mathematical and statistical techniques allowed us to determine the optimal experimental conditions for MAE extraction of agar from *Gracilaria vermiculophylla*. Enzyme-assisted extraction has also been used in the processing of marine algae [22.184] using endoprotease (Neutrase) for enzymatic digestion. A novel enzymatic extraction of marine algal polysaccharides with cellulase using RSM to search for the optimal conditions (enzyme concentration, ratio of medium/raw material, and extraction time) has been developed to reach high yields [22.185].

22.3.2 Purification of Crude Extracts

The crude extracts of algal polysaccharides are usually concentrated, dialyzed against distilled water, and then freeze-dried or precipitated with an excess of ethanol. Crude extracts usually contain a mixture of two or more polysaccharides, for example, pairs of carboxylic and sulfated ones like an alginate/fucoidan mixture from brown algae or an ulvan/glucuronan mixture from green algae. These components are effectively separated by changing the pH or by treatment with Ca^{2+} cations [22.49]. Alginic acids are insoluble due to protonation of carboxyls. In addition, G-blocks in alginates are able to form egg-box complexes with Ca^{2+}, which leads to calcium alginate precipitation. By contrast, fucoidans remain soluble at low pH and weakly interact with divalent cations. Glucuronans are often co-extracted together with ulvans from the cell wall of *Ulva* sp. with the use of hot water that often contains a calcium chelating agent such as sodium oxalate; ion-exchange chromatography has been used to eliminate glucuronans from the ulvan preparations [22.157, 158].

By contrast to ulvans, $(1 \rightarrow 4)$-β-D-glucuronans are insoluble at lower pH. Based on this difference, acidic precipitation (pH 2) has been used to separate ulvan and glucuronan from the hot oxalate buffer extract (0.05 mol L^{-1}, pH 6, 90 °C, 3 h) of *Ulva lactuca* [22.159]. The high amount of uronic acids (94% m/m) and the absence of sulfate groups have confirmed the purity of precipitated glucuronan. Extracts of crude algal polysaccharide have further been fractionalized and/or purified on various preparative columns using gel permeation and/or ion exchange chromatography with isocratic or gradient elution [22.83, 173]. These procedures lead to separation of polysaccharides according to their specific retention times and to removal of impurities like proteins, phenolics, or other polysaccharide admixtures. Proteins are also removed by the treatment with various chemical reagents or proteases; phenolics

by the treatment with oxidative agents. Fucoidan was purified from its natural complex with polyphenols by the treatment with 5% H_2O_2 (pH 8.3–8.7, 18 °C), and this treatment did not lead to marked desulfation of the polysaccharide [22.83, 186]. Fucoidans treated with H_2O_2 have not demonstrated any significant changes in backbones or sulfation degrees compared with the raw polysaccharide [22.83, 187]. However, a decrease in M_w is possible at higher reaction temperatures, H_2O_2 concentration, and reaction time.

22.4 Algal Polysaccharides such as Phycocolloids

Algal matrix cell wall polysaccharides can be assigned as phycocolloids (or phycohydrocolloids) because they are soluble in water and can create colloid systems in aqueous media or participate in their formation. Some of these polysaccharides can form gels and films at appropriate conditions; the films are commonly obtained by solution casting and solvent evaporation. The physical and chemical properties of phycocolloids, including gel formation, have been reviewed in connection with their structure [22.188]. The geometry of glycosidic linkages and the ability to form intra and intermolecular junctions are prerequisites of gel and film formation. These associations may be affected by (i) negatively charged groups (sulfates and carboxylates), which tend to expend a polysaccharide chain due to mutual electrostatic repulsion; (ii) various substituents (methyls, acetyls, pyruvate ketals, glycosylation, etc.), which cause steric hindrance and/or participate in various intra and intermolecular interactions; and (iii) dissolved small molecules or metal cations that influence the hydratation property of water. Conditions such as temperature, pH, and ionic strength may also influence gel formation.

22.4.1 Alginic Acid/Alginate Gels and Films

Alginates of alkali metals and alginic acids form transparent, flexible, and water soluble films by casting of their aqueous solutions. Films prepared from Gul*p*A rich alginate demonstrate lower plasticity and have proved to be better moisture barriers than those prepared from Man*p*A rich alginate under the same conditions [22.189, 190]. Alginate films are effective cation exchangers. Their solubility may significantly decrease in the presence of multivalent metal cations [22.191, 192]. The Ca^{2+} and Zn^{2+} cations make alginate film water insoluble, increase its tensile strength, and decrease elongation. These effects are less pronounced for Cu^{2+} and Al^{3+} cations and little pronounced for Fe^{3+} and Mg^{2+} cations. Metal cations of smaller ionic radius cause tighter cross-linking of alginate films [22.193]. Cross-linking by Ca^{2+} produces significantly thicker and stronger, but less elastic alginate films; water vapor permeability decreases significantly only for highly cross-linking films [22.194]. The incorporation of multivalent metal cations into the alginate films may partially replace the weak polar interactions between polysaccharide chains by stronger cation mediated electrostatic junctions [22.195].

Alginates are able to form two types of gels: (i) alginic acid gel at acidic conditions (pH < pK_a of uronic carboxyls) and (ii) cross-linking thermostable gels at neutral conditions in the presence of Ca^{2+}. Acidic gels are stabilized by intermolecular hydrogen bonds and the junction zones of these gels are probably composed of random aggregates [22.196]. G-blocks are the most important fragments for both acidic and Ca^{2+} gel formation, whereas M-blocks support only acidic gel formation. Alternating or random sequences destabilize alginic acid gel formation due to their lack of repeating structure. In the case of neutral gels, cations of other alkaline earth metals, excluding Mg^{2+} or other divalent metal cations, can be used instead of Ca^{2+}. G-blocks, but not GM or M-blocks, of alginates interact strongly and cooperatively with these cations, forming *egg-box* type structures [22.47, 197]. Ca^{2+} ions can replace carboxylic protons in the G-blocks, which are zipped into cavities [22.198, 199]. The Ca^{2+} binding capacity does not depend on the M/G ratio of alginates, but Gul*p*A exhibit much stronger affinity to these cations than Man*p*A [22.200]. In this model, the cations (*eggs*) are situated in the cavities inside *puckered boxes* formed by four Gul*p*A units of the two superimposed chains. The individual *boxes* potentially possess oxygen ligands from COO^-, 2-OH, 3-OH, O-5 pyranoid rings, and O-4 of glycosidic bonds in the G-blocks; some of these ligands directly interact with Ca^{2+}, others participate in intra and intermolecular hydrogen bonds stabilizing the *egg-box* complex [22.201]. Based on FTIR (Fourier transformed infrared) spectroscopic analysis of ionic alginate gels with various divalent metal cations, different coordination geometry was proposed for the metal-carboxylate complexes in G-blocks (*pseudo-bridged* unidentate) and M-blocks (bidentate

bridging) [22.202]. The strength of alginate gels depends on the M/G ratio and distribution of GG, MM, and GM-blocks in the polysaccharide chain [22.203, 204]. Alginates with a low M/G ratio and a high content of G-blocks form dense and brittle gels, whereas alginates with a high M/G ratio and a high content of M-blocks give more elastic gels [22.12, 13, 205].

There are three distinct and successive steps in the binding of Ca^{2+} to alginate with increased concentration of Ca^{2+} ions [22.206]: (i) interaction of Ca^{2+} with a single Gul*p*A unit forming mono-complexes, (ii) propagation and formation of *egg-box* dimers via pairing of these mono-complexes, and (iii) lateral association of these dimers, generating *egg-box* multimers. In freshly prepared gels, junction zones are assumed to consist of *egg-box* dimers only. At higher Ca^{2+} concentration upon drying, these dimers may associate laterally into ordered domains (*egg-box* multimers) by different ways, including the most probable electrostatic interactions between Ca^{2+} and carboxylates [22.198, 207]. Lateral association of alginate chains is reduced by removing Ca^{2+} excess from the gel beads in a washing step prior to air drying. The *egg-box* multimers are disrupted by noncross-linking cations (Na^+, K^+ or Mg^{2+}), which replace the Ca^{2+} bound to the alginate. The result is swelling of the alginate gel. Ca^{2+} alginate gelation has been described as random cross-linking of multifunctional monochains [22.208]. Alginate gels showed strain-hardening behavior at large deformations, which was explained by (i) the re-orientation of chain segments longer than the distance between cross-links [22.209], (ii) structure densification or highly cross-linked polymer systems [22.205], and (iii) the deformation of rod-like junction zones [22.210].

22.4.2 Gels and Films Based on Sulfated Galactans

Some red algal sulfated galactans (agars and carrageenans) are able to form films and thermally reversible hydrogels by cooling of their hot aqueous solutions. Agar films demonstrate a lower tensile strength, water vapor permeability, swelling ratio, and water solubility than κ-carrageenan films. Each property of the mixture agar/κ-carrageenan films varies depending on the ratio of each component [22.211]. Gelation of sulfated galactans is a complex process that depends on temperature, structure, and concentration of polysaccharide, and on the presence of metal cations, which can induce conformational changes in the polymer chain. As a result, the initial coil-to-helix transition is followed by subsequent aggregation of these helices [22.212–215].

The final gel structure is the result of mutual interactions between conformational transition, molecular cross-linking, and phase-separation processes [22.216]. A honeycomb-like network has been observed for aqueous solutions both of agars and carrageenans at various temperatures. Its structure depends on the gelling stage, the structural type (D- or L- absolute configuration of the 3,6-anhydro-α-Gal*p* units), and the concentration of sulfated galactan. Honeycomb-like structures are gradually replaced by more homogeneous networks during the formation of brittle and strong agar-type hydrogels; they are retained, however, in the case of highly elastic carrageenan-type hydrogels. Therefore, the gelation mechanisms should be different for agars and carrageenans. In addition, sulfated galactan gels of high polysaccharide concentration may be structurally different from those of low polysaccharide concentration. Three possible mechanisms of carrageenan/agar gel formation focussing on the specificity of junction zones have been proposed: (i) the double-helical model based on intertwined double helices [22.217, 218], (ii) the domain model with cation-mediated aggregates of double helices [22.212, 219], and (iii) the nested, single-helix model with cation-specific salt bridges between ordered chain segments [22.220, 221]. To date, the step-by-step mechanism of gel formation as well as corresponding conformational transition, i. e., coil-helix or coil-double helix are still unclear [22.216]. Metal cations may be involved in gel formation depending on the presence and distribution of sulfate semiester groups. The shielding effect of alkali metal cations prevents electrostatic repulsion of sulfate groups in κ-carrageenan gels and thus stabilizes the double helices. Agarose gel is not influenced so much by these cations because of the lack of sulfate groups [22.222].

Carrageenan films have randomly distributed sets of pores [22.223]. Their mechanical properties depend on the sulfate/anhydro substitution of carrageenan. Pure κ-carrageenan films are stronger, less flexible, and exhibit a higher tensile strength than pure ι-carrageenan films. The tensile strength of the mixture of κ/λ and κ/ι-carrageenan films decreases with an increasing contribution of κ-carrageenan and is lower than that of pure λ and ι-carrageenan films. Reciprocal relations have been demonstrated for the elongations and water vapor permeability [22.224]. The gelation properties of carrageenans strongly depend on their structure, first of all on the presence of sulfate semiester and 3,6-anhydro groups. The high density of charged sulfates

supports extensive conformation of the carrageenan chain. The presence of the 3,6-anhydro-link in λ-carrageenan allows the α-D-Gal*p* residues to revert to their $^1C_4$ conformation, which is necessary for the initial double helix formation required for gelling. Alkali treatment of carrageenans or their precursors during processing increases the gelling properties of the final polysaccharide and the strength of gels due to conversion of α-D-Gal*p*6S to the 3,6-anhydride. As a result, κ-carrageenans and furcellaran (κ/β-carrageenan) with less sulfates but more 3,6-anhydro groups form a firm gel with K^+. By contrast, more sulfated but less or no anhydrated ι- and λ-carrageenans are slightly affected by these cations; ι-carrageenan needs Ca^{2+} to form soft elastic gels, and λ-carrageenan cannot form gels at all. Hot solutions of κ and ι-carrageenans form gels after cooling of their hot aqueous solutions to 40–60 °C in the presence of the appropriate metal cations. These gels are stable at room temperature, can be re-melted by heating to 5–20 °C above the gelling temperature, and re-gel after repeated cooling. Highly flexible carrageenan molecules are able to form helical structures when their concentration increases or temperature decreases. The mechanism of κ-carrageenan gelation in the presence of K^+ and other alkali metal cations (Na^+, Rb^+) is thus based on the cation supported aggregation of helical dimers, which consist of two single [22.225] or double [22.210] helices.

In any case, a two-step gelation mechanism has been proposed for both ι and κ-carrageenans [22.212, 214]. Thermally reversible carrageenan gels exhibit hysteresis or a difference between setting and melting temperatures. The gelation of κ-carrageenan solutions is independent of liquid-liquid phase separation due to the rapid formation of cross-linking points [22.226]. Furcellaran (κ/β-carrageenan) and κ-carrageenan gels are relatively rigid and undergo syneresis, but ι-carrageenan yields soft and elastic gels with very little tendency to undergo syneresis. In this case, the 2-*O*-sulfate groups on the 3,6-anhydro-α-D-Gal*p* units prevent the tightly-packed aggregation responsible for the rigidity of κ-carrageenan gels.

Agar is commonly defined as the mixture of neutral agarose and highly sulfated agaropectin. The ratio between these parts depends on the algal source of agar. Agarose demonstrates a much higher gelling ability than amylopectin. Agarose is well soluble in hot water (up to 80 °C) because it has chains at stiff random coil conformation [22.227–229]. Upon cooling below the gelation temperature (40 °C), the coils reorder to form helices that subsequently laterally aggregate into a network of thick bundles to form a gel. It is unclear, however, if the proposed high-ordered structure is made of a single helix [22.228–230] or a double helix [22.218]. Agarose gels (≈ 4 m/v %) are typically rigid and turbid, and undergo hysteresis between the melting and setting temperatures. The length of helices suitable for stable association increases with heating. Slow cooling leads to longer helices than rapid cooling, and longer helices promote better aggregation. As a result, the gels formed by slow cooling are stronger and more turbid than those obtained by rapid cooling [22.231]. The gelation process is followed by liquid–liquid phase separation because the network formation based on aggregated bundles leads to the dilution of solute agarose in the network interspaces [22.226, 232]. The agar chains form bundles of aggregates, generating a network at the temperature of sol–gel transition. Longer agar chains preferably aggregate, while shorter agar chains are retained in the solute and form isolated aggregates upon further cooling. Reheating leads to easy dissociation of the loose aggregates, whereas bundles of aggregates are thermostable like the whole gel [22.233].

22.4.3 Gelling/Film Forming by Fucoidans and Ulvans

In contrast to other phycocolloids, there is a little evidence about the gelling and film forming properties of fucoidans. There are, however, several reports that confirm that this polysaccharide by itself or in a mixture with other polymers is able to form gels and films [22.234]. Varying amounts of fucoidan in a liquid medium may provide specific rheological properties of the resulting gel.

Green seaweed polysaccharide ulvans are well soluble in water. However, despite their high negative charge, they demonstrate condensed conformation and low intrinsic viscosity in aqueous saline or acidic solutions, which is probably due to the hydrophobic contribution of CH_3 in Rha*p* [22.156]. This polysaccharide is probably not fully dissolved but forms free condensed spherical nanostructures (10–18 nm) dispersed in the solvent. At strong alkali conditions (pH 13) these structures collapse into a dense homogeneous network, which is the basis for strong films and gels. Gelation and film formation at lower pH levels needs divalent cations and/or boric acid. Ulvan forms thermoreversible gel in the presence of boric acid, Ca^{2+}, or other divalent metal cations (but not Mg^{2+}), and narrow region of pH around 7.5 [22.11]. The mechanism of gel formation

Table 22.11 Composite algal polysaccharide (phycocolloid) films and gels containing other hydrocolloids

Phycocolloids	Other hydrocolloids	Composite films	Composite gels
Alginate	Starch	[22.235, 236]	[22.235, 237–239]
	Pullulan	[22.240, 241]	
	Cellulose derivatives	[22.241, 242]	
	Pectin	[22.243, 244]	[22.245–247]
	Arabic gum		[22.248]
	Konjac gum	[22.249, 250]	
	Xanthan gum	[22.251]	
	κ- or ι-carrageenan	[22.252–256]	[22.257]
	Phosphorylated chitin	[22.258]	
	Chitosan	[22.259–264]	[22.265–268]
	Mucin		[22.269]
	Proteins	[22.249, 270–274]	[22.248, 275, 276]
Agar	Starch and arabinoxylan	[22.277]	
	κ-carrageenan	[22.211]	
	Galactomannans		[22.278]
	Proteins	[22.279, 280]	[22.281]
Carrageenans	Native or modified starch	[22.282]	[22.283–285]
	Nanocellulose fibres	[22.286]	
	Pectin	[22.287, 288]	
	Galactomannans	[22.289]	[22.290–292]
	Chitosan	[22.293]	[22.294]
	Proteins	[22.273, 295]	[22.296]
Fucoidan	Starch		[22.297]
	Chitosan	[22.298]	
	Protein	[22.299]	
Ulvan	Chitosan		[22.300]

is unique, very complex, and not well understood. The role of borate and cations in ulvan gelation is unclear. Both these reagents probably promote the aggregation of ulvan nanostructures mentioned above, which are interconnected by more hydrophilic parts of non-condensed polysaccharide chains. Ulvan gels should be stabilized by weak reversible interactions, so borate ester bridges between ulvan macromolecules are improbable [22.301, 302]. NMR spectroscopy has not confirmed formation of ulvan borate esters [22.303]. On the other hand, Ca^{2+} may link borate ester, sulfate semi-ester, and/or carboxylate groups forming junction zones. The unique properties of ulvan gel are interesting for systems of controlled gelation, for example, in drug delivery systems [22.303, 304].

22.4.4 Interaction with Other Hydrocolloids

Phycocolloids can interact with other natural and chemically modified hydrocolloids and between each other, forming mixed films, gels, and other structures. Some composite films and gels containing phycocolloids are overviewed in Table 22.11. Among these examples, the alginate–pectin mixtures are interesting due to their ability to form thermoreversible synergistic gels at pH < 3.8 without the addition of sucrose [22.246, 247]. The strength and melting points of these gels increase at lower pH and M/G ratios of alginates. Gelation depends on the sequential distribution of monomeric units in the alginate chain and needs tetrameric or longer G-blocks. Mixtures containing alginates with low M/G ratio and high methoxy (HM) pectins show the strongest synergism, the highest storage modulus, and the fastest kinetics of gel formation [22.245]. By contrast, gels based on alginates with high M/G ratio and low methoxy (LM) or low methoxy amidated (LMA) pectin demonstrate lower storage modulus and slower kinetics. Pectin, but not alginate, affects the network density and the strand characteristics of mixed gels, whereas the homogeneity in the gel microstruc-

ture decreases with the addition of alginate content but is independent of the pectin. Specific interactions between hydrocolloids may significantly change the properties of hydrogels. For example, the addition of starch, alginate, or κ-carrageenan decreases the strength of agar gels, while plant seed galactomannans demonstrate marked synergistic effects on the strength of agar and κ carrageenan gels [22.278, 290, 292].

Rheological properties of starch colloid systems can be regulated by the addition of fucoidan [22.297]. At lower concentrations, fucoidan probably damages the junction zones between starch macromolecules, but these polysaccharides are not mutually excluded. By contrast, at higher concentrations, fucoidan may induce considerable mutual exclusion through a phase separation process. Mixed films and gels can be prepared based on the electrostatic interaction between negatively charged phycocolloids and positively charged chitosan or protein. For example, a combination of sodium alginate and gelatine provides synergism in the rheological properties of the resulting mixed gels [22.276]. Chitosan-alginate films exhibit low solubility, good tensile properties, permeability to water vapor, and biocompatibility, which is important for biomedical applications [22.259, 260]. The addition of fucoidan to chitosan films increases their mechanical robustness and elasticity, making composite chitosan-fucoidan films more suitable as wound dressings than pure chitosan films [22.298]. Ulvan-chitosan ionic complex gels are also prepared and characterized as more stable than alginic acid (alginate)-chitosan gel under the appropriate conditions [22.300].

22.5 Biological Activities and Medicinal Applications

Besides hydrocolloid properties, both sulfated and carboxylated algal matrix cell wall polysaccharides are known to have various specific biological activities, which are prerequisites of pharmaceutical and medicinal applications. Partially sulfated algal polysaccharides exhibit immunomodulatory, anticoagulant, antithrombotic, antimutagenic, anti-inflammatory, antitumor, antiprotozoal, antimicrobial, and antiviral activities [22.305]. Generally, these biological activities are related to the structure, composition, and sulfate substitution of polysaccharide macromolecules. Among the activities mentioned above, the immunomodulating and anticoagulant activities seem to be immediate and, therefore, the most important for understanding the mechanism of action. As potential anticoagulants, algal polysaccharides are able to compete with the commonly used heparin. Because they affect the immune system, they may enhance antitumor, antibacterial, and antiviral resistance. In medicaments they can be used as an active substance if they possess the specific biological activity necessary for curing, or as a drug carrier if they are able to interact with an active substance (commonly small or nonpolymeric molecules), and thus improve its activity or provide its delivery to specific parts of the organism.

22.5.1 Immunomodulation and Related Activities

Immunomodulation is based on the specific enhancement or suppression of the host response with the aim to reach the desired immune effect [22.306]. This approach allows enhanced natural mechanisms of the immune response without the use of therapeutics like antibiotics. Immunomodulators are substances that affect the immune system. They can stimulate, suppress, or modulate any specific part of the immune system, including both adaptive and innate arms of the immune response [22.307]. Immunomodulators can be classified into three categories: (i) immunoadjuvants or specific immune stimulants, (ii) nonspecific immunostimulants, and (iii) immunosuppressants. Immunoadjuvants are true modulators that select between various types and mechanisms of immune responses. Thus, these agents are used to design vaccines or enhance their efficacy. Nonspecific immunostimulants enhance the basic level of immune response and are thus used for prophylactic purposes or for strengthening the impaired immune system. Both specific and nonspecific immunostimulants activate immunity directly or indirectly to be effective against cancer cells and various infection agents (prions, viruses, bacteria, or microscopic *Eukaryota*). Therefore, antitumor, antiprion, antiviral, antimicrobial, antifungal, or antiprotozoal activities of algal polysaccharides are often mediated by immune stimulation. By contrast, immunosuppressants are able to weaken the immune response that is often applied to reduce inflammation in various pathological states. Sulfated algal polysaccharides (fucoidans, galactans, etc.) may stimulate/inhibit cell immunity by activation/inhibition of macrophages and/or other cells of the immune systems and enhance/suppress the production of specific antibodies. Macrophages are critical in reg-

ulating innate immunity as well as adaptive immune responses by producing an array of cytokines such as interleukins (IL) IL-1β and IL-6, tumor necrosis factor (TNF)-α and interferon-γ (INF-γ), and various types of chemokines such as RANTES (regulated on activation, normal T-cell expressed and secreted), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, thymus and activation regulated chemokine (TARC), etc. [22.308, 309]. Polysaccharide macromolecules may interact with specific membrane receptors of macrophages including Toll-like receptor (TLR)-4, cluster of differentiation (CD)-14, complement receptor (CR)-3 and scavenger receptors (SR). As a result, the intracellular signaling pathways are promoted, involving mitogen-activated protein kinases (MAPK) and transcription factors. Activation of macrophages via MAPK induces the production of both nitric oxide (NO) and cytokines. Partially, interleukin-12 (IL-12) stimulates the development of T-cells, which produce interleukin-2 (IL-2) that, in turn, activates proliferation of natural killer (NK) cells, representing a first line of defence organism against both primary tumors and metastases. NK cells produce other cytokines, including interferon (INF)-γ, which can further provoke macrophages in their stimulation of T-cells via induction of IL-12. NK-mediated killing of target cells by apoptosis is facilitated by activation of caspase cascades [22.310]. Many algal polysaccharides have been reported for their immunomodulatory activities as they stimulate the activity of macrophages. Polysaccharides from the red algae *Porphyra yezoensis* [22.311, 312] and *Gracilaria verrucosa* [22.313] are reported to have macrophage-stimulating activity in vitro and in vivo. A fucoidan isolated from the sporophyll of brown alga *Undaria pinnatifida* (Korean *Miyeokgui*, Japanese Mekabu) stimulated macrophages to produce cytokines (IL-6, TNF-α) and chemokines (RANTES, MIP-1α) from macrophages and splenocytes [22.125]. Carrageenan isolated from red algae promotes mice leukocytes to produce TNF-α [22.314]. A sulfated water-soluble polysaccharide isolated from the green alga *Capsosiphon fulvescens* is reported to stimulate macrophage to release TNF-α and IL-6, and induce the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). These enzymes are responsible for the production of NO and prostaglandin E2 (PGE2), respectively [22.315]. A sulfated glucuronorhamnan, an ulvan, of the green algae *Ulva rigida*, is also reported to stimulate macrophage to secrete PGE_2 and induce an increase in COX-2 and NOS-2 expression [22.316]. Immunomodulation and other biological activities of marine algal polysaccharides are reviewed in Table 22.12.

22.5.2 Antitumor and Antimutagenic Activities

The antitumor effect of fucoidans and other sulfated algal polysaccharides that are nontoxic for tumor cells is commonly associated with the following indirect activities:

1. The inhibition of tumor cell proliferation
2. The stimulation of the apoptosis of tumor cells
3. Blocking tumor cell metastasis
4. The inhibition of blood vessel formation
5. The enhancement of various immune responses [22.393].

Partially, antitumor and antimetastatic activity of these polysaccharides is mediated by increased NO production by stimulated macrophages [22.364], activation of NK cells via specific cytokines, and/or induction of caspase-dependent cancer cell apoptosis via inhibition of specific MAPK [22.310, 381, 394]. Fucoidan may also activate caspase-independent apoptosis via activation of ROS-mediated MAPK and regulation of the Bcl-2 family protein-mediated mitochondrial pathway [22.395]. Fucoidan from *Cladosiphon okamuranus* significantly inhibited in vivo and in vitro growth and induced apoptosis of human T-cell leukaemia virus type 1 (HTLV-1) infected T-cells. This fucoidan also inhibited in vivo growth of model HTLV-1-induced tumors based on subcutaneously transplanted infected T-cell lines in immune deficient mice [22.396]. Both Miyeokgui (*Undaria pinnatifida* sporophyll) and commercial (*Fucus vesiculosus*) fucoidans showed antitumor activity against four types of cancer, i. e., PC-3 (prostate cancer), HeLa (cervical cancer), A549 (alveolar carcinoma), and HepG2 (hepatocellular carcinoma) cells [22.137]. Fucoidans blocked tumor cell adhesion that could be important for antimetastatic effects [22.397, 398]. Fucoidan from *Fucus evanescens* produced an inhibitory effect against metastasis and potentiated antimetastatic, but not antitumor, effects of cyclophosphamide [22.348]. Fucoidans suppress angiogenesis and thus reduce the supply of tumor tissues [22.399, 400]. Fucoidans stimulate proliferation of macrophages [22.401] and mediate tumor destruction through Th1 cell and NK cell responses [22.380]. Fucoidans activate lymphocytes and macrophages mediated by the production of NO, H_2O_2, and cy-

Table 22.12 Biological activities of algal cell wall polysaccharides

Polysaccharide	Algal species	Specific biological activities	References
Ulvans	*Enteromorpha prolifera*	Activation of T cells and macrophages	[22.317, 318]
	Ulva lactuca	Antioxidant (biomembrane protection)	[22.319]
	Ulva pertusa, *Ulva rigida*	Activation of macrophages	[22.316, 320]
	Monostroma nitidum	Activation of macrophages, anticancer	[22.321]
Sulfated rhamnan	*Monostroma nitidum*	Anticoagulant	[22.164]
	Monostroma latissimum	Anticoagulant	[22.163, 321]
	Monostroma latissimum	antiviral (HSV-1, HCMV-1, HIV-1)[a,b,c]	[22.322]
Sulfated heterorhamnan	*Gayralia oxysperma*	Antiviral (HSV-1, 2)	[22.166]
Sulfated arabinopyranan	*Enteromorpha clathrata*	Anticoagulant	[22.173]
Sulfated galactan	*Codium fragile*	Activation of macrophages, antiviral (HSV-2)	[22.21, 160]
	Codium cylindricum	Anticoagulant	[22.22]
	Schizymenia binderi	Antiviral (HSV-1, 2)	[22.71]
	Bostrychia montagnei	Antiviral (HSV-1)	[22.147]
	Chaetomorpha aerea	Antibacterial (*Staphylococcus aureus*)	[22.323]
Sulfated xylogalactan	*Nothogenia fastigiata*	Antiviral (HSV-1)	[22.324]
Sulfated xylomannans	*Nothogenia fastigiata*	Antiviral (HSV-1 and other), anticoagulant	[22.168, 169, 325, 326]
	Sebdenia polydactyla	Antiviral	[22.170]
	Nemalion helminthoides	Antiviral (HSV-1)	[22.171]
Sulfated heteromannan	*Capsosiphon fulvescens*	Activation of macrophages	[22.327]
Sulfated mannan	*Codium vermilara*	Anticoagulant	[22.24]
Carrageenans	*Stenogramme interrupta*	Antiviral (HSV-1, 2)	[22.8]
κ-, ι-, λ-carrageenans	*Gigartina acicularis*, *G. pisillata* *Eucheuma cottonii*, *E. spinosa*	Antioxidant (free radical scavenging)	[22.328]
ι-carrageenan	Nonspecified	Antiviral	[22.329–332]
Carrageenan	*Gigartina skottsbergii*	Antioxidant (ABTS, FCA, ORAC, ROS scavenging)[d]	[22.65]
λ, κ/ι and μ/ν carrageenans	*Gigartina skottsbergii*	Antiviral (HSV-1)	[22.333]
$\kappa/\iota/\nu$ carrageenans	*Meristiella gelidium*	Antiviral (HSV-2, DENV-2)[e]	[22.63]
Unusual hybrid carrageenan	*Botryocladia occidentalis*	pro/anticoagulant, antithrombotic	[22.62, 334–338]
Sulfated galactans	*Gelidium crinale*	pro/anticoagulant, antithrombotic	[22.337, 338]
	Gelidium crinale	Anti-inflammatory, antinociceptive	[22.339]
	Grateloupia indica	Antiviral (HSV-1, 2)	[22.340]
	Sphaerococcus coronopifolius *Boergeseniella thuyoides*	Antiviral (HIV-1, HSV-1)	[22.341]
$\kappa/\iota/\nu$ carrageenans	*Cryptonemia crenulata* *Gymnogongrus griffithsiae*	Antiviral (HSV-1, 2; DENV-1-4)	[22.76, 342, 343]
DL-galactans	*Gymnogongrus griffithsiae*		
	Schizymenia binderi	Antiviral (HSV-1, 2)	[22.71]
	Gymnogongrus torulosus	Antiviral (HSV-2, DENV-2)	[22.73]

[a] HSV: herpes simplex virus; [b] HCMV: human cytomegalovirus; [c] HIV: human immunodeficiency virus;
[d] ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ORAC: oxygen radical absorbance capacity, FCA: ferrous ion chelating ability, ROS: reactive oxygen species; [e] DENV: dengue virus

Table 22.12 (continued)

Polysaccharide	Algal species	Specific biological activities	References
Agarocolloid	*Gracilaria birdiae*	Antioxidant (DPPH)[a]	[22.344]
Alginates alginic acid	*Sargassum vulgare*	Antitumor (sarcoma 180)	[22.328]
	Macrocystis pyrifera	Immunostimulant (leucocytes, *Gadus morhua*)	[22.345]
Fucoidan (sulfated fucan)	Nonspecified	Antiprotozoal (*Leishmania donovani*)	[22.346]
	Fucus evanescens	Antitumor, antimetastatic	[22.347, 348]
	Fucus vesiculosus	Antioxidant (ROS scavenging, lipid peroxidation)	[22.328]
	Fucus vesiculosus	Immunostimulant (leucocytes, *Gadus morhua*)	[22.345]
	Fucus vesiculosus	Antitumor (mouse breast cancer)	[22.349]
	Fucus vesiculosus	Anti-inflammatory (suppression of microglia and macrophages)	[22.350–352]
	Fucus vesiculosus	Gastric protection anti-ulcer	[22.353, 354]
	Fucus vesiculosus	Anticancer (induction of apoptosis, activation of NK cells)	[22.310, 355–357]
	Fucus vesiculosus	Anti-obesity (stimulation of lipolysis)	[22.358]
	Laminaria angustata var. *longissima*	Activation of macrophages	[22.359]
	Laminaria japonica	Antibacterial (*Escherichia coli, Staphylococcus aureus*)	[22.360]
	Laminaria japonica	Anti-inflammatory (inhibition of NO production)	[22.361]
	Laminaria japonica	Antioxidant	[22.360, 362, 363]
	Cladosiphon okamuranus	Antitumour, activation of macrophages	[22.364–366]
	Cladosiphon okamuranus	Antiviral (HCV-1b)[b]	[22.367]
	Cladosiphon okamuranus	Prophylactic against prion infection	[22.368]
	Cladosiphon okamuranus	Antibacterial (*Helicobacter pylori*)	[22.369]
	Cladosiphon okamuranus	Anti-inflammatory (inhibition of IL-6 production)	[22.370]
	Cladosiphon okamuranus	Anti-ulcer	[22.371]
	Laminaria cichorioides	Anticoagulant	[22.125]
	Sargassum horneri	Antiviral (HSV-1, HCMV, HIV-1)[c]	[22.372]
	Sargassum hemiphyllum	Anti-inflammatory (inhibition of IL-6 production)	[22.373]
	Sargassum sp.	Anticancer (activation of NK cells)	[22.310]
	Ecklonia cava	Anticoagulant (inhibition of proteases II, VI and X)	[22.374]
	Ecklonia cava	Anti-inflammatory (suppression of macrophages)	[22.375]

[a] DPPH: (2,2-diphenyl-1-picrylhydrazyl); [b] HCV: hepatitis C virus; [c] HSV: herpes simplex virus, HCMV: human cytomegalovirus, HIV: human immunodeficiency virus

tokines (TNF-α, IL-6), contributing to their effectiveness against tumors [22.402].

22.5.3 Anti-Inflammatory Activity

Inflammation is a complex physiological phenomenon that responds to injury, infection, and stress. Anti-inflammatory activity is the ability to reduce inflammation and is used, partially, for pain remedying. Cytokines, such as interleukins IL-1β, IL-6, TNF-α, and NO have been known to play important roles in pro-inflammatory responses involving various types of cells [22.393]. NO is synthesized by inducible nitric oxide synthase (iNOS) isophorm, which is expressed in response to activation of nuclear factor κB (NF-κB) and MAPK induced by various effectors, such as bacterial lipopolysaccharides (LPS) or Concanvalin A (ConA). PGE2, COX-2, and monocyte chemoattractant protein-1 (MCP-1) are also related to inflammatory states. Sulfated polysaccharide from *Sargassum hemiphyllum* significantly reduces secretion of NO and pro-inflammatory cytokines (IL-

Table 22.12 (continued)

Polysaccharide	Algal species	Specific biological activities	References
Fucoidan (sulfated galactofucan)	*Adenocystis utricularis*	antiviral (HIV; HSV-1, 2)	[22.141, 376]
	Undaria pinnatifida (sporophyll)	Antioxidant	[22.377]
	Undaria pinnatifida (sporophyll)	Anti-obesity (suppression of adipogenesis)	[22.378]
	Undaria pinnatifida (sporophyll)	Anti-allergic (regulation of Th1/Th2 cell response)	[22.379]
	Undaria pinnatifida (sporophyll)	Antitumor	[22.137, 380–383]
	Undaria pinnatifida (sporophyll)	Anticoagulant	[22.355, 384]
	Undaria pinnatifida (sporophyll)	Antiprotozoal	[22.380, 385]
	Undaria pinnatifida (sporophyll)	Antiviral (HSV-1,2; human cytomegalovirus)	[22.133, 134, 386]
	Saccharina japonica	Antitumor	[22.383]
	Lobophora variegata	Anticoagulant, anti-inflammatory	[22.387]
	Spatoglossum schroederi	Antithrombotic	[22.142]
Heterofucan	*Spatoglossum schroederi*	Antithrombotic	[22.143]
	Padina gymnospora	Antioxidant, free radical scavenging	[22.388]
	Sargassum filipendula	Antioxidant, antiproliferative	[22.389]
Ascophyllan	*Ascophyllum nodosum*	Activation of macrophages, anticancer	[22.390, 391]
		Growth promoting effect on mammalian cell line	[22.392]

1b, IL-6, TNF-α) by LPS-activated macrophages; it also inhibits LPS-triggered messenger ribonucleic acid (mRNA) expressions of IL-1b, iNOS, and COX-2, which may be attributed to the downregulation of NF-jB in the nucleus [22.373]. Similarly, fucoidan from *Ecklonia cava* inhibits NO and PGE2 production by LPS-stimulated macrophages via the inhibition of enzymes iNOS and COX-2 [22.375]. Microglial activation plays an important role in the pathogenesis of neurodegenerative diseases, and fucoidans significantly inhibit LPS-stimulated microglial production and mRNA/protein expression of the mentioned inflammatory factors involving suppression of specific MAPK [22.350, 361]. Mekabu (*Undaria pinnatifida* sporophyll) fucoidan enhanced Th1 cell response and inhibited Th2 cell response in normal mice, reducing the immunoglobulin E (IgE) level in mice serum [22.379]. Fucoidan also induces IL-10 production in plasma and liver, inhibits the production of ConA-induced proinflammatory cytokines, and thereby prevents liver injury [22.352]. Fucoidan improves murine chronic colitis by inhibition of IL-6 production in colonic epithelial cells [22.370]. The gastro-protective (anti-ulcer) effect of fucoidan against aspirin-induced ulceration may take place through the prevention of the elevation of proinflammatory cytokines IL-6 and IL-12 [22.353]. The anti-ulcer property of fucoidan might contribute to protecting inflammatory cytokine-mediated oxidative damage to gastric mucosa [22.354]. The anti-obesity effect of fucoidan is also related to its anti-inflammatory activity. Miyeokgui (Mekabu) fucoidan suppresses adipogenesis via the inhibition of major markers and inflammation-related cytokines in adipocytes. Hence, it may afford some potential to control or reduce obesity [22.378]. Fucoidan from *Fucus vesiculosus* can be useful for the prevention or treatment of obesity due to its stimulatory lipolysis [22.358].

22.5.4 Activities Against Infection Agents

Marine algal polysaccharides, especially sulfated ones, have attracted considerable attention in recent years for their antiviral activities against animal viruses [22.325, 403, 404], such as HSV types 1 and 2 [22.113, 133, 170, 405], human immunodeficiency virus type-1 (HIV-1) [22.406, 407], the influenza virus [22.408], and the human cytomegalovirus (HCMV) [22.133]. Although the use of these polysaccharides and oligosaccharides as drugs is in its infancy, currently, some are already in various phases of clinical trials [22.409–412]. An *Undaria pinnatifida* extract comprising predominantly sulfated galactofucan (60−70% in mass and with relatively higher molecular weight of over 30−1000 kDa) has been patented for its in vivo antiviral activity including HSV-1 [22.413]. The antiviral activity of algal sulfated polysaccharides may have a direct or an indirect mechanism, i. e., based on (i) hindering the adsorption of viral

particles onto the host cell surface and/or direct inhibition of viral replication, or (ii) activation of immune pathways. Many viruses are able to bind to cell surface proteoglycans (heparan sulfates), which is important for the entrance of viral particles into the cell. Because they have a strong affinity to viruses, algal sulfated polysaccharides may inhibit the adhesion of viral particles onto the cell surface and thus put obstacles into the viral life circle [22.170]. If the antiviral effect of polysaccharides is significant only at the early stage of the viral replication cycle, viral adsorption is proved. By contrast, antiviral activity after infection confirms that the polysaccharide directly inhibits the viral replication and transition of virions between uninfected and infected cells (virucidal effect). For example, sulfated galactans from red algae *Sphaerococcus coronopifolius* and *Boergeseniella thuyoides* inhibit in vitro replication of both HIV and HSV-1. However, in the case of the HSV-1 cycle, these polysaccharides block the adsorption step, while a direct virucidal effect was observed for HIV-1 replication [22.341]. Antimicrobial and antiprotozoal activities of algal sulfated polysaccharides may be caused by direct interaction with microorganisms, blocking of microorganism-binding cell receptors, or by modulation of the immune response. For example, fucoidan (sulfated galactofucan) from *Undaria pinnatifida* sporophyll might inhibit cryptosporidiosis by direct binding to the *Cryptosporidium parvum*-derived functional mediators in the intestinal epithelial cells of neonatal mice [22.385]. This polysaccharide also exhibits anti-malarial activity against cultured *Plasmodium falciparum* parasites in vitro and on *Plasmodium berghei*-infected mice in vivo [22.414].

22.5.5 Anticoagulant and Antithrombotic Activities

The anticoagulant activity of sulfated polysaccharides, including those from algal sources is based on the interactions with plasma cofactors, which are natural inhibitors of coagulation proteases [22.415]. These interactions are complex and depend on the monosaccharide composition, the molecular weight, and the structure and distribution of sulfate groups. Sulfation is always important for both anticoagulant and antithrombotic activities, but the charge density is not always critical. Commonly anticoagulant activity of algal hydrocolloids, i. e., sulfated galactans, mannans, rhamnans, fucans, or more complex polysaccharides, is described as *activated partial thromboplastin time* (aPTT), *thrombin time* (TT), *prothrombin time* (PT), and *antithrombin to anticoagulation factor Xa* activities in comparison with corresponding values of heparin obtained at the same in vitro and/or in vivo experimental conditions [22.416]. The anticoagulant activity of algal sulfated polysaccharides is mainly attributed to thrombin inhibition mediated by antithrombin and/or heparin cofactor II, while other mechanisms have also been proposed. For example, several different fucoidan preparations from various algal species, including *Fucus versiculosus* [22.417, 418], *Laminaria brasiliensis* [22.417], *Ecklonia kurome* [22.418], *Ascophyllum nodosum* [22.419], *Pelvetia caniculata* [22.420], and the sporophyll of *Undaria pinnatifida* [22.384], have been reported for their anticoagulant activity. Fucoidans have potent anticoagulant activity mediated by antithrombin and/or heparin cofactor II [22.421]. The differences in anticoagulation mechanisms could be explained by the structural diversity of these polysaccharides and the interaction with several target proteases. The driving force for the formation of the sulfated polysaccharide/protein complex is the nonspecific electrostatic interaction between these macromolecules; then the complex is stabilized by other (polar or nonpolar) short-range interactions [22.422]. Anticoagulant polysaccharide should undergo appropriate conformational transitions to possess distorted ordered conformation suitable for protein binding. Model invertebrate sulfated α-L-fucans and sulfated α-L-galactans of regular linear structure show in vitro anticoagulant activity; some of them also express antithrombotic activity on in vivo models [22.421]. More complex algal sulfated polysaccharides having similar structural patterns may exhibit similar activities, which are not determined by charge density, but rather depend on monosaccharide composition and sulfation pattern. Partially, disulfated internal monosaccharide residues probably have the highest potential for anticoagulant activity due to their ability to place sulfate groups in spatial positions adequate for interaction with proteins. Slight differences in the proportions and/or distribution of sulfated residues may be critical for the interaction between proteases, inhibitors, and activators of the coagulation system, resulting in a distinct pattern in anti and pro-coagulant activities and in the antithrombotic action of algal sulfated galactans [22.337]. Sulfated galactans need much longer chains for the anticoagulant effect than heparin. Macromolecules of $\approx$15–45 kDa are able to bind antithrombin but are too short to link the plasma inhibitor and thrombin. Binding to different sites on antithrombin, sulfated galactans are less effective than heparin at promoting antithrombin conformational ac-

tivation. A different mechanism predominates over the conformational activation of antithrombin in ensuring the antithrombin-mediated anticoagulant activity of the sulfated galactans. Possibly, sulfated galactan connects antithrombin and thrombin, holding the protease in an inactive form. The conformational activation of antithrombin and the consequent formation of a covalent complex with thrombin appear to be less important for the anticoagulant activity of sulfated galactan than for heparin [22.338]. The $(1 \rightarrow 3)$-α-L-galactan, but not α-L-fucan, sulfated at *O*-2 is a potent thrombin inhibitor mediated by antithrombin or heparin cofactor II; these two polysaccharides show similar inhibitions when factor Xa is used instead of thrombin [22.423]. The proportion and/or distribution of 2,3-disulfated α-Gal*p* units along the galactan chain may be a critical structural motif to promote the interaction of the protease with specific protease and coagulation inhibitors [22.338, 424]. In contrast to the linear fucans from echinoderms and mammalian glycosaminoglycans, which need thrombin inhibitors (antithrombin or heparin cofactor II) for a reasonable anticoagulant effect, branched fucans (fucoidans) from brown algae are direct inhibitors of thrombin [22.425]. The occurrence of 2,4-di-*O*-sulfated units is an amplifying motif for 3-linked α-fucan-enhanced thrombin inhibition by antithrombin. If we replace antithrombin by heparin cofactor II, then the major structural requirement for the activity becomes single 4-*O*-sulfated fucose units, while the presence of 2-*O*-sulfated fucose residues always inhibits anticoagulant activity [22.424]. Therefore, the anticoagulant activities of sulfated galactans and fucans are not determined only by their charge density. The structural requirements for interaction of sulfated galactans and sulfated fucans with coagulation cofactors and their target proteases are stereospecific and not merely a consequence of their charge density and sulfate content [22.423, 424]. The unusual sulfated D-galactan (hybrid carrageenan) from *Botryocladia occidentalis* demonstrate marked anticoagulant activity via enhanced inhibition of thrombin and factor Xa by antithrombin and/or heparin cofactor II [22.62]. Disulfated α-Gal*p* units seem to be necessary for effective anticoagulant action, while high negative charge density itself is not so important. Sulfated galactofucan from *Spatoglossum schroederi* does not show any in vitro anticoagulant activity but demonstrates a potent antithrombotic activity in vivo and strong stimulation of the synthesis of heparan sulfate by endothelial cells. These effects have not bee observed after desulfation [22.142].

22.5.6 Antioxidant Activities

Because they are important for living organisms, enzymatic or nonenzymatic oxidative reactions can be destructive in some cases. Many products, including reactive oxygen species (ROS) and other free radicals are commonly produced by the oxidation of lipids and other biomolecules. These products promote chain reactions that cause the destruction of cellular structures and hence cell death. Antioxidants, in turn, may terminate chain reactions by free radical scavenging. Living organisms, including marine algae, have developed regulator systems based on natural antioxidants, both enzymatic (peroxidases, superoxide dismutase, and catalase) and nonenzymatic (glutathione, vitamins A, C, and E). If such systems do not work well, oxidative stress may occur and lead to various pathological states. Therefore, antioxidants might prevent many diseases, including degenerative ones, and thus promote human health and longevity. Algal antioxidants including sulfated polysaccharides have been reviewed in the context of algal biology and utilization [22.426]. The antioxidant activity of algal polysaccharides is defined as their ability to inhibit model oxidation processes; some examples are given in Table 22.12. The antioxidant properties of algal polysaccharides and associated compounds are commonly established as (i) DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical scavenging, (ii) oxygen radical absorbance capacity (ORAC), (iii) ferric ion reducing antioxidant power (FRAP) or ferrous ion chelating ability (FCA), (iv) ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and (v) ROS scavenging assays [22.427]. Structure and sulfation patterns may influence these antioxidant effects of algal polysaccharides. It has been reported that ι-carrageenan demonstrates higher hydroxyl radical scavenging than λ and κ-carrageenans [22.328]. Alternatively, algal polysaccharides have been tested in accordance with in vitro and in vivo responses to experimental oxidative stress. For example, it has been reported that ulvan-like sulfated polysaccharide from *Ulva lactuca* stabilizes mitochondrial and microsomal membranes by prevention of the oxidative stress induced by D-GalN [22.319].

22.5.7 Medicinal Materials Based on Algal Polysaccharides

Algal cell wall polysaccharides have great potential for medicinal applications because of their biocompatibility with human tissues, their biodegradability, and

their nontoxicity. Among these polysaccharides, commercially available, low-cost alginates are known to be the most suitable for the preparation of biomedical materials based on pure and composite films, gels, and fibers [22.428, 429]. Due to the high water content, porosity, elasticity, permeability, and the ability to create a moist environment, alginate gels are effective in wound healing, drug delivery, and tissue engineering. Partially, calcium alginate hydrogels demonstrate both haemostatic activity due to Ca^{2+} and promotion of the aggregation of platelets and erythrocytes. The hydration, swelling, and erosion behavior of alginate matrices depend on pH, which is also true for drug release mechanisms [22.430]. As was mentioned above, alginic acid/alginate is able to form two types of gel at acidic and neutral conditions, which is a good prerequisite for the construction of controlled-release systems [22.431, 432]. Two types of diffusion systems have been prepared based on alginates [22.431]: (i) the polymer membrane system, in which the drug formulation (solid, suspension, or solution) is inside semipermeable gel-like polymeric capsules, and (ii) the polymer matrix system, in which the drug is homogeneously dispersed in a rate-controlling polymeric matrix of swelling microspheres or conventional tablets. Sodium alginate matrices can effectively sustain drug release even for highly water-soluble drugs [22.433, 434]. Alginate-based dressings are widely used to treat various surgery wounds [22.435–437]. The medicinal effect of calcium alginate as a wound dressing is based on its cation exchange properties [22.435]. After application, Ca^{2+} of the film/fibre surface is partially replaced by Na^{+} from blood serum, which leads to swelling, partial dissolution of the polysaccharide, and gelation. The effectiveness of this process depends on the structure and source of the alginate. Some properties of alginate films like transparency, thermal stability, water absorption, and swelling behavior improve with the presence of various additives, for example *Aloe vera* [22.438]. Composite materials consisting of alginate and other natural or modified polysaccharides have been shown to be potentially useful in drug delivery systems and other medicinal applications [22.131, 439]. Much fewer references in the literature are devoted to using fucoidan for wound healing. Because it has various biological activities, this polysaccharide is interesting as a component of wound dressing materials. Fucoidan-chitosan films might be a potential treatment system for dermal burns [22.298]. Local administration of fucoidan films safely reduced post-surgical adhesion in model animals [22.440]. Fucoidan improves the physical/chemical properties (roughness, hydrophilicity and surface sulfate content) of silk fibroin films, and brings about anticoagulant activity and enhanced endothelial cell affinity to silk fibroin [22.441]. Thus, fucoidan/silk fibroin films can be applied as blood-contacting biomaterials.

22.5.8 How to Improve Algal Polysaccharides

There are many ways to improve initially inactive or weakly active natural algal polysaccharides to fit various medicinal applications. First of all, partial degradation of algal polysaccharides leads to obtaining polymeric molecules of lower molecular mass, smaller oligomeric molecules (oligosaccharides), and small molecules (monosaccharides or their fragments). Smaller fragments of algal polysaccharides are more soluble in water, less viscous, and may interact with various biological targets like cell receptors or viral particles. Marine algal polysaccharides or derived oligosaccharides can be also chemically modified by the addition of polar or nonpolar groups. Partially, sulfation of nonsulfated polysaccharides (alginate) or oversulfation of sulfated algal polysaccharides (galactans, fucoidans) is the way to increase the negative charge density of the macromolecules, which may have a beneficial effect on their immunomodulation, anticoagulant, and other properties.

22.5.9 Partial Degradation

Marine algal polysaccharides can be degraded by the use of chemical or enzymatic treatment. Examples of microbial enzymes and microorganisms that are able to cleavage algal polysaccharides are shown in Table 22.13. Biological functions of oligosaccharides originating from marine algae were reviewed by [22.454]; some of these activities are similar to the corresponding activities of native polysaccharides. For example, both native algal polysaccharides and their oligomeric fragments may stimulate defence responses and protect plants against various pathogens [22.455]. However, in other cases, algal oligosaccharides exhibit novel activities that are nontypical for the initial polysaccharides, even the way of cleavage could be important. Partially, alginate oligosaccharides obtained by enzymatic degradation promote growth and influence the fatty acid composition of the biohydrogen producer of the green algae

Table 22.13 Examples of microbial enzymes and microorganisms degrading marine algal polysaccharides

Polysaccharide	Enzyme	Microbial source	References
Alginate	Alginate lyases	*Vibrio* sp. 510	[22.442]
		Pseudomonas sp. HZJ 216	[22.443]
		Pseudoalteromonas sp. Y-4	[22.444]
Carrageenans	Carrageenases	*Cytophaga* sp. MCA-2	[22.445]
		Pseudoalteromonas carrageenovora IFO12985	[22.446]
		Pseudoalteromonas carrageenovora	[22.4]
		Alteromonas fortis	[22.4, 59, 60]
Agar	Agarase	Unidentified marine bacteria	[22.447]
		Bacillus sp. MK03	[22.448]
		Agarivorans sp. HZ105	[22.449]
		Pseudomonas vesicularis MA103	[22.450]
		Aeromonas salmonicida MAEF108	[22.450]
Fucoidan	Fucoidanase	*Sphingomonas paucimobilis* PF-1	[22.135, 136]
		Fusarium sp. LD8	[22.451]
		Luteolibacter algae H18	[22.452]
	Acetyl esterase	*Luteolibacter algae* H18	[22.452]
Ulvan	Ulvan lyases	*Persicivirga ulvanivorans*	[22.453]

Chlamydomonas reinhardtii, which may significantly enhance biomass production [22.456]. By contrast, partial acidic hydrolysis of alginate does not lead to products that are able to affect the growth of these algal cells. Alginate oligosaccharides also promote the growth of the microalga *Nannochloropsis oculata*, which is used as a feed for sea animals and zooplankton [22.457]. Alginate oligosaccharides stimulate epidermal growth factor (EGF)-dependent growth of human keratinocytes [22.458] and vascular endothelial growth factor (VEGF)-mediated growth and migration of human endothelial cells [22.459]. Alginate oligosaccharides stimulate the in vivo growth of probiotic bacteria of the genus *Bifidobacterium* [22.460]. Alginate oligosaccharides inhibit colonization of *Salmonella enteritidis* in broiler chickens [22.461]. Alginate oligomers affect the growth and quality of biofilms, as well as the state of bacterial cells inside and also reduce the tolerance of wound biofilms to antibiotics [22.462]. Alginate oligosaccharides have a high scavenging •OH activity, while fucoidan oligosaccharides demonstrate good Fe^{2+} chelation [22.463]. Low-molecular weight fuco-oligosaccharides (< 3749 Da) have been prepared by enzymatic degradation of fucoidan (sulfated and acetylated galactofucan) from the Korean *Undaria pinnatifida* sporophyll [22.135, 136]. These fucoidan oligomers exhibit strong anticoagulant activities at which APTT and TT are significantly prolonged. Unlike intact fucoidan, these fuco-oligosaccharides do not affect PT. Therefore, the molecular weight and/or sulfate content may be important factors for the anticoagulant activities of fucoidans. Fucoidan oligosaccharides can markedly reduce the arterial blood pressure and plasma angiotensin II of the resting heart rate (RHR), and the respiration of RHR has the same change trend as blood pressure [22.464]. Carrageenan and agar oligosaccharides have also been prepared and tested on various bioactivities. Various biological activities of these oligosaccharides have been observed, and often these activities depend on the molecular weight and degrees/patterns of sulfation. An oligosaccharide (2 kDa) obtained from κ-carrageenan effectively inhibits influenza A H1N1 virus replication, as well as mRNA and protein expression inside the cell [22.465]. Both κ and λ-carrageenan oligomers demonstrate antitumor activity via a complex modulation of the immune response [22.466, 467] or organ-mediated defence reactions [22.445]. The κ-carrageenan oligosaccharide fraction exhibits a relatively higher antitumor activity against the three types of cancer cells (KB, BGC, and Hela) than the initial polysaccharide [22.466]. The κ-carrageenan oligosaccharides inhibited in vitro growth of five microorganisms, i. e., *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae* (maximal effect), *Penicillium citrinum*, and *Mucor* sp. [22.465]. The short agaro-oligosaccharides (2–4 units) suppress TNF-α production and iNOS expression [22.468], while somewhat longer oligosaccharides (6–8 units) can elicit a physiological response in red algae [22.469, 470]. Agar oligosaccharides obtained by enzymatic cleavage demonstrate antioxidative activities in ROS scavenging (•OH, $\bullet O_2^-$) and inhibit lipid peroxida-

tion [22.447]. Sulfated and longer fragments show stronger antioxidant activities than nonsulfated and shorter ones.

22.5.10 Chemical Modification

Sulfation (or oversulfation) of marine algal polysaccharides is an effective way of obtaining biologically active derivatives that are especially interesting as potential anticoagulants. The patterns of sulfation are often an important factor of expected biological activity. The inactive partially sulfated and 3,6-anhydro-substituted κ, ι, and θ-carrageenans have been oversulfated regioselectively, and the anticoagulant activity of the derivatives obtained has been studied by aPTT *in vitro* assay [22.471]. The results confirmed that sulfation at *O*-2 of 3,6-anhydro-α-D-Gal*p* and at *O*-6 of β-D-Gal*p* increases the anticoagulant activity. The oversulfated κ-carrageenan showed a 30 times higher anticoagulant activity on doubling the PT time in comparison with the native polysaccharide [22.472]. This derivate also gave a threefold enhancement of the glutamic plasminogen (Glu-Plg) activation by tissue plasminogen activator (t-PA) or by urokinase (u-PA), while both native κ-carrageenan and crude heparin were less active. Similarly, oversulfated fucoidan showed four times higher anticoagulant activity in doubling PT time and gave higher stimulations of Glu-Plg activation by t-PA and by u-PA in comparison to the native fucoidan [22.473]. Oversulfation of fucoidan also enhances its antiangiogenic and antitumor activities [22.474]. Similarly what was mentioned above, oligomers of marine algal polysaccharides can be sulfated or oversulfated as well. Alginate oligosaccharides obtained by treatment with alginate lyase from *Vibrio* sp. 510 [22.442] have been sulfated by the formamide-chlorosulfonic acid method [22.475]. One of the derivatives obtained showed significant inhibition of tumor growth without direct cytotoxic effect, which was proposed to be caused by immunomodulation. Oversulfated, acetylated, and phosphorylated derivatives of κ-carrageenan oligosaccharides have been prepared and their in vitro antioxidant activities tested [22.476, 477]. In certain antioxidant systems some of these derivatives exhibit higher activities than the initial poly and oligosaccharides. The κ-carrageenan oligosaccharide derivatives inhibit tumor growth and stimulate macrophage phagocytosis and spleen lymphocyte proliferation [22.478]. Among them, the oversulfated derivative shows the highest antitumor activity expressed both in the decrease of tumor weight and in eliciting NK cell activity. These effects are significantly higher than those of the unmodified oligosaccharides. There are two ways to obtain novel materials based on marine algal polysaccharides: (i) covalent cross-linking via previously added substituents and/or cross-linking agents (dialdehydes, epichlorohydrin, phenolics, etc.), and (ii) interaction with synthetic polymers or their fragments. Porous scaffolds based on alginate derivative bearing phenolics has been prepared using horseradish peroxidase catalyzing cross-linkage formation between phenolic hydroxyls [22.479]. These materials have been shown to be suitable for cell immobilization. Cross-linking carrageenan beads (microgels) have been prepared for controlled release delivery systems using epichlorohydrin as the cross-linker [22.480]. The swelling/shrinking behavior of these cross-linked microgels in saline solutions has shown great potential for the application in food or pharmaceutical products. Highly stretchable and tough hydrogels have been prepared from cross-linked algal polysaccharide and synthetic polymers [22.481]. Both types of macromolecules form networks based on ionic (alginate) and covalent (polyacrylamide) cross-linkages. Because they contain $\approx$ 90% water, composite gels can be stretched beyond 20 times their initial length, and notched samples exhibit a stretch of 17 times. These novel material containing marine algal polysaccharides (cross-linked alginate) significantly enlarge the area of hydrogel applications.

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23. Iodine in Seaweed: Two Centuries of Research

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This paper provides a historic overview of the research on iodine in seaweeds, starting with historic uses of iodine-containing seaweeds in ancient China and Greece. Iodine as a novel element was discovered by Courtois in Napoleon-era France. This triggered research on the chemical properties of the novel element and also started an iodine-producing seaweed industry. However, iodine-containing seaweeds did not receive much academic attention until the end of the nineteenth century, when the release of molecular iodine from red algae was discovered, followed by similar findings in brown algae (kelp) three decades later. The 1950s saw the rise of studies of iodine uptake in kelp, based on the availability of radioactive iodine isotopes. Since the 1970s, a wealth of studies has investigated the emission of iodo- and other halocarbons from seaweeds and their role in the atmospheric and marine environments. Our group has addressed the physiology and biochemistry of iodine uptake in *Laminaria* since the 1990s. This has established the involvement of both hydrogen peroxide and vanadium haloperoxidases in the process and led to the finding of iodine efflux upon oxidative stress. X-ray absorption spectroscopy identified the accumulated form as iodide, constituting an antioxidant protecting the cell wall space and surface of kelp against oxidative stress. Iodide in *Laminaria* constitutes the first inorganic and chemically simplest antioxidant known from a living system. Upon exposure to atmospheric ozone, this leads to the emission of molecular iodine and, together with high irradiance, to the formation of ultrafine particles.

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As early as the third millennium B.C., the goiter-preventing effects of seaweeds had been known to the legendary emperor Shen-Nung in ancient China. The use of burnt seaweeds and sponges as diet supplements for the same purpose was common in the era of the Greek physician Hippocrates (460–370 B.C.; [23.1]). However, iodine as a novel element was discovered in the context of the Napoleonic Wars in Europe. For more detailed historical accounts, several excellent reviews are available (e.g., [23.2–4]). Bernard Courtois, a manufacturer of saltpeter (KNO_3), was trying to contribute to the war effort – and to gain a livelihood – by obtaining saltpeter from novel sources [23.2]. When replacing wood ashes as the raw material for soda by the ashes of brown seaweeds (mainly *Fucus* sp. and *Laminaria* sp., which are abundant on rocky French Atlantic coasts) in (probably November) 1811, his copper vessels not only underwent strong corrosion, but the addition of concentrated sulfuric acid also resulted in the emission of a previously unobserved violet vapor based on the reaction

$$2I^- + H_2SO_4 \rightarrow I_2 + {SO_3}^{2-} + H_2O\,.$$

Tragically, Courtois was not able to pursue his research on this new substance himself due to a lack of money and 2 years passed. Still, he managed to incite Charles Bernard Desormes and Nicolas Clément, two of his chemist friends, to pursue the work, together with André M. Ampère and the most eminent French chemist of that era, Joseph Louis Gay-Lussac. On behalf of Courtois, Clément also presented the findings to the Imperial Institute of France (Conservatoire des Arts et Métier, where he held a professorship) on November 29, 1813, which led to their original publication in the *Annales de Chimie* [23.5]. Based on the Greek *ιώδης* i. e., *violet*, due to the beautiful violet color of its vapor, the name *iode* was already used in this paper for the new substance. It also mentions the metal-like appearance of its solid, elemental

state. Soon afterwards, on December 6 and 20 of the same year, *Gay-Lussac* presented his results about the compounds that this novel element forms with others [23.6, 7].

Amazingly, despite the war in which most of Europe was embroiled in at that time, scientific exchange between opposing powers was little affected. Sir Davy, the leading British chemist of the time, corresponded with his French colleagues (mail was transported between the warring countries by smugglers and cartels). Napoleon himself, enthusiastic about science, gave him free passage to France and onwards to Italy. Ampère gave Davy a sample of iodine, inducing him to conduct his own research on the new substance (Davy was traveling with a portable chest for chemical experiments). After initially believing that it was merely a compound of chlorine, *Davy* ultimately came to conclude that it was an element in its own right [23.8], and competed for a while with Gay-Lussac over priority rights (even though both Davy and Gay-Lussac always acknowledged Courtois as the discoverer of iodine). Despite the scientific fame of the discovery and rising commercial interest in iodine, e.g., for the treatment of wounds, Courtois failed to capitalize on his discoveries and, sadly, died in poverty on September 27, 1838, aged only 62 [23.3]. Iodine production from seaweeds became a major economic activity in coastal regions of Europe – in particular, in parts of Brittany, Normandy, Ireland, and Scotland, and it features in many historic and travel accounts of these places (e.g., [23.9]).

While the chemical knowledge of the novel element progressed, iodine-accumulating seaweeds did not receive much research attention until the end of the nineteenth century. Exemplary for this period are the studies of *Eschle* [23.10], who investigated the iodine content of *Fucus vesiculosus* and *Laminaria digitata*. As early as 1894 *Golenkin* reported on the release of free iodine by the red alga *Bonnemaisonia asparagoides* – detected by a blue stain of starch in paper [23.11]. Several decades passed until this was more widely accepted by the community, in particular by the studies of *Sauvageau* [23.12] on red algae and of *Kylin* [23.13] and *Dangeard* [23.14] in the 1920s. The latter, obviously working in competition, were the first to report the emission of molecular iodine (I_2) from kelp (*L. digitata*) surfaces, termed *iodovolatilization*. Remarkably, their discovery was forgotten or ignored until the re-discovery of molecular iodine emissions in recent years [23.15, 16].

The rise of nuclear physics and the availability of radioisotopes enabled studies of the uptake mechanism of iodine in brown algae, notably those of *Tong* and *Chaikoff* on the Pacific kelp *Nereocystis luetkeana* [23.17], of *Bailey* and *Kelly* on *Ascophyllum nodosum* [23.18], and of Shaw on *Laminaria* [23.19, 20]. In about the same era, the requirement of macroalgae for iodine as a micronutrient was recognized [23.21].

The implications of iodine emissions for atmospheric and marine chemistry were not recognized until the findings of strongly increased methyl iodide concentrations around kelp beds by *Lovelock* [23.22]. The vast majority of these studies focused on organic iodine compounds, curiously ignoring the findings of inorganic, molecular iodine emissions made more than half a century earlier [23.23]. More evidence for the impact of kelp forests on the coastal atmosphere came from research by *Alicke* et al. [23.24], detecting iodine oxide, and *O'Dowd* et al. [23.25], highlighting the role of iodine oxides in aerosol formation in the marine boundary layer (however, postulating diiodomethane as the precursor). Not until the work of *McFiggans* et al. [23.15] and *Palmer* et al. [23.16], however, did the importance of inorganic iodine emissions for coastal, ultrafine particle formation become clear.

Our research on iodine metabolism in *Laminaria* started with a study on its uptake mechanism, based at the Station Biologique de Roscoff in the 1990s. Initially, this work confirmed that iodide (and not iodate, present at roughly equal levels in surface sea water, with a total iodine concentration of around 0.5 µM [23.31, 32]) is the species taken up. Total iodine concentrations of up to over 5% dry weight were observed in young *Laminaria* thalli – effectively constituting the strongest iodine accumulators observed so far among all living systems. It also revealed that an intact cell wall is required for sustained iodine uptake – protoplasts (i. e., algal cells, from which the cell wall has been enzymatically removed) do no take up iodine [23.26]. However, iodine uptake in protoplasts can be restored to levels corresponding to those observed in *Laminaria* tissues under physiological conditions when either exogenous H_2O_2 or a constitutively H_2O_2-producing, enzymatic system are added. These results highlighted the importance of hydrogen peroxide in iodide uptake. Subsequently, experiments were conducted with *Laminaria* tissues, investigating the effect of exogenously added H_2O_2. Remarkably, exogenous H_2O_2 did enhance iodide uptake – but only up to H_2O_2 levels of around 25 µM. Higher hydrogen peroxide levels resulted in a pronounced efflux of previously accumulated iodine – providing first clues of the biological significance of iodine accumulation in

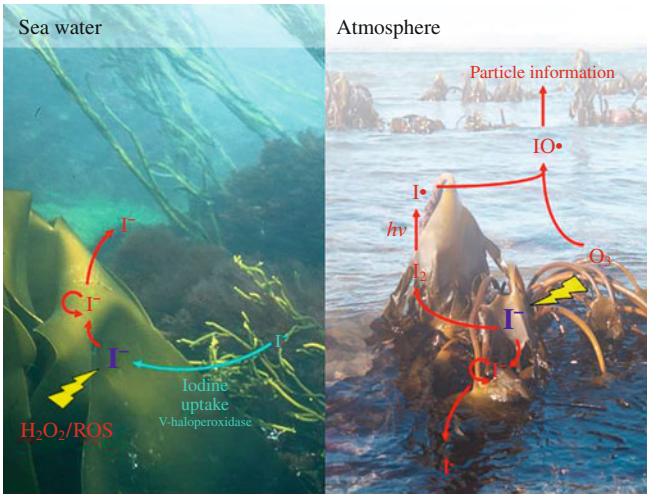

Fig. 23.1 Known pathways of iodine between algal metabolism and the environment in the brown alga *Laminaria digitata*, the strongest accumulator of this element among all living systems. Most of the time, kelp forests are covered by water (*left*). In this largely unstressed steady state (*turquoise*), iodide from sea water is taken up by a mechanism driven by low micromolar levels of hydrogen peroxide and vanadium haloperoxidases [23.26] and stored in the apoplast of cortical cells [23.27]. Iodide is the accumulated species. Upon oxidative stress (*red*) such as an oxidative burst [23.28], this accumulated iodide is mobilized and scavenges hydrogen peroxide by a pseudocatalase-like reaction sequence known as iodide-(halide-)assisted disproportionation of hydrogen peroxide (iodide acts as catalyst, i. e., it is regenerated at the end of a reaction cycle – there is no accumulation of oxidized or organic iodine species [23.29]). When kelp forests are partially exposed (*right*), e.g., during spring tides, significant oxidative stress from various causes can occur, including both atmospheric oxidants such as ozone but also hydrogen peroxide from the photosynthetic Mehler reaction accumulating in the liquid microlayer on the wet algal surface. Ozone reacts directly with iodide, resulting in the release of volatile, molecular iodine (I_2) at flux rates so high that it can easily be detected in the marine boundary layer of the atmosphere photometrically, using starch paper or even by the human nose. Molecular iodine becomes photolyzed rapidly and rapidly reacts further with ozone, forming ultrafine particles consisting of iodine oxides. These are highly hygroscopic and thus provide cloud condensation nuclei [23.30]. In contrast to ozone, hydrogen peroxide from the Mehler reaction is scavenged by iodide-assisted disproportionation as described above

general. This work also established the involvement of vanadium haloperoxidase in iodine uptake: Only the macroscopic *Laminaria* sporophytes take up significant levels of iodine, the haploid, filamentous gametophytes do not. The former have high levels of haloperoxidases, the latter do not. However, iodine uptake can be induced in gametophytes upon addition of exogenous H_2O_2 and haloperoxidase. Subsequently, the Roscoff group identified *Laminaria* iodoperoxidase, a novel subclass of the vanadium haloperoxidases [23.33, 34] which may explain the selectivity for iodide uptake in *Laminaria*.

We joined forces with the Norwich group to investigate iodocarbon emission rates from a range of ecologically relevant seaweed species from the European Atlantic cost [23.35]. These studies went in parallel with a survey of natural *Laminaria* populations on different parts of the European coast. Oral traditions of Breton seaweed gatherers (goémoniers) had suggested that kelp contained more iodine in the winter than in the summer, and also that seaweeds from northerly locations in Europe had more of the precious commodity than in the south (*Kloareg*, personal communication). Interestingly, this could be confirmed; at about 1% dry weight total iodine, fully-grown *Laminaria* thalli contain about twice as much iodine in the winter as in the summer [23.36]. Recently, *Verhaeghe* and the Roscoff group demonstrated that iodine accumulation occurs in the apoplast of cortical *Laminaria* cells [23.27].

Ultimately, we turned our attention to what we considered the core question, which had remained hitherto unanswered, despite almost two centuries of research on iodine in seaweeds: why do these algae actually

have to accumulate this element to such extreme levels? For this, it seemed crucial to first unambiguously clarify the chemical speciation of accumulated iodine. In fact, various and sometimes contradictory opinions existed about the chemical state of iodine in *Laminaria*. To this end, we used a non-invasive technique, X-ray absorption spectroscopy, thus excluding the possibility of artifacts upon extraction [23.37]. This approach revealed that the accumulated form is, indeed, iodide, which is contained in a largely organic (rather than hydrated) environment. Upon oxidative stress such as an oxidative burst [23.28, 38–40], a transition to a more hydrated form occurs. Concomitantly, a strong efflux of accumulated iodide occurs. Altogether and in light of the hypothesis of *Venturi* [23.41], our results suggest that iodide serves the function of an antioxidant, protecting the apoplast (cell wall space) of the cortical cell layers. This constitutes the description of the first inorganic and of the chemically simplest antioxidant known from a living system [23.29]. Indeed, the reaction of iodide with the major reactive oxygen species is both thermodynamically and kinetically favorable. With the involvement of vanadium haloperoxidase and in the absence of organic co-substrates, iodide effectively degrades H_2O_2 (halide-assisted disproportionation of hydrogen peroxide). Furthermore, the millimolar iodide levels on the *Laminaria* surface exposed at low tide effectively scavenge atmospheric ozone, leading to the release of I_2 – at rates up to about five orders of magnitude higher than the combined iodocarbon emissions. These studies [23.16, 29] unambiguously clarify the biochemical origin of particle-forming iodine oxides. Molecular iodine is photolyzed and further oxidized by ozone in the marine boundary layer, producing hygroscopic iodine oxides. The latter form ultrafine particles, leading to aerosol formation and establishing a unique link between a biological antioxidant and climatic processes [23.30].

The current understanding of iodine metabolism is summarized in Fig. 23.1. Using X-ray absorption spectroscopy in combination with other techniques, we have recently demonstrated that bromide complements iodide as an antioxidant in particular with regards to superoxide detoxification and that overall, its function is more diverse than that of iodide in algal physiology [23.42].

In the 200 years since its discovery, research on iodine has developed into multiple areas of science, far beyond marine algae where it was originally found and beyond the scope of this chapter. In this context, we would like to highlight a recent interdisciplinary review article by an international consortium [23.43] that covers the many fascinating facets of iodine science and applications ranging from the history of its discovery to general, marine, atmospheric, environmental, radio- and geochemistry, medicine and physiology, materials science (in particular including semiconductors and solar cells), and organic synthesis.

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24. Marine Macrophytes: Biosorbents

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Biosorption is a biological method suggested as a cheaper and more effective technique for heavy metal ion removal and recapture from aqueous solutions such as industrial wastewater. A large range of biomass, principally bacteria, algae, seagrasses, crab shells, yeasts, and fungi have received increasing attention for heavy metal ion removal and recovery. In particular, through this method, nonliving aquatic macrophytes (i. e., macroalgae and seagrasses) can be used for heavy metal removal due to their large availability, easy regeneration, and low costs. In recent years, macrophytes have been shown to be able to remove pollutants by surface adsorption or by bioaccumulation, incorporating metals into their tissues or storing them in a bound form. The type of biomass used in the treatment procedure can make a significant difference for the removal of pollutants. Furthermore, the knowledge of metal kinetic biosorption parameters for nonliving macrophytes has become crucial for treatment design to improve the efficiency of metal removal in artificial systems for their recycling. In this chapter, the properties of dried macrophytes in the treatment of wastewater and their cell wall and cuticle compartments are described. Moreover, the procedures for biosorption experiments are analyzed.

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24.1 Marine Macrophytes

The term *macrophytes* indicates aquatic organisms capable of performing oxygenic photosynthesis that are sufficiently large-sized to be observed with the bare eye. They typically grow submerged or floating in riverine, estuarine, and marine coastal environments, where they play a key role as primary producers. Marine macrophytes form the bulk of the photosynthesizing biomass in coastal habitats; they belong to two different groups, indicated with the common names of algae and seagrasses.

24.1.1 The Algae

The term algae designates the complex of all oxygenic photosynthesizers other than embryophyte plants [24.1]. As such, algae represent a heterogeneous assemblage of organisms belonging to many separate evolutionary lineages, which in modern classifications are distributed in no less than four eukaryotic supergroups (Archeplastida, Chromalveolata, Excavata, Rhizaria) [24.2]. Due to such vast evolutionary diver-

sity, different groups of algae exhibit striking differences in terms of morphology, ultrastructure, ecology, biochemistry, and physiology.

The benthic macroalgae, or seaweeds, are the most common macrophytes on marine shores where stable substrata exist (natural rock, artificial reefs, concrete, or wood pillars). They belong to three different groups, empirically recognized on the basis of thallus color: the red algae (phylum Rhodophyta), the brown algae (phylum Heterokontophyta, class Phaeophyceae), and the green algae (phylum Chlorophyta, class Ulvophyceae). Although they co-exist in the same coastal habitats, these three groups are phylogenetically distinct and are characterized by different biochemical and physiological traits. This has important implications with regard to their biosorption properties.

24.1.2 Marine Benthic Green Algae (Chlorophyta, Ulvophyceae)

The Viridaeplantae, formed by the embryophyte plants and the complex of algae generally called green algae, is one of the most diverse evolutionary lineages within the eukaryotic supergroup Archaeplastida [24.3, 4]. Molecular and ultrastructural studies conducted in the last 20 years suggest that the Viridaeplantae evolved from a unicellular flagellate ancestor, from which an early separation in two lineages took place [24.3, 4]. One of these, the Streptophyta, radiated in freshwater environments and eventually gave rise to terrestrial embryophyte plants. The other, the Chlorophyta, had a more complex evolutionary history and gave rise to several different groups of green algae. The class Ulvophyceae is the most diverse of these groups in terms of thallus complexity and cellular sophistication, and includes the vast majority of the green benthic macroalgae (about 1600 species) [24.4].

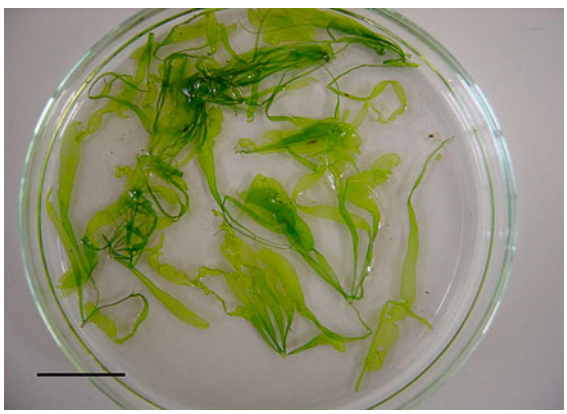

Fig. 24.1 Thallus of *Ulva compressa* (green algae) from Portonovo, Ancona, Adriatic sea (Italy). Scale bar: 2 cm

Species of Ulvophyceae occur in all oceans and are most abundant in the intertidal and shallow subtidal zones of rocky shores. They mostly consist of pluricellular macrophytes (up to 1 m in size) growing attached to stable substrata, but some species (particularly in the genus *Ulva*) (Fig. 24.1, *Ulva compressa* Linnaeus) can get detached and survive floating for a long time. Their morphologies range from microscopic unicells to macroscopic multicellular plants, and giant-celled organisms with unique cellular and physiological characteristics [24.4]. Four main cytomorphological types are recognized in this class [24.5]: nonmotile uninucleate cells (e.g., *Oltmansiellopsis*); multicellular filaments or blades composed of uninucleate cells (e.g., *Ulva*); multicellular filaments composed of multinucleate cells, with nuclei organized in regularly spaced cytoplasmic domains (e.g., *Cladophora, Chaetomorpha*); siphonous thalli, i. e., consisting of a single giant tubular cell, which often contains thousands to millions of nuclei (e.g., *Acetabularia*, *Caulerpa*, *Codium*, and *Halimeda*).

The Ulvophyceae possess chlorophylls a and b and a characteristic set of accessory pigments including the xanthophylls lutein, zeaxanthin, violaxanthin, antheraxanthin, and neoxanthin [24.6]. Siphonein and siphonoxanthin are found in the siphonous representatives. The main reserve polysaccharide is starch, which occurs as grains. The chloroplasts are surrounded by two membranes and may contain one or more pyrenoids. Flagellate cells possess two, four, or numerous flagella, which are similar in structure and in the basal part exhibit a transition zone with stellate structure. Details in the ultrastructure of the flagellar apparatus and mitosis differentiate the Ulvophyceae from other classes of green algae.

24.1.3 The Red Algae (Rhodophyta)

The Rhodophyta are one of the most ancient groups of photosynthetic eukaryotes (fossils of *Bangiomorpha pubescens*, considered the oldest red alga, are approximately 1.2 billion years old [24.7]). This phylum includes about 6200 species, which occur primarily in marine environments (150 species are found in freshwater). Red algae are ubiquitous on rocky shores of all oceans and represent the dominant group in seaweed floras throughout the world. They are also the benthic macroalgae with the widest ecological range, occurring at all levels of the photic zone. Some species occur in

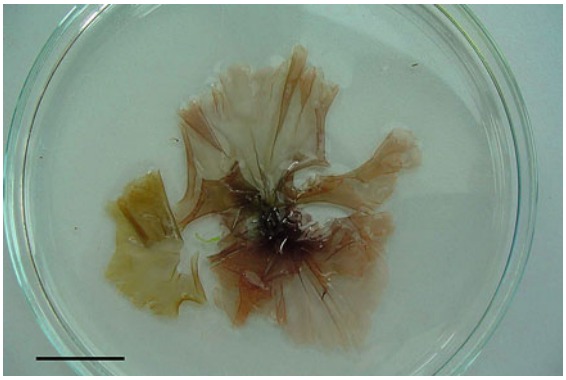

Fig. 24.2 Thallus of *Porphyra leucosticta* (red algae) from Portonovo, Ancona, Adriatic sea (Italy). Scale bar: 2 cm

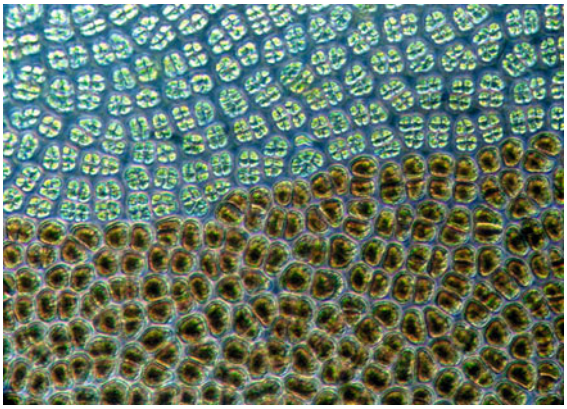

Fig. 24.3 Vegetative and reproductive cells of the thallus of *Porphyra leucosticta* (red algae) from Portonovo, Ancona, Adriatic sea (Italy). Magnification: 40×

the upper intertidal zone, where they are able to tolerate prolonged desiccation (e.g., members of the genus *Porphyra*) (Figs. 24.2, 24.3, *Porphyra leucosticta* Thuret). Most Rhodophyta, however, occur in subtidal habitats, and some species can reach considerable depths. The deepest-living benthic algae known are coralline red algae recorded at −268 m depth off the shores of Bahamas, where the available light irradiance was 0.001% as at the surface [24.8]. From a morphological point of view, this group is equally diverse and includes spherical unicells, thin uniseriate filaments, expanded blades with variable shape, encrusting forms growing attached to the substratum, and corticated macrophytes with parenchymatous structure [24.9].

Chlorophyll a is the only chlorophyll present in this group. Accessory pigments include phycobiliproteins (phycoerythrin, phycocyanin, allophycyanin), carotenoids (α-carotene, β-carotene), and xanthophylls (lutein, zeaxanthin, antheraxanthin, violaxanthin). The main reserve product is a polysaccharide called floridean starch, which is accumulated in grains in the cytoplasm. The chloroplasts are enveloped by two membranes and contains numerous parallel thylakoids, arranged singly and not stacked; numerous phycobilisomes (structures formed by phycobiliproteins) occur on the surface of the thylakoids. A distinctive feature of the red algae is the complete absence of flagella and centrioles in all stages of the life history; the male gametes, called spermatia, are small and spherical and are carried by water currents to the female gamete (called carpogonium). The cytokinesis is brought about by the development of a cleavage furrow that in the majority of the species does not complete the separation between adjacent cells and leaves a protoplasmic connection called pit connection.

24.1.4 The Brown Algae (Heterokontophyta, Phaeophyceae)

The class Pheophyceae, or brown algae, includes approximately 1800 species distributed almost entirely in marine environments (with a few freshwater species). Although they coexist with red algae and ulvophyte green algae, they are not closely related to these groups and belong to a different eukaryotic supergroup, the Chromalveolata [24.10]. They are believed to have arisen between 200 and 150 mya [24.11].

The Phaeophyceae are present in all seas of the world, but are most diverse and abundant in cold seas. This group includes the largest-sized seaweeds and

Fig. 24.4 Thallus of *Cystoseira barbata* (brown algae) from Portonovo, Ancona, Adriatic sea (Italy). Scale bar: 4 cm

Fig. 24.5 Thallus of *Dictyota dichotoma* (brown algae) from Portonovo, Ancona, Adriatic sea (Italy). Scale bar: 2 cm

Fig. 24.6 Transverse section of the thallus of *Dictyota dichotoma* (brown algae) from Portonovo, Ancona, Adriatic sea (Italy). Magnification: 40×

represents a major component of the algal biomass on many rocky shores. In many temperate regions the lower part of the intertidal zone is occupied by dense canopies of brown algae of the order Fucales (*Cystoseira* in the Mediterranean, *Fucus* and *Ascophyllum* in the North Atlantic, *Hormosira* in Australia and New Zealand) (Fig. 24.4, and *Cystoseirabarbata* (Stackhouse) C. Agardh). Species of the order Laminariales (mainly of the genera *Laminaria*, *Ecklonia*, *Lessonia*, and *Macrocystis*) can reach 70 m in length and form large submerged forests in cold seas of both hemispheres.

Brown algae exhibit a great variation in habit, ranging from thin, uniseriate filaments to complex pseudoparenchymatous thalli (Figs. 24.5, 24.6, *Dictyota dichotoma* (Hudson) J.V. Lamouroux). They possess chlorophylls a and c (in the forms c_1, c_2, and c_3), and β-carotene and fucoxanthin as main accessory pigments. The main storage polysaccharide is chrysolaminaran, a β-1,3 glucan deposited in liquid form in special vacuoles. The chloroplasts are surrounded by four membranes, two of which are formed by endoplasmic reticulum; the thylakoids are packed in stacks of three parallel to each other and a peripheral thylakoid called girdle lamella usually encloses the others. The flagellate cells possess two flagella which differ in length and structure; one is long, pleuronematic (i. e., provided with thin tripartite tubular hairs), and directed forward; the other is short, smooth, and possesses a basal swelling in proximity of which the photoreceptor apparatus of the cell is located.

24.1.5 Seagrasses

The phylum Magnoliophyta includes the plants generally known as angiosperms, or flowering plants. This is the most specialized and successful group of vascular plants, which is currently represented by about 275 000 species and dominates the vegetation of most terrestrial ecosystems. The oldest angiosperm fossils date back approximately 135 mya, with the major lineages evolving between 130 and 90 mya [24.12]. Between 100 and 65 mya some lineages of angiosperms moved back to aquatic environments, and a few of them acquired the capability to live in seawater. Marine angiosperms, or seagrasses, are represented today by 60 species belonging to 13 genera and 4 families (Cymodoceaeceae, Hydrocharitaceae, Posidoniaceae, Zosteraceae). As vascular plants, they have a body that can be divided into roots, stem, and leaves, and a vascular apparatus

Fig. 24.7 Thallus of *Cymodocea nodosa* from Gabicce, Pesaro and Urbino, Adriatic sea (Italy). Scale bar: 4 cm

for the transportation of nutrients, water, and gases. Overall all species of marine angiosperms have developed a similar habit, characterized by a prostrate stem (rhizome) supporting bundles of ribbon-like leaves with parallel veins connected by short transversal veins (Fig. 24.7, *Cymodocea nodosa* (Ucria) Asch.).

24.2 Heavy Metals: Definition and Toxicity

Heavy metals such as arsenic (As), cadmium (Cd), chrome (Cr), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn), have a specific gravity (i. e., measure of density) at least five times that of water. Because of their solubility in the water environments, heavy metals can be absorbed by living organisms [24.13–15]. It is known that in small quantities heavy metals are essential for human nutrition, however, when consumed in high amounts they can cause health problems. Indeed, elements like iron, copper, nickel, manganese, selenium, and zinc are common in nature and are normally ingested with food (foodstuffs, fruits, vegetables, multivitamin products) [24.16–18]. The anthropogenic impact on the environment has increased the presence of heavy metals, which is of major concern because of their toxicity and threat to plant and animal life. The main anthropogenic sources of heavy metals released into the environment are:

a) Acid mine drainage (AMD), caused by mining operations
b) Electroplating industry waste solutions (e.g., zinc plating, nickel plating, chrome plating, oxidation)
c) Coal-burning applications for power generation and
d) Nuclear technology for power generation (uranium mining/processing and special waste generation).

Moreover, heavy metals are common in industrial applications like the manufacture of batteries, textile dyes, pesticides, alloys, steel, and others. The risks for human health and the toxicity associated with heavy metals are now well known [24.19–21]. Heavy metals in significant quantities are released into freshwater bodies and seas. These substances can be accumulated and biomagnified along sediment, water, and aquatic food chains, resulting in fatal effects in fish populations, and for humans. They become toxic when they are not metabolized by organisms and accumulate in the soft tissues [24.19, 20]. The Agency for Toxic Substances and Disease Registry (ATSDR), based in Atlanta (Georgia), is one of the largest federal public health agencies of the USA. It provides trusted health information to prevent harmful exposures and diseases related to toxic substances.

24.3 Biosorption

Biosorption is a method that can be used for the removal of pollutants from wastewater, especially those that are not easily biodegradable like heavy metals and industrial dyes. Furthermore, this process can remove low heavy metal concentrations as an inexpensive, simple and effective alternative to conventional methods like activated carbon [24.22–26]. The recapture of heavy metals from industrial wastewater is of dual interest: (i) the prevention of threats to health (metal toxicity) [24.19–21], and (ii) society is realizing the importance of recycling these substances for metal costs [24.27–29]. When the concentrations of wastewater contaminants are above 100 mg/L, the biological treatment processes for removing heavy metals are the most effective [24.30]. The treatments used are precipitation, electrodialysis, and reverse osmosis, which become costly and difficult to manage at low metal concentrations (< 100 mg/L) [24.31, 32]. The potential of metal biosorption by biomass materials has been well established in the last 15 years. This technique uses a variety of biological materials for binding of these pollutants, including fungi [24.33], bacteria [24.34–36], plants [24.37], macroalgae [24.24–26, 38], microalgae [24.39, 40], and agricultural and industrial wastes [24.41–43]. For economic reasons, particular interest must be given to large biomass types, either generated as a waste by-product of large-scale industrial fermentations or certain metal binding macrophytes found in large quantities in the sea. Recent studies showed that aquatic macrophytes (i. e., seaweeds and seagrasses) can be used as potential biosorbents for the removal of heavy metals [24.24–26, 38, 44–46]. This is considered one of the most promising types of biosorbents in view of its high capacity for uptake, its low

cost, and its abundance in many parts of the world. Indeed, a comprehensive collection of algae for food and algal phycocolloids (agar, carrageenans, alginates) is in excess of 3 million tons per year, with a potential estimated at around 2.6 million collected for red algae and 16 million for brown algae [24.47].

The biosorbent ability of the macrophytes resides in the structure of the cell walls of seaweeds and the cuticle of seagrasses, which are responsible for this phenomenon. Indeed, in biosorption, the ability of active sites on the surface of macrophytes to bind and concentrate heavy metals from even the most dilute aqueous solutions is exploited. The cell wall intervenes in the mechanisms of biosorption through physicochemical processes like ion exchange, complexation, microprecipitation, and electrostatic interactions. Specifically, in solution heavy metals can form ions (cations or anions), which create a passive binding with nonliving biomass through a mechanism that is not metabolically controlled, in contrast to bioaccumulation by living cells [24.48–50]. The use of dried nonliving macrophytes as adsorbent material has considerable advantages, such as:

a) High efficiency in detoxification of diluted effluents
b) Minimizing the volume of waste, biological and/or chemical
c) No demand for nutrients, and
d) Low cost.

An additional advantage is represented by the possible recovery of heavy metals: once the binding of metals reaches saturation, the biomass can be regenerated with acid solutions and/or hydroxyl, which release small volumes of heavy metal concentrates [24.51–53]. These may be subsequently treated with techniques of co-precipitation, flocculation, and electro-deposition.

24.3.1 Cell Wall of the Algae

The cell wall is the portion of the algal cell directly involved in biosorption; its chemical structure has a major effect on the mechanisms by which this phenomenon takes place. The electrostatic attraction and the complexation of the groups present on the surface of the cell wall with the metal in solution play a key role in this regard [24.25]. The polysaccharides present in the cell walls of algae contain chemical groups such as carboxyl, hydroxyl, and sulfate, which may act as binding sites [24.54]. They are effective ion exchangers and, therefore, important sites for complexation of metal cations [24.55, 56]. Proteins, lipids and, occasionally, nucleic acids may also be present on the surface of the cell walls. These molecules, however, occur mainly in the plasma membrane and in the cytoplasm; they are, therefore, bound to metal ions through their functional groups (aminic, carboxylic, imidazolic, thiolic, thioesteric, nitrogen, and oxygen in peptidic bonds) mostly within the cell [24.57]. In all algae and plants the cell wall consists of a fibrillar fraction, which is responsible of the mechanical strength of the wall, and an amorphous fraction, which provides flexibility. The composition and relative amounts of these two fractions vary in different taxonomic groups [24.6].

Chlorophyta (Green Algae)

The composition of the cell wall of the Ulvophyceae shows some variation between different orders (in some cases even between different life history stages in the same species) [24.6]. In many groups, the fibrillar component forms an internal layer that lies directly against the plasmalemma, whereas the amorphous portion is predominantly on the outer side of the wall, forming a slimy outer layer and containing relatively few and randomly orientated microfibrils [24.6]. The fibrillar layer consists of microfibrils with diameters varying between 3 and 35 nm. In many ulvophytes, the microfibrils consist of crystalline cellulose and are arranged in numerous parallel layers; in orders with siphonous construction (Bryopsidales and Dasycladales), however, the most abundant constituents are mannans, xylans, and glucans. The structural component is embedded in an amorphous, mucilaginous matrix, which also varies in different orders. This may be formed by acidic, partially sulfated heteropolysaccharides (as in the case of the Ulvales) or highly sulfated arabinogalactans (e.g., in the Cladophorales) [24.6]. Polysaccharides extracted from *Ulva* (order Ulvales) contain 16% sulfates and 15−19% uronic acid [24.58]. Several genera of siphonous ulvophytes (*Acetabularia*, *Avrainvillea*, *Penicillus*, *Halimeda*, *Udotea*) are encrusted with calcium carbonate, which is deposited on the cell wall in the form of aragonite crystals. Members of these genera are among the most important contributors to calcium carbonate deposition on tropical reefs.

Rhodophyta (Red Algae)

In the red algae the fibrillar fraction is constituted by cellulose, which is deposited in the form of microfibrils arranged to produce an irregular felt-like network [24.6, 59] immersed in the amorphous matrix. In some genera, such as *Bangia* and *Porphyra*, cellulose is replaced by xylans and mannans. The amorphous matrix forms up

to 70% of the dry weight of the wall and consists of mucilaginous material that may be extracted with hot water. It is constituted by several types of highly hydrophilic sulfated polygalactans, polymers of β-(1 → 4) galactose, and α-(1 → 3) linked 3,6 anhydrogalactose, which are mostly combined to form two types of polysaccharides: agars and carrageenans. The presence of D-galactose and anhydro-D-galactose distinguishes the more highly sulfated carrageenans from the less highly sulfated agars (anhydro-L-galactose) [24.60]. Agar and carrageenans have colloidal properties and for this reason are widely used in many industrial applications [24.61]. Carrageenans consist of a repeating disaccharide backbone: β-(1 → 4)-D-galactopyranosyl-α-(1 → 3)-D-galactopyranosyl. There are 17 different types of carrageenans, which occur in different species and can also vary within a species. Agars are formed by alternating 3-O-linked β-D-galactopyranose and 4-O-linked 3,6 anhydro-α-L-galactopyranose, but the repetition can be interrupted by blocks of repeated units of either one of the two constituents [24.60].

In all species of the order Corallinales and some species belonging to other orders (Nemaliales, Peyssonneliales), the cell wall is encrusted by calcium carbonate. In the Corallinales this compound is present primarily as crystals of calcite (rhomboid crystals), whereas the calcified members of other orders deposit it mainly as crystals of aragonite (orthorhombic crystals) [24.59]. In other red algal genera (e.g., *Mazzaella*) the surface of the alga is covered by a continuous layer of proteinaceous material [24.60]. This cuticle is neither homologous or biochemically similar to the cuticle of vascular plants.

Phaeophyceae (Brown Algae)

In this group the fibrillar portion of the wall is formed by a felt-like network of cellulose, which is probably strengthened by insoluble alginates [24.6]. The amorphous portion constitutes most of the wall mass and is composed of alginates and fucans. Alginates are salts of alginic acid, a polymer formed by monomers of two acid sugars (D-mannuronic acid and L-guluronic acid) connected by β-1,4 bonds. The residues can form salts with various cations, such as Ca^{++}, Mg^{++}, Na^{+} (this is the predominant form of alginates linked with the cellulose fibrils in the fibrillar portion). Fucans (or fucoidans, or ascophyllans) are sulfated polysaccharides containing, in addition to the monosaccharide L-fucose, varying proportions of galactose, mannose, xylose, and glucuronic acid [24.6]. The amorphous portion is responsible for the elasticity of the cell walls and contributes to prevent desiccation of the thallus during emersion.

Alginates confer flexibility upon the thallus, help prevent desiccation, and function in ion exchange [24.60]. They are used extensively in industry because of their colloidal properties. In alginates, the relative proportion of mannuronic acid and guluronic acid vary, depending on species, age of the thallus, tissue, time of the year, and geographic location. The sequence and the relative amounts of these two sugars produce different chemical structures, and, therefore, different physical properties of these polysaccharides. Haug showed that the affinity of some metal cations (Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}) for alginates differs in relation to the relative amounts of the two sugars, increasing with the content of guluronic acid. The high specificity of alginates containing high amounts of guluronic acid for bivalent cations is explained by their zig-zag structure. In the presence of these ions, the alginates develop a network-like arrangement by dimerization of polyguluronic sequences, which allows the pairing of two chains and the formation of spaces suitable to accommodate Ca^{++} and other bivalent cations similar to Ca^{++} in size. These spaces are produced by the grouping of polyguluronic chains determined by the alignment of carboxylates and other atoms of oxygens of the guluronic units. This structure is known as the egg-box model.

Alginates contain three different functional groups to which metal cations may bind, carboxyl (−COO), ether (C−O−C), and hydroxyl (−OH). Carboxyl groups are usually the most abundant acidic groups in brown algae, and most metals (Cd, Co, Cu, Fe, Ni, Pb) are adsorbed at pH close to the pH of dissociation of carboxyl groups in these algae (pK_0 close to 5) [24.62]. The second most abundant acidic group is the sulfonic acid of fucoidans [24.25]; this compound occurs both in the amorphous matrix and in close association with the cellulose microfibrils in the innermost portion of the wall [24.63–65]. Sulfonic acid plays a secondary role in the bond with metals, except when this happens at low pH. This is also the case for the hydroxyl groups present in all polysaccharides, which are negatively charged only at pH > 10 [24.25].

Proteins represent a minor component in the cell wall of brown algae [24.66]. This implies that the carboxyl groups of the alginates are more abundant than the carboxyl groups and the amine groups of proteins and represent the majority of the binding sites [24.58]. The role of the carboxyl groups in adsorption processes has been clearly demonstrated by the uptake of Cd and

Table 24.1 Binding groups for biosorption in macrophyte

Binding group	Chemical formula	Ligand atom	Occurrence in biomolecules	Phylum
Amine	$-NH_2$	Hydrogen	Protein	Chlorophyta
			Metallothionein	Magnoliophyta
Carboxyl	$-COOH$	Oxygen	Cutin	Magnoliophyta
			Alginic acid	Heterokontophyta
Carbonyl	$> C{=}O$	Oxygen	Alginic acid	Heterokontophyta
			Protein	Chlorophyta
			Alginic acid	Heterokontophyta
Hydroxyl	$-OH$	Oxygen	Polysaccharides	Chlorophyta
			Cutin	Magnoliophyta
			Agarose (agar), carrageenan	Rhodophyta
			Fucoidan (sulfated polysaccharide)	Heterokontophyta
			Agaropectin (agar), carrageenan	
Sulfonate	$-SO_2{=}O$	Oxygen	(Sulfated polysaccharides), porphyran, furcellaran (sulfated galactans)	Rhodophyta
Sulfhydryl (thiol)	$-SH$	Sulfur	Metallothionein	Magnoliophyta

Pb by *Sargassum* following partial or complete esterification of carboxyl sites [24.62].

24.3.2 Seagrass Cuticles

As all vascular plants, the seagrasses possess a wall of microfibrils of cellulose embedded in a matrix that consists mainly of pectins and cross-linking glycans [24.12]. The surface of the leaves is covered by a layer of polymeric material called cuticle, which consists of two types of polymers: cutin and cuticular waxes. This layer serves to avoid water loss, reduce penetration by chemicals and pathogenic organisms, and perhaps prevent adhesion of organ surfaces during development [24.12]. Since seagrasses live mostly submerged, they do not need protection against desiccation (which is the main function of cuticle); in these plants the cuticle is, therefore, thinner than in terrestrial angiosperms and the stomata are absent. The thin cuticle also facilitates uptake of ions and carbon; seagrasses are able to uptake nutrients and carbon directly through the leaves. In order to facilitate the exchange of solutes and gases, the cuticle of seagrasses is porous and perforated [24.67].

The cuticle forms an external layer around the surface of the leaves. Cutin and cuticular waxes, the main constituents of the cuticle, are large molecules with a complex tridimensional arrangement. Cutin is a complex compound formed by omega hydroxy acids and their derivatives interlinked via ester bonds and forming a polyester polymer of indeterminate size [24.68, 69]. Waxes are esters of fatty acids and long-chain alcohols; within the cutin network they exist in an amorphous state, but at the outer surface they crystallize to form plates and protrusions that may be very elaborate [24.12]. The cuticle may also contain several nonlipidic components such as carbohydrates and phenols [24.70]. It is also known that the polymeric matrix includes a considerable amount of dissociable groups [24.71] and is a polyelectrolyte with an isoelectric point around 3 [24.71].

24.3.3 Macrophytes: Key Chemical Functional Groups

Biosorption is based on the ability of biological materials to sequester metals [24.25, 53] through chemical-physical mechanisms [24.72] ranging from electrostatic forces, or Van der Waals interaction to ionic and covalent bonds [24.32]. Macrophytes show several chemical groups that have been suggested as being responsible for biosorption of heavy metals, such as carboxyl, hydroxyl, carbonyl, sulfhydryl, and sulfonate (Table 24.1) [24.24, 25, 38, 73, 74]. In particular, the matrix of the brown algal (phylum Heterokontophyta) cell wall consists mainly of alginic acid with carboxyl groups and a smaller amount of sulfated polysaccharides, such as fucoidan with sulfonic acid. In red algae (phylum Rhodophyta) the amorphous portion is formed by a number of sulfated galactans such as carrageenan, agar, furcellaran, porphyran. Green algae (phylum Chlorophyta) may have an external capsule

that is composed of protein or polysaccharides or both (Table 24.1) [24.60]. In the leaves of seagrasses, carboxylic groups present on the cutin layer are probably responsible for the binding of heavy metal ions [24.24]. The biosorption ability of different chemical groups depends on many factors: the number of sites on the adsorbent material, accessibility of the site, their chemical status (i. e., availability), and the affinity between site and metal ions (i. e., bond strength) [24.25, 58, 75]. So it is important to understand what kind of interaction takes place between the functional groups present on the adsorbent material and ionic metal speciation in solutions; MEDUSA software can be used for this [24.76].

24.3.4 Chemical and Physical Mechanisms

Mechanisms involved in the biosorption process have a chemical-physical nature. More specifically, heavy metal chelation depends on the constituents of the outer layers of macroalgae and seagrasses. The biosorption capacity of different chemical groups depends on several factors: the number of sites on the adsorbent material, accessibility, their chemical status (availability), and the affinity between site and metal (bond strength) [24.77]. In particular:

1) Key functional groups (Table 24.1)
2) Ion-exchange processes, and
3) Ionic speciation of metals

have a fundamental role in biosorption studies. The carboxylic groups are abundant in brown algae, due to the presence of alginic acid, and in the cutin of seagrasses. The adsorption capacity of the macrophytes is directly associated with the presence of these sites on cell walls and on the cuticles of the nonliving biomass. Ionic speciation of metals in solution is important for metal uptake and frequently depends on the pH of the sorption system. The MEDUSA software [24.76] creates theoretical predictions of ionic dissociation of metals as a function of pH. The most common metals investigated, such as Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+}, show a maximal or nearly maximal sequestration at pH near the apparent dissociation constant of carboxylic acids (R−COOH), which indicates adsorption on carboxyl groups. The role of carboxylic groups in the adsorption process has been widely demonstrated through the adsorption capacity of lead and other metals cations by dried biomass of *Cystoseira*, *Scytosiphon*, *Sargassum*, *Ascophyllum* (brown algae), *Posidonia*, and *Cymodocea* (seagrasses) following partial or complete esterification of the carboxylic sites [24.24, 45, 75, 78–81]. The other acidic functional group that is important in biosorption activity is the sulfonate group of fucoidans (brown algae) and sulfated polysaccharides (red algae) (Table 24.1). Sulfonic acid groups have an important role when metal binding occurs at low pH values. Hydroxyl groups present in all macrophytes studied except brown algae (Table 24.1) become negatively charged at high pH values (about pH > 10), and consequently have a minor role at low pH [24.25, 75, 80]. Concerning heavy metals that have negative ionic speciation in solution, such as As(V), recent studies have demonstrated that red algae (phylum Rhodophyta), which are known to be bad binders of cationic metals, showed very good arsenic sorption performance at pH values higher than 7 [24.38].

Ion-exchange is an essential mechanism at the base of biosorption. It is the process in which ions are exchanged between a solution and an insoluble solid, usually a resin. During the biosorption activity, dried macrophytes can be viewed as natural ion-exchange materials that contain weakly acidic and basic groups (Table 24.1). In the pH range 2.5−5, the binding of heavy-metal cations is determined primarily by the state of dissociation of the weakly acidic groups (acid–base equilibrium theory) [24.25, 41]. In studies of biosorption, maintaining a low pH (around pH 4.5−5) in the sorption system, microprecipitation of metals and their contribution to the uptake are avoided. pH control in the system is important both for its effects on the configuration of the active ion-exchange sites and for the ionic state of the metals in the solution. In solution, at low pH the concentration of protons is high, and the ion-exchange sites, therefore, become solidly protonated. This allows displacing the metals sequestered on the biosorbent by a simple acidic wash. The regeneration of the biosorbent biomaterial allows its multiple recycling, further increasing the economy of its use [24.82, 83]. Consequently, the use of a biosorbent such as dried algae and seagrasses in wastewater treatment often depends not only on the biosorptive capacity, but also on how well the biosorbent can be regenerated and recycled.

24.4 Biosorption Experiments: Procedure

24.4.1 Biosorption: Kinetic Curves

The kinetic curves of adsorption describe the time evolution of the concentration of metal in solution, in the presence of the adsorbent biomaterial. A stock solution containing a known amount of metal salts is prepared to test the ability of the adsorbent macrophytes. Dried biomass of the macrophytesis is suitably fragmented to increase the adsorbing surface area and is placed in a beaker with deionized water. The pH of the solution is adjusted using solutions of NaOH and HCl (in general, 0.1 M) according to the experimental plan followed. Indeed, pH check control is important to avoid metal precipitation in solution. Subsequently, a stock solution with metals is added according to experimental conditions. Aliquot amounts of solution are then periodically sampled to determine the concentration of metal. In general, the kinetic curves are set for investigation of different pH values.

24.4.2 The Langmuir Adsorption Isotherm

The equilibrium isotherms represent the concentration of the metal on the solid (q, mg/g) as a function of the respective concentration in solution at equilibrium (C_{eq}, mg/L). The determination of isotherms is essential to define the capacity of the adsorption of metal ions by the adsorbent material, such as dried macrophytes. For the determination of curves, a known amount of dried and fragmented biomass is added to a solution of deionized water with varying concentrations of metals established from the experimental plan. The pH of the solutions is adjusted to predetermined values with solutions of NaOH (general 0.1 M) and HCl (general 0.1 M) [24.24, 38]. 1 mL aliquots of test solution are sampled periodically and centrifuged for metal determination. Metal uptake, q (mg g^{-1}), is calculated as the difference in metal concentration in the aqueous phase before and after sorption, according to (24.1)

$$q = \frac{V\left(C_i - C_{eq}\right)}{W}\,, \tag{24.1}$$

where V is the volume of metal solution (L), C_i and C_{eq} are the initial and equilibrium concentration of metal in solution (mg/L), respectively, and W is the mass of dry macrophytes (g).

Through regression analysis, the Langmuir adsorption isotherm [24.84] given in (24.2) was adapted to the experimental data

$$q = \frac{q_{max} b C_{eq}}{1 + b C_{eq}}\,, \tag{24.2}$$

where q_{max} (mg/g) and b (L/mg) are the Langmuir constants, which represent the maximum adsorption capacity and the affinity between the metal ion and adsorbent dried biomass, respectively.

24.5 Conclusions

Macrophytes represent particularly efficient biosorbents compared with other biomass types for the treatment of wastewater [24.24, 26, 37, 38, 45, 46]. Furthermore, due to their economic value in many food, cosmetic, and pharmaceutical applications, there is a large bulk of information about their cell wall structure and biochemical composition of the thalli [24.85–89]. Brown algae, in particular the orders Laminariales and Fucales, are the most important algae with respect to biosorption because of the abundance of their cell wall matrix polysaccharides and extracellular polymers (Table 24.1). Alginates and fucoidans (sulfated mucopolysaccharides) are mainly responsible for the natural ion exchange ability of brown algae. In fact, previous studies support the possibility that the biosorption process consists essentially of ion exchange, which involves the carboxyl group in the alginate polymer and sulfonic acid in the fucoidan [24.24, 26, 38]. The cuticle of seagrasses is also rich in carboxyl groups, which are involved in the bond with the protonated metal, while red and green algae have a lower capacity to immobilize protonated metals due of the presence of groups other than carboxylic ones (Table 24.1). In general, it is assumed that the number of binding sites identified decreases in the order brown algae > seagrasses > green algae > red algae.

Moreover, recent literature confirms that the structure of the thallus affects the adsorption performance of the algae [24.24–26, 38, 45]. *Pennesi* et al. [24.24] concluded that the sorption abilities of the red algae *Porphyra* and *Polysiphonia* might be related to the simple structure of the thallus (i. e., monostromatic blades

and uncorticated filaments, respectively) compared to species of *Gracilaria*, which possess a more complex, parenchymatous thallus. Accordingly, it may be supposed that there is a direct relationship between the complexity of the thallus and the adsorption of lead [24.90].

As a whole, these results have opened new perspectives for the utilization of marine macrophytes as low-cost sorbents in the removal of heavy metals from wastewater; this is a key step towards the implementation of biosorption technology in industrial and environmental remediation.

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25. Marine Algae Biomass for Removal of Heavy Metal Ions

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The increase of industrial activities has accentuated environmental pollution problems, due to the accumulation of harmful pollutants, such as heavy metals. In regard to its simplicity, biosorption on nonconventional and low-cost materials has gained credibility in the last few years, because it offers an efficient and cost-effective alternative to the conventional decontamination techniques. From this perspective, numerous applications of marine algae biomass have been developed for the efficient removal of heavy metal ions from aqueous environments. In this chapter, the biosorptive characteristics of marine algae biomass (marine algae and marine algae wastes) for different heavy metal ions from aqueous solution are described, as a function of experimental conditions (solution pH, biosorbent dose, contact time, temperature, etc.) and the experimental procedure used in the biosorption process. Application of isotherm and kinetic models used for the mathematical description of experimental data is also reviewed. The biosorption mechanism has been found quite complex, including adsorption, surface precipitation, ion exchange and complexation.

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25.1 General Remarks

Nowadays, the intensification of industrial activities has intensified more environmental problems as seen for example in the deterioration of several ecosystems due to the accumulation of dangerous and harmful pollutants, such as heavy metals [25.1]. Industrial and municipal wastewater frequently contain heavy metal ions, and represent the major sources of natural water pollution with heavy metals.

Toxic heavy metals are still used in various industries, such as textile, pigments, plastics, mining, plating, chemical, and smelting, due to their technological importance, and are considered stable and persistent environmental contaminants, because they cannot be destroyed or degraded [25.2, 3]. In addition, these metal ions are reported as priority pollutants due to their mobility, persistency, and accumulation tendency in natu-

ral water ecosystems [25.4, 5], and water contaminated by such pollutants remains an important problem with serious ecological and human health consequences.

The presence of some heavy metals, such as copper, zinc, cadmium, lead, mercury, cobalt, nickel, and others, has a potentially damaging effect on human health and other biological systems, when the tolerance levels are exceeded [25.6, 7]. In order to prevent the deterioration of environment quality, legislation governing the levels of heavy metals, discharged from waste stream is becoming progressively stricter [25.8].

Under these conditions, it is desirable for the benefit of environment to eliminate the heavy metal ions from industrial waste stream, and this could also be important from economical considerations, due to the high value of these metals. Together with pollution prevention and detection, the elimination of a wide range of pollutants from aqueous waste stream is an absolute requirement for sustainable development [25.9].

The most widely employed methods for removing heavy metals from metal-bearing effluents include chemical precipitation, ion exchange, adsorption, flocculation, solvent extraction, membrane-related processes, electrochemical and biological techniques, and different combination of these [25.10–14]. Low efficiency performances, particularly when are used for small concentrations of heavy metal ions, low selectivity, the necessity of using expensive chemicals in some methods, and the generation of high amount of toxic sludge or high energy consumption, are the main drawbacks of these conventional methods [25.13, 15]. Therefore, the development of new methods for the removal of heavy metal ions from aqueous environments that minimize these disadvantages is an actual scientific issue, which has great applicative importance.

In regard to its simplicity and high-efficiency characteristics, the adsorption is looked upon as a better method that is recommended for the removal of low concentrations of heavy metal ions in wastewater (1−100 mg/l dissolved metal ions) [25.16], because it is rapid, reversible, economical and environmental friendly in contrast with the mentioned conventional methods [25.17, 18]. Well-designed adsorption processes have high efficiency, resulting in high-quality effluents after treatment, which can be recycled.

The adsorption process, generally implies the presence of solid material, called *adsorbent*, that binds metal ions from contaminated aqueous solutions by physical attractive forces, ion exchange, or/and chemical binding. Cheap and efficient adsorbents are advisable for the treatment of aqueous effluents containing heavy metal ions. Thus, natural materials available in large quantities or waste products from industry or agriculture, generally designated *low-cost adsorbents*, have been proved suitable for this purpose. This is due to the fact that most such materials have high number and variety of superficial functional groups (e.g., carboxyl, hydroxyl, amino, phosphate, etc.), which can retain the metal ions through different kinds of mechanisms. Moreover, because such cost adsorbents are cheap and easily regenerable, they are an attractive alternative over commonly used adsorbing materials, such as activated carbon, which is costly to use for the removal of such dangerous chemicals from industrial wastes [25.12, 19].

In the past few decades, biosorption using microorganism biomass as the adsorbent has emerged as a potential alternative method in the removal of contaminants from aqueous effluents. The use of biological materials, including living and nonliving microorganisms for the removal of heavy metals from industrial wastewater, has gained important credibility in the last few years, because of the good efficiency, minimization of secondary (chemical or biological) wastes, and low cost of these materials [25.16, 18]. The microorganisms, such as bacteria, algae, fungi, or yeasts, can uptake heavy metals from aqueous solutions, both actively (metabolically mediated – bioaccumulation) and passively (by physical–chemical pathways, named biosorption) [25.20, 21].

Although in the beginning most studies have been focused on heavy metal accumulation and concentration within living organisms [25.22, 23], upon noticing that dead biomass possesses high metal retention capacity [25.24], the interest was shifted to biosorption [25.25, 26]. This is mainly due to the fact that the nonliving biological materials are easier and cheaper to obtain, and their utilization as biosorbents has been shown to be an economical and effective method to remove the heavy metals from wastewater.

The term *biosorption* indicates a metabolism-independent binding of heavy metals by dead (nonliving) biological materials, and is defined as the ability of these materials to bind the metal ions to the surface of cellular walls or membrane, in the equilibrium process [25.21, 27, 28]. In the biosorption processes, the nonliving biological materials (biomass) act as a chemical substrate of biologic origin and the main advantages of their utilization are: (i) the variety of functional groups from the biosorbent surface, (ii) relatively small and uniform distribution of binding sites on biosorbent surface, and (iii) the preference of alkali and alkali-

earth metal ions for the binding sites is less than in the case of ion exchange resins [25.16].

The marine algae are available in large quantities in many regions of the world, and are a kind of promising biological resources [25.29]. Even, though the red and brown algae have been the most used biosorbents for the removal of heavy metals [25.30], their utilization may cause serious secondary pollution due to organic substances release, such as alginate dissolving from biosorbents, during of the biosorption process. Another problem of brown algae utilization is that the leaching of some retained compounds may also lead to a loss of biosorption capacity [25.31]. These secondary processes drastically limit their industrial application.

In the case of green algae, the effect of secondary pollution is significantly lower, and many studies from the literature [25.17, 32, 33] have shown that these posses high metal binding capacities, due to the presence of polysaccharides, proteins, or lipids from the cell walls surface. The marine green algae are mainly cellulose, and high percentages of the cell wall are proteins bonded to polysaccharides to form glycoproteins. These compounds contain several functional groups (amino, carboxyl, sulfate, hydroxyl, etc.) which could play an important role in the biosorption process [25.34–36]. In addition, the marine green algae are particularly useful in the biosorption process of heavy metals due to their relatively simple structure, high surface area, and small and uniform distribution of binding sites [25.37, 38].

However, the marine algae are frequently used for the extraction of oil in the cosmetic industry and for the production of biofuels [25.39, 40]. This utilization is more economically efficient than the removal of heavy metal ions from wastewater, and for this reason high quantities of marine algae are used for this purpose. After the extraction process, the remained marine algae biomass is considered to be a waste and is discharged or incinerated, becoming a serious problem for environmental protection. In order to avoid this problem, the marine algae waste biomass can also be used as biosorbent for the removal of heavy metals ions from wastewater. Recent studies performed by *Bulgariu* and *Bulgariu* [25.41, 42] have shown that the biosorption capacity of the marine green algae waste biomass obtained after extraction of oils from marine green algae (*Ulva lactuca* sp.) can be successfully used for the removal of heavy metal ions from aqueous solution, and that the metal uptake capacity is not significantly affected by the extraction step.

In this chapter, the biosorptive characteristics of marine algae biomass (marine algae and marine algae wastes) for different heavy metal ions from aqueous solution, as a function of the experimental procedure used in the biosorption process and experimental conditions (solution pH, biosorbent dose, contact time, temperature, etc.) are presented. The most important isotherm and kinetic models used for the mathematical description of experimental biosorption data are also reviewed. The biosorption mechanism has been found to be quite complex, including adsorption, surface precipitation, ion exchange, and complexation.

25.2 Chemical Characteristics of Marine Algae Biomass

Marine algae (or seaweeds) are relatively undifferentiated simple biological organisms that fall into the general category of *plants*. Their size varies from tiny microscopic unicellular forms (3−10 μm) to large macroscopic multicellular forms (up to 70 m long). Most of the marine algae are photosynthetic biological organisms that have chlorophyll, but they also contain additional pigments, that are the basis of classification [25.43].

Even if they are considered the oldest member of the plant kingdom (existing back many hundreds of millions of years), the marine algae do not form a homogeneous group. They are found throughout the world's oceans and seas, and are divided into several categories, namely: (i) brown algae (*Phaeophyta* division), (ii) red algae (*Rhodophyta* division), and (iii) green algae (*Chlorophyta* division), as a function of the color of additional pigments. The mixture of pigments in their chloroplasts lends characteristic colors to the marine algae, and many of their scientific names are based on these colors [25.44]. The differences between these types of marine algae are mainly in the structure of cell walls, where the retention of heavy metal ions takes place.

The cell walls of brown algae generally contain three main components: cellulose (that is the structural support), alginic acid (a polymer of mannuronic and guluronic acids, and the corresponding salts of sodium, potassium, magnesium and calcium and represent 14−40% of the dry weight of the biomass)

Table 25.1 The main constituents of some marine algae (after [25.45])

Constituent (brown algae)	*Fucus visoides* (brown algae)	*Cystoseira barbata* (red algae)	*Gracillaria compressa* (red algae)	*Gracillaria confervoides*	*Ulva lactuca* (green algae)
Humidity (%)	10.50	9.40	11.30	10.80	11.09
Lipids (%)	3.20–4.70	1.34–2.36	–	–	–
Proteins (%)	12.31	13.51	17.71	29.52	13.91
Ash (%)	17.72	20.41	23.55	24.01	16.6
P (%)	0.14	0.20	0.14	0.39	0.12
I (mg/g)	0.31	0.40	0.17	0.50	0.11
Br (mg/g)	0.17	0.10	0.69	0.50	–
K (mg/g)	42.71	86.61	50.81	0.18	23.14
Ca (mg/g)	20.03	27.31	33.12	30.51	31.80
Mg (mg/g)	12.11	10.0	7.75	4.34	18.91
Zn (mg/g)	173.05	145.0	49.01	32.75	43.17
Mn (mg/g)	147.05	91.03	92.05	1693.01	–
Cu (mg/g)	11.23	18.21	7.22	22.86	10.12

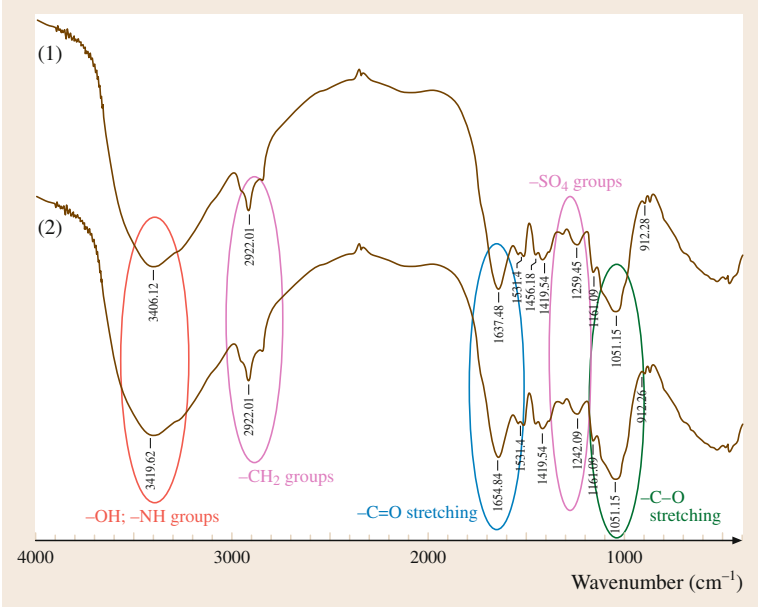

Fig. 25.1 FT-IR spectra of *Ulva lactuca* sp. marine algae before (1) and after extraction of oil (2)

and sulfated polysaccharides [25.35, 46]. Red algae also contain cellulose as structural support, but their biosorptive properties are mainly determined by the sulfated polysaccharides made from galactanes (agar and carragenates) [25.35, 47]. Green algae are mainly cellulose and a high percent of cell walls are proteins bonded to polysaccharides to form glycoproteins [25.35, 48]. These constituents contain numerous reactive functional groups (e.g., amino, hydroxyl, carboxyl, sulfate, etc.) that can be involved in chemical binding with metal ions, and could play an important role in the biosorption processes. Table 25.1 summarizes the main constituents that are present in the structure of some marine algae.

Besides these main constituents, the marine algae also contain several extractive compounds (such as fat, fatty acids, fatty alcohols, phenols, terpenes, steroids, resin acids, and waxes) [25.21], that have also reac-

tive functional groups in their structure. Unfortunately, these components are little involved in chemical binding with metal ions, because they are easily extracted in organic and/or aqueous solvents, being responsible for the secondary pollution observed during of the biosorption process [25.16]. Thus, by removing such compounds by extraction with organic solvents or alkaline treatment [25.49, 50], the main classes of functional groups are not affected, and the obtained marine algae waste biomass has comparable or even higher uptake capacity, for different metal ions from aqueous solutions.

The presence of different functional groups in the structure of marine algae and marine algae waste biomass can be easily proved using FT-IR spectra, recorded for dry samples. For exemplification, Fig. 25.1 illustrates the FT-IR spectra obtained for *Ulva lactuca* sp. (marine green algae) before and after extraction of oils (marine algae waste biomass).

A detailed analysis of the FT-IR spectra of these biosorbents has indicated that in the structure of marine algae, numerous functional groups (such as hydroxyl, amine, carbonyl, carboxylic, sulfate) are present and these can interact with heavy metals during the biosorption process. The same functional groups can also be identified in the FT-IR spectra of all types of marine algae, whatever class they belong [25.26, 29, 30, 35, 48]. It was also observed that the nature of functional groups is not changed significantly after the extraction step. These low-cost biosorbents will act as a chemical substrate and will bond heavy metal ions from aqueous solutions by characteristic chemical (electrostatic and/or complexation) interactions.

Although the constituents of the cell walls provide an extensive array of ligands with various functional groups capable of binding metal ions, the role that any functional group has in the biosorption process depends on several factors. Thus, the number of active sites on the biosorbent material surface, their accessibility and chemical state, and the affinity of metal ions for a given functional group play an important role in metal binding interactions, and will influence the efficiency of the biosorption process.

25.3 Experimental Methodology

Generally, the marine algae used as biosorbents in the experimental studies are collected from their natural environment (seawater), washed several times with distilled water to remove the impurities and dried in air at 50–60 °C for 6–10 h, and then crushed and sieved to a given granulation (0.5–1.5 mm). In the case of marine waste algae biomass obtained after extraction steps with organic solvents and an additional washing step with a NaOH solution is required, in order to remove the trace of organic solvents. The obtained materials are stored in desiccators and can be directly used as biosorbent, without any additional pretreatment. Because these kinds of biosorbents are easily obtained and require only few numbers of operations, they can be included in the category of low-cost biosorbents.

From the experimental point of view, the assessment of a biosorption system is usually based on two types of methodologies: equilibrium batch biosorption tests and dynamic continuous-flow biosorption studies. Both have several important advantages and disadvantages, and choosing a specific methodology depends on the objective of the study.

25.3.1 Equilibrium Batch Biosorption Technique

The batch (or discontinue) methodology is relatively simple and easy to use in the laboratory, especially for the experimental study of single-metal ions biosorption. This methodology is preferred when we want to establish the optimum conditions for retention of a given metal ion on certain biosorbent, or to test the biosorptive performances of a new biosorbent.

In this case, a small amount of marine algae biomass (biosorbent) is mixed with well-measured volume of aqueous solution containing a given metal ion, in certain conical flasks, with intermittent or continuous stirring for a required period of time. At the end of the biosorption procedure, the biosorbent is separated through filtration, and the concentration of the studied metal ion in filtrate is analyzed, using an adequate analysis method.

It should be mentioned that in the biosorption systems, the experimental parameters (solution pH, biosorbent dose, metal ion concentration, contact time, temperature, etc.) should be carefully controlled at the

Table 25.2 Some utilizations of marine algae biomass for the removal of heavy metal ions from aqueous solutions, by the batch technique

Marine algae	Type of algae	Heavy metal ions	References
Fucus serratus	Brown algae	Cu(II), Zn(II), Pb(II), Ni(II), Cd(II), Ce(III)	[25.51, 52]
Laminaria sp.	Brown algae	Pb(II), Cd(II), Cu(II), Co(II), Ni(II), Mn(II)	[25.30, 53]
Bifurcaria bifurcata	Brown algae	Cu(II), Zn(II), Pb(II)	[25.53]
Sargassum muticum	Brown algae	Cu(II), Zn(II), Pb(II), Ni(II), Cd(II)	[25.53, 54]
Fucus spiralis	Brown algae	Cu(II), Zn(II), Pb(II), Ni(II), Cd(II)	[25.53]
Gracilaria sp.	Red algae	Cd(II), Cu(II), Pb(II), Zn(II), Ni(II)	[25.54, 55]
Gelidium sp.	Red algae	Pb(II), Cu(II)	[25.56]
Chaetomorpha linum	Green algae	Cu(II), Zn(II)	[25.57]
Chlorella vulgaris	Green algae	Cu(II), Cd(II), Fe(III), Sn(IV)	[25.58]
Caulerpa lentillifera	Green algae	Cu(II), Cd(II), Pb(II)	[25.32]
Spirogyra sp.	Green algae	Cr(III), Pb(II), Cu(II)	[25.59, 60]
Ulva lactuca	Green algae	Cr(III), Pb(II), Cd(II), Co(II), Cu(II), Ni(II),	[25.38, 54, 61, 62]
Ulva fasciata	Green algae	Cu(II), Zn(II)	[25.33, 63]

given value over the entire period of contact, until the biosorption equilibrium is reached. This may take few minutes or few hours, depending on the nature of marine algae biomass used as biosorbent. The contact time required to attain the biosorption equilibrium can be establish by a preliminary test, and represents the time period after that the metal ion concentration in the solution remains unchanged.

Table 25.2 presents several examples of marine algae biomass utilization for the removal of heavy metal ions from aqueous solutions, by the batch technique.

In each of the presented examples, the performances of marine algae biomass in the biosorption process were quantitatively evaluated using the amount of heavy metal ions retained on weight unit of biomass (q, mg/g) and the percent of heavy metal removed (R, %), calculated from experimental data with the following equations:

$$q = \frac{(c_0 - c) \cdot (V/1000)}{m} \tag{25.1}$$

$$R, \% = \frac{(c_0 - c)}{c_0} \cdot 100 \tag{25.2}$$

where c_0 is the initial concentration of heavy metals solution (mg/l), c is the equilibrium concentration of heavy metals solution (mg/l), V is the volume of solution (ml), and m is the biosorbent mass (g).

25.3.2 Dynamic Continuous-Flow Biosorption Technique

Unfortunately, the batch-technique biosorption has limited applicability to the pilot or industrial scale. The industrial utilization of biosorption processes required the use of continuous systems, where the marine algae biomass can be used in multiple biosorption–desorption cycles [25.64]. In addition, the heavy metal ions biosorption is more effective if it is carried out in a packed-bed column and the requirement of biosorbent and the operation cost of the process are substantially reduced [25.65].

Generally, the experimental study of biosorption processes in dynamic conditions is performed in cylindrical columns, with variable dimensions (do not typically exceed 1.5 m in diameter and 5 m in height), which are filled with a layer of biosorbent material. In this case, aqueous solution containing heavy metal ions is normally passed in up-flow mode through the column, and at the bottom of this is collected aqueous solution where the heavy metal ions were removed (named the effluent solution). By plotting the variation of heavy metal ion concentration of the effluent solution as a function of the operating time of the column, or the volume of the treated aqueous solution, the breakthrough curves are obtained (Fig. 25.2).

One the main tools used in the investigation of the performances in the biosorption column is the breakthrough analysis, which is based on the calculation of several parameters from the experimental breakthrough curves [25.66].

The breakthrough time (t_b) and volume (V_b) represent the time and volume at the breakthrough point, where the effluent concentration of the heavy metal ion reaches a value of $0.1 \cdot c_0$ (breakthrough concen-

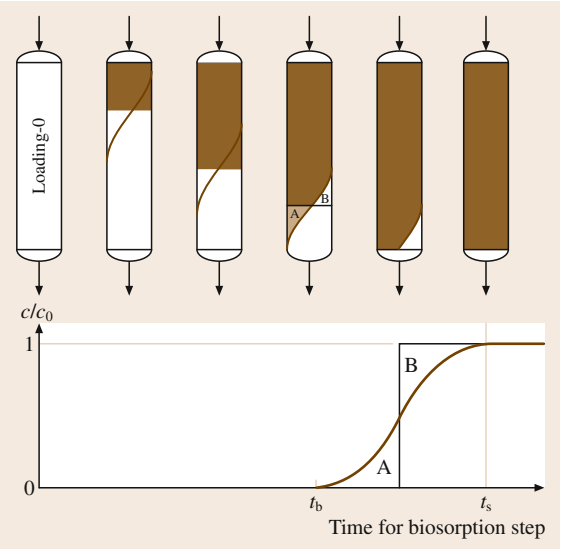

Fig. 25.2 Schematic representation of the breakthrough curves for the biosorption processes in dynamic continuous-flow conditions

tration). The saturation time (t_s) and volume (V_s) are the time and volume at saturation point, and were selected as the time and volume when the effluent concentration achieved a constant value around $0.95 \cdot c_0$ (where c_0 is the initial concentration of the heavy metal ion), although the biosorbent bed was not fully saturated.

The efficiency in the overall use of the biosorbent mixture in the column is indicated by the mass transfer zone (MTZ), which is defined as the length of the biosorption zone in the column, and can be calculated by using the following equation:

$$\mathrm{MTZ} = H \cdot \frac{(t_s - t_b)}{t_s} \tag{25.3}$$

where MTZ is the length of the mass transfer zone (cm); H is the length of the biosorbent bed (cm); t_b is the time required to reach the breakthrough point (min); and t_s is the time required to reach the saturation point (min).

The longer the mass transfer zone, the more biosorbent is not fully utilized at the end of the biosorption process. So, for the process to be economical it is important to minimize the length of the transfer zone and increase, thus, biosorbent utilization.

Practically, the dynamic continuous-flow biosorption is accomplished by using batteries of multiple columns that work in parallel and/or in series, to optimize the performance of the process. Some examples of using marine algae biomass in continuous-flow biosorption systems are presented in Table 25.3.

Generally, the smaller the biosorbent particles, the more difficult is the passing of aqueous solution through the column. However, the small dimension of biosorbent particles makes the metal uptake process to be more effective and rapid. When, the particles of biosorbent from the packed bed are larger, aqueous solution of metal ions can pass through the column easily, but the efficiency of biosorption

Table 25.3 Some utilizations of marine algae biomass for the removal of heavy metal ions from aqueous solutions, by dynamic continuous technique

Marine algae	Biosorbent bed	Heavy metal ions	References
Symphoricarpus albus	Biomass bed	Pb(II)	[25.67]
Caulerpa lentillifera	Biomass bed	Cu(II), Cd(II), Pb(II)	[25.32]
Sargassum sp.	Biomass bed	Cu(II), Cd(II), Pb(II), Zn(II), Ni(II), Fe(III)	[25.66, 68–72]
Gelidium sp.	Biomass bed	Pb(II), Cu(II), Cr(III)	[25.73, 74]
Ulva reticulata	Biomass bed	Cu(II), Co(II), Ni(II), Zn(II)	[25.75, 76]
Posidonia oceanica	Biomass bed	Cu(II)	[25.77]
Sargassum baccularia	immobilized onto polyvinyl alcohol (PVA) gel beads	Cu(II)	[25.78]
Laminaria japonica	Biomass gel	Re(VI)	[25.79]
Gelidium sp.	Algae waste from the agar extraction process immobilized with polyacrylonitrile	Pb(II), Cu(II), Cr(III)	[25.73, 74]
Ulva lactuca	Agar matrix	Cu(II), Zn(II),Cd(II), Pb(II)	[25.80]
Ulva lactuca	Mixture: algae waste: Purolite A-100 = 1 : 2	Pb(II)	[25.81]

process is lower due to the decrease of the specific surface area. Thus, for the best mass transfer and utilization, the biosorbent granules should be as small as possible. Practically, the recommended biosorbent dimension rages between 0.5 and 2.0 mm.

The main disadvantage of using marine algae biomass in dynamic continuous-flow biosorption systems is the easy with which the column is clogged, due to the small size of biosorbent. In consequence, the flow rate of aqueous solution through the column will be obstructed, and thus the efficiency of the biosoption process will be drastically affected. To solve this problem, some studies from the literature have proposed the conversion of marine algae biomass into granules by including them in various organic polymer matrices (see Table 25.3). But, by using this procedure, some functional groups from the marine algae biomass surface become unavailable for the heavy metal ions from aqueous solution, because they are blocked by interactions with the polymer matrix. This makes the biosorption capacity of marine algae biomass decrease, and the efficiency of the biosorption process in continuous-flow conditions less than those obtained in batch systems [25.65]. In order to avoid this inconvenience, *Bulgariu* and *Bulgariu* [25.42] propose the mixing of marine algae waste biomass with commercially available anion exchanger resin (Purolite A-100), and the obtained biosorbent mixture has proven to be efficient in the removal of Pb(II) ions from aqueous media. Because on this resin the retention of Pb(II) ions is insignificant, it can be considered an inert material, which has the role to prevent the clogging of the column, due to the larger size of its particles. In addition, the Purolite A-100 resin has an excellent retention capacity for some inorganic anions (such as nitrate or sulfate) [25.81], which are also present in wastewater and are also pollutants for aquatic environment.

25.4 Influence of Experimental Parameters

The removal of heavy metal ions from aqueous solutions by biosorption on marine algae biomass takes places with maximum efficiency only in well-defined experimental conditions. In consequence, an important part of the studies related to the removal of heavy metal ions by biosorption on low-cost materials such as marine algae biomass was the study of the influence of several experimental parameters on the retention process efficiency. As a function of the experimental technique used in the biosorption studies, the experimental parameters that should be considered in the experimental studies are as follows:

i) For batch technique – solution pH, biosorbent dose, initial metal ion concentration, contact time between the two phases, and temperature.
ii) For the dynamic continue-flow technique – height of biosorbent bed, aqueous solution flow rate, and initial concentration of heavy metal ions.

The experimental results obtained in these studies have provided a complete view of the biosorption process and allowed us to establish the optimal conditions for that the removal efficiency of metal ions from aqueous solutions is maximum.

25.4.1 Influence of Experimental Parameters on Batch-Technique

Effect of Solution pH

The pH of heavy metal ions solutions is the most important experimental parameter that governs the biosorption process. When designing a process for metal ions removal from wastewater, both optimal pH value for metal biosorption and pH value required for effluent discharge to comply with regulation must be taken into consideration before making a final decision [25.82].

Many studies from the literature [25.16, 28–38] have shown that the low-cost materials, including marine algae and marine algae waste biomass, act as a good biosorbent in a rather narrow pH range. The solution pH strongly influences not only the dissociation degree of functional groups from biomass surface, considered as biosorption sites, but also the solution chemistry of heavy metal ions (hydrolysis, complexation by organic and/or inorganic ligands, redox reactions, precipitation, etc.), the speciation and the biosorption availability of the heavy metals [25.83–85].

In Sect. 25.2 it was already shown that the marine algae and marine algae biomass contains in their structure various functional groups (such as carboxyl, hydroxyl, amino, sulfate). With the change in the so-

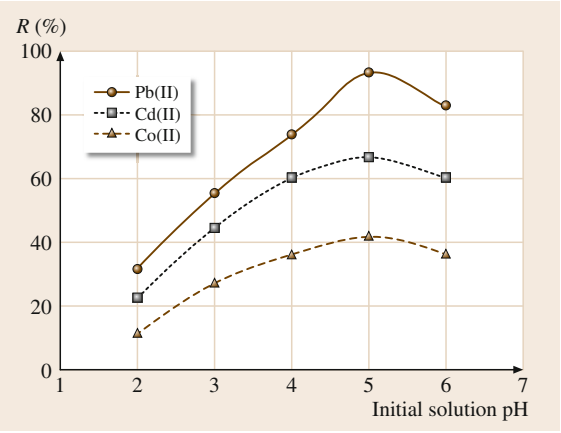

Fig. 25.3 Influence of solution pH on the removal efficiency of some heavy metal ions on marine algae – *Ulva lactuca* sp. (after [25.41, 49, 88])

lution pH, the behavior of each of these groups is changed. Thus, at highly acidic pH values most of these functional groups are protonated and act as a positively charged species [25.86]. Deprotonation of functional groups occurs due to increasing pH, when they behave as negatively charged groups, and start to interact with positively charged metal ions. On the other hand, certain functional groups such as amino contain lone pairs of electrons and thus can form with the metal ions coordinate bonds. The coordination process is highly pH dependent and occurs only at some specific pH value [25.87]. Thus, a change of initial solution pH can affect the achievement of complexing interactions and can determine a change in the biosorption efficiency.

The speciation and solubility of heavy metal ions are also affected by the value of solution pH. Generally, the biosorption efficiency of heavy metal ions gradually increases with increasing solution pH, but not in a linear relationship. In most of cases, this effect is more pronounced as initial heavy metal ion concentration of solution also increases. On the other hand, a too high pH value may cause the precipitation of metal species, and in consequence it should be avoided during experiments. Figure 25.3 illustrates a typical effect of solution pH on the biosorption efficiency of some heavy metal ions onto marine algae biomass (*Ulva lactuca* algae).

Such biosorption behaviors may be attributed to competition between heavy metal ions and protons for the binding sites from the biomass surface. At low pH values (< 4.0), the excess of protons from the systems can compete with heavy metal ions, and in consequence the amount of heavy metals retained is low. Increasing the solution pH determined a reducing of proton concentration and an increase of dissociation degree of functional groups from the biosorbent surface, which make the amount of retained heavy metal ions increase, until a maximum is attained. This pH value corresponding to the maximum of biosorption represents the optimum value and a further increase of pH makes that the biosorption efficiency subsequently decrease. The strong pH dependence of the biosorption process suggests that the electrostatic interactions between negatively charged functional groups and positively charged heavy metal ions may be one of the specific biosorption mechanisms [25.89].

For most biosorption systems, the uptake of heavy metal ions from aqueous solutions using marine algae or marine algae waste biomass as biosorbent occurs with maximum efficiency in a pH range between 4.0 and 6.0 (see Table 25.4). This is because, in this pH range most of heavy metals exist in solutions predominantly as free metal ions (M^{2+}) [25.83], and the ionization degree of functional groups from adsorbent surface is higher enough to allow the electrostatic interactions [25.29, 38].

However, metal anions, such as Cr(VI), exhibits different pH features in comparison with heavy metal cations. In general, a low pH value is favorable for the biosorption, and a much lower optimum pH (1.5–2.0) has been reported for different types of brown, red or green marine algae biomasses (Table 25.4). This is determined by the fact that the anions could be retained only by the protonated active sites of the biosorbent, primarily by electrostatic interactions. At very low pH values where the concentration of protons is very high, the most of functional groups are undissociated or positively charged. This will enhance the interactions of Cr(VI) with the binding sites from the biosorbent surface, by attractive electrostatic forces. As the solution pH increased, the overall surface charge on the biosorbent became negative, and decreased the biosorption efficiency [25.108].

In most of experimental studies presented in the literature, the optimum value of solution pH is adjusted by adding small volume of mineral acids (HCl, HNO_3, H_2SO_4) or alkaline base (NaOH, KOH) solutions. But, due to the interactions between heavy metal ions and functional groups from biomass surface during biosorption process protons are released. In consequence, the solution pH decreases (with 0.5–1.0 units) and this make that the biosorption efficiency to decrease, mainly

Table 25.4 Optimum experimental conditions for the biosorption of some heavy metal ion onto marine algae biomass

Heavy metal ion	Biosorbent	Type of marine algae	pH	Biosorbent dose (g/l)	Contact time (min)	Temperature (°C)	Reference
As(III)	*Maugeotia genuflexa*	Green algae	6.0	4.0	60	20	[25.90]
	Ulothrix cylindricum	Green algae	6.0	4.0	60	20	[25.91]
	Fucus ceranoides	Brown algae	4.5	2.5	25	25	[25.92]
	Fucus vesiculosus	Brown algae	4.5	2.5	25	25	[25.92]
			6.0	1.0	120	25	[25.93]
	Fucus serratus	Brown algae	4.5	2.5	25	25	[25.92]
	Bifurcaria bifurcata	Brown algae	4.5	2.5	> 60	25	[25.60]
	Saccorhiza polyschides	Brown algae	4.5	2.5	> 60	25	[25.60]
Cd(II)	*Ascophyllum nodosum*	Brown algae	4.5	2.5	> 60	25	[25.60]
	Laminaria ochroleuca	Brown algae	4.5	2.5	> 60	25	[25.60]
	Pelvetia caniculata	Brown algae	4.5	2.5	> 60	25	[25.60]
	Cystoseira baccata	Brown algae	4.5	2.5	17	25	[25.49, 94]
	Ceramium virgatum	Red algae	5.0	0.1	60	20	[25.95]
	Ulva lactuca	Green algae	5.0	0.1	60	20	[25.38]
			5.0	8.0	30	20	[25.48]
	Ulva lactuca waste[a]	Waste biomass	5.0	8.0	60	20	[25.87]
	Gelidium waste[b]	Waste biomass	5.3	3.0	20	20	[25.96]
Co(II)	*Ptredocladia capillacea*	Red algae	5.0	10.0	< 60	25	[25.97]
	Galaxaura oblongata	Red algae	5.0	10.0	< 60	25	[25.97]
	Ulva lactuca	Green algae	5.0	8.0	–	23	[25.62]
	Ulva lactuca waste[a]	Waste biomass	5.0	8.0	60	20	[25.41]
Cr(III)	*Spirogyra* spp.	Green algae	5.0	3.0	45	25	[25.59]
	Chlorella sorokiniana	Green algae	4.0	1.0	15–20	25	[25.98]
	Fucus vesiculosus	Brown algae	2.0	2.0	120	Room temperature	[25.99]
	Fucus spiralis	Brown algae	2.0	2.0	120	Room temperature	[25.99]
Cr(VI)	*Palmaria palmate*	Red algae	2.0	2.0	30	Room temperature	[25.99]
	Polysiphonia lanosa	Red algae	2.0	2.0	30	Room temperature	[25.99]
	Ceramium virgatum	Red algae	1.5	0.1	90	20	[25.100]
	Ulva lactuca	Green algae	1.0–1.5	3.0	120	25	[25.61]
	Fucus serratus	Brown algae	5.5	0.9	350	25	[25.51]
	Fucus vesiculosus	Brown algae	5.0	1.0	120	23	[25.93]
	Sargassum sp.	Brown algae	5.5	1.0	30	25	[25.33]
	Fucus spiralis	Brown algae	5.0	0.5	120	Room temperature	[25.35]
	Ascophyllum nodosum	Brown algae	4.0	1.0	120	Room temperature	[25.35]
Cu(II)	*Asparagopsis armata*	Red algae	5.0	0.5	120	Room temperature	[25.35]
	Chondrus crispus	Red algae	4.0	0.5	120	Room temperature	[25.35]
	Ulva fasciata	Green algae	5.5	1.0	30	22	[25.33, 101]
	Chaetomorpha linum	Green algae	5.0	20	120	23	[25.57]
	Spirogyra spp.	Green algae	5.0	1.0	30	Room temperature	[25.60]
	Cladophora spp.	Green algae	5.0	1.0	30	Room temperature	[25.60]
	Fucus spiralis	Brown algae	5.0	0.5	120	Room temperature	[25.35]
	Ascophyllum nodosum	Brown algae	5.0	0.5	120	Room temperature	[25.35]
	Cystoseira baccata	Brown algae	4.5	2.5	47	25	[25.46, 60]
	Fucus vesiculosus	Brown algae	5.0	1.0	120	23	[25.93]
	Asparagopsis armata	Red algae	4.0	0.5	120	Room temperature	[25.35]

Table 25.4 (continued)

Heavy metal ion	Biosorbent	Type of marine algae	pH	Biosorbent dose (g/l)	Contact time (min)	Temperature (°C)	Reference
Pb(II)	*Chondrus crispus*	Red algae	4.0	0.5	120	Room temperature	[25.35]
	Cladophora fascicularis	Green algae	5.0	2.0	30	25	[25.36]
	Spirogyra spp.	Green algae	5.0	0.5	100	20	[25.60, 102]
	Cladophora spp.	Green algae	5.0	1.0	30	Room temperature	[25.60]
	Ulva lactuca	Green algae	5.0	0.1	60	20	[25.38]
			5.0	8.0	30	22	[25.49]
	Ulva lactuca waste[a]	Waste biomass	5.0	8.0	60	20	[25.41]
	Gelidium waste[b]	Waste biomass	5.3	4.0	30	20	[25.103]
	Sargassum glaucescens	Brown algae	6.0	1.0	120	30	[25.104]
	Padina australis	Brown algae	6.0	1.0	120	30	[25.105]
	Fucus spiralis	Brown algae	6.0	0.5	120	Room temperature	[25.35]
	Ascophyllum nodosum	Brown algae	6.0	0.5	120	Room temperature	[25.35]
Ni(II)	*Asparagopsis armata*	Red algae	6.0	0.5	120	Room temperature	[25.35]
	Chondrus crispus	Red algae	6.0	0.5	120	Room temperature	[25.35]
	Codium vermilara	Green algae	6.0	0.5	120	Room temperature	[25.35]
	Cladophora spp.	Green algae	5.0	5.0	–	23	[25.106]
	Ulva lactuca	Green algae	4.5	2.0	60	30	[25.106]
Se(IV)	*Cladophora hutchinsiae*	Green algae	5.0	8.0	60	20	[25.107]
	Fucus spiralis	Brown algae	6.0	0.5	120	Room temperature	[25.35]
	Ascophyllum nodosum	Brown algae	6.0	0.5	120	Room temperature	[25.35]
	Asparagopsis armata	Red algae	6.0	0.5	120	Room temperature	[25.35]
	Chondrus crispus	Red algae	6.0	0.5	120	Room temperature	[25.35]
Zn(II)	*Codium vermilara*	Green algae	6.0	0.5	120	Room temperature	[25.35]
	Spirogyra insignis	Green algae	6.0	1.0	120	Room temperature	[25.35]
	Chaetomorpha linum	Green algae	5.0	20	120	25	[25.57]
	Ulva fasciata sp.	Green algae	5.0	0.1	20	30	[25.21, 63]

[a] Marine green algae (*Ulva lactuca* sp.) waste biomass obtained after extraction of oils; [b] waste algae biomass obtained from agar extraction industry.

due to the change of dissociation degree of superficial functional groups of marine algae biomass.

In order to maintain a constant dissociation degree of superficial functional groups of marine algae biomass during biosorption process, buffered solutions can be used. These are obtained by adding certain buffer solution to aqueous solutions of heavy metal ions before mixed with the solid biosorbent. Even if this procedure can be successfully applied in the case of peat moss, when the presence of acetate buffer improves the adsorption capacity for Pb(II) ions [25.109], in the case of marine algae biomass an opposite effect is observed. The experimental results obtained by *Lupea* [25.110] have shown that in the presence of buffer solutions (such as acetate buffer) the amount of heavy metal ions retained on weight unit of *Ulva lactuca* sp. marine algae is lower compared to unbuffered solutions, regardless of the solution pH value (Fig. 25.4).

The decrease of the biosorption efficiency of heavy metal ions in buffered solutions is mainly determined by the fact that the marine algae biomass has in its structure a high number of hydroxyl and carboxyl groups that can be involved in specific interactions (such as esterification) with organic ligands (acetate, in this case) from buffer. These interactions drastically decrease the availability of reactive functional groups from the biosorbent surface to bind heavy metal ions, and thus the efficiency of the biosorption process decreases. Under these conditions, it is recommended that

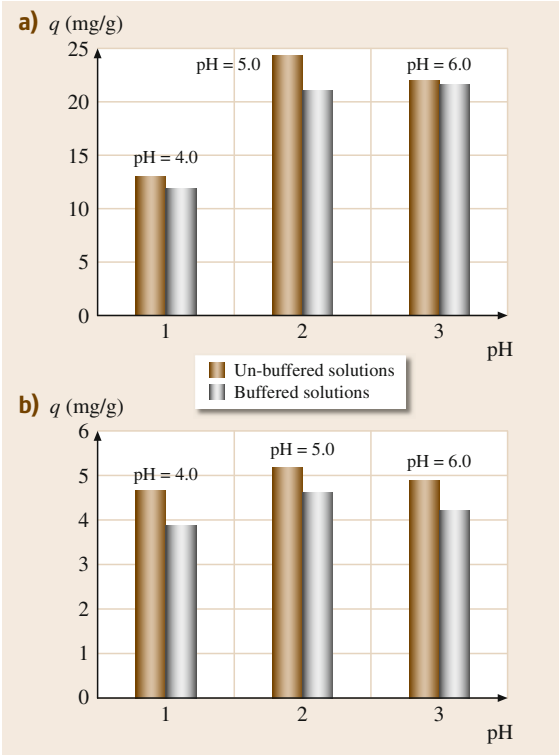

Fig. 25.4a,b Effect of buffer solution on the biosorption efficiency of Pb(II) **(a)** and Cd(II) **(b)** ions onto *Ulva lacuta* sp. marine algae [25.88, 110]

the removal of heavy metal ions from aqueous solutions by biosorption onto marine algae and marine algae waste biomass be done at the optimal value of pH in unbuffered solution.

Effect of Biomass Dose

The biomass dose is another important experimental parameter which has a great influence on the biosorption process and determined the potential of biosorbent through the binding sites available to remove heavy metal ions, at a specific concentration [25.16]. Thus, in order to optimize the experimental conditions for the removal of heavy metal ions by biosorption onto a given low-cost biosorbent, it is necessary to establish the optimum dose, and this could also be advantageous from economical considerations.

In general, it has been shown that a given initial heavy metal ion concentration, the higher efficiency of biosorption process is obtained for a lower dose of biosorbent. This is because the lower the dose of biosorbent, the higher will be the metal ion/biosorbent ratio, and the metal ion is retained onto the biosorbent unit, unless the biosorbent reaches saturation. High dose of biosorbent can exert a shell effect, by protecting the functional groups from being occupied by heavy metal ions. The result of this is a lower specific heavy metal ion uptake that means smaller amount of metal ion retained per biosorbent unit [25.87]. On the basis of these considerations, for all the biosorption systems the influence of the biosorbent dose should be studied and the obtained results analyzed carefully.

In the experimental studies, the marine algae and marine algae waste biomass dose usually varied from one hundred milligrams to few tens of grams for 1 l of aqueous solution. A typical representation of the influence of biosorbent dose on the removal efficiency of heavy metal ions obtained in the case of biosorption of Pb(II) and Co(II) ions from aqueous solution onto marine algae and marine algae waste biomass is illustrated in Fig. 25.5.

At equilibrium, the metal biosorption capacity (q, mg/g) decreases with the increasing biosorbent dose, in both the cases. This decrease can be due to the concentration gradient between the biosorbent and metal ions; an increase in the biosorbent concentration causes a decrease in the amount of heavy metal ion retained onto a unit weight of the biosorbent material. But, in the selection of optimal biosorbent dose, the variation of removal percent (R, %) must be also taken into account. It can be observed (Fig. 25.5) that the removal percent generally increases with the increase of the biomass dose. It reaches a maximum at certain value of biosorbent dosage, and then remained almost constant (as is the case of metal ions biosorption onto *Ulva lactuca* sp. marine algae) or varies very little (as is the case of metal ions biosorption onto other marine algae biomass). By analyzing these dependences the biosorbent dose that is sufficient for the quantitative removal of heavy metal ions from aqueous solutions can be selected, and this value is considered to be the optimum.

Similar trends have been reported in the literature for different adsorption systems using low-cost biological materials [25.16, 18, 82, 111], and the positive correlation between biosorbent dose and heavy metals removal efficiency, observed experimentally, can be related to the increase in the number of active sites and available surface area.

Therefore, in order to design an economical biosorption system for heavy metal ions removal, the optimum biosorbent dose should be chosen after a careful comparison of the experimental variations obtained for both parameters (q, mg/g and R, %). Table 25.4

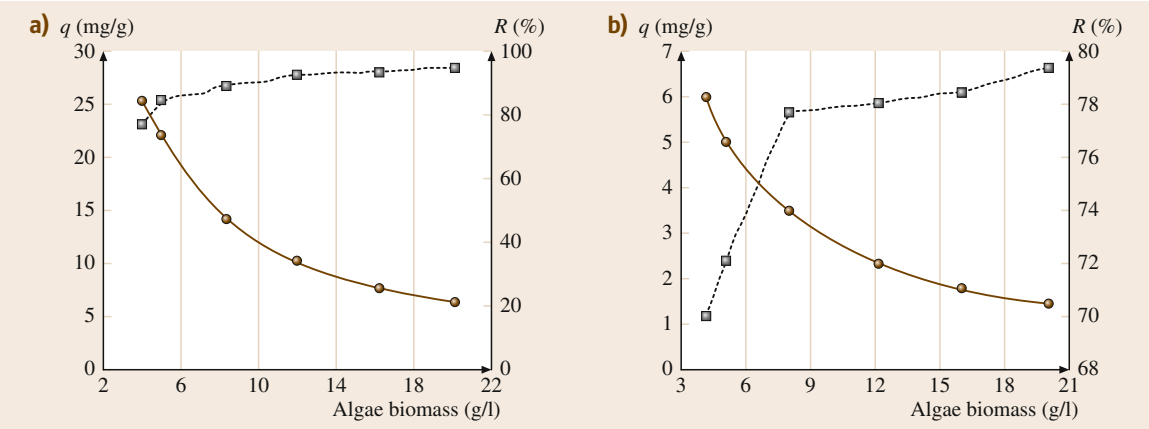

Fig. 25.5a,b Effect of biosorbent dose on the removal efficiency of Pb(II) – **(a)** and Co(II) – **(b)** ions from aqueous solution on *Ulva lactuca* sp. marine algae, in optimum experimental conditions (*q – continue line*; *R*, *% – dotted line*) (after [25.49, 88])

summarizes the values of optimal biosorbent dose used for the removal of heavy metal ions from aqueous environments, in the case of different types of marine algae biomasses.

Effect of Contact Time

As the biosorption proceeds, the biosorbent reaches the saturation state and then the retained metal ions tend to desorbs back into solution. When the system reaches this state of equilibrium, no further net biosorption occurs [25.16]. For this reason it is necessary to determine the time at which the biosorption equilibrium is attained.

The contact time between the two phases required to reach the equilibrium state is a very important experimental parameter for the biosorption process optimization of heavy metal ions from aqueous solution. The unsatisfactory value of this parameter drastically limits the practical use of a given biosorption process, even if its efficiency in the heavy metal ions removal is high. This is the main reason why in all the biosorption studies, the effect of contact time is included in the experimental protocol.

The effect of contact time between the solid phase – biosorbent material (marine algae or marine algae waste biomass) – and aqueous phase – heavy metal ions solution is generally investigated at room temperature, in considered optimum conditions (solution pH and biosorbent dose), for a given value of initial concentration of metal ions. The biomass is kept in contact with metal-bearing solution for different periods of time (usually no more than 3 h), and the general shape of

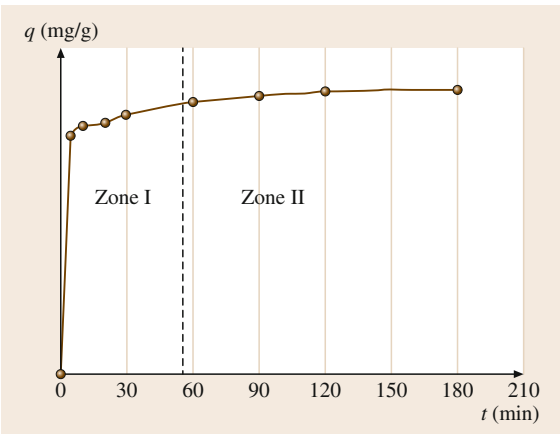

Fig. 25.6 General shape of the experimental dependences obtained in the study of the contact time influence on the removal efficiency by biosorption onto marine algae or marine algae waste biomass, in optimum experimental conditions

experimental dependencies obtained under these conditions is illustrated in Fig. 25.6.

In all the cases, the amount of heavy metal ions retained onto certain biomass increase with the increase in contact time, and the biosorption process reaches the equilibrium state after no more than 180 min. The biosorption process of heavy metal ions from aqueous solutions onto marine algae biomass generally occurs in two steps, a fast initial step (zone I) where the amount of heavy metal ions retained increase sharply (up to 90% in the first 20–60 min), followed by a much slower

gradual biosorption, near to the equilibrium (zone II). In most of the biosorption studies using marine algae or marine algae waste biomass as biosorbent, no further significant biosorption is noted after 3 h [25.24].

By analyzing the experimental dependences obtained in the study of the influence of contact time on the removal efficiency of heavy metal ions from aqueous solutions, the optimum value of this parameter can be establish. In Table 25.4 are summarized the values of contact time required for the efficient removal of heavy metal ions from aqueous environments, in the case of different types of marine algae and marine algae waste biomass. No more than 120 min of contact time is necessary in these biosorption processes. Such low values of contact time required to attain the equilibrium state indicate that the biosorption of heavy metal ions onto marine algae biomass is mainly controlled by the chemical (electrostatic) interactions between positively charged metal ions and negatively charged functional groups form biomass surface; the share of elementary diffusion processes, especially the intraparticle type, is much smaller [25.112].

Such two-steps adsorption behavior has been reported in most studies of metal ion retention in various low-cost materials [25.16, 18, 112–114], and is mainly determined by the presence of sites with different binding affinities for metal ions on the adsorbent surface, thereby yielding different binding sites. According to *Qin* and co-workers [25.25, 89], the rapid initial biosorption step is generally the result of the fast transfer of metal ions to the surface of the biosorbent particles, while the subsequent slow biosorption process is a consequence of the slow diffusion on metal ions into the pores of the biosorbent particles. The very fast biosorption and settling of marine algae biomass make these materials suitable for continuous flow wastewater treatment systems.

Effect of Temperature

Temperature is also an important experimental parameter for the removal of heavy metal ions, dealing with the thermodynamics of the biosorption process. This parameter is directly related to the kinetic energy of the metal ions, and an increase or decrease in temperature should cause a change in the amount of heavy metal ions removed or retained by biosorbent [25.87].

In general, the temperature affects the removal efficiency of the heavy metal pollutants from aqueous solution, but to a limited extent in a certain range of temperature. In case of marine algae biomass the most experimental studies were performed in the temperature range of 5–50 °C, at a contact time lower than 4 h. This is because in this temperature range, the biosorbent materials do not suffer secondary processes of thermal degradation [25.16], and the low value of contact time do not make the volume of aqueous solutions vary significantly, due to the evaporation phenomena.

The effect of temperature on the heavy metal removal by biosorption onto marine algae biomass depends on the nature of the heavy metal ion and on the nature of biomass used as biosorbent, and usually was observed in two stages. The biosorption equilibrium occurs rapidly at lower heavy metal ion concentrations, for all temperature values, and becomes relatively constant at higher concentrations. Table 25.4 presents the values of temperature considered as optimum for the removal of some heavy metal ions from aqueous solution, using different types of marine algae and marine algae waste biomass as biosorbents.

Such processes are normally exothermic, so biosorption capacity increases with decrease of temperature, and indicate that the ion exchange interactions exist in the biosorption process to some extent. The decrease of biosorption capacity of marine algae biomass at higher temperatures may be due to the damage of active binding sites in the biomass [25.115]. When the temperature is too high, a distortion of some available functional groups from the cell surface occurs, and in consequence the efficiency of the biosorption process decreases.

However, *Gupta* and *Rastogi* [25.102] have found that the Pb(II) biosorption on *Spirogyra* sp. marine algae increases with increasing temperature, in the range of 25–45 °C. They explained that higher temperature would lead to higher affinity of sites for metal ions, and the energy of the systems facilitates the binding of Pb(II) ions on the functional groups from cell surface to some extent. Such behavior has been observed and for the other removal processes of heavy metal ions using marine algae and marine algae waste biomass [25.76, 110].

The variation of temperature does not always significantly change the biosorption capacities of marine algae biomass. *Bulgariu* and *Bulgariu* [25.41] have shown that temperature (in the 10–40 °C range) has minor effect on the removal of the Pb(II), Cd(II), and Co(II) ions from aqueous solution onto marine green algae waste biomass. Thus, the increase of temperature with 30 °C determined a decrease of biosorption

capacity of the biomass with 0.07 mmol/g in the case of Pb(II), with 0.06 mmol/g in the case of Cd(II) and with 0.03 mmol/g in the case of Co(II), respectively.

On the basis of these experimental results, it is recommended that at large scale, the removal of heavy metal ions from aqueous solutions by biosorption on marine algae and marine algae waste biomass to be performed at ambient temperature, and this is dictated especially by economical considerations [25.116].

On the other hand, the change in temperature causes a change of thermodynamic parameters, such as free Gibbs energy change (ΔG), enthalpy change (ΔH) or entropy change (ΔS), which contribute to help understand the biosorption mechanisms [25.117]. Temperature data are usually used to determine these parameters, because they provide valuable information about the biosorption process. The negative value of free Gibbs energy change (ΔG) shows that the process is feasible and spontaneous. The increase of the ΔG value, in the negative direction, with temperature is an indication of the increase of biosorption process probability [25.24]. Also, the standard enthalpy change (ΔH) shows the route of energy in the system, and has an important contribution to deciding whether a certain biosorbent can be used for the removal of heavy metal ions at elevated temperature or not. A positive value indicates an endothermic process that will be favored by high temperatures, while a negative value of this parameter shows an exothermic process, which is more efficient at low temperatures.

Table 25.5 summarizes the values of thermodynamic parameters for some removal processes of heavy metal ions from aqueous media, using marine algae biomass as biosorbents.

The positive value of the entropy change indicates the increasing randomness at the solid/liquid interface during the biosorption process, whereas the negative value of this parameter suggests a decrease in the randomness at the solid/liquid interface during the biosorption process [25.76]. Thus, even if some biosorption processes are endothermic, in some conditions these can be spontaneous because of the positive entropy change.

All these parameters should be considered in the experimental studies of heavy metal ions removal by biosorption onto marine algae biomasses, and the obtained results will allow us to establish the optimum conditions, to ensure the maximum efficiency of the biosorption process.

25.4.2 Influence of Experimental Parameters in a Dynamic Continuous-Flow Technique

The process of heavy metal ions biosorption in a dynamic continuous-flow technique is mainly influenced by three key experimental parameters: bed height, flow rate of aqueous solution, and initial concentration of heavy metal ions.

As the heavy metal ions solution passes through the column, the biosorption zone (where the bulk of biosorption takes place) starts moving out of the column and the effluent concentrations start rising with time. As a consequence, the variation of these parameters will change the characteristics of the breakthrough curve (breakthrough point and saturation point), and this will also affect the applicability of the heavy metal ion removal process. For this reason, in the experimental studies, for each of these parameters the optimal values that should be established to ensure the efficient delivery of the biosorption process.

Effect of Bed Height

Accumulation of heavy metal ions in a packed-bed column is largely dependent on the quantity of biosorbent (marine algae biomass) inside the column. This is mainly due to the higher contact time between heavy metal ion solution and available biomass in the column, and also due to more number of binding sites and functional groups available for biosorption of heavy metal ions [25.118].

Generally, the characteristics of the breakthrough curves (breakthrough time (volume) and saturation time (volume)) increases with the rise in the bed height, and this can be easily observed from Fig. 25.7 chosen for exemplification. This may be due to an increase in the surface area of the biosorbent as the quantity of biomass packed in the column increase.

However, the increase in the quantity of algae biomass deposited in the column also results in a broadened mass transfer zone, which makes breakthrough curves less steeper. The more gradual shape of the curves as the bed height increased implies that the biosorbent bed was more difficult to be completely saturated, and the decrease in the slope of the breakthrough curves with the increase of this parameter clearly reflects this fact [25.66]. Even if a high biosorbent bed height increases the usage time of the column, a too high value of this parameter has several disadvantages, the most important being the increase in the difficulty of solution flow through the column and the quantity of

Table 25.5 The values of thermodynamic parameters obtained for some biosorption processes of heavy metal ions onto marine algae biomass

Heavy metal ion	Marine algae biosorbent	T (°C)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/molK)	Reference
As(III)	*Maugeotia genuflexa*	20	−18.39	−29.69	−38.43	[25.56]
	Ulva lactuca sp.	20	−16.40	−32.80	−55.0	[25.38]
Cd(II)	*Ceramium virgatum*	20	−19.50	−31.80	−42.40	[25.62]
	Gymnogongrus torulosus	22	−15.65	−23.00	−25.00	[25.92]
Cu(II)	*Gymnogongrus torulosus*	22	−15.64	−33.00	−59.00	[25.92]
	Ulva lactuca sp.	20	−16.70	−30.20	−45.8	[25.38]
Pb(II)	*Spirogyra* sp.	25	−20.45	4.00	83.50	[25.73]
	Gymnogongrus torulosus	22	−15.25	−24.00	−32.00	[25.92]
	Waste algae biomass	20	−16.60	26.69	34.43	[25.93]
Se(IV)	*Cladophora hutchinsiae*	20	−18.39	−45.96	−70.92	[25.78]
Zn(II)	*Gymnogongrus torulosus*	22	−10.44	28.00	129.0	[25.92]
	Ulva reticulate sp.	25	−14.70	4.45	64.00	[25.89]

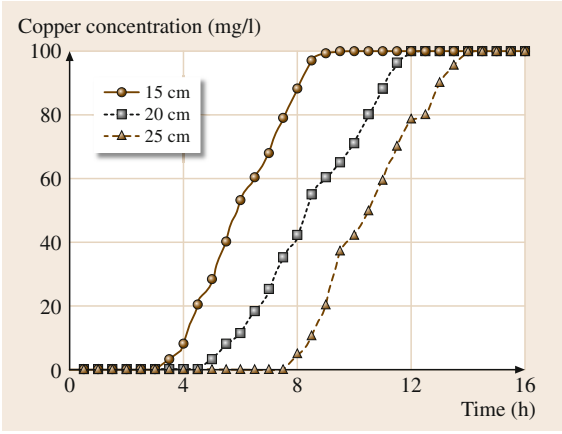

Fig. 25.7 Breakthrough curves for biosorption of copper by *Ulva reticulate* at different bed heights (flow rate = 5 ml/min; pH = 5.5; initial concentration = 100 mg/l; temperature = 30 °C) (after [25.75])

biosorbent required for the biosorption process. On the other hand, if the biosorbent bed height is too low, the column becomes quickly saturated, and biosorbent bed must be regenerated before another use.

Table 25.6 presents the experimental values of the biosorbent bed height used for the removal of some heavy metal ions from the aqueous environment on various algae biomasses, in dynamic continuous-flow conditions.

Lodeiro et al. [25.66] have shown that in the case of Cd(II) ions removal on protonated *Sargassum muticum* marine algae the breakthrough curves showed no *tail* toward saturation time when the biosorbent mass inside of column was low. Moreover, when the quantity of algae biomass is increased a slower increase of the breakthrough parameters toward saturation of the bed can be observed. The broad tailing edge of breakthrough curves could be a consequence of the rate limiting intraparticle diffusion mechanism or flow nonidealities, which may occur when an excessive quantity of algae biomass is present in the column [25.75].

Effect of Solution Flow Rate

The solution flow rate is an important parameter affecting biosorption of metal ions in a packed-bed column, because the retention process, especially at low metal ions concentrations, is usually controlled by the mass transfer process [25.123].

Generally, the characteristics of the breakthrough point (breakthrough time and volume) decrease when the solution flow rate through the biosorbent bed increases. In addition, the volume of solution which is efficiently treated until a breakthrough point, decreases significantly with the increase of flowrate, and therefore the useful time of biosorbent bed is significant reduced. Initially, the biosorption process is very fast at lower solution flow rate, probably associated with the availability of functional groups from the biosorbent surface. During the use, these functional groups are gradually occupied, and the uptake process becomes less effective. The breakthrough curves become steeper when the solution flow rate is increased, and the breakthrough point and the retained metal ion concentration decreases.

For exemplification, in Fig. 25.8 is illustrated the effect of solution flow rate obtained in the case of

Table 25.6 Experimental conditions for biosorption of some heavy metal ions onto marine algae biomass, in dynamic continuous-flow conditions

Heavy metal ion	Marine algae biomass	Bed height (cm)	Flow rate (ml/s)	Initial concentration (mg/l)	Reference
Cd(II)	*Sargassum muticum*	6.9	10.0	50	[25.66]
	Sargassum glaucescens	40	–	25	[25.69]
	Gelidium	15	4.0	50	[25.119]
	Ulva lactuca	–	3.5	65	[25.80]
	Ulva lactuca – fixed in agar	–	3.5	65	[25.80]
Co(II)	*Ulva reticulata*	25.0	10.0	100	[25.75]
	Ulva reticulata	25.0	10.0	100	[25.75]
	Gelidium	15	4.0	50	[25.119]
Cu(II)	*Sargassum fluitans*	20	7.5	100	[25.120, 121]
	Sargassum – Ca form	50	4.0	200	[25.122]
Ni(II)	*Ulva reticulata*	25.0	10.0	100	[25.75]
	Sargassum filipendula	30.5	6.0	125	[25.68]
	Gelidium	15	4.0	50	[25.73, 119]
	Sargassum glaucescens	40	–	245	[25.69]
Pb(II)	*Ulva lactuca*	–	3.5	65	[25.80]
	Ulva lactuca – fixed in agar	–	3.5	65	[25.80]
	Ulva lactuca: A-100	2.5	3.5	135	[25.42]
Re(VII)	*Laminaria japonica*	100 mg biosorbent	6.0	20	[25.79]
Zn(II)	*Sargassum* – Ca form	50	3.0	200	[25.122]
	Ulva reticulata	25	5.0	100	[25.76]

Pb(II) removal on biosorbent mixture (marine algae waste biomass: Purolite A-100) bed [25.42], but similar results have been reported for the most dynamic continuous-flow systems that use marine algae biomass as biosorbents.

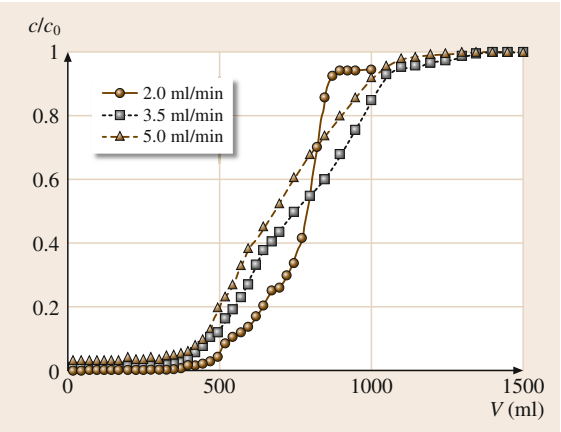

Fig. 25.8 Breakthrough curves for biosorption of Pb(II) by the biosorbent mixture-bed column (*Ulva lactuca*: Purolite A-100 = 1 : 2), at different flow rates (pH = 5.0; $c_0 = 0.34$ mmol/l; $H = 2.5$ cm; temperature = 20 °C) [25.42]

Lower solution flow rate favor biosorption of heavy metal ions, and as a consequence the biosorption capacity of marine algae biomass is significantly improved. This is because at lower solution flow rate the metal ions have more time in contact with the biosorbent and the diffusion of metal ions to the binding sites from the algae waste biomass surface occurs. If the solution flow rate is higher, the metal ions have a short diffusion time and will interact predominantly with the functional groups for which they have higher affinity and availability. When these binding sites are occupied, the biosorbent bed becomes saturated early, and the biosorption process is stopped. On the other hand, when the flow rate is lower, the contact time inside the column is longer, and the diffusion process becomes effective. The heavy metal ions have more time to diffuse amidst the biosorbent particles and will interact with the available superficial functional groups that have lower affinity for metal ions. In consequence, a longer breakthrough time is obtained and better biosorption efficiency.

Therefore, the choice of the best solution flow rate values should be a compromise between these two opposite situations [25.124]. Several such *compromise* values of solution flow rate, used for the removal of some heavy metal ions onto marine algae biomass in dynamic continuous-flow conditions, are presented in Table 25.6.

It should also be noted that a too little solution flow rate has two undesirable consequences, from the applicative point of view: (i) the saturation point not reached, which means that the obtained breakthrough curves cannot be modeled and characteristic parameters cannot be calculated, and (ii) the reach of the saturation point is so slow and the metal ions concentration in the effluent becomes early high for that the removal process is considered to be efficient, even if the biosorbent is not exhausted. Under these conditions, the biosorption process becomes unfeasible from economical considerations.

Effect of Initial Heavy Metal Ions Concentration

The variation of initial heavy metal ions concentration changes the shape of breakthrough curves. An increase in the initial concentration of heavy metal ions, when other experimental parameters are kept constant, significantly affects the breakthrough curves, as is illustrated in Fig. 25.9.

In all the studied biosorption systems that use marine algae biomass as biosorbent, the increase of the initial heavy metal ions concentration caused a faster breakthrough as expected. In addition, at a high initial heavy metal ions concentration, the breakthrough curves were steeper in comparison with those at a low initial heavy metal ions concentration, with a shorter mass transfer zone (MTZ). The steepness of the curve (dc/dt) is a measure of the efficiency of the column to reach the saturation, and a high value of this parameter is better for the column performances [25.65].

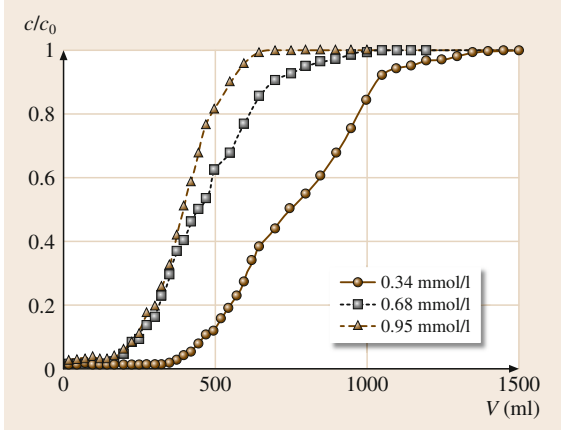

Fig. 25.9 Breakthrough curves for biosorption of Pb(II) by biosorbent mixture-bed column (*Ulva lactuca*: Purolite A-100 = 1 : 2), at different initial metal ions concentrations (pH = 5.0; flow rate = 3.5 ml/min; H = 2.5 cm; temperature = 20 °C) [25.42]

The experimental results demonstrate that an increase in the initial heavy metal ions concentration changes the biosorption rate through the packed bed and increases the biosorbent-bed capacity. Thus, biosorption of heavy metal ions at breakthrough and saturation was greater at high initial heavy metal ions concentration, because higher metal concentration provides better driving force to the metal biosorption process during continuous column run [25.42].

25.5 Modeling of Biosorption Process in Batch Conditions: Isotherm and Kinetic Models

Biosorption of heavy metal ions is a passive non-metabolically mediated process of metal ions binding by biosorbent. Many kinds of marine algae or marine algae waste biomass can be used as biosorbents for the removal of heavy metal ions from aqueous solution. Biosorption can also be defined as a number of passive accumulation processes, which may include, in any particular cases, ion exchange, coordination, complexation, adsorption, and microprecipitation [25.18]. For this reason, biosorption is considered to be a fast physical/chemical process, and its rate is determined by the type of elementary interactions.

Proper analysis and design of biosorption removal processes requires relevant equilibrium and kinetic data, as the vital information. In equilibrium, a certain relationship prevails between heavy metal ions concentration from aqueous solution and biosorbent state. So, it is both fundamentally and economically important to correlate the equilibrium and kinetic data between various heavy metal ions and biosorbents, in order to asses the biosorption capacity for different heavy metal

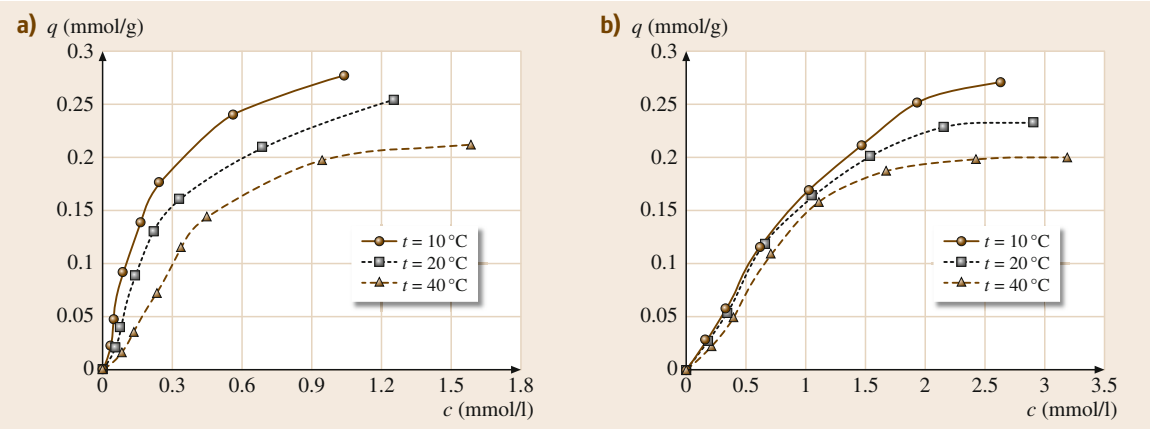

Fig. 25.10a,b Biosorption isotherms for retention of Pb(II) **(a)** and Cd(II) **(b)** on green algae waste biomass (pH = 5.0; biosorbent dose = 8 g/l; contact time = 4 h) (after [25.41])

ions and as a prerequisite for the design of commercial treatment systems. The more complex is the wastewater composition, the more accuracy is required in describing and selecting the biosorption systems [25.18, 112].

25.5.1 Equilibrium Modeling of Biosorption

The uptake capacity of a certain biosorbent (marine algae or marine algae waste biomass) for a given heavy metal ion from aqueous solutions, in specified experimental conditions, can be described by the equilibrium biosorption isotherms. The biosorption isotherms represent the equilibrium distribution of the studied heavy metal ions between the phases of the solid biosorbent and aqueous solution. Because the heavy metal ions equilibrium concentrations are function of temperature, obtaining equilibrium biosorption isotherms for different temperatures are necessary for the thermodynamic modeling of the biosorption process. Figure 25.10 illustrates, for exemplification, the biosorption isotherms obtained for Pb(II) and Cd(II) ions retention on *Ulva lactuca* sp. marine algae, but similar dependences have been obtained for almost all biosorption systems, which use marine algae biomass as biosorbents.

Most of biosorption isotherms obtained experimentally have a similar shape with those presented in Fig. 25.10, being nonlinear and composed from two regions. In the first region – corresponding to low concentrations of heavy metal ions, the biosorption equilibrium arises rapidly, while in the second region, the biosorption equilibrium occurs more slowly, and this is a characteristic of the high concentrations of heavy metal ions. The value of metal ions concentration that separates the two regions depends on the nature of marine algae biomass used as biosorbent and the metal ion involved in the biosorption process. Therefore, for the optimization of the biosorption process design, it is necessary to obtain the appropriate correlation for the equilibrium biosorption isotherms. These isotherms are characterized by definite parameters, whose values express the surface properties and affinity of biosorbent for different heavy metal ions, and these are obtained by using equilibrium isotherm models.

The equilibrium isotherm models are usually classified in two categories [25.16, 125]:

a) *Empirical equations* – These equations are used to describe the biosorption equilibrium, and can provide information of metal-uptake capacity of various biosorbent materials, and can be used to describe mono- or multimetal ions biosorption systems. In Table 25.7 are presented some well-known empirical models for single and multicomponents systems. However, the empirical models do not reflect any mechanism of metal ions uptake and can hardly explain the biosorption behavior with adequate physical interpretation. In addition, predictive conclusions cannot be drawn, for the systems operating in different conditions.
b) *Mechanistic models* – These models are based on the mechanism of metal ions biosorption, and these can be used not only to represent experimental behavior but also to explain and predict the biosorption behavior; based on the understanding of biosorption mechanisms. The most important mechanistic models are the ion exchange model and

Table 25.7 Frequently used equilibrium isotherm models (after [25.15, 18])

Isotherm model	Mathematical equation	Observations
Single-component isotherm models		
Freundlich	$\ln q_e = \ln K_F + \frac{1}{n} \ln c_e$	q_e is the equilibrium metal biosorption capacity; c_e is the equilibrium metal ions concentration in solution; K_F is a biosorption equilibrium constant, representative of the biosorption capacity; n is a constant related to the biosorption intensity
Langmuir	$q_e = q_{max} \cdot \frac{K_L \cdot c_e}{1 + K_L \cdot c_e}$	q_{max} is the maximum adsorption capacity upon complete saturation of biosorbent surface; K_L is the Langmuir constant, related to the biosorption/desorption energy
Dubinin–Radushkevich	$\ln q = \ln q_{max}^{DR} - \beta \cdot \varepsilon^2$	q_{max}^{DR} is the maximum amount of heavy metal retained on weight unit of biosorbent; β is a constant related to the adsorption energy and ε is the Polanyi potential, defined by the mathematical relation: $\varepsilon = RT \ln(1 + 1/c_e)$; where: R is the gas constant; T is the absolute temperature, c_e is the equilibrium concentration of heavy metals in solution.
Temkin	$q_e = \frac{R \cdot T}{b} \ln(a \cdot c_e)$	R is the gas constant; T is the absolute temperature; a, b are constants directly correlated to the biosorption heat
BET model	$q = \frac{K_B \cdot q_{e,max} \cdot c_e}{(c_s - c_e) \cdot [1 + (K_B - 1) \frac{c_e}{c_s}]}$	K_B is BET constant, indicating the energy of interaction between metal ions and the biosorbent surface; $q_{e,max}$ is a constant indicating the amount of metal ions retained to form a complete monolayer; c_s is the saturation concentration of the biosorbed component
Redlich–Peterson	$q_e = \frac{K_{RP} \cdot c_e}{1 + a_{RP} \cdot c_e^{\beta}}$	K_{RP}, a_{RP} and β are the Redlich–Peterson parameters. The exponent β lies between 0 and 1. For $\beta = 1$, the equation converts to the Langmuir form
Sips	$q_e = q_{max} \cdot \frac{(K_S \cdot c_e)^{\gamma}}{1 + (K_S \cdot c_e)^{\gamma}}$	K_S and γ are Sips parameters; q_{max} is the maximum adsorption capacity upon complete saturation of biosorbent surface
Multiple-component isotherm models		
Langmuir (multicomponent)	$q_{e,i} = \frac{K_{L,i} \cdot q_{max,i} \cdot c_{e,i}}{1 + \sum_i K_{L,i} \cdot c_{e,i}}$	$c_{e,i}$ and $q_{e,i}$ are the concentration and the retained quantity of metal ion per weight unit of biosorbent for each component; $q_{max,i}$ and $K_{L,i}$ are derived from the corresponding individual Langmuir isotherm equations
Combined Langmuir–Freundlich model	$q_{e,i} = \frac{a_i \cdot c_{e,i}^{1/n_i}}{1 + \sum_i b_i \cdot c_{e,i}^{1/n_i}}$	a_i and b_i are phase concentration of a single biosorbed component in equations
Competitive Redlich–Peterson model	$q_e = \frac{K_{RP,i} \cdot c_{e,i}}{1 + \sum_i a_{RP,i} \cdot c_{e,i}^{\beta_i}}$	$K_{RP,i}$, $a_{RP,i}$ and β_i are the Redlich–Peterson parameters derived from the corresponding individual Redlich–Peterson isotherm equations
IAST: Ideal Adsorbed Solution	$\frac{1}{q_t} = \sum_i \frac{Y_i}{q_i^0}$	Y_i is the solute concentration of component i in the solid phase; q_i^0 is the phase concentration of a single retained component, with concentration c_i^0.

surface complexation model [25.108]. Both these models take into account the nature of interaction between metal ions and functional groups of biosorbent, and modeling the biosorption process considering that metal biosorption mechanism involve ion exchange or complexation interactions [25.126, 127]. By using these models, they have predicted the isotherms of different metal ions biosorption (e.g., Cd(II), U(IV), etc.) on different types of nonliving marine algae biomass, in various experimental conditions [25.126, 128]. Unfortunately, the use of these models is limited to a small number of biosorption systems, because they assume the existence of a single type of interaction between metal ion and functional groups of biosorbent, which does not happen in the case of marine algae biomass.

In the equilibrium modeling studies, two classical empirical isotherm models, Freundlich and Langmuir, were most frequently selected to fit the experimental data obtained for various biosorption systems that use marine algae biomass for the removal of heavy metal ions from aqueous solution. Although the basic assumptions of these models were not fulfilled due to

the heterogeneity of the biosorbent surface, they were quite successful in predicting the practical biosorption capacity and optimization of the biosorption system design [25.129]. Generally, the linear regression is used to find the most adequate model that could describe the biosorption isotherms of heavy metal ions on marine algae biomass. Even though nonlinear method provides better results, the linear least-square method is still often preferred in favor of its simplicity and convenience.

The Freundlich isotherm model is originally of empirical nature (Table 25.7). This model is one of the the most widely used isotherm model for description of adsorption equilibrium, both for organic and inorganic compounds on a wide variety of adsorbents, including biosorbents [25.18]. The Freundlich isotherm model has been interpreted as biosorption to heterogeneous surface or surface supporting sites of different affinity, and assumed that the stronger binding sites are occupied first and that the binding strength decreases with increasing degree of occupation [25.87]. Generally, the Freundlich isotherm model is used to estimate the biosorption intensity of heavy metal ions toward a given biosorbent.

The graphical representation of $\ln q_e$ versus $\ln c_e$ has a slop with the value of $1/n$ and an intercept magnitude of $\ln K_F$. A favorable biosorption process tends to have the Freundlich constant n between 1 and 10 [25.18]. Larger value of n (smaller value for $1/n$) indicates strong interactions between biosorbent and heavy metal ions, while a value of n equal to 1, suggests linear biosorption process, leading to identical biosorption energy for all sites [25.130].

The Freundlich isotherm model can be successfully used to analyze most of the experimental biosorption data, and is especially excellent for fitting data from systems that use highly heterogeneous biosorbents, as listed in Table 25.8. Even, the biosorption capacities for marine algae biomasses (obtained from the Freundlich K_F constant) summarized in Table 25.8 are quite low in comparison with commercially activated carbons, these biosorbents are still attractive due to its biosorption advantages and cost-effectiveness for the removal of heavy metal ions.

Nevertheless, in some cases, the Freundlich isotherm model could not fit the experimental data well due to the low values of obtained correlation coefficients, or is not suitable to describe the biosorption equilibrium. Thus, the Freundlich isotherm model cannot be use to predict the biosorption equilibrium data at extreme concentrations of heavy metal ions. This is because the mathematical equation is not reduced to a linear expression at very low concentrations, and do not has limit expression at very high concentrations [25.18]. Fortunately this is not a problem, because a moderate concentration range is frequently used in most of biosorption studies.

The Langmuir isotherm model is probably the best known and the most widely used sorption isotherm for describing heavy metals biosorption on marine algae biomass, which produced good agreement with a wide variety of experimental data. In the simplest case, the Langmuir model is based on the following assumptions [25.4, 18]:

i) The biosorption process is limited to monolayer coverage,
ii) The surface of biosorbent is homogeneous and all surface sites (constant heat of biosorption);
iii) There is only one sorbate,
iv) One sorbate molecule reacts with one active site, and
v) The ability of a molecule to be retained on a given site is independent of the surface occupancy.

This model assumed that biosorption forces are similar to the forces in chemical interactions, and can be used to estimate the maximum biosorption capacity (q_{max}, mg/g), corresponding to biosorbent surface saturation. Usually, the parameters of the Langmuir isotherm model (q_{max} and K_L) are evaluated from the intercepts and the slopes of linear plots of c_e/q versus c_e. Table 25.8 summarizes a number of studies that draw upon the Langmuir isotherm to analyze the biosorption equilibrium data of heavy metal ions onto marine algae biomass.

According to the Langmuir model, the maximum biosorption capacity (q_{max}, mg/g) should coincide with saturation of the monolayer coverage, and as a consequence it should be independent of temperature. The experimental results have shown that a relatively small increase or even decrease in the maximum biosorption capacity is usually observed in the case of heavy metal ions biosorption on marine algae biomass (Table 25.8). This is because in biosorption processes that use marine algae biomass as biosorbents, saturation of certain biomass depends on the accessibility of the binding sites, the chemical state, and the affinity of studied metal ions for these sites. The temperature variation may affect one or more of these factors, and this will lead to the variation of the maximum biosorption capacity.

Table 25.8 Freundlich and Langmuir parameters for the removal of some heavy metal ions by various kinds of algae biomass

Heavy metal ion	Algae biomass	Freundlich model			Langmuir model			Reference
		R^2	n	K_F (mg/g)	R^2	q_{max} (mg/g)	K_L (L/mg)	
Cd(II)	*Laminaria* sp.	0.847	4.98	64.07	0.972	104.53	0.01	[25.131]
	Oedogonium sp.	0.934	1.63	4.89	0.995	88.20	0.02	[25.132]
	Lobophora variegata	0.906	1.27	17.02	0.982	167.91	0.15	[25.133]
	Pelvetia caniculata	0.941	2.80	13.03	0.992	75.00	0.07	[25.46]
	Cystoseira baccata	0.970	3.10	75.31	0.980	77.56	0.09	[25.94]
	Gelidium	–	–	–	0.998	18.0	0.19	[25.96]
	Ulva lactuca	0.910	1.43	0.56	0.988	41.66	0.003	[25.88]
	Algae waste biomass[a]	0.942	1.22	14.31	0.987	34.60	0.01	[25.41]
	Algae waste biomass[b]	–	–	–	0.998	9.7	0.16	[25.96]
Cr(III)	*Spirogyra* sp.	0.911	3.57	10.51	0.911	28.16	0.03	[25.59]
	Ulva lactuca	0.987	4.76	1.01	0.997	2.03	1.15	[25.61]
	Cladophora sp.	0.998	2.45	84.38	0.961	47.02	0.14	[25.134, 135]
		0.937	6.91	6.29	0.993	14.71	0.06	[25.60]
	Fucus serratus	0.978	2.83	161.54	0.993	101.73	0.26	[25.51]
	Undaria pinnatifida	0.947	1.52	6.65	0.965	78.88	0.06	[25.136]
Cu(II)	*Ulva fasciata* sp.	0.960	0.45	2.22	0.999	26.88	0.25	[25.63, 101]
	Spirogyra sp.	0.958	4.15	9.67	0.995	38.61	0.04	[25.60]
	Laminaria sp.	0.878	4.43	35.56	0.990	61.59	0.02	[25.131]
	Turbinaria ornata	0.981	1.49	1.26	0.992	125.10	0.03	[25.137]
	Undaria pinnatifida	0.941	2.52	7.17	0.984	28.89	0.10	[25.136]
	Oedogonium sp.	0.997	2.05	1.83	0.985	40.98	0.03	[25.138]
	Laminaria sp.	0.845	3.78	36.39	0.954	66.33	0.02	[25.131]
Ni(II)	*Cystoseria indica*	0.957	1.77	2.75	0.999	47.62	0.03	
	Nizmuddinia zanardini	0.973	1.66	2.09	0.999	50.00	0.02	[25.104]
	Sargassum glaucescens	0.974	1.63	2.04	0.998	52.63	0.02	
	Padina australis	0.969	1.72	1.08	0.999	23.8	0.02	
	Algae waste biomass[a]	0.890	1.33	61.14	0.998	66.43	0.01	[25.41]
	Spirogyra sp.	0.981	2.66	4.71	0.990	140.84	0.02	[25.139, 140]
		0.981	2.66	9.22	0.991	90.91	0.02	[25.60]
	Cladophora sp.	0.997	3.62	163.47	0.998	200.42	0.04	[25.134]
		0.980	3.03	6.63	0.996	46.51	0.03	[25.60]
	Laminaria japonica	0.916	4.91	213.42	0.916	237.50	0.03	[25.140]
Pb(II)	*Cladophora* sp.	0.997	3.62	37.46	0.998	198.50	0.04	[25.36]
	Oedogonium sp.	0.925	1.75	8.07	0.998	144.92	0.02	[25.141]
	Nostoc sp.	0.949	2.28	8.34	0.990	93.46	0.02	[25.141]
	Lobophora sp.	0.928	1.03	93.24	0.974	580.16	0.11	[25.133]
	Cystoseira baccata	0.840	6.00	153.33	0.980	182.33	0.37	[25.94]
Zn(II)	*Ulva fasciata* sp.	0.983	0.44	1.42	0.998	13.50	0.09	[25.21, 101]
	Laminaria sp.	0.921	2.92	23.53	0.983	54.26	0.01	[25.131]

[a] Marine green algae (*Ulva lactuca* sp.) waste biomass obtained after extraction of oils; [b] waste algae biomass obtained from agar extraction industry

In addition, the variation of the Langmuir constant (K_L) with temperature indicates whether the biosorption process is exothermic or endothermic. Thus, the decrease of K_L value with temperature rise signifies the exothermicity of the biosorption process (physical biosorption), while the decrease of K_L with temperature indicates that the biosorption process need supplementary (thermal) energy (is endothermic), leading to chemosorption. These observations are very useful in the optimum design of biosorption systems. Thus, in the case of physical biosorption, the bonds between heavy metals and active sites of biosorbent are weak, and desorption occurs simply, by treating the loaded biosorbent with water. In the case of chemosorption, the bonds between heavy metals and active sites of biosorbent are stronger, and for desorption are needed more aggressive chemical reagents (mineral acids, complexing agents, etc.).

In order to appreciate the nature – physical or chemical – of the biosorption process, the Dubinin–Raduskevich model (Table 25.7) is more adequate for the analysis of equilibrium biosorption data. This model takes into account the energetically nonuniform surface of biosorbent, and the model parameters (q_{max}^{DR} and β) are obtained from the graphical representation of $\ln q_e$ versus ε^2. Using the value of β, the mean biosorption energy (E, kJ/mol) can be calculated using the following equation,

$$E = \frac{1}{\sqrt{2\beta}} . \qquad (25.4)$$

The mean energy of biosorption (E, kJ/mol) gives information about the biosorption mechanism. Therefore, if the biosorption process has a mean energy of biosorption in the range of 1−16 kJ/mol, then it indicates that the physical electrostatic interactions are potentially involved in the biosorption mechanism [25.32, 143]. Some values of the Dubinin–Raduskevich model parameters obtained for biosorption of heavy metal ions onto marine algae biomass are given in Table 25.9.

One of the main advantages of the Dubinin–Raduskevich model is that it is temperature dependent. If the biosorption data at different temperatures are graphical represented, all the suitable data shall in general lay on the same characteristic curve. This curve can be used to measure the applicability of the Dubinin–Raduskevich model in analysis of the biosorption equilibrium data, together with the value of correlation coefficient. When the fitting procedure gives high correlation coefficient, but the analyzed data does not lie in the same characteristic curve, the validity of calculated parameters is questionable. Anyway, the characteristic curve for a given biosorption systems cannot be examined if the experiments were performed for a single value of temperature [25.18].

The other isotherm models presented in Table 25.7, even commonly appearing in the biosorption literature, have a much lower applicability in the analysis of equilibrium experimental data. This is mainly determined by the fact that these models do not always reflect the physicochemical principles of the biosorption process, and in most of the cases, are not well understood. From the practical point of view, they are just mathematical models that describe the experimentally observed relation between amount of heavy metal retained on weight unit of biosorbent (q_e) and its equilibrium concentration from solution (c_e), without to offer information about biosorption mechanism, or taking into account the influence of the experimental parameters.

Most of biosorption studies of heavy metal ions by diverse kinds of biosorbents, including marine algae biomass, have been performed on the single metal uptake. In contrast to this ideal situation, the real wastewater commonly contain a mixture of metal ions, and therefore the modeling of multimetal biosorption systems is essential in the design of treatment systems. The biosorption of heavy metal ions in real systems that contain more than one component (multicomponent systems) involves the study of the biosorption equilibrium in conditions of competition between different types of heavy metal ions, and are required for better understanding of the system and for design purpose.

Only few isotherm models have been developed to describe the biosorption equilibrium in such systems. The most used multimetal biosorption models applied for the modeling of biosorption equilibrium are listed in Table 25.7. These models range from simple equations associated with the individual isotherm parameters (nonmodified biosorption models) to more complex models; exploiting the individual isotherm parameters along with correction factors (modified biosorption models) [25.144].

The most frequent use of multicomponent biosorption models is the multicomponent Langmuir model including its modification as well as multicomponent Freundlich models. *Lee* and *Chang* [25.60] have use the multicomponent Langmuir and Freundlich models to describe the simultaneous biosorption of Pb(II) and Cu(II) ions into two types of marine algae: *Spirogyra* sp. and *Cladophora* sp. They noticed that the co-ion

Table 25.9 Dubinin–Raduskevich parameters for biosorption of some heavy metal ions on various kinds of marine algae biomass

Heavy metal ion	Marine alga biomass	Dubinin–Raduskevich model parameters				Reference
		R^2	q_{max}^{D-R} (mmol/g)	β (mol^2/kJ^2)	E (kJ/mol)	
Cd(II)	*Caulerpa lentillifera*	0.974	0.035	−0.020	4.95	[25.32]
	Ulva lactuca	0.997	0.370	–	9.00	[25.38]
	Cystoseira indica	0.730	0.108	−0.225	4.76	[25.16]
	Gymnogongrus torulosus	0.834	0.403	−0.007	26.73	[25.142]
Cu(II)	*Caulerpa lentillifera*	0.968	0.076	−0.029	4.14	[25.32]
	Gymnogongrus torulosus	0.861	0.611	−0.007	26.73	[25.142]
Ni(II)	*Cystoseira indica*	0.870	0.268	−0.006	9.22	[25.16]
	Caulerpa lentillifera	0.979	0.120	−0.015	5.75	[25.32]
Pb(II)	*Ulva lactuca*	0.993	0.200	–	10.40	[25.38]
	Cystoseira indica	0.520	0.035	−0.005	6.91	[25.16]
	Gymnogongrus torulosus	0.647	0.164	−0.002	50.00	[25.142]
Se(IV)	*Cladophora hutchinsiae*	0.997	0.008	–	10.9	[25.107]
Zn(II)	*Gymnogongrus torulosus*	0.862	0.504	−0.009	7.54	[25.142]

effect on the equilibrium uptake became more significant as the concentration of metal ions in solution is increased. In addition, they also showed that these two types of marine algae displayed stronger biosorption inhibition of Cu(II) compared to Pb(II), which can be explained by the higher affinity of Pb(II) ions toward the functional groups from marine algae biomass surface.

Other studies of binary biosorption of heavy metal ions on marine algae biomass were carried out by *Sing* et al. [25.146] and *Kleinubing* et al. [25.72]. *Sing* et al. [25.146] examined single and binary component biosorption of Pb(II) and Cu(II) from aqueous solution using *Spirogyra neglecta* green algae. Equilibrium uptake of Pb(II) and Cu(II) is improved by increment of its initial concentration up to 100 mg/l. In contrast, the presence of increasing concentration of other metal ions brought about the deterioration in equilibrium uptake value. Also, they observed that Pb(II) ions are more severely inhibited Cu(II) biosorption than vice versa; thus reflecting greater affinity of Pb(II) ions for the marine algae biomass.

The binary biosorption of Cu(II) and Ni(II) ions onto dried *Sargassum filipendula* algae was investigated by *Kleinubing* et al. [25.72]. Biosorption data in their binary systems showed that the retained amount of one metal ion declines as the concentration of the other competitive metal ion increases in the solution. The consequence is quite substantial for Ni(II) biosorption as the process is strong suppressed by the presence of higher Cu(II) ions in solution.

Nevertheless, the researches on co-ions biosorption models are only in their preliminary stage. How to use the mathematical models with multiparameters to describe the competitive biosorption of heavy metal ions from aqueous solution is a future direction of biosorption research [25.15]?

25.5.2 Kinetic Modeling of Biosorption

The studies of biosorption equilibriums are very important to determine the efficiency of biosorption systems in the removal processes of heavy metals from aqueous media. Besides this, it is also necessary to identify the biosorption mechanism type in a given systems, and this can be achieved by kinetic studies. The information obtained from kinetic studies is used in the design of adsorption systems because they provide valuable information about the biosorption mechanism and its potential rate-controlling step, as either mass transfer or chemical interactions, in order to obtain the optimum operating conditions for industrial-scale batch metal removal processes [25.147].

In batch systems, the kinetics of biosorption processes is described by a number of models based on biosorption equilibrium [25.16, 18, 112], which are used to fit the experimental results obtained for a given initial metal ion concentration, biosorbent dose, solution pH, temperature, and different values of contact time between biosorbent and metal ions from aqueous solution. These include the pseudo-first Lagregen model, pseudo-second order

Table 25.10 The mathematical expressions of the pseudo-first order Lagregen and pseudo-second order Ho kinetic models (after [25.16, 18, 112, 145])

Kinetic model	Differential equation	Integral equation	Significance of terms
Pseudo-first order Lagregen model	$\frac{dq_t}{dt} = k_1(q_e - q_t)$	$\ln(q_e - q_t) = \ln q_e - k_1 \cdot t$	q_e, q_t are the amounts of heavy metals retained on weight unit of biosorbent at equilibrium and at time t, (mg/g); k_1 is the rate constant of the pseudo-first order kinetics equation (min^{-1})
Pseudo-second order Ho model	$\frac{dq_t}{dt} = k_2(q_e - q_t)^2$	$\frac{t}{q_t} = \frac{1}{k_2 \cdot q_e^2} + \frac{t}{q_e}$	q_e, q_t are the amounts of heavy metals retained on weight unit of biosorbent at equilibrium and at time t, (mg/g); k_2 is the rate constant of pseudo-second order kinetics equation (g/mg min)

Table 25.11 Kinetic data for the use of some marine algae and marine algae waste biomass for the removal of heavy metal ions

Heavy metal ion	c_0 (mg/l)	Biosorbent	Best kinetic model	Kinetic parameters			Reference
				R^2	q_e (mg/g)	k_2 (g/mg min)	
Cd(II)	75	*Laminaria hyperborea*	Pseudo-second order	0.997	31.3	0.024	[25.53]
	75	*Sargassum muticum*	Pseudo-second order	0.997	38.4	0.004	[25.53]
	225	*Lobophora variegata*	Pseudo-second order	0.995	94.2	0.006	[25.133]
	225	*Cystoseira baccata*	Pseudo-second order	0.999	56.2	0.007	[25.94]
	100	*Fucus vesiculosus*	Pseudo-second order	0.999	52.8	0.051	[25.93]
	50	*Ceramium virgatum*	Pseudo-second order	0.999	116.9	0.003	[25.95]
	67.4	Algae waste biomass[a]	Pseudo-second order	0.999	5.83	0.056	[25.41]
	45.5	Algae waste biomass[b]	Pseudo-second order	0.999	7.60	9.200	[25.96]
Cr(VI)	50	*Ulva lactuca* sp.	Pseudo-second order	0.999	12.9	0.010	[25.61]
	10	*Ulva fasciata*	Pseudo-second order	0.999	9.7	0.013	[25.33]
Cu(II)		*Sargassum* sp.	Pseudo-second order	0.999	9.6	0.010	
	100	*Fucus vesiculosus*	Pseudo-second order	0.999	41.9	0.002	[25.93]
	225.4	*Cladophora fascicularis*	Pseudo-second order	0.999	144.9	0.004	[25.36]
	100	*Laminaria hyperborea*	Pseudo-second order	0.996	50.3	0.020	[25.53]
	100	*Sargassum muticum*	Pseudo-second order	0.997	38.2	0.017	[25.53]
	200	*Spirogyra* sp.	Pseudo-second order	0.998	111.1	0.032	[25.102]
	200	*Oedogonium* sp.	Pseudo-second order	0.996	117.6	0.064	[25.141]
Pb(II)	200	*Nostoc* sp.	Pseudo-second order	0.992	89.3	0.038	[25.141]
	414	*Lobophora variegata*	Pseudo-second order	0.967	229.5	0.010	[25.133]
	414	*Cystoseira baccata*	Pseudo-second order	0.999	142.9	0.001	[25.94]
	100	*Fucus vesiculosus*	Pseudo-second order	0.999	102.2	0.009	[25.93]
	133	Algae waste biomass[a]	Pseudo-second order	0.999	18.1	0.022	[25.41]
	–	Algae waste biomass[b]	Pseudo-second order	0.915	41.4	–	[25.103]
Zn(II)	75	*Laminaria hyperborea*	Pseudo-second order	0.991	19.2	0.075	[25.53]
	75	*Sargassum muticum*	Pseudo-second order	0.995	34.1	0.012	[25.53]
	100	*Ulva fasciata*	Pseudo-second order	0.976	3.2	0.168	[25.21]

c_0 is the initial concentration of heavy metal ions; [a] marine green algae (*Ulva lactuca* sp.) waste biomass obtained after extraction of oils; [b] waste algae biomass obtained from agar extraction industry.

Ho model [25.145], Weber–Morrison kinetic model, Adam–Bohart–Thomas kinetic model [25.148], first-order reversible kinetic model [25.149], external mass transfer model [25.32], etc. The first-order Lagregen model and the pseudo-second order Ho model are the most frequently used kinetic models to study the

biosorption kinetics of heavy metal ions on marine algae biomass, and they are listed in Table 25.10.

A plot of $\ln(q_e - q_t)$ versus t should generate a straight line, according to the pseudo-first order Lagregen model, and the kinetic parameters can be calculated from the slope (k_1) and the intercept ($\ln q_e$). In the case of the pseudo-second order Ho model, the graphical representation of (t/q_t) versus t will give a straight line with the intercept of $1/k_2$, and slope of $1/q_e$. The calculated values of q_e, from both the kinetic models are then compared with that experimental values.

The shape of graphical dependences and comparison of experimental and calculated values of biosorption capacity at equilibrium (q_e) can help deciding which kinetic model is followed by the biosorption process. On the other hand, to obtain the best-fitting kinetic model, the linear regression is used to analyze the experimental data [25.145]. So, the value of the linear regression coefficient (R^2) is another factor that influences such decision. A value of $R^2 > 0.98$ shows that the model is suitable for describing the biosorption kinetics [25.150].

Table 25.11 summarizes the values of kinetic parameters of the pseudo-first and pseudo-second order kinetic models together with the corresponding correlation coefficients (R^2) for biosorption of some heavy metal ions on various types of marine algae and marine algae waste biomass.

It is clear that most of biosorption processes that use marine algae or marine algae waste biomass for the removal of heavy metal ions from aqueous solutions are very well described by the pseudo-second order kinetic model.

The pseudo-second order kinetic model is based on the assumption that the rate-controlling step in the adsorption process, are the chemical interactions between superficial functional groups of biosorbent and heavy metal ions from aqueous solution, and similar behaviors have been reported for various types of adsorbent materials [25.15, 16, 18]. The great advantage of this model is its great accuracy in describing the whole kinetic experimental data. In addition, the high values of rate constants (k_2, g/mg min) obtained in the case of these biosorbents (Table 25.11) indicate that the rate of the biosorption process is limited by the availability of heavy metal ions and functional groups from the biosorbent surface to interact. When the availability of the superficial functional groups is higher, the rate of adsorption process is also higher.

In the biosorption system where the possibility that the intraparticle diffusion to be the rate-controlling step, the intraparticle diffusion approach described by Weber and Morrison is used. The kinetic equation of the intraparticle diffusion model [25.151] is

$$q_t = k_{dif} \cdot t^{1/2} + c \qquad (25.5)$$

where: k_{dif} is the intraparticle diffusion rate constant (mg/g min$^{1/2}$), and c is the concentration of heavy metals from solution at equilibrium (mg/l).

If the intraparticle diffusion process is the rate-controlling step, the amount of heavy metal retained on weight unit of biosorbent (q_t, mg/g) varied with the square root time. The graphical representation of q_t versus $t^{1/2}$ dependences obtained in the case of Pb(II) and Cd(II) biosorption onto *Lobophora variegate* brown algae [25.133] or in the case of Pb(II), Cd(II), and Co(II) biosorption on green algae waste biomass (Fig. 25.11) does not go through origin, and two separated region exists, in all cases.

The deviation of the straight line from the origin indicates that the intraparticle diffusion process is not the only rate-controlling step [25.151, 152], and the boundary diffusion controls biosorption up to a certain degree, in all cases. The first region (Region 1) is attributed to the mass transfer of heavy metals from the bulk solution to biosorbent surface, while the second region (Region 2) indicates the intraparticle diffusion. The significant differences between these two regions is given by their slopes, when the first region has a pronounced slope, the slope of the second region is much lower. This indicates that the binding sites are located on biosorbent surface and are readily accessible for the heavy metal

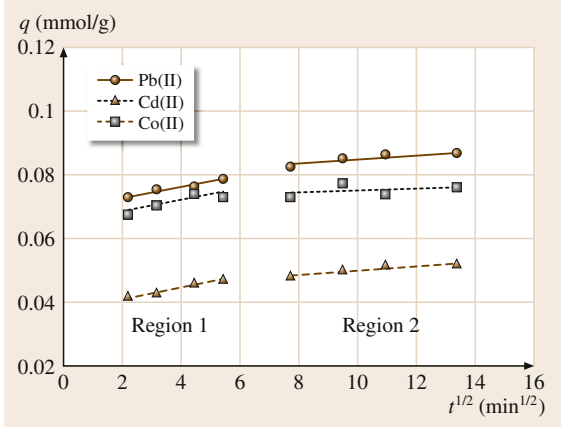

Fig. 25.11 Intraparticle diffusion kinetics for biosorption of Pb(II), Cd(II), and Co(II), respectively, onto green algae waste biomass, at a temperature of 20 °C (after [25.41])

ions, or at external intralayer surface. Hence, the experimental data suggest a limited contribution of mass transfer and boundary layer diffusion in the biosorption process of heavy metal ions, onto marine algae biomass, and that the intraparticle diffusion influenced the biosorption process up to a certain degree.

25.6 Modeling of Biosorption Process in Dynamic Continuous-Flow Conditions

The large-scale utilization of biosorption processes for the removal of heavy metal ions from aqueous environments requires the use of continuous systems, where the biosorbent (marine algae biomass) can be used in multiple biosorption–desorption cycles [25.64]. In addition, the heavy metal ions biosorption is more effective if it is carried out in a dynamic continuous-flow column, and these systems allow the treatment of very large volumes of wastewater and have lower operating cost [25.146]. The recording of the variation of heavy metal ions concentration of the effluent solution, as a function of the operating time of the column, or the volume of treated aqueous solution, gives usually a typical S-shaped breakthrough curve whose shape and slope is a result of the equilibrium biosorption isotherm relationship, the mass transfer to and throughout the biosorbent in the column, and operation macroscopic flow parameters.

Any optimized column system is based on the accurate prediction of the characteristic of the breakthrough curve under given specific operating conditions. When the heavy metal ions concentration in the effluent solution reaches a predefined level, the column operation is terminated. At this point, the regeneration process may begin before activation for the next cycle of operation. Therefore, successful design of a column biosorption process required prediction of breakthrough curves for the effluent, and for this purpose various mathematical models can be used [25.65, 153, 154].

The Bohart–Adams, Thomas, and Yoon–Nelson models have been frequently used for modeling of the metal ions biosorption process on various types of materials in continuous systems. These models are able to describe the breakthrough curves with accuracy in most of the cases, and at the same time, they provide important system parameters that can be used to predict the useful time of the column for the scale-up of the experiments.

The Bohart–Adams model is mainly used when the effluent concentration is lower than $0.35 \cdot c_0$ [25.155], so to describe the initial part of the breakthrough curve. This model assumed that the biosorption rate is proportional to the residual capacity of the solid and the concentration of the sorbent substances [25.156]. The mathematical expression of this model is as follows,

$$\ln \frac{c_t}{c_0} = k_{BA} \cdot c_0 \cdot t - k_{BA} \cdot N_0 \cdot \frac{H}{v} \tag{25.6}$$

where c_0 is the initial heavy metal ions concentration, c_t is the effluent heavy metal ions concentration at time t, k_{BA} is the kinetic constant of the Bohart–Adams model, N_0 is the saturation concentration of column, H is the bed height of column, and v is the linear velocity calculated by dividing the flow rate by the column section area.

The parameters k_{BA} and N_0 of the Bohart–Adams model can be estimated from the slope and intercept of $\ln c_t/c_0$ versus t dependence.

The Thomas model is one of the most general and widely used to describe the biosorption process in a packed-bed column [25.65, 156]. This model assumes that biosorption is not limited by the chemical interactions, but by the mass transfer at the interface [25.66, 69]. The linear expression of the Thomas model is

$$\ln\left(\frac{c_0}{c_t} - 1\right) = \frac{k_T \cdot q_0 \cdot m}{F} - \frac{k_T \cdot c_0}{F} \cdot V_{eff} \tag{25.7}$$

where k_T is the Thomas rate constant, m is the biomass packed in the column, F is the solution flow rate through the column, q_0 is the maximum concentration of solute in the solid phase, and V_{eff} is the effluent volume.

Most commonly, the Thomas model is applied to analyze the obtained data between breakthrough and saturation points of the experimental breakthrough curves, and the characteristic parameters (k_T and q_0) were determined from the slope and intercept of the linear plot $\ln(c_0/c_t - 1)$ versus V_{eff}.

The Yoon–Nelson model is based on the assumption that the rate of decrease in the probability of adsorption for each adsorbate molecule is proportional to the

Table 25.12 Bohart–Adams, Thomas, and Yoon–Nelson model parameters at the removal of lead(II) ions from aqueous solutions by biosorption on various kinds of marine algae biomass

	Experimental conditions		Bohart–Adams model		Thomas model		Yoon–Nelson model		Reference
Marine algae biomass	c_0 (mg/l)	Flow rate (ml/min)	k_{BA} (l/mg min)	N_0 (mg/l)	k_T (l/mg min)	q_0 (mg/g)	k_{YN} (min^{-1})	τ (min)	
Spirogyra neglecta	10	0.6	14.00×10^{-3}	310.60	0.23	29.50	0.14	81.95	[25.65]
	25	0.6	8.86×10^{-3}	411.11	0.22	46.24	0.33	51.37	
	50	0.6	4.00×10^{-3}	495.50	0.09	53.52	0.27	29.74	
	50	3.0	25.60×10^{-3}	303.70	0.47	33.05	1.41	3.67	
	50	5.0	26.10×10^{-3}	387.60	0.50	36.83	1.52	2.45	
Marine algae waste biomass: Purolite A-100 = 1 : 2	70	3.5	4.95×10^{-4}	114.00	0.17	73.95	0.42	183.54	[25.42]
	141	3.5	2.88×10^{-4}	149.95	0.06	110.54	0.42	119.02	
	197	3.5	1.88×10^{-4}	204.94	0.04	157.37	0.44	116.34	
	70	2.0	5.28×10^{-4}	131.34	0.22	71.32	0.31	282.18	
	70	5.0	3.71×10^{-4}	103.85	0.15	78.96	0.62	121.41	

probability sorbate and the probability of sorbate breakthrough on sorbent [25.156]. The linear equation of this model can be written as

$$\ln\left(\frac{c_t}{c_0 - c_t}\right) = k_{YN} \cdot t - \tau \cdot k_{YN} \qquad (25.8)$$

where k_{YN} is the Yoon–Nelson model rate constant and τ is the time required for 50% sorbate breakthrough.

The model parameters (k_{YN} and τ) can also be calculated from the slope and intercept of linear dependence $\ln(c_t/(c_0 - c_t))$ versus t.

Table 25.12 summarizes, for exemplification, the values of Bohart–Adams, Thomas, and Yoon–Nelson models parameters, obtained at the removal of Pb(II) ions by biosorption on various kinds of marine algae biomass.

The values of Bohart–Adams model parameters indicate that the saturation concentration of column (N_0) increases with the increase in initial metal ions concentration and decreases with increase in the solution flow rate. In addition, the kinetic constant (k_{BA}) decreases with increase in initial heavy metal ions concentration, but increases with the increase in the flow rate. Therefore, for better saturation concentration (N_0) and lower kinetic constant, which means minimum resistance of the column, the initial concentration of heavy metal ions should be higher, while the flow rate needs to be lower [25.42, 65].

The values of the Thomas model parameters obtained in the case of Pb(II) ions biosorption on various types of marine algae biomass (Table 25.12) indicate that the equilibrium biosorption capacity (q_0, mg/g) is higher at higher initial lead(II) concentrations and higher flow rates of metal solution through the column. Also, the Thomas kinetic constant (k_T) was generally higher at lower initial metal ions concentrations.

The Yoon–Nelson constant (k_{YN}) usually increases with increase in initial Pb(II) concentration and the solution flow rate. The greatest value of Yoon–Nelson constant when the initial Pb(II) concentration is higher could be related to the increase in the forces that control the mass transfer in the liquid phase [25.65]. The value of τ, which represent the time required for 50% breakthrough, decreases with the increasing in initial Pb(II) concentration and flow rate. It should be noted that in all cases these values are close to the values obtained from experimental breakthrough curves.

However, the most complete column model is the mass transfer model. This model take into account the dominant intraparticle mass transfer and was developed by *Tan* and *Spiner* [25.157] for the ion exchange processes. In principle, the mass transfer model can predict the breakthrough curves for most of chemical species that can be removed by the biosorbents, and even also for the elution curves obtained during the biosorbent regeneration [25.122]. Unfortunately, the mathematical methodology of this model is quite complex, and to solve it, some simplifications are necessary.

The use of different models to analyze the experimental breakthrough curves yields important informa-

tion from the biosorption process feasibility stand-point by providing the time intervals between the breakthroughs of the different marine algae biomass used as biosorbents, and the magnitude of the overshoots that inside the column translate as concentration extremes [25.4].

In the design of the biosorption process for the removal of heavy metal ions, the biosorption/desorption performances of marine algae biomass and some of these characteristic properties (such as mass transfer, chemical stability, etc.) should be well known. While batch-biosorption experiments yield some basic information, the possible applicability of biosorption systems at the industrial level should be tested under dynamic continuous-flow conditions. The performances assessment of biosorption systems and prediction based on both equilibrium as well as dynamic studies eventually leads to sizing of the equipment.

25.7 Mechanism of Biosorption

The influence of any of the experimental parameters on the efficiency of biosorption process can be quantitatively evaluates when the biosorption mechanism is known. The binding of heavy metal ions to the functional groups of marine algae biomass can be done by several types of elementary mechanisms, such as ion exchange, complexation, chelation adsorption, microprecipitation, etc. [25.24]. But, the biosorption processes are quite complex, and the overall metal uptake mechanisms are obtained by combination of different elementary mechanisms that take place at the same time.

As a generalization, the uptake of heavy metal ions from aqueous solution by biosorption on marine algae biomass can be done via two major mechanisms:

a) Ion exchange – when the heavy metal ions are binding by ionisable functional groups (such as carboxyl, hydroxyl, phosphate, sulfate, etc.) from marine algae biomass surface;
b) Complexation – in this case the metal ions from aqueous solution form complexes (even chelates) with functional groups of biosorbents, by electron pair interactions. The most likely electron donor atoms in marine algae biomass are nitrogen atoms, neutral oxygen, or sulfur atoms.

Even if, for the removal of heavy metal ions by biosorption on marine algae biomass, numerous types of marine algae and marine algae waste biomass were used as biosorbent in various experimental conditions; a little attention has been paid to investigation of the biosorption mechanism. The understanding of the metal uptake mechanisms can help to quantify the heavy metal ions – marine algae biomass interactions that are fundamental for the evaluation of potential implementation methodology.

The key factors that controll and characterized the biosorption mechanism are: (i) the type of functional groups present on the surface of biosorbent, (ii) chemical speciation of metal ions present in aqueous solution, and (iii) characteristics of heavy metal ions solution (such as pH, presence of other inert or competing ions, etc.) [25.158].

25.7.1 Ion Exchange

According to *Davis* et al. [25.30], the ion exchange is an important type of interactions which may occur in the biosorption process, and that can explain many of the observations made during the heavy metal ions uptake experiments. It should be noted that the ion exchange does not clearly identify the mechanism of heavy metal ions retention on marine algae biomass. This is because the ion exchange interactions may range from physical (such as electrostatic or van der Waals forces) to chemical (such as ionic or covalent), but they are useful in describing the experimental observations [25.4].

In the most general sense, the ion exchange mechanism assumes that the functional groups from the biosorbent surface can bind the heavy metal ions from aqueous solution, according to the reaction

$$R^- - X^+ + M^+ \leftrightarrows R^- - M^+ + X^+ \qquad (25.9)$$

where R^- is the binding site from the biosorbent surface, X^+ is a mobile cation (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+}, etc.), and M^+ is the heavy metal ions present in aqueous solution.

However, a one-to-one stoichiometry is not complied with as typical two mobile ions are released upon the binding of one divalent heavy metal ion; the ion exchange approach is considered to be the most sim-

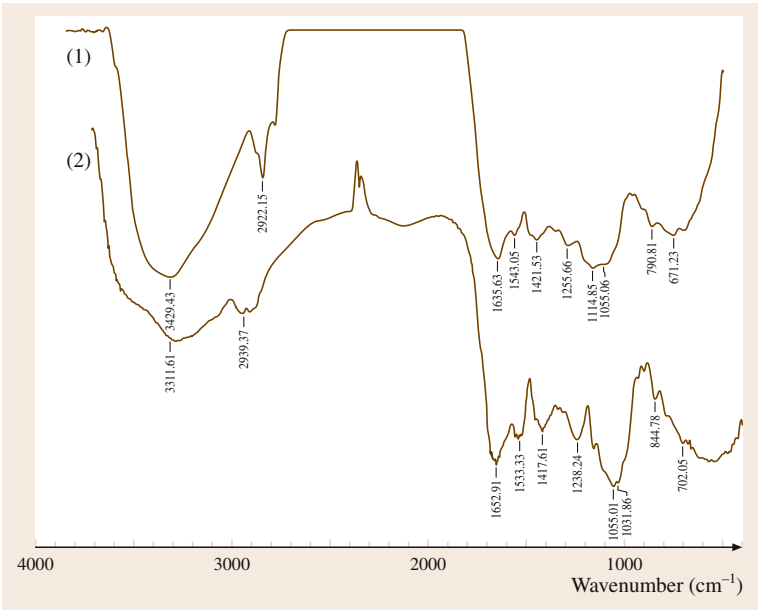

Fig. 25.12 FT-IR spectra dry Ulva lactuca marine algae before (1) and after (2) Cd(II) ions biosorption

ple but not very accurate to describe the biosorption mechanism [25.60].

The marine algae biomass can be considered as a natural complex material that contains various functional groups, with weakly acid and basic character. These functional groups can be ionized in aqueous solution, at a certain value of pH, and can bind the heavy metal ions from aqueous solution, mainly by electrostatic interactions. In this case, it is observed that: (i) the biosorption efficiency is strongly influenced by the solution pH, and (ii) in the FT-IR spectra recorded for the marine algae biomass before and after retention of considered heavy metal ions, no supplementary bands appear. In most of the cases are observed only some shifted of the maximum absorption wave number, corresponding to the functional groups involved in the uptake process.

For exemplification, Fig. 25.12 illustrates the FT-IR spectra of *Ulva lactuca* marine green algae before and after retention of Cd(II) ions from aqueous solution. The change in the wave number of some absorption bands suggests that several functional groups (such as amino, C=O, and C−O) are involved in the biosorption process of Cd(II) ions [25.88].

On the other hand, it is known that marine algae biomass generally contains alkali and alkaline earth metal ions (such as Na^+, K^+, Ca^{2+}, and Mg^{2+}), that are abundant in the seawater. Thus, untreated algae biomass can retain heavy metal ions from aqueous solution, and release these mobile cations. In this case, the solution pH varies very little during the biosorption process, but a significant increase in the concentration of these ions is observed. Thus, *Kuyucak* and *Volesky* [25.159] have reported an enhancement release of Ca^{2+} ions from the *Ascophyllum nodosum* brown algae when is in contact with a cobalt aqueous solution, rather than cobalt-free solution. In addition, when this marine alga was treated with $CaCl_2$ and HCl, a 2 : 3 stoichiometric relationship between Co^{2+} uptake and Ca^{2+} release was observed [25.60]. This means that the alkali and alkaline earth metal ions play a key role in the ion exchange properties of the marine algae biomass, regardless the nature of metal ion from aqueous solution to be retained.

The importance of the ion exchange mechanism in the biosorption process of heavy metal ions on marine algae biomass has been demonstrated by various studies from the literature, performed both in batch- and dynamic continuous-flow conditions [25.4, 17, 26, 28, 30, 35]. However, it has been suggested by many researchers that ion exchange is neither the sole nor the main mechanism for heavy metal ions biosorption on marine algae biomass [25.158]. The ion exchange mechanism is very fast and predominant at low concentration of heavy metal ions in aqueous solution, while at higher concentration the binding of heavy metal ions can occurs and by complexation.

Table 25.13 pH value for the maximum biosorption of some heavy metal ions on brown marine algae biomass (after [25.161–163])

Heavy metal ion	Brown algae biomass	pH	Biosorption capacity (mg/g)
Cd(II)	*Ascophyllum nodosum*	4.9	214.68
	Sargassum natans	3.5	131.51
	Fucus vesiculosus	3.5	73.06
Cu(II)	*Sargassum vulgare*	4.5	59.05
	Sargassum filipendula	4.5	56.52
	Fucus vesiculosus	4.5	74.93
Pb(II)	*Ascophyllum nodosum*	3.5	271.43
	Sargassum natans	3.5	253.15
	Fucus vesiculosus	3.5	229.91
Zn(II)	*Sargassum fluitans*	4.5	77.17
	Fucus vesiculoses	4.5	52.32

25.7.2 Complexation

Complexation is another possible mechanism that plays an important role in the biosorption processes of heavy metal ions, from aqueous environments [25.60]. In general, the complex formation of heavy metal ions with functional groups from marine algae biomass involves the presence of an atom or atoms having lone pair electrons to donate (such as RNH_2). The complexes are formed by electrostatic or covalent interactions, and can be neutral, positively, or negatively charged, and may have different coordination numbers (most common coordination number ares 4 and 6) [25.24]. When one functional group interact with the heavy metal ions through two ore more coordination atoms, the formed complex is called chelate, and its formation process is named chelatization.

To explain the formation of the complexes between heavy metal ions from aqueous solution and functional groups from marine algae biomass surface, the acid–base (Pearson) classification is used. According to this classification, the hard acids (metal ions such as Na^+, K^+, Ca^{2+}, Mg^{2+}, etc.) will bind preferentially to the oxygen containing functional groups, such as HO^-, HPO_4^{2-}, $R\text{-}COO^-$, C=O, etc. On the other hand, the soft acids (most of heavy metal ions) are bonded covalently to the functional groups that contain nitrogen and sulfur donor atoms, named soft bases [25.160].

The marine algae biomass surface can be considered a heterogeneous ligand, which contains various functional groups. The binding of different heavy metal ions on such biosorbent depends on ionic properties, such as electronegativity, ionization, redox potential, and ionic radius of these metals. Thus, the more electronegative heavy metal ion will be, the more strongly is attracted to the surface, and the formed complex is more stable. In this case, it is observed that: (i) the biosorption process occur with maximum efficiency at a given pH value of aqueous solution, where the complex formed between metal ions and functional groups of biomass has higher stability (Table 25.13), and (ii) some changes in the characteristics of biomass surface can appear, visible by the microscopic technique.

The importance of complexation interactions in the biosorption processes of heavy metal ions from aqueous solutions have been evidenced; especially in the case of brown algae biomass. Thus, *Davis* et al. [25.30] review some studies from the literature and concluded that some of heavy metal ions, such as Pb(II), Cu(II), Cd(II), Co(II), Ni(II), Mn(II), etc. are covalent bonded on brown algae biomass, and in these interactions the functional groups from alginic acid are predominant involved. This preferential binding of heavy metal ions to the functional groups of alginic acid is mainly due to the stereochemical effects, since larger ions might better fit a binding site with two distant functional groups. This hypothesis has been experimentally proved by X-ray diffraction and NMR spectroscopic analysis. Also, in the study of *Tsezos* et al. [25.158] it has been shown that the complexation interactions have an important role in biosorption of precious metals on brown algae biomass. The biosorption mechanisms in this case, involve two steps: first the precious metal ions are covalent bonded on functional groups of marine algae biomass, and then the in-situ reduction of these occurs.

Nevertheless, the complexation interactions have been evidenced as elementary interactions in many biosorption processes on various types of marine algae biomass; especially at high initial concentration of heavy metal ions.

25.8 Final Remarks

Biosorption can be successfully used for the removal of heavy metal ions from aqueous environments. The major advantages of this method over the conventional treatment methods include:

- Economic viability
- High efficiency of heavy metal removal from dilute solutions
- Minimization of chemical and/or biological waste
- Easy regeneration of biosorbents
- Possibility of heavy metal ions recovery.

The marine algae are available in large quantities in many regions of the world, being a kind a promising biological resources, and their utilization as biosorbents in biosorption processes are mainly due to: (i) the variety of functional groups from the biosorbent surface, (ii) relatively small and uniform distribution of binding sites on biosorbent surface, and (iii) the preference of alkali and alkali-earth metal ions for the binding sites is less than in the case of ion exchange resins. In addition, the wastes obtained after extraction from marine algae of different compounds, can also be used as biosorbents, because their biosorptive characteristics are not very much changed.

In the design of the biosorption process for the removal of heavy metal ions, the biosorption/desorption performances of marine algae biomass and some of their essential characteristic (such as mass transfer, chemical stability, etc.) should be well known. Thus, the experiments must be started in batch conditions, when some basic information is obtained, and then the possible applicability of biosorption systems at large level should be tested under dynamic continuous-flow conditions. The performances assessment of biosorption systems and prediction based on both equilibrium as well as dynamic studies eventually leads to sizing of the equipment.

In the future development of the biosorption processes for the removal of heavy metal ions from aqueous environments, two trends can be mentioned, according to the studies from the literature. The first one concern to the use of hybrid technologies, where the biosorption processes will be a step in the overall removal process of heavy metal ions from aqueous environment. This trend is mainly determined by the difficulties existing in the utilization of biosorption processes, that made to be considered the applying the hybrid technologies to treat industrial wastewater. Unfortunately, until now no one variant was found to be economical viable. The second trend is to develop good commercial biosorbents derived from marine algae biomass, just like a kind of ion exchange resins that can be easily sale in the market. The failure of the marine algae biomass commercialization as biosorbents is mainly due to nontechnical barriers involved in commercialization of technological innovations. For implementation of innovative processes, a solid capitalization is always required, and this makes that choice of partners to be critical.

However, the utilization of biosorption processes along with other conventional methods (including chemical precipitation, flotation, membrane-related processes, electrochemical techniques, etc.) may also be helpful in the treating of industrial effluents at large scale, even for the simultaneous removal of organic substances and heavy metal ions from aqueous environments.

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Marine Part E M

Part E Marine Microbiology and Biotechnology

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26. Biotechnological Potential of Marine Microbes

Gian M. Luna

The world's oceans, from the coasts to the abysses, harbor an incredible level of microbial diversity. This marine microbial biosphere is an enormous, untapped resource of biotechnological interest. This chapter reviews the potential of marine microbes in biotechnology. The biotechnological potential is considerable, ranging from the synthesis of bioactive molecules to the production of biofuels, cosmeceuticals, nutraceuticals, and biopolymers; from the engineering of marine microbes for biomedical purposes to the degradation of pollutants, and the use of microbial biosensors as sentinels for environmental quality. Marine viruses have great biotechnological potential, yet the exploration of the marine virome, and the associated gene and protein pool, is only beginning. Marine archaea have so far been exploited for the isolation of enzymes, yet many biotechnological exploitations can be foreseen. Bacteria and microbial eukaryotes, especially fungi and photosynthetic protists, provide an important contribution to biotechnology; the combination of *omics-driven* technologies and improved cultivation techniques is widening the knowledge on their biological diversity, paving the way for new biotechnological exploitations. The exploration of the marine microbial biosphere, and its extraordinary genetic and physiological diversity, will undoubtedly continue to offer chances for the development of new and sustainable blue biotechnologies, helping to solve important societal challenges of the twenty-first century.

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26.1 Microbial Diversity in the World's Oceans and Biotechnological Applications of Marine Microbes

The world's oceans are the largest ecosystem on Earth. Life in global oceans is dominated by unicellular microbes, accounting for the largest fraction of biomass and biodiversity [26.1]. Although microbes play a fundamental role in the productivity, ecosystem functioning, and global biogeochemical cycles of the major elements [26.2], we still know little about marine microbial diversity, the patterns and driving forces, the genetic and metabolic repertoire, and the ecological role each microbial species plays in the ecosystem [26.3]. Little is known about the functions of marine microbes in the ecosystem and their metabolic capabilities because of the current inability to assign any function to a large proportion of their genes [26.4]. The vast majority of marine microorganisms cannot be cultured in the laboratory under standard laboratory conditions [26.5, 6]. It was only the development of molecular methods that made it possible to identify marine microbes [26.7–9], but scientists are merely beginning to gain a comprehensive understanding of the full extent of microbial biodiversity and functions in the world's oceans [26.10].

In the United Nations Convention on Biological Diversity, biotechnology was defined as [26.11]:

> *any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use.*

There is, however, no agreed upon definition of biotechnology, and definitions may range from *the uses of biology for the benefit of man* to *the use of biology to make money* [26.12]. Biotechnology is nowadays experiencing an increasing importance at the planetary scale, and is expected to increasingly contribute in addressing important socio-economic issues of our societies. The term *blue biotechnology* is typically utilized to describe the aquatic, either marine or freshwater, applications of biotechnology [26.4].

Marine organisms can be utilized as biotechnological agents to provide products and services that have useful applications for humans and their well-being. Among these, there are new medical technologies, food and feed ingredients (e.g., nutraceuticals), molecules useful in many industrial sectors, and biofuels [26.13]. Marine biotechnology has the potential to contribute significantly to key societal challenges, such as the sustainable supply of healthy and high-quality food, the production of bioenergy (such as biofuel production from many microalgae), the discovery and exploitation of pharmaceuticals or diagnostic tools for improving human health, the isolation of biopolymers useful in the cosmetic, food and health industries, and the synthesis of enzymes, proteins, or biomaterials of interest in several industrial processes. Marine bioresources (organisms and molecules) can be helpful in increasing stock production in aquaculture processes, in controlling infectious diseases of farmed organisms, as well as in addressing key environmental issues such as marine pollution (e.g., by the development of biosensing technologies for environmental monitoring, the use of microbes for degradation of pollutants, or the identification of natural antifouling technologies).

This chapter reviews the potential of marine microbes in biotechnology. Marine microbes include representatives from three domains of life: archaea, bacteria and eukarya [26.14]. Bacteria and archaea are the most abundant cells in all known marine ecosystems. Microbial eukaryotes, among which unicellular microbes (protists) and fungi (including either unicellular or multicellular forms), are ecologically important members of marine microbiota, and many studies are documenting their once-unimaginable diversity [26.15, 16]. This review also covers the biotechnological potential of marine viruses. Viruses do not have an organized cell structure, but represent the most abundant biological entities in the oceans. Phages are believed to be the most diverse biotic component in marine systems, and they are potentially interesting resources for biotechnology.

26.2 Why Do Marine Microbes Matter in Biotechnology?

In the twentieth century, research on marine natural products mainly involved the collection of macroorganisms from the sea, their extraction, and the analysis of the extracts [26.17]. Most frequently, algae and marine invertebrates were investigated. Several molecules with biological activity were isolated, and most of them were typically obtained from corals, sponges, or other invertebrates. The first marine bioactive compounds, spongouridine and spongothymidine, were isolated from the sponge *Cryptotheca crypta* in the Caribbean [26.17]. In the following decades scientists proved that these compounds had anticancer and antiviral activities ([26.18] and references therein). These nucleosides were the basis for the synthesis of Ara-C, the first anticancer agent derived from a marine organism, which is currently used in the treatment of patients affected by leukemia and lymphoma, and the antiviral drug Ara-A [26.19]. An overwhelming number of bioactive compounds of marine origin have subsequently been isolated and described, and more than 15 000 marine products have been described so far [26.20]. Recent estimates report some 20 000 marine high-value-added compounds, including a range of proteins, carbohydrates, and lipids [26.4, 21]. There are many examples in the marine pharmacology literature of antitumor and cytotoxic compounds isolated by marine animals (tunicates, nudibranchs, sponges, octocorals, bryozoans), algae, fungi, and bacteria [26.22]. Some of these substances have created new and exciting means for disrupting tumor specific cell signaling, cell division, energy metabolism, or gene expression, and have the potential to revolutionize cancer treat-

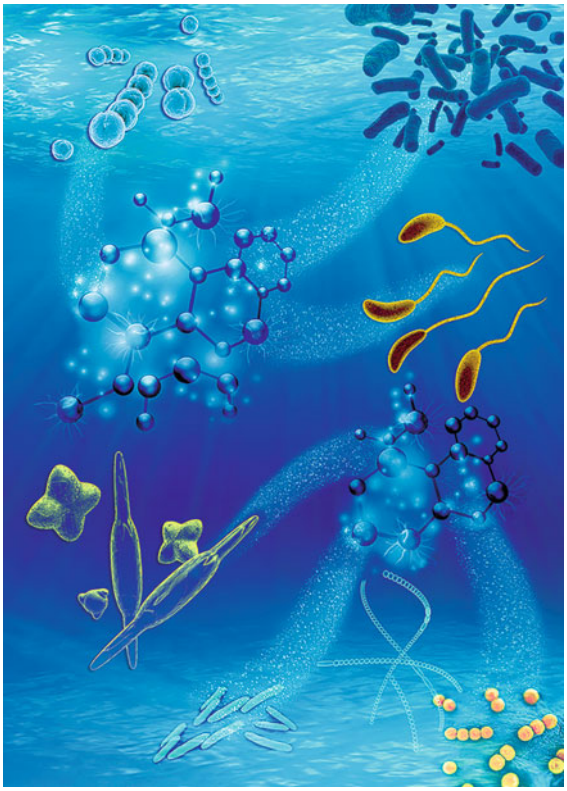

Fig. 26.1 Marine microbes are a source of bioactive molecules and secondary metabolites, many of which offer potential biotechnological opportunities. Photo courtesy of Marta Scandali (Ancona, Italy)

ment [26.22, 23]. Marine organisms can be important sources of useful molecules for treating infectious diseases, such as HIV-AIDS (HIV: human immunodeficiency virus; AIDS: Aquired Immuno Deficiency Snydrom; [26.22] and references therein). However, the application of many substances from marine macroorganisms has been hampered by difficulties regarding their reproduction and scaling up, or problems of supplying sufficient amounts of the pure substance [26.17]. Efforts in trying to cultivate marine macroorganisms, such as in the case of sponges, have been difficult [26.24], despite recent advances in the ability to cultivate organisms, cells, or their microbial symbionts [26.25]. Therefore, although a large number of marine natural products isolated from macroorganisms has been described from marine biota, only a few of them have so far entered preclinical or clinical trials [26.17].

The marine environment is incredibly heterogeneous, being characterized by a wide variability in the main physical and chemical variables. Temperature can range from values close to (or below) 0 °C to up to 370 °C in hydrothermal vents [26.26]. Salinity is typically more uniform across the ocean, but can vary significantly in specific areas, such as coastal transitional environments and estuaries, or reach nearly saturating concentrations in hypersaline environments, such as in the deep-hypersaline anoxic basins in the Mediterranean Sea [26.27]. The seabed, historically considered to be a flat, uniform, and biologically inert territory, where hydrostatic pressure can reach values up to 1100 bar, and light is typically absent, is now known to include a wide variety of habitats [26.28]. Despite these apparently inhospitable conditions for supporting life, the seafloor has been recognized as the largest biome on Earth, and hosts a significant proportion of global biomass and biodiversity.

Marine organisms, including microbes, have evolved to occupy a broad range of ecological niches, and their adaptation across the evolutionary time scale has originated an enormous array of physiological, genetic, and metabolic diversity. The marine biosphere, and particularly its microbial component, is an immense source of biodiversity, of bioactive compounds and secondary metabolites with potential applications in biotechnology (Fig. 26.1). Marine microbes have evolved specific adaptations to survive under different environmental challenges, resulting in different survival mechanisms, growth strategies, and genetic adaptations [26.29]. Some examples have been described recently. The photosynthetic pelagic bacterium *Prochlorococcus marinus* has adopted a minimalist approach and has reduced the size of its genome size, to gain a competitive advantage, given that conditions within its environmental niche vary only a little [26.29, 30]. *Pelagibacter ubique*, a marine α-proteobacterium, has no transposons, extrachromosomal elements, pseudogenes, or introns, and its genome, consisting of less than 1400 genes, is the smallest described for a free-living microbe [26.31].

The ability of marine microbes to adapt to changes in environmental conditions and to thrive under extremes conditions, coupled with their high genetic plasticity, have influenced their prowess to produce compounds and secondary metabolites. The marine microbial biosphere represents an important opportunity for bioprospecting, but the exploration of the potential of marine microbes in terms of the exploitation of molecules is still at an early stage [26.4]. As opposed to macroorganisms, which need to be collected from the sea for sufficient amounts of the searched metabolite to be extracted, marine microbes can be cultivated in the

laboratory in large quantities [26.17, 32]. Therefore, microbial blue biotechnology has the advantage of being a more sustainable activity, by avoiding the collection of marine organisms from their habitat.

26.3 Biotechnology of Marine Microbes, from Viruses to Microbial Eukaryotes

26.3.1 Marine Viruses

Viruses are ubiquitous biotic components of pelagic and benthic ecosystems. They influence lateral gene transfer, genetic diversity, and bacterial mortality [26.33, 34]. Marine viruses were not studied until 1989, but are now recognized as the most abundant biological entities in the sea (typically 10^7 viruses ml^{-1}; [26.35]. Phage diversity studies were initially restricted by the requirement for cultivated hosts, but recent applications of culture-independent techniques have revealed an enormous, and unexpected, viral diversity in marine ecosystems [26.35, 36]. Archaeal, bacterial, and eukaryotic viruses are cosmopolitan and abundant throughout the world's oceans [26.37]. Their host range covers all known marine organisms, from prokaryotes to fishes and mammals. Phages are particularly abundant in marine sediments, and viral infection represents a substantial source of mortality in deep-sea benthic ecosystems [26.38]. Many lines of evidence have suggested that phage diversity is virtually immense, but studies describing marine phage diversity are nowadays limited [26.39].

The genetic pool of marine viruses has enormous potential for biotechnology, but the use of marine viruses as biotechnological agents is still at an early stage. One potential application can be in medicine, to provide new solutions to sanitary problems for humans or other organisms. Potential applications of viruses in biomedicine, some of them already explored, include the use of viral vectors (gene transfer, gene therapy and vaccine development) and the use of viruses for protein expression and oncolytic viruses (e.g., virotherapy for treating tumors). There are currently no studies involving marine viruses but, given the magnitude of the diversity of marine phages, potential biotechnological exploitations can be expected.

Phages were used in early forms of biotechnology to control bacterial infections [26.33, 40]. Phage therapy was abandoned due to contrasting results and the discovery of antibiotics. It is, however, currently the subject of revived interest because of the emergence of antibiotic-resistance as either a biomedical and environmental problem. Some authors have explored the biotechnological usefulness of marine viruses as agents for curing coral diseases. The rapid emergence of infectious diseases in tropical stony corals is causing serious damage to coral reef ecosystems [26.41]. *Efrony* et al. [26.42] recently isolated two phages of known coral bacterial pathogens (*Vibrio coralliilyticus* and *Thalassomonas loyana*) and used them in controlled aquarium experiments to explore their utility in coral *phage therapy*. The results showed that diseases could be successfully controlled by the use of pathogen-specific phages, suggesting the possible usefulness of phage therapy. *Cohen* et al. [26.43] and other authors recently confirmed the potential application of marine viruses in the treatment of coral diseases. Other potential biotechnological applications of marine viruses are in the control of harmful algal blooms. *Onji* et al. [26.44] reported on the isolation of two virus-like agents that are able to suppress the growth of *Gymnodinium mikimoto*, a red-tide-forming marine dinoflagellate.

The exploration of the diversity of the marine virome has just begun. The discovery of novel genes and proteins with unknown functions, with potential biotechnological applications, can be expected, and deserves further in-depth investigations.

26.3.2 Marine Archaea

Archaea are evolutionarily unique prokaryotes, as genetically distant from bacteria as they are from eucarya [26.14]. They were historically assumed to live only in *extremophilic* habitats, but two decades of studies have revealed that they are abundant, widespread, and ecologically important members of the marine biota [26.45, 46]. The discovery of archaea was a significant breakthrough in the history of biology, leading to the replacement of the prokaryote/eukaryote dichotomy by a trinity of domains, the archaea, bacteria and eukarya [26.47].

Marine archaea are an interesting source for biotechnology, however only few fields of biotechnological exploitations have so far been successfully

investigated. One was the isolation of enzymes from some hyperthermophilic archaea. The vent polymerase was isolated from the marine archaeon *Thermococcus litoralis*, and is currently marketed as a useful alternative to the widely used *Taq* deoxyribonucleic acid (Taq DNA: a thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus*; Pfu DNA: a thermostable DNA polymerase named after the thermophilic bacterium *Pyrococcus furiosus*) polymerase. The *Pfu* DNA polymerase, isolated by the hyperthermophilic archaeon *Pyrococcus furiosus*, is commercially available and possesses a higher fidelity compared with other conventional Taq DNA polymerases [26.48]. Research into high-fidelity enzymes from marine archaea is still ongoing and is continuously yielding new findings [26.49].

Marine archaea have been also exploited in several industrial sectors. A heat and acid stable α-amylase, Valley Ultra-Thin, discovered from a deep-sea hydrothermal vent archaeon, has been developed to facilitate the processing of corn into ethanol [26.17]. Marine archaea can be important producers of biopolymers and bioplastics, such as PHA (polyhydroxyalkanoate), and a source of new secondary metabolites [26.17]. Recent studies have pointed out the importance of aquatic archaea as useful agents for hydrocarbon biodegradation and decontamination of polluted areas [26.50], indicating a potential, underexploited opportunity for biotechnological investigations.

26.3.3 Marine Bacteria

Bacteria are typically the most abundant and diverse members of the microbial biota in pelagic and benthic ecosystems. They typically outnumber archaea, despite exceptions in certain habitats [26.51, 52] and are the key players in biogeochemical processes and the fluxes of energy and matter in the ocean. Many bacterial species are distributed across marine ecosystems worldwide. *Candidatus Pelagibacter ubique*, a marine bacterium, is recognized as being one of the most abundant organisms on Earth [26.31].

Marine bacteria have been largely exploited for several biotechnological applications. Most of the marine bioactive compounds that have been successfully screened originate from bacteria [26.4]. Marine bacteria, especially actinomycetes, are important producers of antimicrobial secondary metabolites and antibiotics (see [26.32] for a review). Marine actinomycetes produce different types of secondary metabolites, a large fraction of which possess biological activities and have the potential to be developed as therapeutic agents [26.53]. Many other marine bacteria display significant antibacterial activity [26.54], and they are believed as an exciting resource for discovering new classes of therapeutics within the areas of oncology and infectious diseases [26.55]. Marine cyanobacteria also produce bioactive and cytotoxic molecules that are useful as drugs [26.56, 57].

Unique biosynthetic enzymes from marine bacteria have begun to emerge as powerful biocatalysts in medicinal chemistry and total synthesis [26.58]. Marine bacteria are important producers of biopolymers and biodegradable plastics [26.59], pigments (including melanin, which is potentially exploitable for sunscreens, dyes, and coloring), cohesive molecules that are useful as marine cements, and extracellular substances that are useful as surfactants ([26.22] and references therein).

The surface of many marine macroorganisms such as, to mention only a few, sponges [26.60], algae [26.61], tropical stony corals [26.62, 63], cold-water corals [26.64], hydroids [26.65], crabs [26.66, 67], and fishes, is a particularly interesting niche to study marine bacteria and other microorganisms with respect to biotechnology. The surface of virtually all marine macroorganisms hosts abundant and diverse communities of microbes [26.68]. For instance, the tissue of stony corals typically harbors an associated and diversified microbial community (Fig. 26.2), consisting of archaea, bacteria, and eukaryotes [26.69, 70]. Similarly, marine sponges typically contain diverse and abundant microbial communities, made up of bacteria, archaea, microalgae, and fungi, which comprise up to 40% of the sponge volume and contribute significantly to the host metabolism [26.60]. Microbial associates are believed to have several functions for the sponge, such as stabilization of the sponge skeleton, nutrient uptake, processing of metabolic waste, and secondary metabolite production. An increasing number of studies is documenting the existence of close associations between prokaryotes and higher eukaryotic organisms, typically forming mutualistic or symbiotic relationships [26.17]. Chemically-driven interactions are thought to be important in the establishment of relationships between epibiotic microorganisms and their eukaryotic hosts. For instance, soft-bodied marine organisms lack obvious structural defense mechanisms and rely on chemical defense, by production of bioactive compounds (either by themselves or the associated microflora), to survive [26.71]. Marine invertebrates are a rich source of bioactive metabolites, yet recent stud-

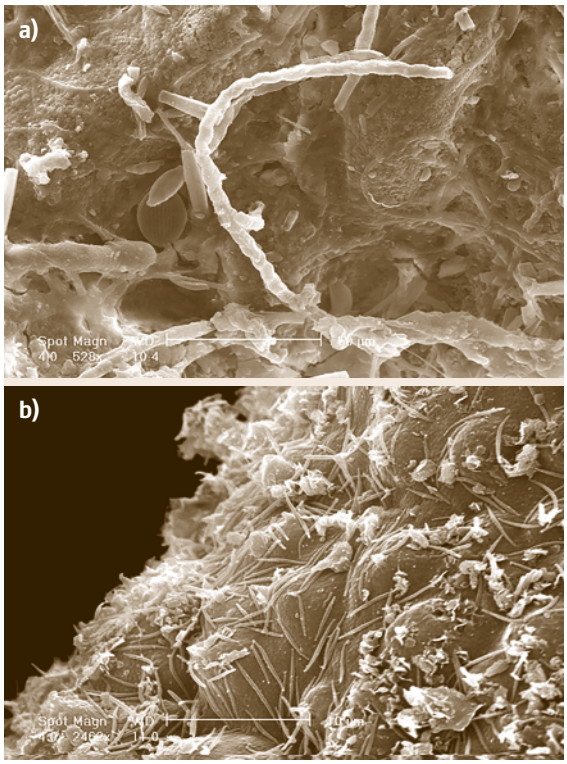

Fig. 26.2a,b Scanning electron microscopy (SEM) images of **(a)** the surface of the stony coral *Merulina ampliata* and **(b)** the colonial hydroid *Ectopleura crocea*. The surfaces of marine macroorganisms are typically hotspots of microbial diversity, and host a myriad of prokaryotic and eukaryotic microbes with interesting, underexplored biotechnological potentialities. Photo in b) courtesy of Dr. Cristina Gioia Di Camillo (Polytechnic University of Marche, Italy)

ies have shown that some bioactive compounds that were previously ascribed to the host are produced by the microbial symbionts [26.72]. Epibiotic microbes can produce a plethora of bioactive compounds, including antibiotics, antiviral, antitumor, and biopolymers. The investigation of the epibiotic microbial communities clearly deserves further study. Similarly, the study of other underexplored niches, such as the intestines of marine organisms [26.73, 74], is expected to provide important contributions to biotechnological developments.

Marine bacteria have enormous potential applications as biotechnological agents to remediate pollution in marine systems. These applications can range from the use of hydrocarbon-degrading bacteria for oil degradation [26.75–77] to the biosynthesis of surfactant and emulsifier molecules to be used in industrial processes, for environmental remediation or as drugs [26.78]. Hydrocarbon contamination is an important environmental issue, especially in coastal areas subjected to high anthropogenic input. Hydrocarbons, and similarly other pollutants such as pesticides, toxic metals, or herbicides, reach the marine environment from a wide variety of anthropogenic sources, including oil spills, urban runoff, shipping, and industrial activities. Hydrocarbons typically accumulate in the sediments, posing serious concerns for both the environment and human health. The success of petroleum bioremediation strategies relies on the ability to provide the optimal conditions to stimulate metabolism of those bacteria that are able to degrade hydrocarbons. Autochthonous bacterial communities can be used in the bioremediation of contaminated sediments, by stimulating their degradation processes with the addition of adequate substrates [26.77].

The aquaculture industry can also benefit from several biotechnological applications deriving from marine bacteria. They can contribute as probiotics, producers of functional foods and additives to increase biomass yields, to fight potentially pathogenic bacteria and to manage infectious diseases, or may serve as biodegraders of aquaculture wastes and organic pollutants [26.79]. Exploring the potential of marine bacteria and their metabolites will help in transforming aquaculture into a more sustainable and efficient industry.

26.3.4 Marine Microbial Eukaryotes

Protists (including photosynthetic protists or microalgae) and fungi are abundant and ecologically relevant members of marine microbial biota. A plethora of studies have documented their functional role, and recent studies are documenting their under-recognized genetic diversity and the spatial distribution across the ocean [26.80, 81].

Marine fungi form a taxonomically heterogeneous group, including *obligate* marine fungi (those able to grow and sporulate exclusively in seawater) and *facultative* marine fungi, which have a freshwater or terrestrial origin but can grow and sporulate in marine ecosystems [26.82]. There are currently about 800 described species of obligate marine fungi, mostly belonging to ascomycetes, anamorphs, and a few basidiomycetes [26.82]. Many fungi, such as thraustochytrids, play an important ecological and biogeochemical role in marine ecosystems, and mediate the

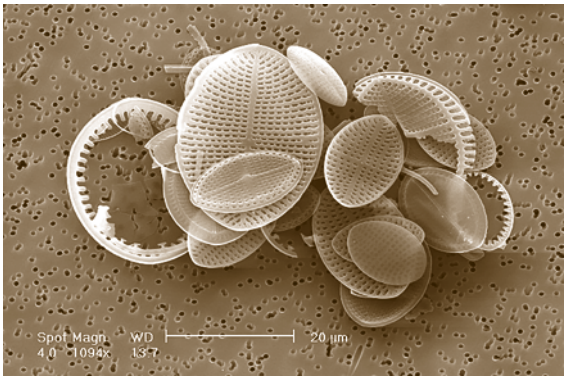

Fig. 26.3 SEM image of benthic diatoms (*Cocconeis* spp.). Marine microalgae can be important targets for biotechnology, especially for biofuel production. Photo courtesy of Dr. Chiara Pennesi (Polytechnic University of Marche, Italy)

degradation of organic matter by the production of extracellular degradative enzymes [26.83]. Historically, fungi have been believed to be rare in marine environments, but recent studies, based on molecular and metagenomics approaches, are revealing an unexpected diversity of fungal communities in coastal and deep-sea ecosystems [26.84, 85]. Recent authors reported on the discovery of 36 novel marine lineages of marine fungi [26.85]. Marine fungal biotechnology, or *blue mycotechnology*, is consequently an exciting and promising area of investigation [26.86]. The number of bioactive compounds isolated from marine fungi is increasing [26.32]. Marine fungi produce a plethora of compounds, among which are anticancer, antibiotic, antiangiogenesis, and antiviral compounds, and molecules having antiproliferative activity [26.87]. Deep-sea fungi have been less described in terms of their abundance, diversity, and ecological role, but are potentially important and productive sources of bioactive products. *Li* et al. [26.88] reported about the isolation of *Phialocephala* sp. in deep-sea sediments, which synthesizes new sorbicillin trimers with cytotoxic properties. *Damare* et al. [26.89] isolated barotolerant fungi in deep-sea sediments, and reported that some of them produce proteases active at low temperatures, with biotechnological potential for waste digestion, food processing, detergents for washing at cold temperatures, and preservation. Marine fungi may also serve as useful pollutant degraders [26.90].

Microalgae have an important biotechnological potential. Microalgal biotechnology is currently based on the production and synthesis of food and feed, additives, cosmetics, pigments (carotenoids), and biofuel [26.91]. They are used as additives in products for human consumption (to enrich the protein content or the nutritional value in food) or as live feed in the aquaculture industry for a variety of farmed organisms. Marine microalgae are important producers of bioactive compounds [26.92], which are useful as pharmaceuticals and cosmetics. An noteworthy line of biotechnological exploitation of microalgae is their use for the production of biofuels [26.93]. Combined with their fast growth rate, microalgae are considered one of the few realistic sources for the production of biofuels and as being superior to agricultural crop-derived bioethanol [26.94]. Many microalgae, such as diatoms and dinoflagellates [26.95] naturally accumulate large amounts of hydrophobic compounds, which can exceed 80% of the algal dry weight. Diatoms are among the most productive and environmentally flexible eukaryotic microalgae on the planet (Fig. 26.3). They are responsible for 20% of global carbon fixation by primary production [26.96, 97] and have several characteristics that make them useful for large-scale biofuel cultivation [26.98]. There has been substantial progress in the development of algal biofuels in the last decade, but more fundamental research is needed to better understand their physiology and metabolism and to develop efficient large-scale culture systems to grow algae and produce biofuel [26.99]. The biotechnological potential of diatoms and other marine microalgae also cover the production of pharmaceuticals, health foods, biomolecules, materials relevant to nanotechnology, and their use as bioremediators of contaminated waters [26.100]. The dinoflagellate *Karlodinium veneficum* synthesizes karlotoxins, a group of potent toxins that cause membrane permeabilization, having possible opportunities to construct new molecules to fight tumors and other human pathologies [26.101, 102].

26.4 Conclusions and Future Perspectives

Oceans are an enormous, untapped and sustainable source of biotechnological opportunities, yet are unexplored for the most part. Marine microbial diversity is almost unlimited, and offers a huge potential for biotechnological exploitations. Marine microbes may pose solutions to a variety of issues relevant for humans,

by favoring the discovery of more efficacious drugs, the production of biofuel from sustainable sources, the isolation of enzymes that are useful for the industry, the synthesis of biopolymers and biodegradable plastics, the remediation of environmental pollution, and the development of a sustainable aquaculture. The combination of improved cultivation techniques with new culture-independent, *omics-driven* approaches (such as metagenomics, metatranscriptomics, metaproteomics, and metabolomics) will multiply the possibilities of successfully exploring the biotechnological potential of marine microbes, with the hope of solving important societal challenges of the twenty-first century.

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27. Marine Actinomycetes in Biodiscovery

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Marine natural products will be the driving force for interdisciplinary advancements bridging medicine, chemistry, and biology for sustainable future biotechnological advancements. Recent advances in sequencing technology and bioinformatics have revealed that actinomycete genomes encode far more secondary metabolite gene clusters than originally thought. The development of new culturing techniques from marine environments has enabled the isolation of previously uncultured actinomycetes resulting in the identification of new bioactive compounds deriving from these bacteria, including species-specific bioactive compounds. Advances in genomic technologies have renewed interest in marine actinomycetes as an attractive source of new therapeutic agents. Genome sequences of bioactive marine actinomycete taxa will continue to provide information on the bioactive potential of the genus/family members, and selective isolation protocols will be designed based on the taxon-specific information generated. This chapter provides an overview on the above highlighted advances from an eco-taxonomical perspective reflecting on the diversified natural product chemistry provided by marine actinomycetes.

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27.1 Overview

27.1.1 Marine Actinomycetes in Biodiscovery

Advances in molecular ecology clearly indicate that novel bacterial taxa are present in marine environments including surface seawater and tidal mud flats [27.1–3]. These novel taxa can also be a source for novel chemistry leading to the discovery of, for example, new aminofuran antibiotics from *Verrucosispora maris* [27.4].

The impact of marine microorganisms can be seen from the annual reviews of marine natural products [27.5]. The review covering the 2005 literature showed that 45% of the accumulated literature on marine microorganisms had been published in the period 2001–2005 (as compared to 1965–2005). While analysis of the distribution of articles, compounds and species to the end of 2006 showed that 5% of the marine species examined originate from bacteria and > 95% are from marine Eukaryota [27.6] comparison of 2007 against the historic average for the period 1965–2005 showed a rise of 600% in the microorganism category [27.7].

The journey to establish that marine actinomycetes are unique and a source of diverse secondary metabolites has been long but it serves to illustrate that unique environments in particular the marine ones produce unique species and confirms that such natural product sources of new biologically active compounds will continue to be productive [27.1, 3, 8, 9]. Unique biosynthetic enzymes discovered from marine-derived bacteria also emerge as powerful biocatalysts in medicinal chemistry and total synthesis. The increasingly interdisciplinary field of marine natural product chemistry provides impetus for future developments in medicine, chemistry, and biology [27.10].

Pioneering work dates back to 1970s when *Okami* and co-workers at the Institute of Microbial Chemistry in Tokyo detected bioactive actinomycetes from shallow- and deep-water sediments from the coasts of Japan [27.11]. One of these bioactive actinomycetes was *Chainia purpurogena* SS-228, isolated from mud samples obtained from Sagami Bay, producer of a benzanthraquinone antibiotic [27.8, 12]. The search for marine bioactive actinomycetes continued and from the mid-1980s in Fenical's laboratory resulted in the isolation of the first true marine obligate bacteria in 1991 [27.8, 13]. A new marine actinomycete cluster was reported by *Mincer* et al. [27.14] and the genus *Salinispora* was established as seawater obligate marine actinomycete in 2005 [27.15]. In subsequent studies over 1000 *Salinispora* strains have been isolated from sediments collected from subtropical Atlantic, the Red Sea and the Sea of Cortez all requiring seawater and sodium for growth. In a study of a collection of 288 samples from around the island of Guam, actinomycete growth was obtained with 223 (77%) of samples. The most abundant actinomycetes recovered belonged to the genus *Salinispora* with 58% of the 984 strains obtained in pure culture assigned to this genus [27.16].

The species of *Salinispora* have been shown to be producers of potent compounds [27.17–21] and the genus has been the forerunner of the identification of other unique marine genera. These efforts resulted in the development of new approaches to sample the deep oceans. An autonomous bounce corer was developed that allowed replicate sampling from sediment cores from surface vessels [27.20].

The genus *Salinispora* has become a model concept to address species and species-specific metabolite production [27.21–23]. *Salinispora tropica* and *Salinispora arenicola* have been described as new species and the description of another new species *Salinispora pacifica* is underway [27.23, 24]. The close phylogenetic relationships among the three species is shown by the genus as a whole having 99% 16S rRNA (ribosomal ribonucleic acid) sequence identity and the pair-wise comparison differ by as few as five nucleotides [27.23]. The three currently known species of *Salinispora* co-occur at six widely separated and distinct locations and the strains produce different compounds [27.25].

Bioactive compounds reported from this genus include: arenimycin, an antibiotic effective against rifampin- and methicillin-resistant *Staphylococcus aureus* from *S. arenicola* [27.26] as well as cyanosporasides A and B, chloro- and cyano-cyclopenta[α]indene glycosides from *S. pacifica* [27.27].

Besides *Salinispora* there are other new bioactive marine genera described to date, examples include *Salinibacterium* [27.28], *Sciscionella* [27.29], *Serinicoccus* [27.30], and *Demequina* [27.31].

Marine-sourced actinomycetes have been shown to be a remarkable resource for the discovery of natural products showing potent biological activities. New antibiotics have been reported from marine actinomycetes such as the streptogramin antibiotic etamycin derived from a marine coastal-line-associated actinomycete from Fiji produced activity against methicillin resistant *S. aureus* [27.32]. Caboxamycin, a new antibiotic of the benzoxazole family has been isolated from the deep-sea *Streptomyces* species [27.33]. Indole alkaloid marine natural products have also been shown to contain anticancer activity [27.34] and three new chlorinated dihydroquinones were reported from a marine-derived streptomycete species by *Soria-Mercado* et al. [27.35].

Other examples include: thiocoraline, a new anticancer drug produced by a marine *Micromonospora* strain [27.4, 36], which is under preclinical assessment and the antiviral drug cyclomarin A produced by a marine *Streptomyces* strain [27.4, 37]. Chandrananimycins A, B, and C with anticancer activity were isolated from a marine-derived *Actinomadura* species [27.38]. Abyssomicin A–C polycyclic antibiotics from a marine *Verrucosispora* strain inhibit *p*-aminobenzoic acid/tetrahydrofolate biosynthesis pathway by blocking the *p*-aminobenzoate formation in the bacterial folate biosynthetic pathway [27.39]. The antimalarial potential of marine actinomycete-derived compounds has also recently been reported [27.40].

27.1.2 Novel Chemistry from Marine Actinomycetes

Marine-derived actinomycetes have been a prolific source of natural products containing diverse chemi-

cal structures and unique biological activities [27.9]. Listed below are some recent examples of bioactive natural products isolated from marine-sourced actinomycetes exemplifying the diversity of novel structures and molecular scaffolds.

A new marine actinomycetes genus, *Marinospora* (strain CNQ-140) was isolated from a sediment sample at 56 m offshore of La Jolla, California and shown to contain marinomycin A–D [27.41]. Marinomycin A was the most potent antibacterial agent showing in vitro minimum inhibitory concentrations (MIC_{90}) of 0.13 mM against both methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF).

The marine actinomycetes *V. maris*, isolated from sediment collected in the Raune Fjord (Norway) produced the nonribosomal peptide synthetase (NRPS)-derived polypeptides proximicin A, B, and C [27.42]. Collection was achieved by box grab at 250 m. 16S rRNA gene sequencing showed similarity of 99.6% to the type strain *Verrucosispora gifhornensis* [27.43]. The proximicins contain a previously unknown γ-amino acid (4-aminofuran-2-carboxylic acid).

The lynamicins were discovered from a *Marinispora* sp. from sediment collected from Mission Bay, San Diego. Lynamicins A–E are chlorinated bis-indole pyrroles with broad spectrum activity against Gram-positive and Gram-negative organisms, in addition to activity against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VREF) [27.44].

Marinopyrroles A and B are halogenated axially chiral bispyrroles obtained from culture of a *Streptomyces* sp. from marine sediment collected near La Jolla, California. The marinopyrroles were isolated as single atropo-enantiomers and can be racemized at a higher temperature. Marinopyrrole A had a MIC_{90} of 0.61 mM and marinopyrrole B had a MIC_{90} of 1.1 mM against methicillin-resistant *S. aureus* (MRSA) [27.45].

A novel *Micromonospora* species was isolated from the marine ascidian *Didemnum proliferum* and produced diazepinomicin. Diazepinomicin represents a unique molecular class with a dibenzodiazepine core linked to a farnesyl side chain. Diazepinomicin showed modest activity against Gram-positive bacteria [27.46].

Lipoxazolidinones A, B, and C have been isolated form new marine actinomycetes, *Marinospora* sp. from a sediment sample collected in Cocos Lagoon, Guam. Lipoxazolidinones A had broad spectrum activity against Gram-positive bacteria (MIC values ranging from 0.5 to 5 mg/mL) and MIC values of 12 mg/mL against two strains of *Haemophilis influenzae*. The antibacterial spectrum and potency were similar to that of linezolid [27.47]. The lipoxazolidinones are 4-oxazolidinones while linezolid has a 2-oxazolidinone skeleton.

Cyclic meroterpenoid antibiotics merochlorins A–D have been isolated from a *Streptomyces* sp. (strain CNH-189) from a marine sediment sample collected from Oceanside, California. Merochlorins A and B showed strong inhibition or several methicillin-resistant *S. aureus* strains with MICs in the range of 2–4 μg/mL [27.48].

Salinosporamide A, containing an unusual γ-lactam-β-lactone bicyclic ring structure, was isolated from *S. tropica* [27.17]. The compound was shown to be a reversible inhibitor of the 20S proteasome and entered clinical trials for the treatment of cancer only three years after its initial publication. Moreover, the entire supply of salinisporamide A required for the preclinical developments and clinical trials has been manufactured by saline fermentation, bypassing the need for total synthesis [27.21]. Salinisporamide A exemplifies that marine-sourced actinomycetes have been shown to be abundant producers of new and novel structural classes of natural products and as such will continue to be an attractive resource for the discovery of new therapeutic agents [27.19].

27.2 Advances in the Field of Biodiscovery: Genomics and Genome Mining for Discovery of New Antibiotics

The genome sequencing studies of actinomycetes indicate that they possess larger genomes compared to other bacteria [27.49] and only a fraction of their coding capacity is used for the production of mostly cryptic secondary metabolites. Additionally, the genes responsible for production of many compounds can be found in the genomes of nonproducing strains. Their functions are still under investigation to determine whether these genes are nonfunctional, are not expressed under standard growth conditions or perhaps these genes require external signalling to turn them on [27.50]. Current techniques may not be powerful enough for

detection of the functions of these genes, and perhaps more efficient biochemical and chemical methods as well as genomic approaches will allow the discovery of large numbers of novel compounds coded by these genes. The new mass spectrometry (MS)-guided genome-mining method that connects the chemotypes of peptide natural products to their biosynthetic gene clusters by iteratively matching de novo tandem MS (MS^n) data to genomics-derived structures is an example of current effective approaches [27.51].

In a recent review, *Lane* and *Moore* [27.52] highlighted the advances made over the last decade (2000–2010) to improve understanding of the genetic basis of marine prokaryotic natural product biosynthesis. Initial studies focussing on identification and sequencing of individual gene clusters provided valuable insights, with examples including enterocins [27.53, 54] and wailupemycins [27.55]. However, in-depth understanding on the total quantity and diversity of biosynthetic pathways has not been achieved as exemplified in these early studies. The true genetic capabilities of marine microorganisms have only been revealed when genome sequencing has become an effective tool to explore the biosynthetic potential of marine microorganisms [27.52].

The first released genome sequences for marine microbes revealed that some of these organisms harbor genes for biosynthesis of far more natural products than previously recognized through cultivation-based studies. These orphan pathways, biosynthetic pathways for which corresponding natural products are unknown, bring an intriguing possibility that these pathways might be accessed for discovery of structurally unique, biomedically useful compounds [27.52].

Bioinformatics studies on the genome sequence of the first seawater-requiring marine actinomycete, *S. tropica*, revealed that this marine bacterial species owns a large proportion of genes (about 9.9%) responsible for natural product biosynthesis, while the corresponding numbers in *S. coelicolor* and *S. avermitilis* are 4.5 and 6%, respectively [27.56, 57].

The genome of *S. tropica* contains an amazingly large size of genes (516 kb) dedicated to polyketide synthase (PKS) and/or nonribosomal peptide synthetase (NRPS) biosynthesis, which are megasynthases that are responsible for many active natural product biosynthesis [27.57]. Genome sequence of the abyssomicin- and proximicin-producing marine actinomycetes *V. maris* AB-18-032 has also been completed [27.58]. Genome mining studies can reveal gene clusters for all known molecules from an organism, as well as biosynthetic gene clusters for natural products yet to be identified. Only through these studies will the total potential of an organism as a novel chemistry supplier be identified [27.52]. Discovery of salinosporamide K from *S. pacifica* by genome mining has provided insight into pathway evolution [27.59].

Recent advances in encoding precursor peptides or biosynthetic proteins, in silico mining of genomes combined with molecular biology approaches has also guided the discovery of a large number of new ribosomally derived natural products. Effective strategies were then designed for the identification of these ribosomally synthesized and post-translationally modified peptides (RiPPs) and the elucidation of their structures [27.60].

27.3 Ecological and Physiological Perspectives

27.3.1 Metagenomic Approaches

Marine microbial communities have been investigated using cultivation-independent genomic approaches [27.61, 62]. Large scale sequencing of environmental genomes has brought higher resolution insights into microbial diversity [27.61]. These studies are thus currently providing a more comprehensive description of the organisms and processes which define microbial community structure, function, and dynamics in the sea [27.52].

A more comprehensive view of uncultivated microbial species, gene, and biochemical pathway distributions, and naturally occurring genomic variability is also becoming clearer as well as fundamental principles that drive microbial ecological and evolutionary processes are being defined [27.52, 63, 64], with an ultimate goal of sustained development of marine biotechnology [27.63].

27.3.2 Exploring Marine Physiological Adaptations of Actinomycetes for Selective Recovery

Penn and *Jensen* [27.65] recently studied marine adaptations of *Salinispora* species using comparative ge-

nomics. They observed relatedness between marine and nonmarine actinobacteria through constructed phylogenic trees which might indicate recent introduction to marine environments.

Sponga et al. [27.66] indicated that the presence of a few antibacterial activities expressed when marine-derived actinomycetes were incubated in a fermentation medium diluted with sea water might be an indication of adaptation of these isolates to the marine environment rather than originating from those environments. The establishment of the marine affinity of organisms isolated from the sea requires generation of scientific evidence beyond laboratory studies [27.67]. Initial growth on complex laboratory media prepared with sea water, or on media made isotonic with sea water by the addition of NaCl, may not be an absolute criterion of marine adaptation as many terrestrial origin microorganisms also grow on such media [27.67]. True marine bacteria might be differentiated from their land counterparts through their need for Na^+ in the medium for growth but not by having requirements for sea water [27.67, 68]. Our knowledge is still poor about specific, natural nutrients and growth factors required for their cultivation. As stated by *Fenical* [27.8] common media constituents such as peptone, simple sugars etc. are unrealistic marine nutrients. These are apparently replaced with complex carbon sources such as chitin, sulfated polysaccharides, and marine proteins in the marine environment. In addition, there is still little understanding of the effects of uncommon inorganic elements, such as lithium, silicon, etc., which are also abundant in marine sediments. All these factors are still today greatly limiting our ability to isolate and culture the majority of the interesting and new bacterial forms present [27.8].

There is a need to develop a full understanding on the survival and marine adaptations of bacteria which can then be translated into development of effective selective isolation protocols [27.69]. One successful approach for the terrestrial ones has been the use of data deriving from physiological and growth requirements of terrestrial streptomycetes to design target-specific selective isolation methods [27.70]. Adaptation of such an approach for marine-derived taxa would result in effective recovery as well as generating information on the physiology and growth requirements of marine bacteria. *Imada* et al. [27.71] investigated the growth and antimicrobial compound production of marine actinomycetes in the presence of seawater. While the optimal concentration of growth ranged from 10 to 30 % (v/v), the production of antibacterial substances was observed in the concentration range 60–110 % (v/v) despite poor growth, indicating that the production of antibiotics is seawater-dependent.

27.3.3 Bacteriophage-Guided Route to Detection and Recovery of Marine Actinomycetes

When detection of rare actinomycetes is targeted, particularly the marine derived ones with low abundance frequency unless highly selective pressures are used, these taxa could not easily be isolated [27.69]. One approach has been the use of indicator phages specific towards targeted taxa using their close relatives as propagation hosts, and provided clues whether such species would be present in those environments [27.72–75].

An interesting finding was the occurrence of polyvalent *Salinispora* phages infecting members of *Micromonosporacea* including the type strains of terrestrial origin in Germany [27.74].

Stadler et al. [27.76] isolated cinnabaramides A–G, analogs of lactacystin and salinosporamide from a terrestrial streptomycete. These observations and findings might be in agreement of the above mentioned fact by *Penn* and *Jensen* [27.65] that a recent introduction of genera like *Salinispora* occurred into the marine environments and terrestrial relatives of these genera might also carry similar biosynthetic genes coding for the production of similar bioactive compounds. *Udwary* et al. [27.77] however, have indicated that due to the current lack of information on the other *Micromonosporacea* genome sequences, it is unclear whether this natural product diversity is common to this family or whether the diversity in *Salinispora* derives from environmental pressures present in marine environments.

Once the presence of bioactive taxa is indicated by bacteriophages in an environmental sample, they can also be used to reduce the numbers of unwanted taxa on isolation plates making the growth medium available for consumption by targeted organisms without competition [27.73, 75, 78].

Bacteriophages can also indicate the relatedness of unknown bacteria to their closest relatives [27.72]. In recent reviews *Kurtböke* [27.75, 79] provided insights into bacteriophage–host interactions. Examples included multiple strategies evolved by bacteriophages to interfere with bacterial growth. Such host conquering strategies used by the bacteriophages can provide clues towards detection of new bioactive compounds such as turning phage endolysins to enzybiotics [27.80].

27.4 An Australian Example: Exploring the Biosynthetic Potential of a Marine-Derived Streptomycete

Through systematic screening of marine environments in the Sunshine Coast Region of Australia using the information derived from indicator phages, a novel marine streptomycete species USC-633 was isolated from near shore marine waters [27.81] with an optimum growth temperature at 28 °C. Growing very slowly (over 3–4 weeks) in both salt dependent and independent media this near shore marine actinomycete was highly susceptible to low temperatures and lost viability immediately in cold storage temperatures (e.g. 4 °C) indicating it was an organism truly deriving from subtropical marine waters. Following the 16S rRNA sequencing investigation it occupied a distinct position on the phylogenetic tree (Fig. 27.1) and displayed only 68% similarity to other previously described bioactive marine streptomycete species such as the DQ448742 [27.82].

27.4.1 Morphology, Physiology, and Secondary Metabolite Production

The isolate displayed a range of growth characteristics when grown on different media. Only on oatmeal agar (3% yeast extract, 20% oats, and 20% agar) scarce aerial mycelium formation and sporulation were observed both visually and through electron microscopy studies (Fig. 27.2, 27.3a,b). On all other growth media including the Marine agar (Difco) used to grow the microorganism, extensive aerial mycelium development and subsequent sporulation were absent even after 4–6 weeks of incubation (Fig. 27.4).

However, well-defined chemical profiles were obtained on bold cultures of the isolate such as when it was grown on glucose yeast extract and malt extract agar (GYM; medium 65, www.dsmz.de) (Figs. 27.5, 27.6). These observations might correlate with the findings of *Ng* et al. [27.83] where increased rifamycin production was recorded after 31 days of growth with darkened color colony with *S. arenicola*.

In most actinomycetes including the streptomycetes the vegetative mycelia directly develops by germinated spores in a parallel disposition of the mycelium [27.83]. Such growth structure allows for the efficient utilization of the nutrients in the agar due to the hydrolytic enzymes secreted by the microorganisms allowing the efficient use of polymers [27.83–85].

Production of hydrolytic enzymes has also been linked to bioactivity. Of 116 chitinolytic actinomycetes isolated from marine sediments, 85 were found to possess antimicrobial activity by *Pisano* et al. [27.86]. They noted a high correlation between chitinolysis and bioactivity. Furthermore, addition of $CaCO_3$ by *Macedo* et al. [27.87] resulted in higher values of maximum lignin peroxidase activity. GYM agar contained $CaCO_3$ which might be triggering increased enzyme activity and in turn bioactivity. Deep dark green color produced on the GYM agar by the USC-633 might be related to the presence of such hydrolytic ability resulting in increased bioactivity.

Dark coloration was also evident with co-culture inoculations with other bacteria and fungi on the same solid state fermentation environment. Perhaps a wide array of characteristic demonstrated by the isolate making it adaptable to different substrates including marine ones highlights the fact that occurrence and adaptation is an ecological factor perhaps in response to environmental stresses rather than a true taxonomical separation [27.66, 67]. As soon as the culture was given the marine conditions a brominated compound was produced as a response to its new environment.

A large variety of aromatic and aliphatic carbon centers have been reported to be halogenated during natural product biosynthesis, with over 95% of the cases involving chlorine or bromine. In the marine environment where bromine is in higher concentration than in fresh water a comparable bromination of aromatic and heteroaromatic molecules as well as bromination of isoprenoid metabolites have been reported [27.88].

Magarvey et al. [27.89] investigated the obligate growth requirements of the PNG1 and UMM518 clades and concluded that they do not have an obligate requirement for salt like the true marine clade MAR1, but do complete a full developmental cycle in submerged cultures (liquid saltwater growth medium containing 3% NaCl). Both of these slowly growing actinomycetes were capable of sporulating under submerged conditions in the presence of a salt concentration of 3%, typical of seawater, consistent with their ability to grow and divide in the marine environment.

It has frequently been reported that small changes in culture conditions can have a major influence in the spectrum of secondary metabolites [27.50]. In-depth information gained from physiological studies can further be used to manipulate secondary metabolite production in conjunction with the reflected information deriving from secondary metabolism. Co-culture exper-

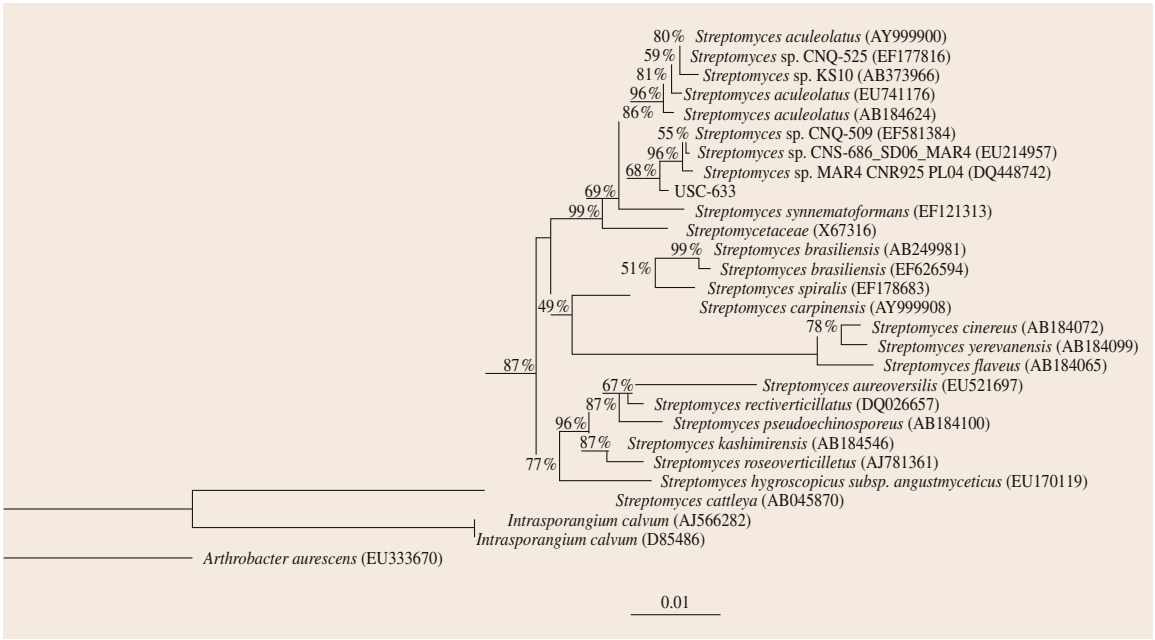

Fig. 27.1 Phylogenetic tree of the 16S rRNA gene sequence obtained from isolate USC-633 and closest relatives from the genus *Streptomyces* and other members of the order *Actinomycetales* (adapted from *Kurtböke* [27.81]). *Numbers* at branch node points representing values (only values > 50% are shown). The *scale bar* represents 1% sequence divergence. GenBank accession numbers of reference sequences are presented in parentheses. *Arthrobacter aurescens* was used as an outgroup sequence to root the tree

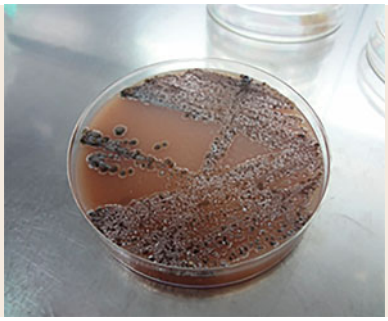

Fig. 27.2 Aerial mycelium formation on oatmeal agar plates

iments can also provide novel insight into secondary metabolism and facilitated development of new vessels for stable mixed-culture fermentation, microbial con-

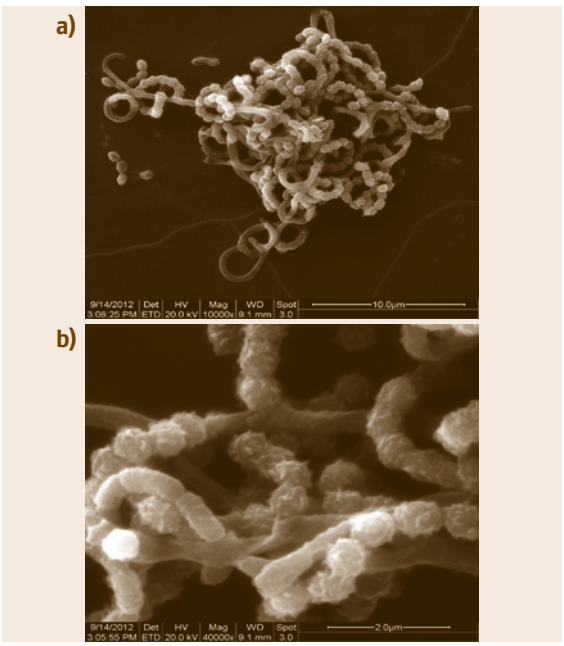

Fig. 27.3a,b Electron micrographs of the **(a)** spore chains at the tips of **(b)** aerial mycelium and mature spores produced on oatmeal agar after 4 weeks of incubation (*bar* indicates **(a)** 10 μm, **(b)** 2 μm) ►

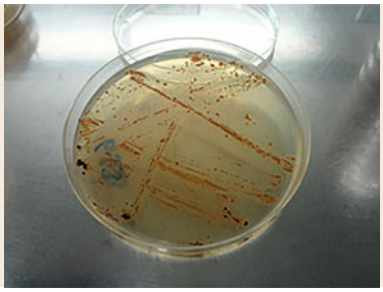

Fig. 27.4 Growth characteristics of USC-633 on marine agar (Difco) after 4 weeks of incubation

Fig. 27.5 Growth characteristics of USC-633 on GYM agar after 4 weeks of incubation

sortium as a whole [27.50]. Microorganisms co-exist in tiny ecosystems. As a result, these microbial communities can hold the potential for production of novel compounds through diverse signalling, and cross-feeding between organisms that might elicit production of novel compounds [27.50, 90, 91]. *Burgess* et al. [27.92] showed that bacteria associated with starfish and seaweed have the ability to produce antimicrobial compounds against other marine bacteria and also against terrestrial pathogenic bacteria. Furthermore, when culture supernatants were added, antibiotic production in marine surface-associated bacteria was elicited. Following cross-species induction enhanced microbial activity in marine bacteria has also been shown by *Mearns-Spragg* et al. [27.93] as well as by *Slattery* et al. [27.94] on the competition-mediated antibiotic induction in the marine bacterium *Streptomyces tenjimariensis*.

27.4.2 Natural Product Chemistry

In order to explore the secondary metabolite production of the marine-derived actinomycete USC-633, the strain was grown on several different agar-based media, and the ethyl acetate extracts analyzed by high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC-MS), and proton nuclear magnetic resonance (^{1}H NMR). Isolation and compound identification work revealed that the strain produces phenazine-related natural products, with the secondary metabolite production being media dependent. When cultured on oatmeal agar medium, USC-633 afforded 1-methoxyphenazine as a main natural product while the GYM agar culture afforded *N*-monoterpenoid substituted phenazine lavanducyanin and 2-chlorolavanducyanin. Marine agar culture yielded lavanducyanin and 2-chlorolavanducyanin as well as 2-bromolavanducyanin indicating that the microbe is adapting to the new nutrient environment. The diversity of USC-633 secondary metabolite production is depicted in Fig. 27.7.

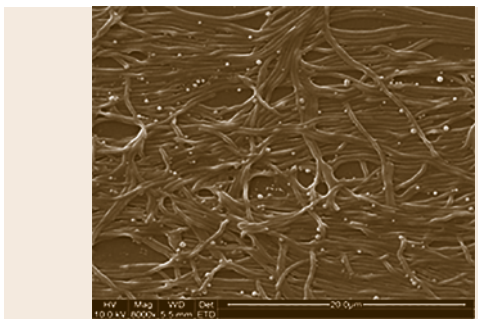

Fig. 27.6 Electron micrograph of the growth characteristics of USC-633 on GYM agar after 4 weeks of incubation (*bar* indicates 20 μm)

Phenazines are produced by many different groups of bacteria including *Streptomyces* [27.95, 96]. They are reported to be involved in pathogenesis and competi-

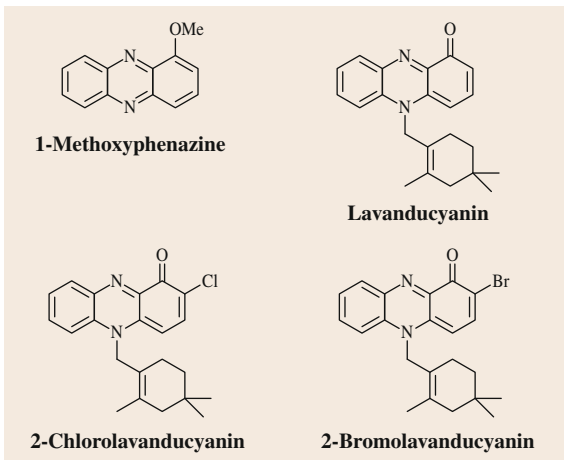

Fig. 27.7 Natural products identified from USC-633

tive fitness of the bacteria attributed primarily to their ability to generate reactive oxygen in other organisms and tissues [27.96]. The competitive environment of the near shore marine environments including pollution from human activities and presence of marine macroorganisms might be triggering the production of these compounds.

Penn et al. [27.97] has recently identified that genomic islands can harbor functional traits that differentiate ecologically distinct populations of environmental bacteria. Their comparative analysis of the complete genome sequences of the marine actinobacteria *S. tropica* and *S. arenicola* revealed that 75% of the species-specific genes were located in 21 genomic islands. These islands were enriched in genes associated with secondary metabolite biosynthesis providing evidence that secondary metabolism linked to functional adaptation. Brominated and chlorinated compounds produced by the USC-633 might also be as a result of such functional adaptation.

27.5 Future Prospects

Traditional approaches have been successful at identifying many successful therapeutic agents since the discovery of penicillin. However, growing antibiotic resistance and need for potent agents to combat many incurable diseases indicate the urgent need for new approaches. The application of genome mining, metabolomics, and proteomic tools has recently been a leading force in developing novel advances in the drug discovery area. They will continue to be the driving forces for discovery and design of potent therapeutic agents from microbial sources in the years to come. Through these advances the true biosynthetic potential and metabolic diversity of marine microorganisms will be revealed [27.98, 99]. Cross-country advances in bioinformatics tailored for the marine field, consistent data acquisition and exchange and design of relevant marine bacterial models is now a recognized priority in Europe resulting in an EU funded series of workshops and projects [27.100].

Genome sequences will continue to provide valuable insights for activation of silent gene clusters [27.99]. One of the most successful outcomes via the use of genomic approaches has so far been through the studies conducted on *Salinispora* species. Examples include the exploration and engineering of biosynthetic pathways in the marine actinomycetes *S. tropica* [27.101] and the discovery and assembly line biosynthesis of the lymphostin pyrroloquinoline alkaloid family of mammalian target of rapamycin (mTOR) inhibitors in *Salinispora* [27.102].

High-throughput genome scanning methods will also facilitate discovery of metabolic loci-independent of expression. Genome sequence tags (GSTs) are genes involved in natural product biosynthesis and they can be utilized as probes for screening of specific genes within clonal libraries. Subsequently, any clone that contains a GST can then be screened for novel natural product gene clusters such as the identification of more than 450 natural product clusters [27.50, 103]. Genome inspired-technologies will also unveil new metabolites which were overlooked under standard fermentation and detection conditions.

Advances in the chemistry field will continue to facilitate rapid discovery of bioactive molecules using improved ability to dereplicate, isolate, and elucidate the structure of natural products from reduced sample quantity [27.3]. Emerging physiology-oriented engineering strategies based on improved understanding into microbial physiological responses to environmental stresses, will also result in productive physiological performances of industrial microorganisms [27.104].

Microbial *ecotypes* [27.105] or *geovars* [27.106] can also be specific to a geographical–ecological region, for example more than 200 secondary metabolites have been isolated from various *Streptomyces hygroscopicus* strains of different geographical origin. Moreover, strains of the same subspecies may produce different compounds [27.50]. *Jensen* and *Mafnas* [27.25] following their studies using tropical and subtropical ocean sediments conclude that *Salinispora* species are globally distributed and speciation within this genus is driven by ecological selection not geographical isolation. The reported presences of indicator bacteriophages specific to *Salinispora* type strains in different geographical regions by *Kurtböke* [27.74] also support their conclusions.

Biogeographical information combined with metagenomics-derived data will give insight into population dynamics of predominantly unculturable microbial communities. It will also provide information into the capabilities of microorganisms that allow them to collaborate and compete to survive in a wide range of environments [27.107]. The application of new molec-

ular and genomic techniques to the ocean will be driving a scientific revolution in marine microbiology by defining marine *genotypes* representing the potential biological capacity, and *phenotypes* reflecting the complex interactions of individuals and populations within their physical and chemical environment and with each other [27.108]. Furthermore, information derived from marine adaptational responses of bioactive taxa will enable development of effective search strategies to identify sites of bioactivity. Bacteria in marine environments are often under extreme conditions in terms of pressure, temperature, salinity, and depletion of micronutrients. Microbial ability to survive and proliferate is dependent on the ability to produce biologically active compounds [27.109]. Bacteria can produce molecules that prevent the attachment, growth and/or survival of challenging organisms in competitive environments [27.109] and such molecules can be of interest for therapeutic reasons especially against antibiotic resistant bacteria. There is now an opportunity to pair metagenomic analysis with other genomic approaches to evaluate gene expression and bioactive compound production which will in turn pave the way to unprecedented discoveries [27.62]. This paired approach will also challenge our perception of community structure and function in environmental systems as well as offering opportunities for novel gene discovery and biotechnological advancements. Community diversity and ecosystem functions of microorganisms will be revealed. As a result, information deriving from these molecular approaches will sharpen our focus during design of selective isolation techniques to culture bioactive microorganisms. Detection of environmental function-related bioactivity and its exploitation for pharmaceutical and biotechnological purposes will be achieved in a relatively shorter time span. When such approaches are combined with an improved understanding of the natural product metabolome, application of rational approaches for more effective exploitation of natural products will be facilitated [27.110].

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28. Biotransformation of Nitriles by Marine Fungi

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In this study, a screening of 12 marine fungi (*Penicillium miczynskii* CBMAI 930, *Penicillium raistrickii* CBMAI 931, *Aspergillus sydowii* CBMAI 933, *Aspergillus sydowii* CBMAI 934, *Aspergillus sydowii* CBMAI 935, *Bionectria* sp. CBMAI 936, *Penicillium oxalicum* CBMAI 1185, *Penicillium citrinum* CBMAI 1186, *Penicillium decaturense* CBMAI 1234, *Penicillium raistrickii* CBMAI 1235, *Cladosporium* sp. CBMAI 1237, and *Aspergillus sydowii* CBMAI 1241) was conducted in order to evaluate the enzymatic potential of these microorganisms for biotransformation of phenylacetonitrile **1**. These microorganisms were isolated from sponges and algae collected on the northern part of the coast of São Paulo State, Brazil. The screening was carried out on a solid mineral medium supplemented with glucose and phenylacetonitrile **1** as the only source of nitrogen. Afterwards, the microorganisms adapted were tested in a liquid medium containing 20, 40, and 60 μL of phenylacetonitrile **1**. The phenylacetonitrile **1** was biotransformed into the 2-hydroxyphenylacetic **1a** acid by eight of the marine fungi selected. The enzymes which hydrolyzed nitriles in these catalytic systems were inducible. Since the mycelium of *A. sydowii* CBMAI 934 grew strongly in solid and liquid mineral media in the presence of phenylacetonitrile **1**, this fungus was selected for the enzymatic hydrolysis reactions using other nitriles, such as 4-fluorophenylacetonitrile **2**,4-chlorophenylacetonitrile **3**, 4-methoxyphenylacetonitrile **4**, cyclohexenylacetonitrile **5**, and 2-cyanopyridine **14**, yielding their corresponding carboxylic acids: 4-fluorophenylacetic **2a** (51%), 4-chlorophenylacetic **3a** (55%), 4-methoxyphenylacetic **4a** (43%), cyclohexenylacetic acid **5a** (28%), and the amide, 2-pyridinecarboxamide **14a**, respectively. This chapter reports on the study on the biotransformation of nitriles by marine microorganisms which is summarized in the context of directions for future research.

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28.1 Overview

28.1.1 Biocatalysis and Biotransformation

Biocatalysis and biotransformation refer to processes in which enzymes are used to catalyze chemical reactions, but outside their normal biochemical systems, with non-natural substrates for their metabolic pathways. The term biotransformation is applied to specific structural modifications or interconversions of compounds in multiple stages by living organisms [28.1, 2].

Biocatalysis is an area of biotechnology utilized in chemistry to promote organic reactions with one or more steps, to produce compounds of interest, especially of high enantiomeric purity. In addition to the importance of biocatalysis for synthesis in chiral blocks, focused on the production of chiral drugs, there has been much stress lately on its applicability to the production of raw materials for products in the cosmetics, polymers, agrochemicals, and fuel industries [28.3, 4].

To establish an effective process of biotransformation, it is necessary to make a detailed analysis of the factors that influence the development and optimization of the integrated biotechnological process, which depend on: i) the biocatalyst, ii) the bioconversion, and iii) the isolation and purification of the product. The purpose of this chapter is to investigate the biotransformation of nitriles using marine fungi isolated on the Brazilian coast.

28.1.2 Marine Microorganisms

While most industrial enzymes are derived from terrestrial sources, the potential of marine microorganisms is being explored with a view to industrial use. In recent years, marine microorganisms have attracted the interest of researchers aiming to exploit various enzymes in biotechnological applications. An example is proteases, a very important industrial-type enzyme, which are used in the processing of leather, food, and detergents [28.5].

The occurrence of fungi in the marine environment was recorded in the late nineteenth century; the oceans cover over 70% of the land surface. Nevertheless, the known species of marine fungus are far fewer than known terrestrial species [28.6]. The first facultative marine fungus, *Phaeosphaeria typharus*, was described in 1849, and the first obligate marine fungus, *Halotthia posidoniae* in 1869 [28.7]. Marine fungi can be divided into two major groups. The obligate marine fungi grow and sporulate exclusively in marine or estuarine habitats, while the facultative group inhabits inland water ecosystems and terrestrial environments, but also grow and survive in marine environments [28.7]. Marine fungi, among other organisms present in the complex and vast array of marine ecosystems, represent a valuable source of new and rarely studied species for the scientific laboratory and for ecological, evolutionary, and biotechnological study. These fungi can be found associated with various marine organisms, such as sponges, fishes, cnidarians, tunicates, and algae. The isolation of fungi from the ocean does not necessarily signify that these organisms are active in the marine environment. Therefore, it is possible to isolate a terrestrial fungus that has adapted or is simply present in the marine environment. Also, the fungus could be isolated in the dormant form of spores or fragments of hyphae, awaiting conditions in the laboratory that are favorable for germination, growth, and sporulation. Several strains of fungi isolated from the marine environment are not demonstrably marine fungi, having been identified as facultative or optional. Therefore,

a generalized expression *marine-derived fungi* has been used [28.8].

Recently, studies were performed to assess methods of isolating marine-derived fungi on various culture media, resulting in a set of 688 fungal strains isolated from ascidians, sponges, and algae. In addition, with the advance of techniques in molecular biology, it has been possible to isolate and identify obligate marine species of bacteria and fungi from the oceans [28.9].

Marine microorganisms have specific biochemical properties that can provide new enzymes with unique characteristics, which can be more active and stable than the corresponding enzymes of terrestrial organisms [28.10]. The complexity of the marine environment with its high salinity and pressure, low temperature, and special lighting may give rise to the significant differences between the enzymes produced by marine microorganisms and the homologous enzymes from terrestrial microorganisms. Such differences boost the technological potential of enzymes from marine microorganisms in the production of valuable compounds of scientific and economic interest [28.10].

28.1.3 Nitriles

Organic compounds of various classes containing the cyano group occur naturally in the environment and are synthesized by plants, fungi, bacteria, seaweed, sponges, insects, etc. Plants and microorganisms are able to produce both aliphatic and aromatic nitriles, such as cyanoglucosides, cyanolipids, ricinine, and phenylacetonitrile (Fig. 28.1) [28.11]. These compounds can serve not only to store nitrogen, but also to protect the organism against herbivory [28.11, 12].

The enzymatic pathways leading to nitriles are little known. *Kato* et al. showed that the aldoxime dehydratase is responsible for the formation of nitriles from aldoximes-degrading microorganisms, e.g., *Bacillus* sp. OxB-1 and *Rhodococcus* sp. YH3-3, from soil [28.13]. It is known that the metabolism of compounds containing the cyano group involves a wide spectrum of reactions, among which stand out hydrolysis by nitrilase or nitrile hydratase, followed by amidase, reactions catalyzed by oxygenase and oxynitrilase, and reductions catalyzed by nitrogenase (Fig. 28.2) [28.14].

Oxygenases of plants and insects catalyze the oxidation of nitriles to cyanohydrins (α-hydroxynitriles), which are converted to aldehydes and hydrogen cyanide (HCN) by oxynitrilases (hydroxynitrile lyases) [28.15].

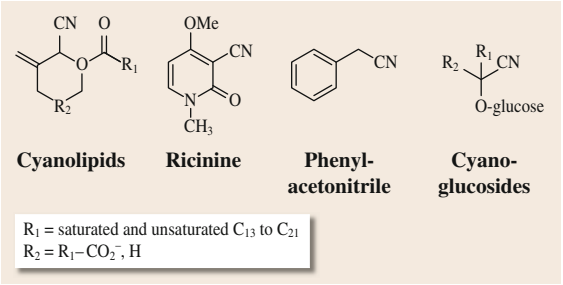


Fig. 28.1 Organic nitriles of natural occurrence

HCN can also be converted by cyanase to CO_2 and NH_3 or by cyano hydratase to formamide, while cyanide dihydratase produces formic acid and NH_3. Nitrogenases present in nitrogen-fixing organisms in soil are capable of reducing organonitrile compounds to the corresponding hydrocarbons and NH_3 (Fig. 28.2) [28.14].

Most nitriles found in nature are toxic, mutagenic, and carcinogenic. In general, the toxicity of organonitriles to humans causes gastric problems, nausea, shortness of breath, and bronchial irritation. Despite the high toxicity of most nitriles, they are widely used as intermediates in organic chemistry for the synthesis of amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones, and heterocyclic compounds (Fig. 28.3) [28.14, 16].

The chemical hydrolysis of nitriles requires the use of strong bases or acids, salts of toxic metals, and elevated temperatures. However, enzymatic hydrolysis provides a means of promoting reactions under mild conditions and does not produce any by-products [28.17–19].

Enzymatic Hydrolysis of Nitriles

Enzymatic hydrolysis is the commonest pathway for microbial metabolism of nitriles; it occurs by two routes:

i) *Nitrilase* (NLase): directly hydrolyzes the cyano group of organonitriles to the corresponding carboxylic acid, via the addition of two molecules of water.
ii) *Nitrile hydratase* (NHase): converts the cyano group of organonitriles to the corresponding amide, via the addition of one water molecule. Next, an *amidase* converts the amide group to the corresponding carboxylic acid via the addition of one water molecule [28.14]. The two pathways for the enzymatic hydrolysis of nitriles are shown in Fig. 28.4.

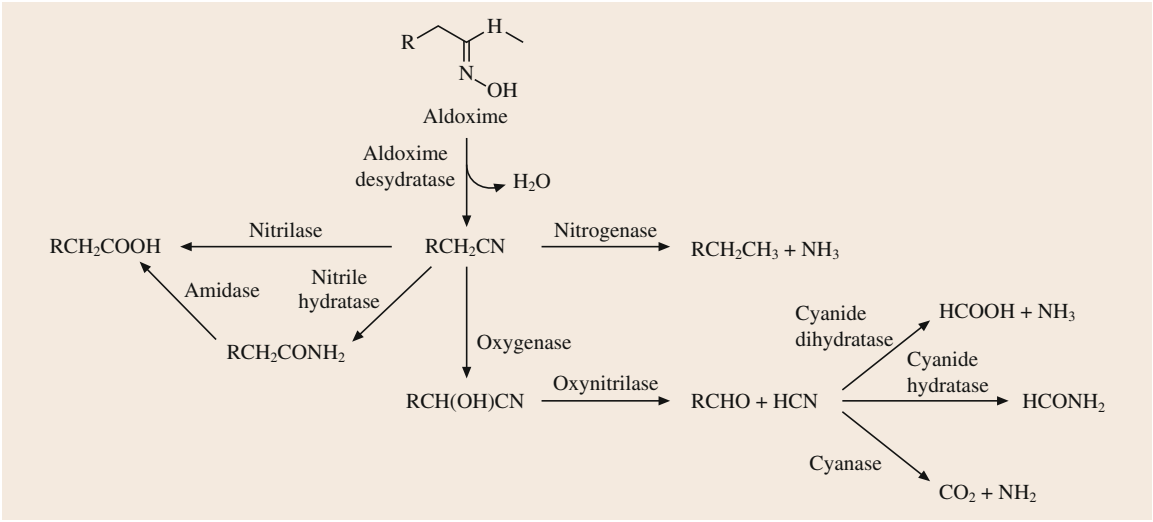


Fig. 28.2 Different pathways for biotransformation of nitriles

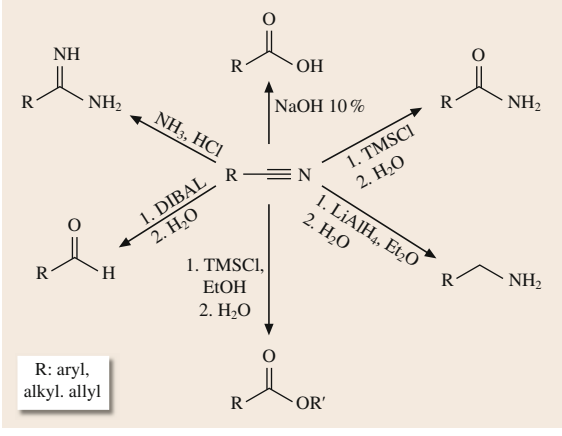


Fig. 28.3 Syntheses of organic compounds from nitriles

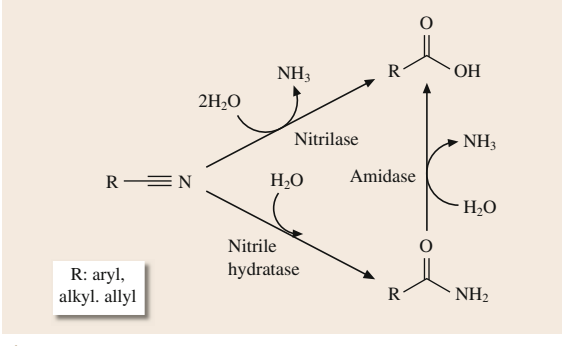


Fig. 28.4 Enzymatic hydrolysis of nitriles

A simplified proposal for the mechanism enzymatic of hydrolysis of nitrile by *nitrilases* is shown in Fig. 28.5. The main steps are:

a) At the catalytic site of the enzyme, there are three amino acid residues, glutamic acid (Glu), cysteine (Cys), and lysine (Lys), and a water molecule.
b) Initially, an interaction occurs via hydrogen bonding between the substrate (nitrile) and the Lys protonated in the catalytic site. In addition there is hydrogen bonding between the water and the nitrile, Cys and Glu. Glu is hydrogen-bonded to the Lys and water, further stabilizing the active site.
c) In the first step of the reaction, the electrophilic carbon of the cyano group undergoes nucleophilic attack of the sulfhydryl group of cysteine for forms an enzyme-covalent complex thioimidate. The nucleophilic character of cysteine is enhanced by hydrogen-bonding interactions with water.
d) The thioimidate-enzyme complex is subsequently hydrolyzed by a water molecule to form a tetrahedral intermediate.
e) The nitrogen of the tetrahedral intermediate is protonated by capturing a proton from glutamic acid.
f) Subsequently, the protonation of this nitrogen favors the elimination of ammonia and promotes the formation of a covalent thioester-enzyme complex.
g) Finally, the thioester-enzyme complex is hydrolyzed by a water molecule to form the corresponding free carboxylic acid.

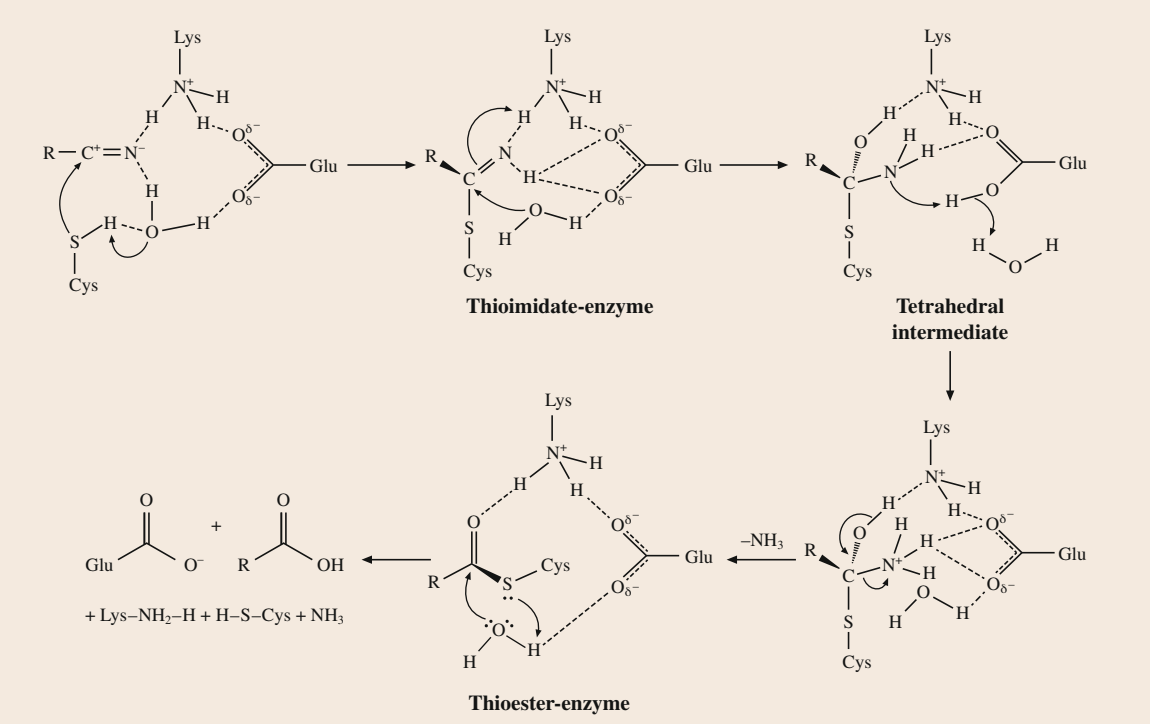


Fig. 28.5 Mechanism proposed for hydrolysis of nitriles by nitrilase [28.20]

In the active site of the nitrilases, a cysteine is involved in the catalysis of the cyano group. The nitrile hydratases have a metal cation in the active site that is believed to play a catalytic role [28.21].

The NHases carry the metal ions, Fe^{3+} or Co^{2+} at the active site. Thus, they can be classified into two groups: NHases dependent on iron(III) and NHases dependent on cobalt(II) [28.14]. For example, NHases from *Pseudomonas chlororaphis* B23, *Brevibacterium* sp. R312, and *Rhodococcus* sp. require iron, while those from *R. rhodochrous* J1 and *Pseudomonas putida* NRRL 18668 require cobalt [28.22]. However, the NHases from *Agrobacterium tumefaciens* need both metal ions. There are two main reasons for the presence of the metal ion at the active site of the enzyme. First, these metal ions are great catalysts for hydration of the cyano group. Second, the metal ions are essential to enhance the flexibility (bending) and stability of the polypeptide chain (α and β-subunit) of the NHases [28.14, 16].

The activity of NHase has unique features when the enzyme is exposed to light. The chromophore involved in this photo-activation is an iron complex in the β-subunit. Light irradiation of the complex induces a conformational change in the β-subunit. Because of this, an endogenous NO molecule that is bound to the non-heme iron(III) center in the inactive NHase is released, resulting in the recovery of the original NHase activity [28.14, 23]. Two possible mechanisms of action of NHase are shown in Fig. 28.6.

All ligands coordinated to the metal are in the α-subunit of the protein; these ligands are three cysteine thiolates (Cys110, Cys112, Cys114) and two nitrogen atoms (Ser113, Cys112). Five amino acids occupy the vertices of the octahedron and the sixth position can be occupied by a hydroxide ion. The main steps of these mechanisms are:

- *Mechanism I*: The electrophilic carbon of the cyano group undergoes nucleophilic attack by the hydroxide ion that is coordinated to the metal ion at the catalytic site of the enzyme, leading to the formation of an imidate. The imidate undergoes rearrangement to the more stable amide.
- *Mechanism II*: The hydroxide ion coordinated to the of the catalytic site metal ion enzyme acts as a base,

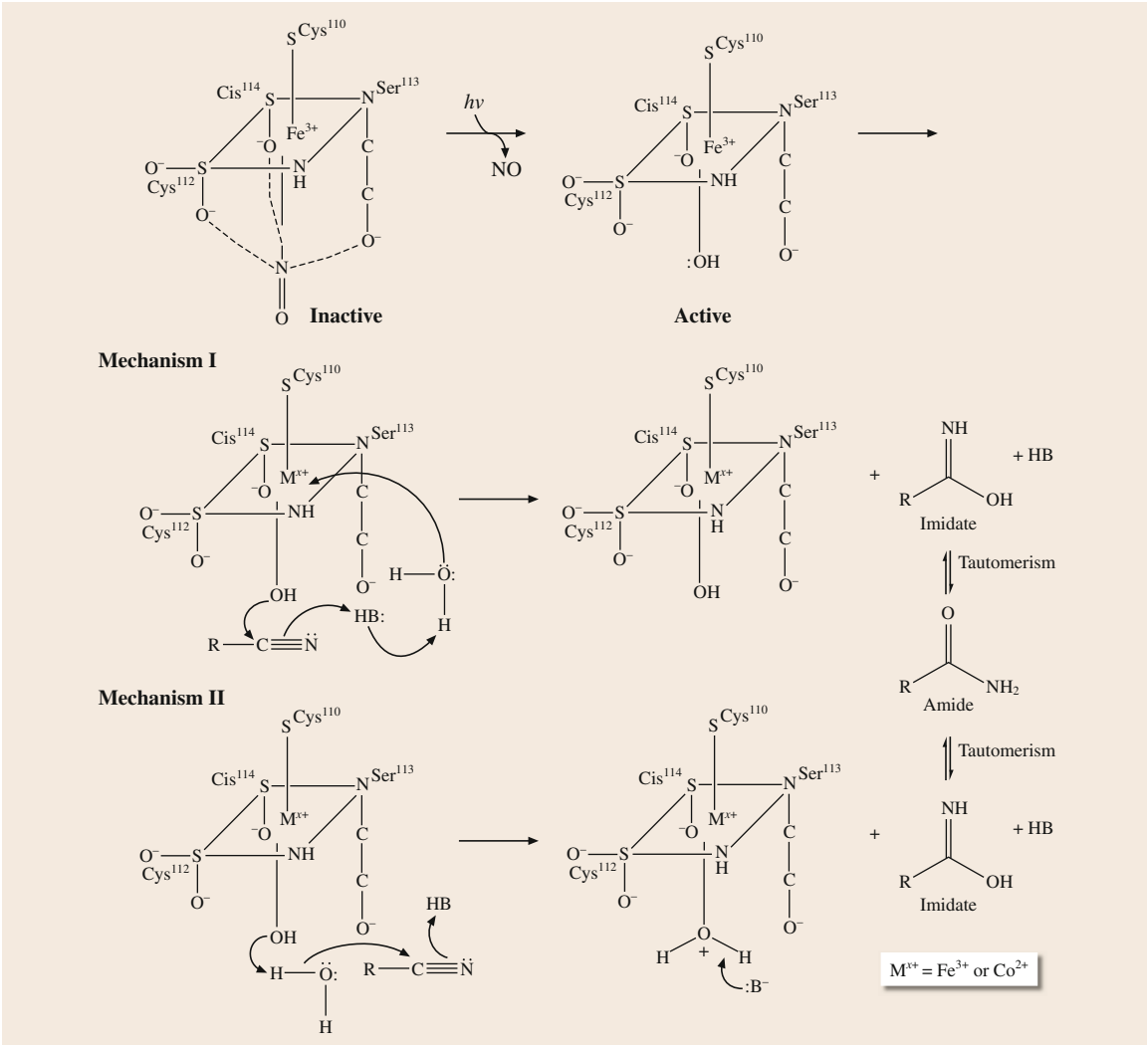


Fig. 28.6 Mechanism proposed for NHase. Photoactivation and enzymatic catalysis [28.14]

activating a water molecule to promote nucleophilic attack on the cyano group carbon to form an imidate. The imidate forms the amide as in mechanism I, by tautomerization.

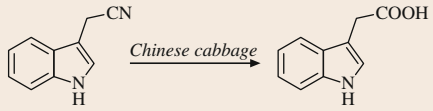
28.1.4 Nitrilases, Nitrile Hydratases, and Amidases

The nitrilases (EC 3.5.5.1) and amidases (EC 3.5.1.4) are enzymes that belong to the class of hydrolases, while the nitrile hydratases (EC 4.2.1.84) belong to the class lyases [28.24, 25].

Nitrilases (EC 3.5.5.1)

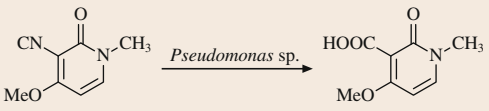
Since their discovery in plants (in 1958) and in bacteria (in 1964), over 30 nitrilases have been reported in many organisms, including fungi [28.11]. The nitrilase enzyme was first recognized by the biotransformation of indoleacetonitrile (IAN) to indoleacetic acid (IAA) in barley leaves; the enzyme responsible was named indole acetonitrilase (Fig 28.7) [28.26]. It is also found in the cabbage family.

The first bacterial nitrilase to be purified and partially characterized was isolated from *Pseudomonas* sp.; this catalyzed the biotransformation of *N*-methyl-3-



Indoleacetonitrile **Indoleacetic acid**

Fig. 28.7 Biotransformation of indole acetonitrile by *Chinese cabbage*



***N*-methyl-3-cyano-4-methoxy-2-pyridone** ***N*-methyl-3-carboxyl-4-methoxy-2-pyridone**

Fig. 28.8 Biotransformation of *N*-methyl-3-cyano-4-methoxy-2-pyridone by *Pseudomonas* sp.

cyano-4-methoxy-2-pyridone to *N*-methyl-3-carboxy-4-methoxy-2-pyridone (Fig. 28.8) [28.26].

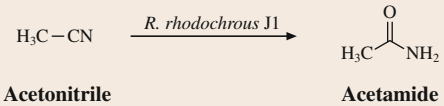
The biochemical and biological properties of nitrilases and their substrates (nitriles) indicate that the functions of these enzymes are probably protection, detoxification, and the use of the nitrogen atom as a nutrient source and for synthesis of plant hormones [28.11].

The nitrilases of plants are better characterized than microbial nitrilases with respect to their biological functions, particularly NIT1, NIT2, NIT3, and NIT4 of *Arabidopsis thaliana*. Plant nitrilases form two distinct groups according to the type of substrate they catalyze: nitrilases with high activity for hydrolysis of arylacetonitriles (phenylpropionitrile) and those with high hydrolytic activity for aliphatic nitriles (β-cyano-L-alanine; Fig. 28.9) [28.11].

Most of the nitrilases characterized to date are of bacterial origin and were isolated on a selective medium that allows only Gram-positive strains to develop in the presence of nitrile as the sole nitrogen source [28.27, 28]. Culturing microorganisms on selective media is an efficient way to isolate strains capable of hydrolyzing nitriles. The nitrilases and amidases convert the cyano group in their respective substrates to ammonia, which is readily used as a nitrogen source. Thus, these microorganisms, which produce the enzymes of interest, can grow in the presence of nitriles or amides as the sole source of nutrients. In some cases, the carboxy-

Phenylpropionitrile **β-ciano-L-alanine**

Fig. 28.9 Structures of natural compounds containing the cyano group



Acetonitrile **Acetamide**

Fig. 28.10 Biotransformation of acetonitrile by nitrile hydratase

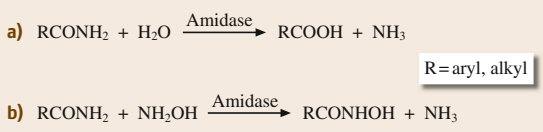


Fig. 28.11 **(a)** Enzymatic hydrolysis of amides. **(b)** Biotransformation of amides by amidases in the presence of hydroxylamine

lates derived from hydrolysis of nitriles or amides are also utilized by the microorganisms, thereby promoting the complete degradation of the substrate [28.29].

Bacteria of the genera *Rhodococcus, Alcaligenes*, *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Corynebacterium*, *Arthrobacter*, and *Nocardia* are sources of nitrilases with a variety of substrate specificities, which have been purified and characterized [28.14, 26, 30]. Fungal nitrilase was first isolated from *Aspergillus niger* and was characterized and sequenced by biochemical methods. Other genera were then identified as producers of these enzymes, such as *Fusarium, Gibberella*, and *Penicillium* [28.20, 31].

Nitrile Hydratase (EC 4.2.1.84)

In 1980, the novel enzyme acetonitrile hydratase was detected, degrading aliphatic nitriles [28.32]. The first occurrence of this nitrile hydratase was reported in the bacterium *Rhodococcus rhodochrous* J1, initially identified as *Arthrobacter* sp. J1, which catalyzed the biotransformation of acetonitrile to acetamide (Fig. 28.10) [28.16].

Nitrile hydratases were initially considered specific for aliphatic nitriles, but later these enzymes were re-

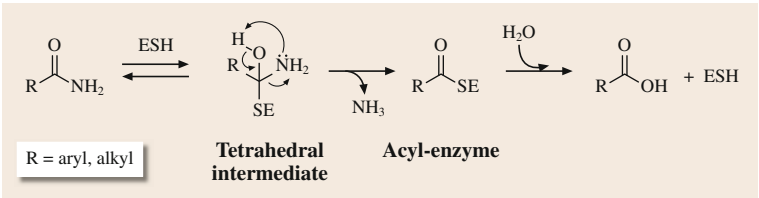


Fig. 28.12 Mechanism of catalysis of amide hydrolysis by amidases [28.33]

ported to act on heteroaromatic nitriles [28.29]. Like the nitrilases, the nitrile hydratases are also usually inducible. It is noteworthy that the metal ions Fe^{3+} and/or Co^{2+} are essential to their catalytic activities [28.16].

Amidases (EC 3.5.1.4)

The amidases are enzymes that catalyze the hydrolysis of amides to the corresponding carboxylic acids and ammonia. Analogously, amidases catalyze the biotransformation of amides to the corresponding hydroxamic acid in the presence of hydroxylamine (Fig. 28.11a,b) [28.16].

The steps of the reaction catalyzed by amidases are shown in Fig. 28.12:

i) The carbonyl group of the amide undergoes a nucleophilic attack by the enzyme, leading to formation of a tetrahedral intermediate.
ii) The tetrahedral intermediate is converted to the acyl-enzyme complex, releasing a molecule of ammonia.
iii) The carbonyl carbon of the acyl-enzyme complex undergoes nucleophilic attack by a water molecule, yielding carboxylic acid.

The amidases are involved in the metabolism of both prokaryotes and eukaryotes and can be divided into:

i) *Aliphatic amidases* that catalyze the hydrolysis only of short-chain aliphatic amides and that catalyze the hydrolysis of medium-chain aliphatic amides.
ii) *Arylamidases* that catalyze the hydrolysis of aromatic amides [28.33].

The amidases may also catalyze other reactions, such as ester hydrolysis, conversion of esters to hydroxamic acids, conversion of carboxylic acids to hydroxamic acids, and hydrolysis of isonicotinic acid hydrazide (Fig. 28.13a–d).

It is important to note that the majority of amidases are enantioselective and have been used in the synthesis of chiral carboxylic acids, by combination with the nitrile hydratases. Reactions catalyzed by enantioselective amidases have provided optically pure compounds of pharmaceutical importance such as amino acids and 2-arylpropionic acid [28.16].

Recently, a number of products that can be used as intermediates for pharmaceuticals, chemical additives in foods, and so on, have been obtained by enzymatic reactions. The enzymes that convert nitriles (nitrilases and/or nitrile hydratases and amidases) are potential catalysts for the production of carboxylic acids and amides of interest for industrial applications.

28.1.5 Application of Nitrilases, Nitrile Hydratase, and Amidases to Biocatalysis

Nitrilases, nitrile hydratases, and amidases are important biocatalysts in the fine chemicals and pharmaceuticals industry for their high specificity due to chemoselectivity, regioselectivity, and enantioselectivity. In addition, they are used in the detoxification of industrial waste and degradation of herbicides containing the cyano group [28.34].

Hydrolysis of Racemic Nitriles by Nitrilases

Chemoenzymatic resolution of *rac*-nitriles has been carried out with the aim of producing α-arylpropionic acids of *S*-configuration, such as ketoprofen, ibuprofen, and/or naproxen, which are widely used as anti-inflammatory drugs [28.12].

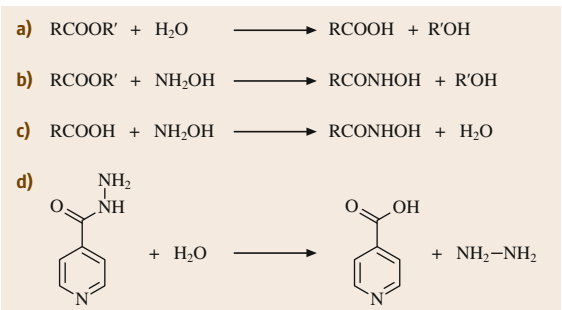


Fig. 28.13a–d Reactions catalyzed by amidases

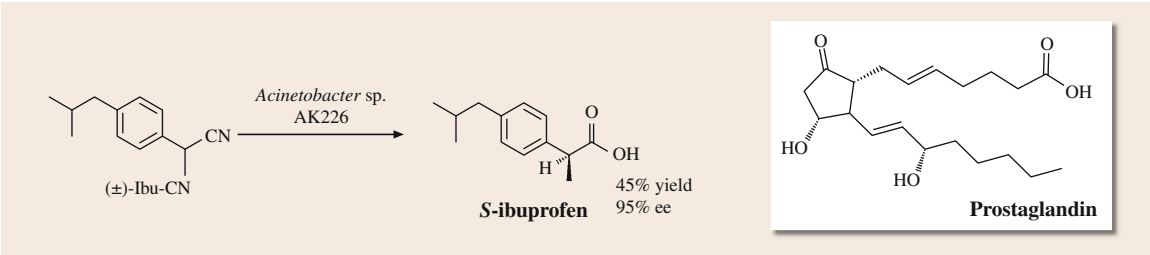


Fig. 28.14 Production of *S*-ibuprofen by the nitrilase of *Acinetobacter* sp. AK226

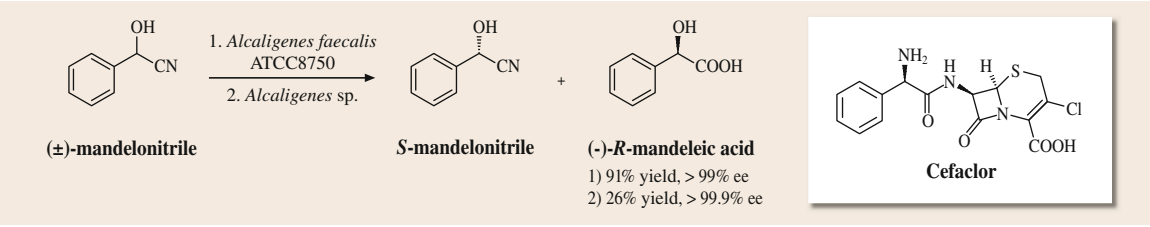


Fig. 28.15 Hydrolysis of (±)-mandelonitrile catalyzed by bacterial nitrilase

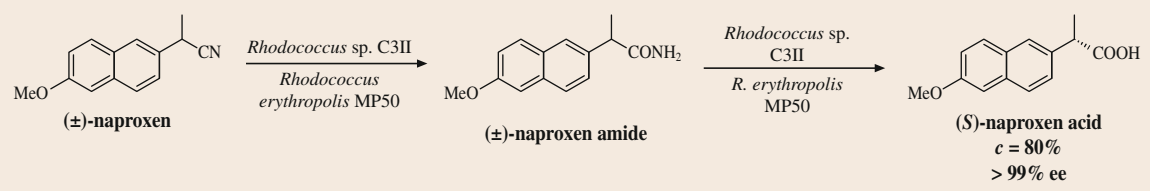


Fig. 28.16 Hydrolysis of the (±)-naproxen by *Rhodococcus* sp. C3II and *Rhodococcus erythropolis* MP50

The (+)-(*S*)-2-(4′-isobutylphenyl)propionic acid (*S*-ibuprofen) was produced by kinetic resolution of (±)-2-(4′-isobutylphenyl)propanonitrile (Ibu-CN) by *Acinetobacter* sp. AK226, with 95% of enantiomeric excess (ee) and 45% yield (Fig. 28.14) [28.35]. Reports have shown that *S*-ibuprofen is more active than *R*-ibuprofen in vitro inhibition of prostaglandin biosynthesis [28.36].

Another important commercial application of nitrilases is in the production of (-)-*R*-mandeleic acid by the bacteria *Alcaligenes faecalis* ATCC8750 (91% yield and > 99% ee) [28.34] and *Alcaligenes* sp. ECU0401 (26% yield and > 99.9% ee; Fig. 28.15) [28.37]. (-)-*R*-Mandeleic acid has wide application as an intermediate for the production of pharmaceutical products such as Cefaclor, a cephalosporin-type antibiotic [28.38].

Hydrolysis of Racemic Nitriles by Nitrile Hydratase

In general, nitrile hydratases are associated with amidases in living organisms and, in most cases, the formation of amides and optically pure acids is due to the higher enantioselectivity of amidases compared to nitrile hydratases. A number of acids and amides have been obtained by enzymatic methods; for example, *S*-naproxen was synthesized by *Rhodococcus* sp. C3II and *Rhodococcus erythropolis* MP50. The two bacterial strains, by combining nitrile hydratase and amidases, were able to convert the cyano group of the substrate into *S*-naproxen acid [(+)-(*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid] with an enantiomeric excess > 99% and conversion 80% (Fig. 28.16) [28.16].

Another interesting example is the enantioselective hydrolysis of (±)-1,4-benzodioxane-2-carbonitrile under mild conditions to (+)-(*S*)-1,4-benzodioxane-2-carboxylic acid (46% yield and 99% ee) and (-)-(*R*)-1,4-benzodioxane-2-amide (50% yield and 94% ee) by the bacterium *Rhodococcus* sp. AJ270 (Fig. 28.17) [28.16]. These compounds are important for the pharmaceutical industry, as intermediates in the synthesis of drugs [28.16]. (-)-(*S*)-1,4-Benzodioxane-2-carboxylic

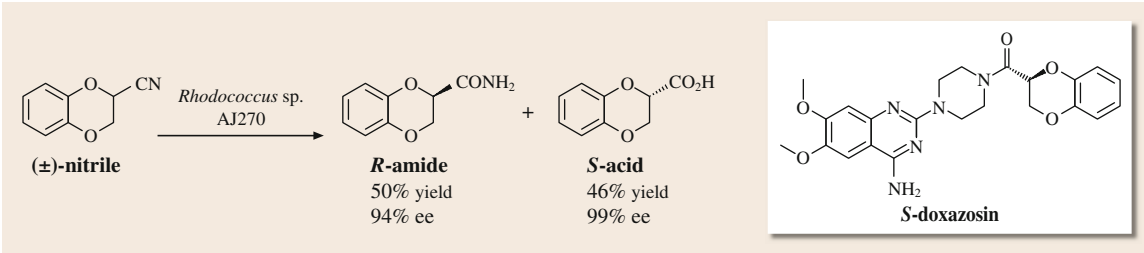


Fig. 28.17 Biotransformation of the (±)-1,4-benzodioxane-2-carbonitrile by *Rhodococcus* sp. AJ270

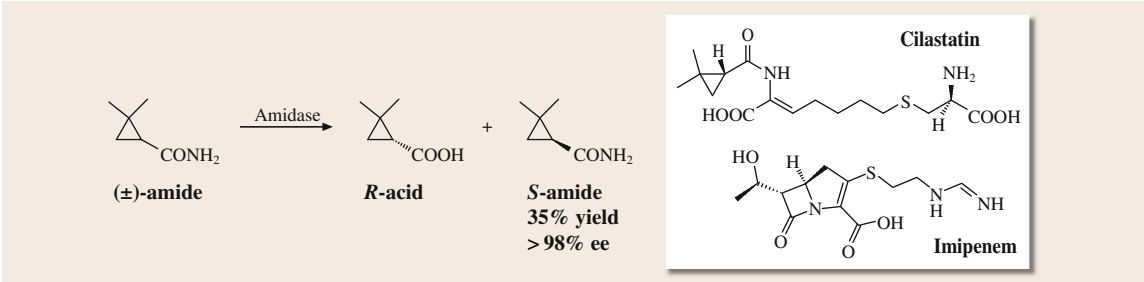


Fig. 28.18 Biotransformation of the (±)-2,2-dimethylcyclopropanecarboxamide by amidase from *Comomonas acidivorans*

acid is a chiral intermediate in the synthesis of *S*-doxazosin. (±)-Doxazosin is a drug produced by Pfizer named Canduran, indicated for the treatment of hypertension. *S*-enantiomer doxazosin has been shown to be effective in the treatment of benign prostatic hyperplasia [28.39].

Hydrolysis of Racemic Nitriles by Amidases

One example of industrial biotransformation catalyzed by the amidase from *Comomonas acidivorans* is the enzymatic resolution of (±)-2,2-dimethylcyclopropanecarboxamide yielding the (*S*)-isomer (35% yield and > 98% ee; Fig. 28.18) [28.40]. The *S*-isomer is used as an intermediate in the production of the antibiotic cilastatin, an inhibitor of renal dehydropeptidase. Cilastatin is commonly administered together with the antibiotic imipenem in a formulation named Tienam, indicated for the treatment of urinary tract infections [28.41]. This combination has the advantage of inhibiting the renal biotransformation of imipenem, making the treatment more effective [28.40].

Stereoselective amidases, isolated from bacterial cells, have been used in the kinetic resolution of racemic piperazine-2-carboxamide to the (*S*) and (*R*)-piperazine-2-carboxylic acids. The bacterial strain *Klebsiella terrigena* DSM 9174 catalyzed the biotransformation of (±)-piperazine-2-amide to (-)-(*S*)-piperazine-2-carboxylic acid (41% yield, 99.4% ee), while *Burkholderia* sp. DSM 9925 yielded the (+)-(*R*)-piperazine-2-carboxylic acid (22% yield and 99% ee; Fig. 28.19) [28.42].

The acids obtained belong to the class of non-proteinogenic amino acids and are used as precursors for numerous bioactive compounds [28.16]. For example, (-)-(*S*)-piperazine-2-carboxylic acid is an intermediate in the synthesis of Crixivan (Merk), a drug used in the treatment of HIV virus infections [28.42]. The biocatalytic synthesis of (-)-(*S*)-piperazine-2-carboxylic acid by *Klebsiella terrigena* DSM 9174 for commercial purposes was developed by Lonza AG.

Regioselective Hydrolysis of Dinitriles

Nishise et al. reported the synthesis of *trans*-4-cyanocyclohexane-1-carboxylic acid (*t*-MCC) from *trans*-1,4-dicyano cyclohexane by nitrilases of the bacterium *Acremonium* sp. (Fig. 28.20). *t*-MCC is an intermediate used in the synthesis of tranexamic acid (*trans*-4-aminomethyl-cyclohexane-1-carboxylic acid), which is a drug used to treat fetal hemorrhage [28.43].

A similar process is the regioselective biotransformation of adiponitrile to 5-cyanovaleric acid, which is used as an intermediate in the synthesis of the polymers

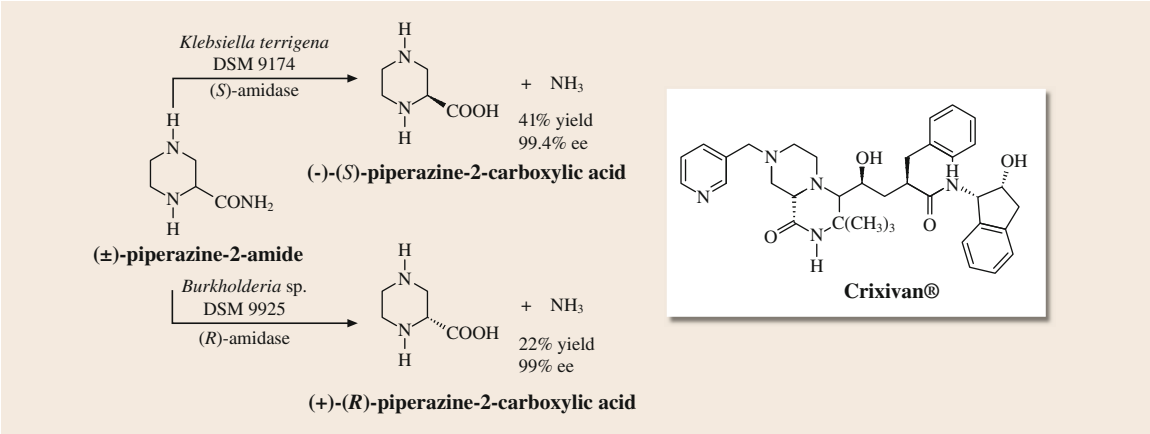


Fig. 28.19 Enantioselective hydrolysis of the (±)-piperazine-2-amide by bacterial amidases

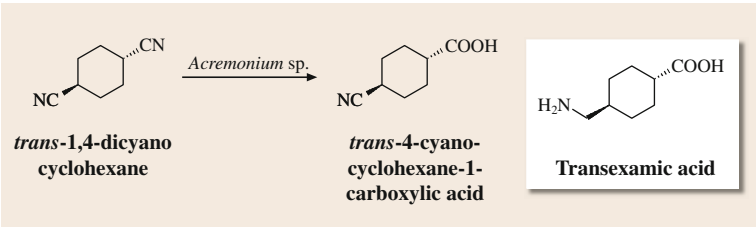


Fig. 28.20 Regioselective hydrolysis of the trans-1,4-dicyano cyclohexane by *Acremonium* sp.

nylon-6 e nylon-6,6. This reaction was catalyzed by nitrilases of the bacterium *Rhodococcus rhodochrous* K22 (Fig. 28.21) [28.14].

Desymmetrization of Dinitriles

One of the most important features of enzymes that metabolize nitriles is their ability selectively to convert one cyano group of a prochiral dinitrile; this reaction is very difficult to achieve by conventional chemical methods [28.14].

An example of enantioselective desymmetrization was the biotransformation of 3-hydroxyglutaronitrile to (*R*)-4-cyano-3-hydroxybutyric acid, catalyzed by the *R*-specific nitrilase BD9570, which was expressed in *Pseudomonas fluorescens*. This acid was obtained with enantiomeric excess ranging between 98.6–99.4%, and a conversion of approximately 99% (Fig. 28.22) [28.44]. (*R*)-4-Cyano-3-hydroxybutyric acid after esterification is used as an intermediate for the synthesis of Lipitor, a drug marketed by Pfizer to control cholesterol [28.20].

Desymmetrization of nitriles is not restricted to nitrilases. It can also be achieved with nitrile hydratases. For example, the transformation of 3-(benzyloxy)glutaronitrile to 3-(*S*)-(benzyloxy)pentanoic acid was catalyzed by the combined action of nitrile hydratase and amidases in *Brevibacterium* sp. B312 with 65% yield and 88% enantiomeric excess. 3-(Benzyloxy)pentanoic acid is an intermediate in the synthesis of the drug Lovastatin, used in the treatment of hypercholesterolemia and cardiovascular disease (Fig. 28.38) [28.45].

Chemoselective Hydrolysis of Nitriles

As for the performance of nitrile hydratases, two significant industrial applications are based on the bacteria *Rhodococcus rhodochrous* J1. More than 30 000 t/yr of acrylamide are produced by Nitto Chemical Industry Ltd. (Yokohama, Japan) from acrylonitrile. The reaction is extremely efficient, producing more than 7 kg of acrylamide per g cells in 99.97% conver-

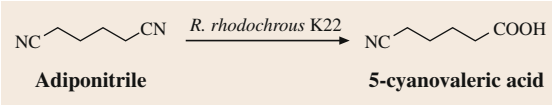


Fig. 28.21 Regioselective hydrolysis of adiponitrile by nitrilase from *Rhodococcus rhodochrous* K22

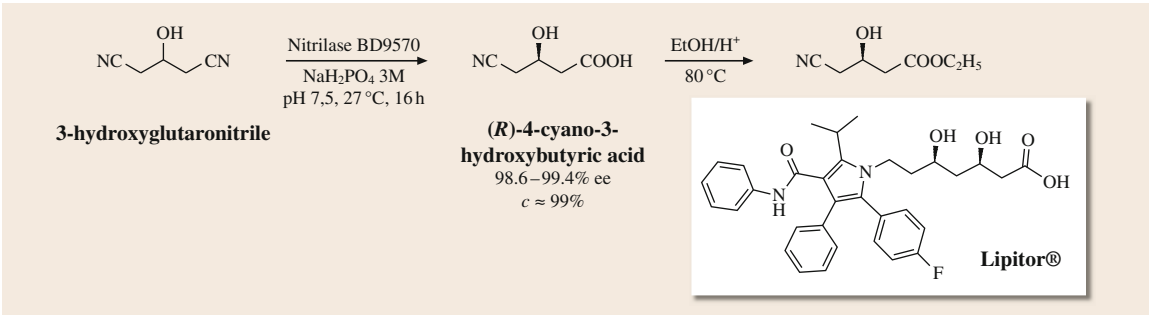


Fig. 28.22 Desymmetrization of the 3-hydroxyglutaronitrile by nitrilase BD9570

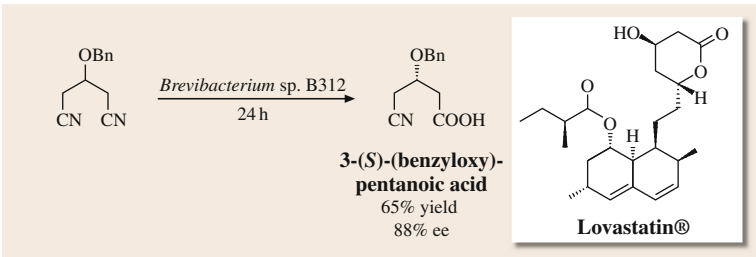


Fig. 28.23 Desymmetrization of the 3-benzyloxyglutaronitrile by *Brevibacterium* sp. B312

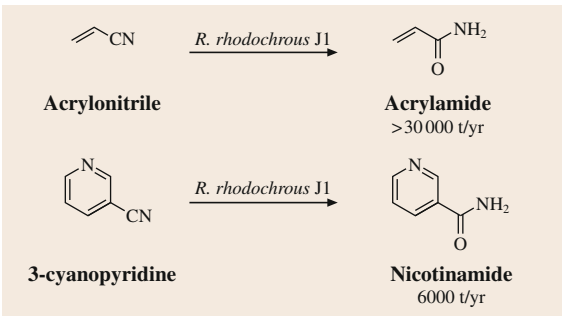


Fig. 28.24 Production of acrylamide and nicotinamide by nitrile hydratases from *Rhodococcus rhodochrous* J1

sion (Fig. 28.24). In addition to this bacterial strain, acrylamide was also produced by the bacteria *Pseudomonas chlororapis* and *Brevibacterium* sp., with > 99% yield [28.21, 22]. Acrylamide is an important chemical commodity for the synthesis of various polymers.

Another large-scale industrial process is the production of nicotinamide by the conversion of 3-cyanopyridine, which is marketed by Lonza. This process reaches 6000 t/year (Fig. 28.24). Nicotinamide is an essential nutrient in the diet of humans and animals. In contrast to the chemical process, the enzymatic process does not to form nicotinic acid as a by-product [28.12, 22].

The nitrile hydratase of *Rhodococcus rhodochrous* J1 was also effective in catalyzing the biotransformation of 4-cyanopyridine to isonicotinamide ($1.099\,g \cdot L^{-1}$), a precursor of isonicotinic acid hydrazide, a tuberculostatic agent (Fig. 28.25) [28.12, 46].

The bacterium *Rhodococcus rhodochrous* J1, induced by ε-caprolactam, activated its nitrilases to produce acrylic acid from acrylonitrile, providing $390\,g\,L^{-1}$ of the biotransformed compound (Fig. 28.26) [28.47]. Acrylic acid is a chemical commodity and the scale of this biotransformation exceeds 30 000 t/year [28.12]. Acrylic acid and its esters are used in paints, polymers, and paper [28.16].

The bacterium *Alcaligenes* sp. ECU0401 produced the glycolic acid (2-hydroxy-acetic acid) from glycolonitrile (2-hydroxy-acetonitrile) by the action of nitrilases, with a yield of 96.5% (Fig. 28.27). Gly-

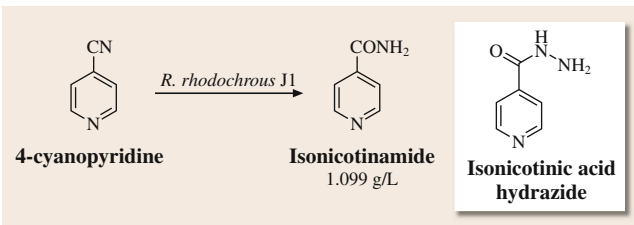


Fig. 28.25 Hydrolysis of the 4-cyanopyridine by *Rhodococcus rhodochrous* J1

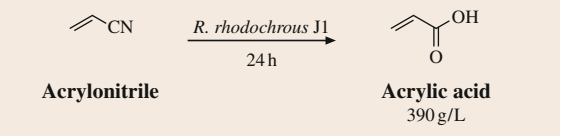


Fig. 28.26 Hydrolysis of acrylonitrile by *Rhodococcus rhodochrous* J1

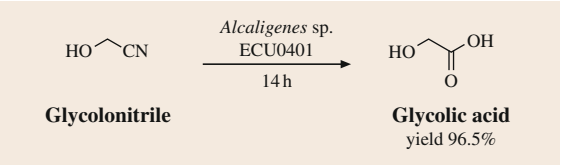


Fig. 28.27 Hydrolysis of glycolonitrile by *Alcaligenes* sp. ECU0401

colic acid has many applications in cosmetic products and is also an important ingredient in the processing of metals, automotive oil additives, and biodegradable polymers [28.48].

28.1.6 Biodegradation of Nitriles

Nitriles are highly toxic, carcinogenic, and mutagenic. Therefore, it is necessary to control the disposal of organonitriles into the environment and to monitor their presence there.

Typically, compounds such as acrylonitrile, acetonitrile, benzonitrile, and so on are widely used in laboratories and industry as solvents or as intermediates for pharmaceuticals (chiral blocks), plastics, synthetic rubber, and pesticides and herbicides, such as bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), ioxynil (3,5-diiodo-4-hydroxybenzonitrile and dichlobenil (2,6-dichlorobenzonitrile; Fig. 28.28) [28.16].

Many experiments have been performed on the biodegradation of nitriles, harnessing the huge potential demonstrated by enzymes that convert the cyano group. For example, prolonged exposure to the herbicides bromoxynil and dichlorobenil (Fig. 28.28) may result in symptoms such as weight loss, vomiting, fever, or headache [28.14]. Thus, enzymes that metabolize nitriles may be used to prevent these herbicides entering the food chain and causing harm to human health. The herbicides bromoxynil, ioxynil, and dichlobenil were degraded by the soil bacterium *Agrobacterium radiobacter*, by means of its nitrile hydratase. The efficacy of degradation was enhanced by adding iron or cobalt ions to the culture medium [28.49, 50].

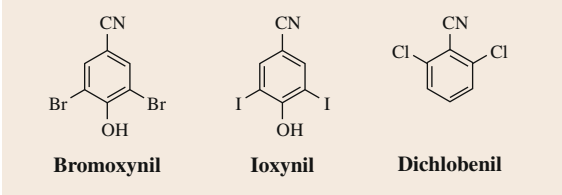


Fig. 28.28 Examples of herbicides containing the cyano group

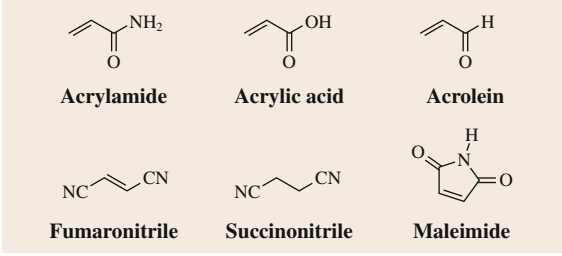


Fig. 28.29 Structures of the compounds present in industrial waste waters from acrylonitrile synthesis

The effluents from the synthesis of acrylonitrile contain acrylamide, acrylic acid, acrolein, cyanopyridine, fumaronitrile, succinonitrile, and maleimide, in addition to acrylonitrile (Fig. 28.29). *Wyatt* and *Knowles* [28.51] performed a study on degradation of acrylonitrile in industrial wastewater by microorganisms. A mixed culture of bacteria was grown in the presence of industrial effluents from the synthesis of acrylonitrile. In this study was observed that all components of the residual mixture were degraded by the mixed bacterial culture.

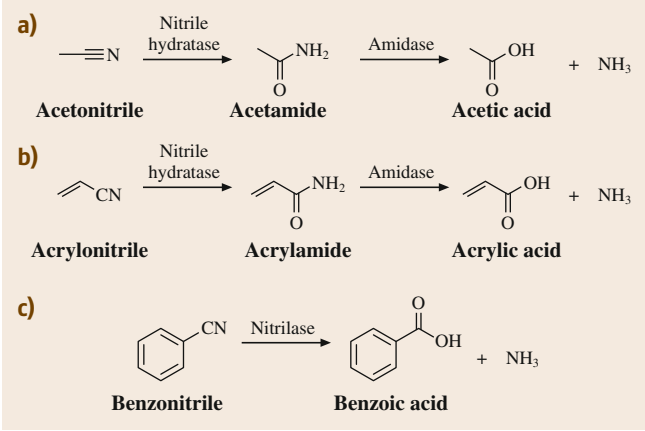


Fig. 28.30a–c Biodegradation of nitriles by a consortium of microorganisms

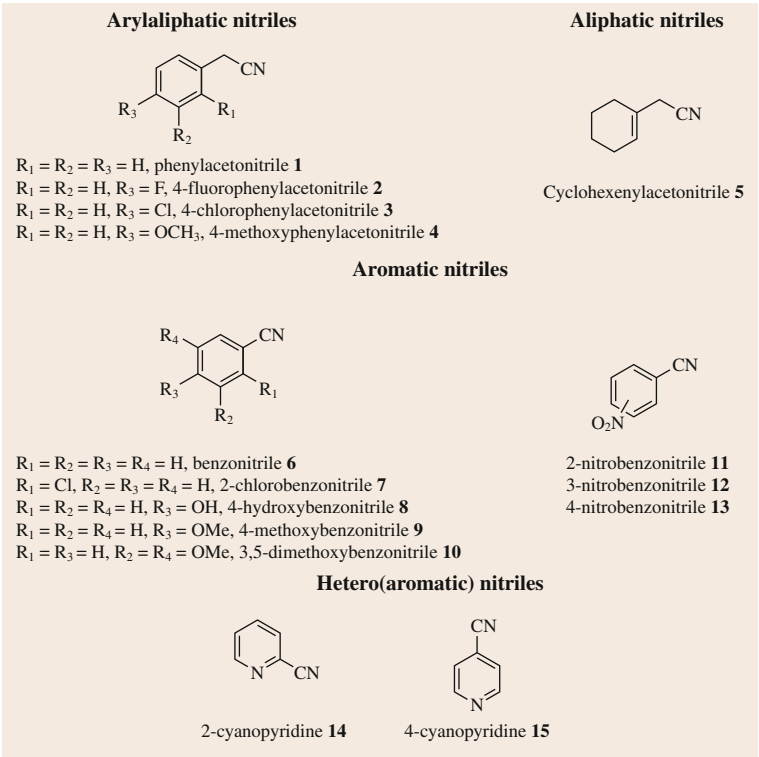


Fig. 28.31 Organonitriles selected for biotransformation by marine fungi

Recently, a process for microbial degradation of organonitriles (acetonitrile, benzonitrile, and acrylonitrile) in waste water from the pharmaceutical industry was assessed [28.16]. The degradation of these nitriles was investigated with microorganisms present in activated sludge in pharmaceutical the sewage water waste, whose growth was adapted in the presence of acetonitrile as the sole source of carbon and nitrogen [28.52]. The biodegradation of acetonitrile and acrylonitrile to their corresponding carboxylic acids occurred in two steps. Initially, the corresponding amides (acetamide and acrylamide) were obtained, then the acrylic and acetic acids, both reactions forming ammonia. On the other hand, biodegradation of benzonitrile occurred in a single step, with the direct production of benzoic acid and ammonia (Fig. 28.30).

The results obtained by *Li* et al. showed that irrespective of the substrate, a mixed culture of these microorganisms can develop distinct pathways of degradation, either via the nitrile hydratase followed by amidase or via nitrilase [28.52].

In most studies reported in the literature, the enzymatic hydrolysis of nitriles is carried out by microorganisms (fungi, yeasts, and bacteria) or plants. Recently, we reported the biotransformation of the phenylacetonitrile to 2-(2-hydroxyphenyl)acetic acid by marine fungi. This compound is often used in the pharmaceutical industry as an intermediate in the preparation of some biologically active products, such as antihypertensives [28.53, 54].

In view of the shortage of studies on biotransformation of organonitriles by marine microorganisms, in this chapter we aim to describe the methodology applied in our laboratory to select marine fungi capable of biotransforming nitriles. The methods will be described in detail and then the results achieved. The types of nitriles selected for enzymatic hydrolysis reactions by marine fungi are shown in Fig. 28.31.

28.2 Experimental Methods

28.2.1 Isolation, Identification, and Preparation of Stock Cultures of Marine Fungi

The fungal strains *Aspergillus sydowii* CBMAI 933 and *Penicillium miczynskii* CBMAI 930 were isolated from the marine sponge *Geodia corticostylifera*. *A. sydowii* CBMAI 934, *A. sydowii* CBMAI 935, *Bionectria* sp. CBMAI 936, and *P. raistrickii* CBMAI 931 were isolated from marine sponge *Chelonaplysilla erecta*. *A. sydowii* CBMAI 1241, *P. raistrickii* CBMAI 1235, *P. decaturense* CBMAI 1234, and *Cladosporium* sp. CBMAI 1237 were isolated from marine sponge *Dragmacidon reticulate*. *P. oxalicum* CBMAI 1185 and *P. citrinum* CBMAI 1186 were isolated from the marine alga *Caulerpa* sp. The sponges and algae were collected in the South Atlantic Ocean off the northern coast of the state of São Paulo at São Sebastião, Brazil. The fungi were identified by both conventional and molecular methods at the Chemical, Biological and Agricultural Pluridisciplinary Research Center at UNICAMP, São Paulo, Brazil (http://www.cpqba.unicamp.br/). Stock cultures of the fungi were stored on a solid culture medium [K_2HPO_4 (1.0 g L^{-1}), $MgSO_4 \cdot 7\ H_2O$ (0.5 g L^{-1}), KCl (0.5 g L^{-1}), $FeSO_4 \cdot 7\ H_2O$ (0.01 g L^{-1}), $CoCl_2 \cdot 6H_2O$ (0.001 g L^{-1}), $ZnSO_4 \cdot 7\ H_2O$ (0.0067 g L^{-1})], glucose (15.0 g L^{-1}), phenylacetonitrile **1** (0.2, 0.4 or 0.6 g L^{-1}) and agar (15.0 g L^{-1}) in Petri dishes maintained at 4 °C in the refrigerator.

28.2.2 Cultivation of Marine Fungi on a Solid Medium in the Presence of Phenylacetonitrile 1

Marine fungi were cultured on Petri dishes (10 × 1 cm) containing solid mineral culture medium (glucose) supplemented with phenylacetonitrile **1** 5.0 µL (0.04 mmol), 10.0 µL (0.08 mmol), 15.0 µL (0.12 mmol), and 20.0 µL (0.17 mmol) per plate. Phenylacetonitrile **1** was added to the agar at 40–45 °C and the homogenous mixture was poured into Petri dishes. Fungal mycelia were inoculated to the surfaces of the agar plates with an inoculating loop. Next, the fungi were incubated at 32 °C, and the tolerance to phenylacetonitrile **1** was estimated by the size of the colony formed on the surface of the plates after 8 days. Control plates of mineral medium and glucose were prepared, without the addition of phenylacetonitrile **1**.

28.2.3 Biotransformation of Phenylacetonitrile 1 by Marine Fungi in a Liquid Medium

Small slices of solid medium (0.5 × 0.5 cm) bearing the mycelia of eight marine fungal strains were cut from the solid stock cultures and used to inoculate 100.0 mL of liquid culture mineral medium containing 15.0 g glucose in 250 mL Erlenmeyer flasks. The cultures were incubated at 32 °C on a rotary shaker (124 rpm), with various volumes of phenylacetonitrile **1** added to the medium (20.0 µL, 0.17 mmol; 40.0 µL, 0.34 mmol; 60.0 µL, 0.52 mmol; Table 28.1). After the growth of the strains in liquid medium for 8 days, the mycelia were filtered on a Buchner funnel and the mycelial dry weight was determined after drying at 70 °C for 24 h. The results are summarized in Table 28.2. The biotransformation of phenylacetonitrile **1** was monitored every 2 days by collecting 2.0 mL samples, which were extracted by stirring with ethyl acetate (2.0 mL), centrifuged (6000 rpm, 6 min), and analyzed by analyzed by GC-FID (Gas Chromatography-Flame Ionization Detector) and GC-MS (Gas Chromatography-Mass Spectrometry) (Table 28.3).

28.2.4 Isolation of 2-Hydroxyphenylacetic Acid 1a from Culture Media Produced by *A. sydowii* CBMAI 934

An additional experiment was performed with the fungus *A. sydowii* CBMAI 934 for isolation and purification of the biotransformed product. Biocatalytic reactions were carried out for 96 h in quintuplicate, with 60.0 µL (0.52 mmol) of phenylacetonitrile **1** in 100.0 mL medium per Erlenmeyer flask. Next, another 60.0 µL of phenylacetonitrile **1** was added and the reactions were maintained on the orbital shaker (124 rpm, 32 °C) for a further 96 h. The reaction mixtures were filtered, the combined filtrates were extracted with EtOAc (3 × 50.0 mL), and the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum. The mycelia (16.0 g) were transferred to an Erlenmeyer and stirred for 30 min with 50.0 mL of distilled water. Next, 50.0 mL of EtOAc was added, and the mixture was stirred for 30 min. The mycelia were filtered off, and the filtrate was extracted with ethyl acetate (3 × 50.0 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and the solvent evaporated

Table 28.1 Growth of marine fungi on a solid medium with various amounts of phenylacetonitrile **1** (32 °C, 8 days)

Marine fungi	Colony diameter (cm)			
	5 µL (0.04 mmol)	10 µL (0.08 mmol)	15 µL (0.12 mmol)	20 µL (0.17 mmol)
Penicillium miczynskii CBMAI 930	[a]	–	–	–
Penicillium raistrickii CBMAI 931	1.5	1.5	1.5	0.5
Aspergillus sydowii CBMAI 933	1.5	2.0	2.0	[a]
Aspergillus sydowii CBMAI 934	1.5	1.8	1.8	[a]
Aspergillus sydowii CBMAI 935	1.5	2.0	2.0	0.5
Bionectria sp. CBMAI 936	2.5	3.5	3.5	[a]
Penicillium decaturense CBMAI 1234	1.5	1.5	1.5	[a]
Penicillium raistrickii CBMAI 1235	1.0	1.0	1.0	[a]
Cladosporium sp. CBMAI 1237	[a]	–	–	–
Aspergillus sydowii CBMAI 1241	1.2	1.5	1.5	[a]
Penicillium oxalicum CBMAI 1185	[a]	–	–	–
Penicillium citrinum CBMAI 1186	[a]	–	–	–

[a] No colony formed

Table 28.2 Growth of marine fungi in liquid mineral medium (100.0 mL) with various amounts of phenylacetonitrile **1** (32 °C, 124 rpm, 8 days)

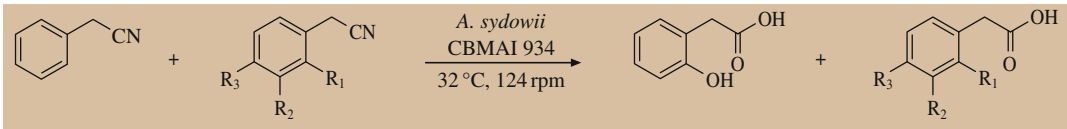
Marine fungi	Volume of phenylacetonitrile 1 (µL)/Erlenmeyer flask		
	20.0 (0.17 mmol)	40.0 (0.34 mmol)	60.0 (0.52 mmol)
	Total cell dry weight (g)		
Aspergillus sydowii CBMAI 935	0.61	0.75	0.90
Aspergillus sydowii CBMAI 934	0.61	0.66	0.94
Aspergillus sydowii CBMAI 1241	0.72	0.95	1.10
Aspergillus sydowii CBMAI 933	0.69	0.72	0.72
Bionectria sp. CBMAI 936	0.46	0.59	0.63
Penicillium decaturense CBMAI 1234	0.41	1.10	1.46
Penicillium raistrickii CBMAI 931	1.10	1.10	1.10
Penicillium raistrickii CBMAI 1235	0.88	0.95	1.28

under vacuum. The extracts obtained were analyzed by GC-FID and GC-MS. The extracts obtained from the filtered medium and the mycelia were combined and purified by flash column chromatography (CC) over silica gel to yield 2-hydroxyphenylacetic acid **1a** (51% yield). The spectroscopic data of the isolated 2-hydroxyphenylacetic acid **1a** were in agreement with those reported in the literature [28.55].

28.2.5 Biotransformation and Isolation of Phenylacetonitrile Derivatives 2–4 by *A. sydowii* CBMAI 934

Small slices of solid medium (0.5 × 0.5 cm) bearing mycelia of the fungal strain were cut from the solid stock cultures and used to inoculate 100.0 mL of liquid culture mineral medium with glucose (15.0 g L^{-1}), containing 60 µL of phenylacetonitrile **1** (0.52 mmol), in 250 mL Erlenmeyer flasks. The marine fungus was grown at 32 °C on a rotary shaker (124 rpm). After growth of the strain in liquid medium for 96 h, the following nitriles were added separately to each experiment: 4-fluorophenylacetonitrile **2** (20.0 µL (0.17 mmol), 40 µL (0.34 mmol), 60 µL (0.51 mmol)), 4-chlorophenylacetonitrile **3** (20.0 µL (0.16 mmol), 40.0 µL (0.32 mmol), 60.0 µL (0.48 mmol)), and 4-methoxyphenylacetonitrile **4** (20.0 µL (0.15 mmol), 40.0 µL (0.30 mmol), 60.0 µL (0.45 mmol)). The biotransformations of nitriles **2–4** were monitored every 24 h by collecting

Table 28.3 Biotransformation of phenylacetonitrile **1** and its derivatives **2–4** by marine fungus *A. sydowii* CBMAI 934[a]



1 | $R_1 = R_2 = H, R_3 = F$ (**2**); $R_1 = R_2 = H, R_3 = Cl$ (**3**); $R_1 = R_2 = H, R_3 = OMe$ (**4**) | **1a** | **2a-4a**

Time (h)	c (%)	c (%)	*c* (%)	*c* (%) [Yield] (%)
#	**1**	**2**	**1a**	**2a**
24	100	–	–	–
96[b]	–	100	100	–
120	–	–	12	88 [28.51]
#	**1**	**3**	**1a**	**3a**
24	100	–	–	–
96[b]	–	100	100	–
120	–	–	10	90 [28.55]
#	**1**	**4**	**1a**	**4a**
24	100	–	–	–
96[b]	–	100	100	–
120	–	50	28	22
192	–	–	12	88 [28.43]

[a] (Sect. 28.2).
[b] The nitriles **2–4** were added (Sect. 28.2).
c = concentration of unreacted nitriles **1–4** determined by GC-FID analysis.
c = conversion determined by GC-FID analysis.

2.0 mL of samples, which were extracted by stirring with EtOAc (Ethyl Acetate) (2.0 mL) and then centrifuged (6, 000 rpm, 6 min) and analyzed by GC-FID (Fig. 28.32).

28.2.6 Biotransformation of Cyclohexenylacetonitrile 5 by *A. sydowii* CBMAI 934

Initially, the fungal strain was cultured as described in Sect. 28.2.5. After the initial growth of the mycelia, cyclohexenylacetonitrile **5** (20.0 µL (0.16 mmol), 40.0 µL (0.32 mmol), and 60.0 µL (0.48 mmol)) was added to the flasks. In a second experiment, after fungal growth as in Sect. 28.2.5, the mycelia were pooled (3 in 1) and transferred to a 1 L Erlenmeyer flask, and then further cyclohexenylacetonitrile **5** (60.0 µL, 0.48 mmol) was added. The biotransformation of nitrile **5** was monitored every 24 h for 96 h by collecting 2.0 mL samples, which were extracted by stirring with ethyl acetate (2.0 mL), centrifuged (6000 rpm, 6 min) and analyzed by GC-FID (Fig. 28.33).

28.2.7 Biotransformation of Aromatic Nitriles

After the growth of the fungal strain in 100.0 mL liquid culture mineral medium supplemented with glucose (15.0 g/L) and phenylacetonitrile **1** (60.0 µL, 0.52 mmol) for 96 h in 250 mL Erlenmeyer flasks, the respective aromatic nitriles were added: benzonitrile **6** (20.0 µL, 0.19 mmol), 2-chlorobenzonitrile **7** (20.0mg, 0.15 mmol), 4-hydroxybenzonitrile **8** (20.0 mg, 0.17 mmol), 4-methoxybenzonitrile **9** (20.0mg, 0.20 mmol), 3,5-dimethoxybenzonitrile **10** (20.0mg, 0.12 mmol), 2-nitrobenzonitrile **11** (20.0mg, 0.14 mmol), 3-nitrobenzonitrile **12** (20.0mg, 0.13 mmol) and 4-nitrobenzonitrile **13** (20.0mg, 0.14 mmol). The reactions were monitored every 24 h by collecting 2.0 mL aliquots, which were extracted by stirring with EtOAc (2.0 mL), fol-

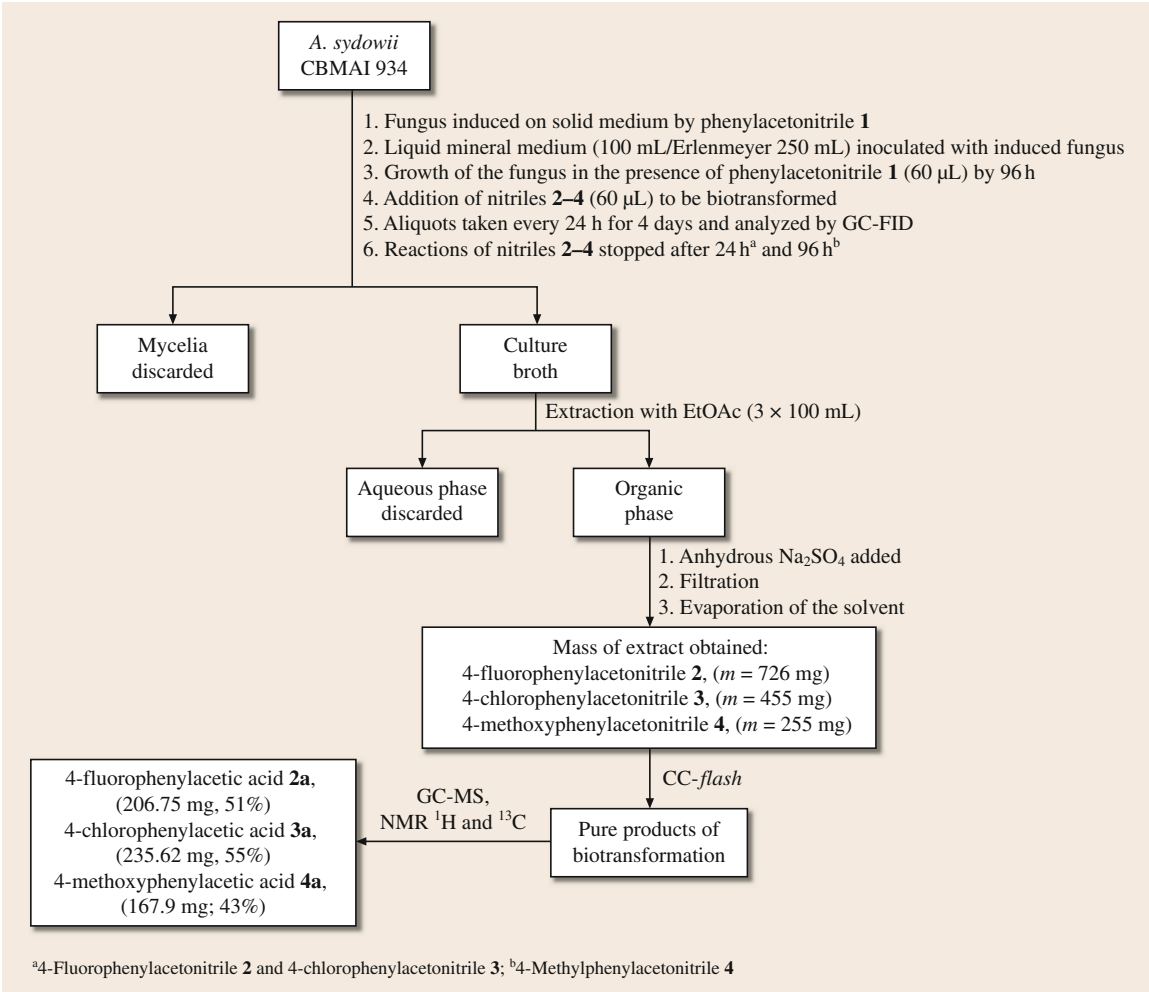


Fig. 28.32 Biotransformation and isolation of phenylacetonitrile derivatives **2-4** by *A. sydowii* CBMAI 934

lowed by centrifugation (6000 rpm, 6 min), and analyzed by GC-FID.

28.2.8 Growth of Marine Fungus *A. sydowii* CBMAI 934 in the Presence of 2-Cyanopyridine 14 in Solid and Liquid Media

Marine fungus was cultured on plates (10 cm × 1 cm) of solid culture mineral medium, with glucose (15.0 g L^{-1}) and supplemented with 5.0 µL 2-cyanopyridine **14** (0.052 mmol) per plate as follows. The 2-cyanopyridine **14** was added to the agar at 40–45 °C and the homogenous mixture was harvested in Petri dishes. Fungal mycelium was transferred to the surface of the agar with an inoculating loop, and the plates were incubated at 32 °C for 8 days. The tolerance to 2-cyanopyridine **14** was estimated by the size of the colony formed on the surface of the plate after 8 days. Next, small slices of solid medium (0.5 × 0.5 cm) bearing mycelium of the marine fungal strain were cut from the solid stock cultures and used to inoculate 100.0 mL of liquid culture mineral medium containing glucose (15.0 g L^{-1}) and 2-cyanopyridine **14** (20.0 µL, 0.21 mmol) in a 250 mL Erlenmeyer flask. The marine fungus was grown at 32 °C on a rotary shaker (124 rpm) for 96 h. The reactions were monitored every 2 days by collecting 2.0 mL samples, which were extracted by stirring with EtOAc (2.0 mL), followed by centrifugation (6000 rpm, 6 min), and analyzed by GC-FID.

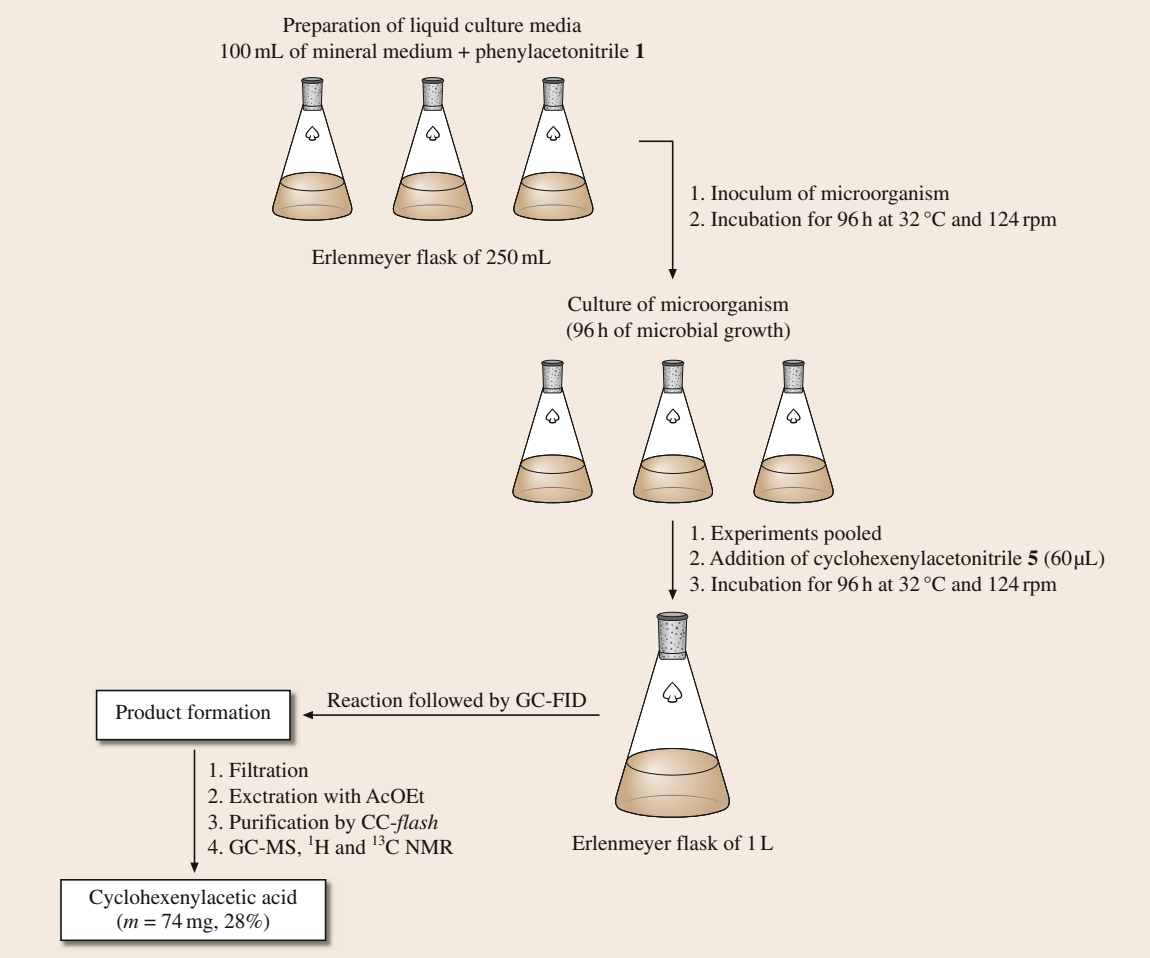


Fig. 28.33 Biotransformation of cyclohexenylacetonitrile **5** by *A. sydowii* CBMAI 934

28.2.9 Biotransformation of Benzonitrile 6 and 2-Cyanopyridine 14

Two different experiments were carried out with these nitriles. In the first, after the fungus had grown in 100.0 mL of the liquid culture mineral medium in Erlenmeyer flasks (250 mL) for 96 h in the presence of glucose (15.0 g L^{-1}) and phenylacetonitrile **1** (60.0 µL, 0.52 mmol), benzonitrile **6** (20.0 µL, 0.19 mmol) and 2-cyanopyridine **14** (20.0 µL, 0.21 mmol) were added simultaneously. In the second experiment, after the fungal growth in the presence of glucose (15.0 g L^{-1}) and phenylacetonitrile **1** (60.0 µL, 0.52 mmol) for 96 h, 2-cyanopyridine **14** (20.0 µL, 0.21 mmol) alone was added to the liquid culture mineral medium. The reactions were monitored every 24 h by collecting 2.0 mL samples, which were extracted by stirring with EtOAc (2.0 mL), centrifuged (6000 rpm, 6 min), and analyzed by GC-FID and GC-MS.

28.3 Results and Discussion

28.3.1 Screening of Marine Fungi with Phenylacetonitrile 1 in Solid and Liquid Media

Initially, the biotransformation of phenylacetonitrile **1** by marine fungi was conducted on solid culture media. The microorganisms were grown on plates of a mineral medium supplemented with glucose and phenylacetonitrile **1**, as carbon and nitrogen sources, respectively. The growth of the fungal colonies was observed on the surface of agar containing phenylacetonitrile **1** (5.0 μL, 0.04 mmol) per plate at 32 °C for 8 days. Table 28.1 summarizes the results of the qualitative growth test of 12 marine fungi on the solid culture media in the presence of phenylacetonitrile **1**. Eight strains exhibited good colony formation, while four isolates (*P. miczynskii* CBMAI 930, *P. oxalicum* CBMAI 1185, *P. citrinum* CBMAI 1186, and *Cladosporium* sp. CBMAI 1237) did not grow under these conditions (Table 28.1).

Control experiments were conducted on the 12 strains by inoculating the mineral agar medium supplemented with glucose and without addition of phenylacetonitrile **1**. In these experiments, more of the marine fungi grew on the agar. This result was important for the selection of marine fungi with a potential for biotransformation of phenylacetonitrile **1**, since there strains used **1** effectively as a nitrogen source. The conditions described here were responsible for the excellent results obtained in the biotransformation of phenylacetonitrile **1**. The marine fungi cultured with phenylacetonitrile **1** at a concentration of 5.0 μL per plate were evaluated with 10.0 μL (0.08 mmol), 15.0 μL (0.12 mmol), and 20.0 μL (0.17 mmol) per plate (Table 28.1). Several strains showed better growth at intermediate concentrations of phenylacetonitrile **1** (10.0 and 15.0 μL), while the growth of the strains of *Penicillium* remained unchanged from 5.0 to 15.0 μL (Table 28.1). These results showed that increasing the concentration of phenylacetonitrile **1** as the sole nitrogen source favored the growth of the marine fungi. However, all the microorganisms were inhibited at 20.0 μL of phenylacetonitrile **1** per plate (Fig. 28.34).

In order to investigate the biotransformation of phenylacetonitrile **1**, experiments were conducted in liquid mineral medium in the presence and absence of nitrile **1** (Table 28.2).

To confirm the biotransformation of the phenylacetonitrile **1**, aliquots of liquid medium were collected every 24 h, extracted, and analyzed by GC-FID. The chromatograms showed a product peak, with a retention time of 10.4 min, with a total consumption of phenylacetonitrile **1** at 96 h of reaction (Fig. 28.35). However, no biotransformation of nitrile **1** occurred in the malt extract medium, and the added nitrile was recovered unchanged and in quantitative yield. In conclusion, the marine fungi catalyzed the biotransformation of phenylacetonitrile **1** to 2-hydroxyphenylacetic acid **1a**, only when previously cultured in a liquid mineral medium. The marine fungi cultured in the liquid mineral medium in the absence of the nitrile showed no biotransformation of the nitrile added later. When the fungi were cultured under nutrient-rich conditions, as in 2% malt

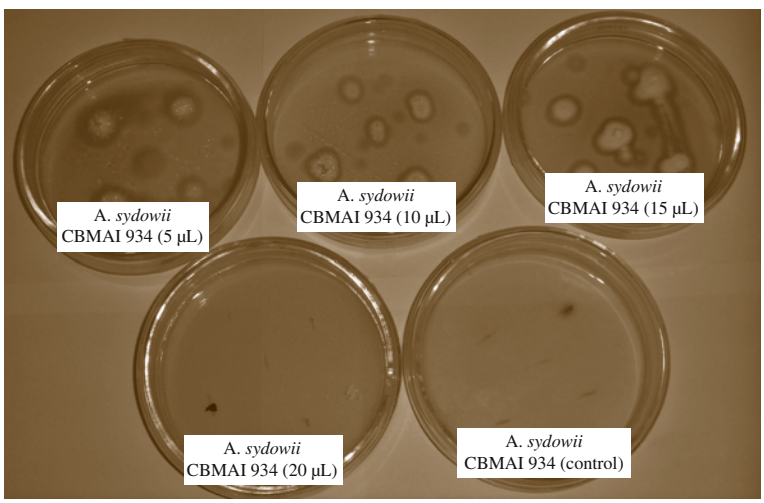


Fig. 28.34 Growth of the fungus *A. sydowii* CBMAI 934 on a solid medium with various amounts of phenylacetonitrile **1** (5, 10, 15, and 20 μL/plate) and without phenylacetonitrile **1** (control)

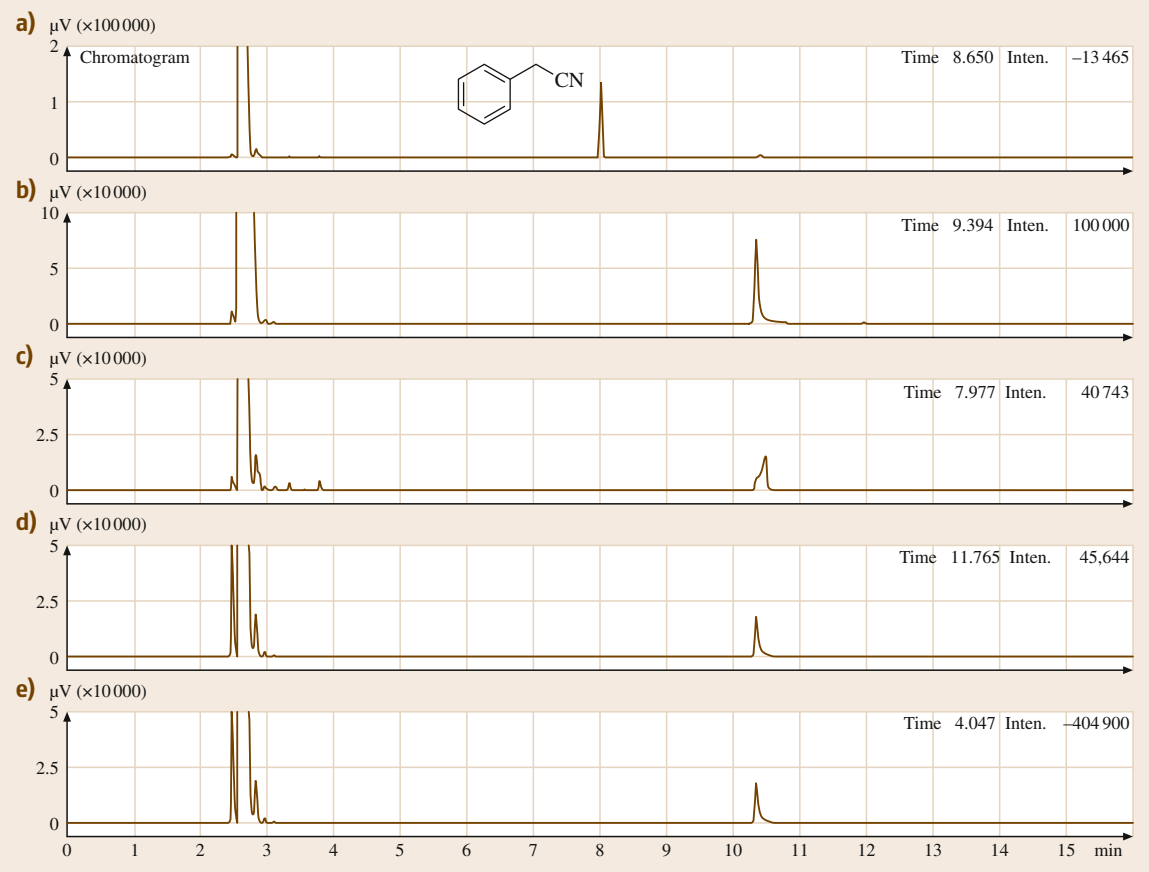


Fig. 28.35a–e GC-FID Chromatograms. **(a)** Reaction with the fungus *A. sydowii* CBMAI 934 in 60 µL of phenylacetonitrile **1** at 48 h. **(b)** Reaction with the fungus *A. sydowii* CBMAI 934 in 60 µL of phenylacetonitrile **1** at 96 h. **(c)** Reaction with the fungus *A. sydowii* CBMAI 934 24 h after having added another 60 µL of phenylacetonitrile **1** at 96 h. **(d)** Extract of the culture broth. **(e)** Extract of mycelial cells

medium, the enzymes capable of the biotransforming phenylacetonitrile **1** were probably not active. Thus, enzymes capable of hydrolyzing the cyano group present in the marine fungi are probably inducible enzymes, which require an inducer (such as nitrile **1**) in the growth medium of the microorganism.

For the isolation and characterization of 2-hydroxyphenylacetic acid (**1a**), experiments were carried out in quintuplicate in a liquid culture medium inoculated with *A. sydowii* CBMAI 934. This fungus showed favorable growth on the Petri plate within 8 days and in liquid culture, and compared to other fungi adapted to growth in a mineral medium supplemented with glucose and phenylacetonitrile **1**.

Initially, phenylacetonitrile **1** (60.0 µL, 0.52 mmol) was added to each flask, and the fungus was inoculated and incubated on a rotating orbital shaker platform (96 h, 124 rpm, 32 °C). Next, a further 60.0 µL of phenylacetonitrile **1** was added per Erlenmeyer flask, and the reactions were maintained in the rotary shaker (96 h, 124 rpm, 32 °C). The mycelia were harvested by filtration and the extract obtained was purified. The product was identified and characterized as 2-hydroxyphenylacetic acid **1a** by NMR (Nuclear Magnetic Resonance), IR (Infrared Spectroscopy) and HRMS (High Resolution Mass Spectrometry) analyses [28.53].

The production of 2-hydroxyphenylacetic acid **1a** from phenylacetonitrile **1** by marine fungi involved a sequence of enzymatic reactions. In this case, the fungi cultured in the presence of phenylacetonitrile **1**, in a liquid mineral medium, developed an efficient enzy-

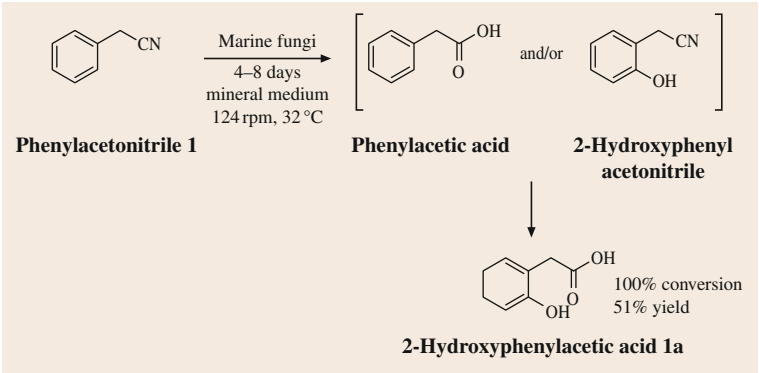


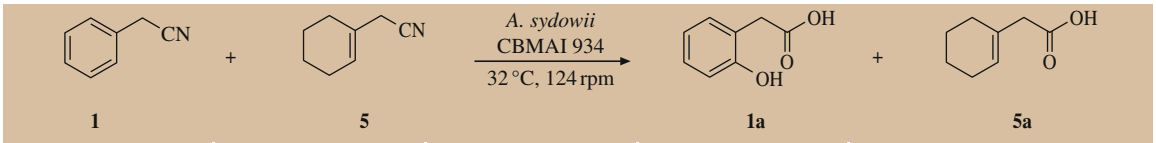
Fig. 28.36 Possible pathway for the biotransformation of phenylacetonitrile **1** to 2-hydroxyphenylacetic acid **1a** by induced marine fungi in liquid medium containing mineral salts, glucose, and phenylacetonitrile **1** (after [28.53])

matic pathway to produce 2-hydroxyphenylacetic acid **1a** (Fig. 28.36). Two pathways are possible: phenylacetonitrile **1** may be hydrolyzed to the carboxylic acid, after which enzymatic hydroxylation occurs at the *ortho* position of the aromatic ring, leading to 2-hydroxyphenylacetic acid **1a**. In an alternative route, the hydroxylation could occur first and subsequently the hydrolysis of the nitrile group. Intermediate compounds such as phenylacetic acid or 2-hydroxyphenylacetonitrile were not observed (Fig. 28.36).

Possibly, the phenylacetonitrile **1** is first hydrolyzed to the corresponding carboxylic acid by a nitrilase and/or nitrile hydratase followed by amidase. The phenylacetic acid obtained may then be hydroxylated regioselectively at the *ortho* position by a mono-oxygenase providing 2-hydroxyphenylacetic acid **1a**. *Mason* et al. observed that mono-oxygenases are often involved in hydroxylation of aromatic rings [28.56].

Selective hydroxylation of aromatic compounds catalyzed by enzymes is of great interest to the pharmaceutical and agrochemical industries [28.57]. Recently, a pathway for the total degradation of phenylacetonitrile **1** by the yeast *Exophiala oligosperma* R1 was investigated and two key intermediates were

Table 28.4 Biotransformation of phenylacetonitrile **1** and cyclohexenylacetonitrile **5** by marine fungus *A. sydowii* CBMAI 934[a]



1 **5** **1a** **5a**

Time (h)	c (%) 1	c (%) 5	*c* (%) 1a	*c* (%) [Yield] (%) 5a
Phenylacetonitrile (**1**, 60.0 µL, 0.52 mmol) and cyclohexenylacetonitrile (**5**, 60.0 µL, 0.48 mmol)[c]				
24	100	–	–	–
96[b]	–	100	100	–
120	–	91	6	3
192	–	85	3	12
Phenylacetonitrile (**1**, 60.0 µL, 0.52 mmol) and cyclohexenylacetonitrile (**5**, 60.0 µL, 0.48 mmol)[d]				
24	100	–	–	–
96[b]	–	100	100	–
120	–	49	13	28
192	–	24	4	72 [28.28]

[a] (Sect. 28.2)
[b] The nitrile **5** was added (Sect. 28.2)
[c] Biotransformation of the cyclohexenylacetonitrile **5** (60.0 µL, 0.47 mmol) when cultures were not pooled (Sect. 28.2).
[d] Biotransformation of the cyclohexenylacetonitrile **5** (60.0 µL, 0.47 mmol) cultures pooled in sets of three (Sect. 28.2).
c = concentration of unreacted nitriles **1** and **5** determined by GC-FID analysis.
c = conversion determined by GC-FID analysis.

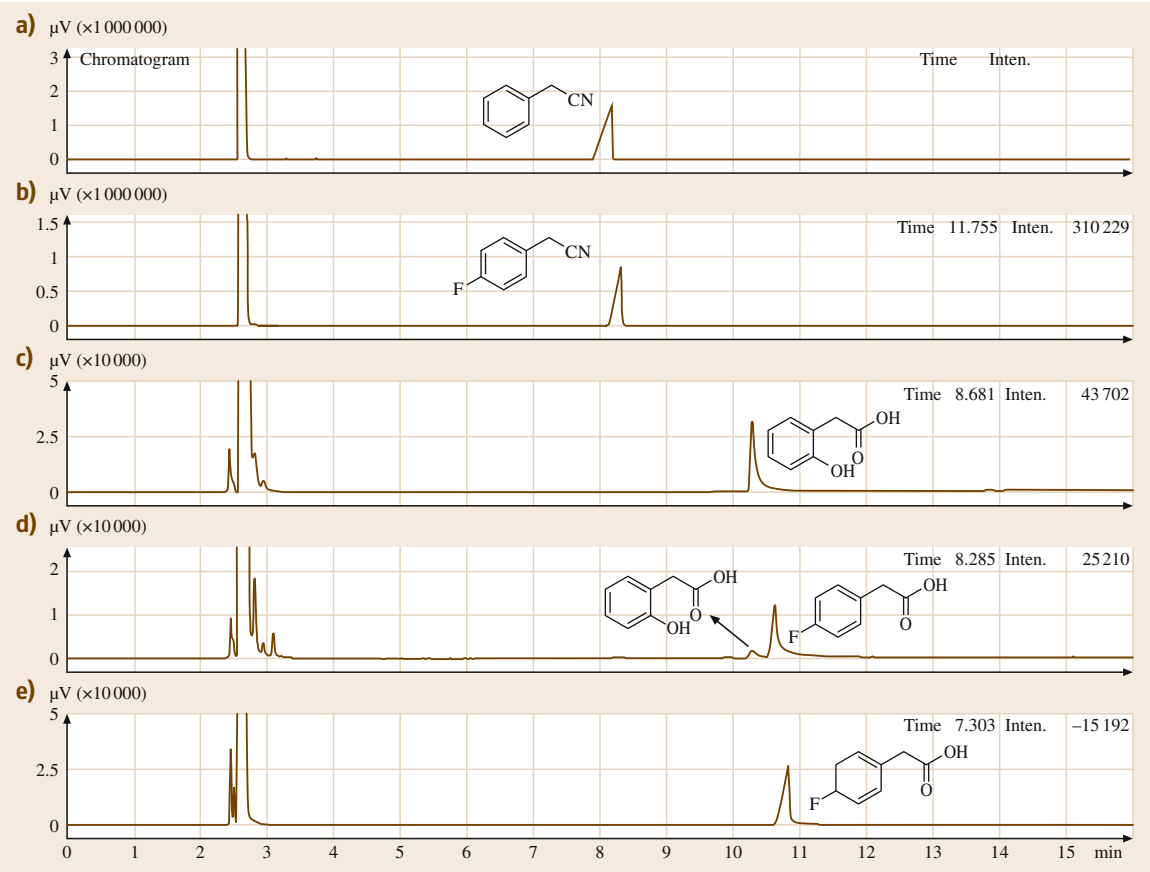


Fig. 28.37a–e GC-FID Chromatograms. **(a)** Standard of phenylacetonitrile **1**. **(b)** Standard of 4-fluorophenylacetonitrile **2**. **(c)** Standard of 2-hydroxyphenylacetic acid **1a**. **(d)** Biotransformation of 4-fluorophenylacetonitrile **2** (60.0 µL, 0.47 mmol) by *A. sydowii* CBMAI 934 in 24 h. **(e)** 4-Fluorophenylacetic acid **2a** purified by flash chromatography. Conditions of GC-FID analysis: $T_i = 100\,^{\circ}C$ (2 min), $T_f = 160\,^{\circ}C$ (2 min), $r = 5\,^{\circ}C/min$, $t_c = 16$ min

found, phenylacetic acid and 2-hydroxyphenylacetic acid **1a** [28.58, 59]. This type of reaction catalyzed by microorganisms in a single step is a promising method to synthesize products of interest under mild conditions. 2-Hydroxyphenylacetic acid **1a** is often used in the pharmaceutical industry as an intermediate in the preparation of some biologically active products, such as antihypertensives [28.54].

28.3.2 Biotransformation of Phenylacetonitrile Derivatives 2–4 by the Marine Fungus *A. sydowii* CBMAI 934

Selective biotransformations of the phenylacetonitrile derivatives, 4-fluorophenylacetonitrile **2**, 4-chlorophenylacetonitrile **3**, and 4-methoxyphenylacetonitrile **4**, as well as cyclohexenylacetonitrile **5**, which contains the nitrile group linked to an sp^3 carbon atom, were investigated in the presence of *A. sydowii* CBMAI 934 (Tables 28.3 and 28.4). The aim of this experiment was to test whole mycelia of this fungus, to assess its behavior with arylaliphatic and aliphatic nitriles, so as to catalyze the biotransformation of this class of compounds. The nitrile-converting enzyme activity was first induced in *A. sydowii* CBMAI 934 by phenylacetonitrile **1**, in solid and liquid mineral media [28.53].

The nitriles **2** and **3** were completely converted into carboxylic acids **2a** and **3a** (20.0, 40.0, and 60.0 µL nitrile per reaction) in 24 h, after total conversion of phenylacetonitrile **1** (which took 96 h), while nitrile **4**

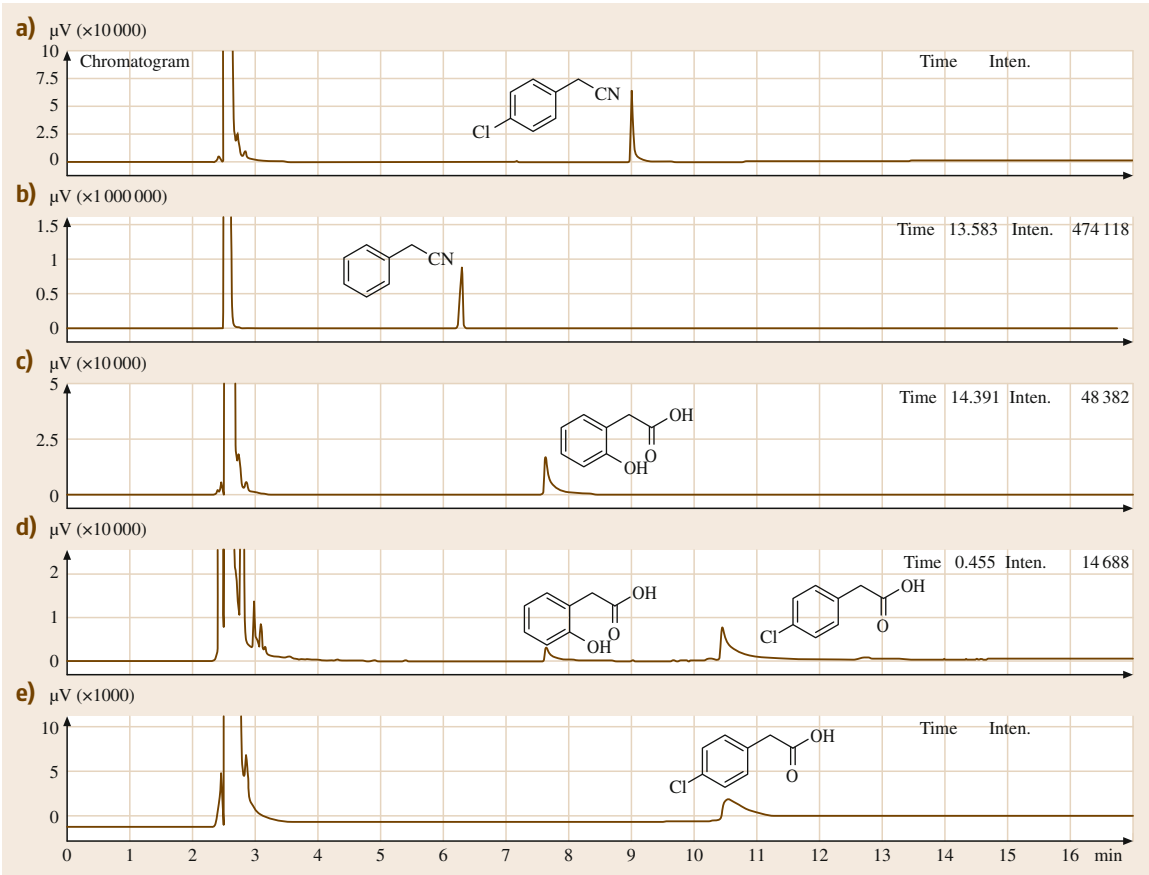
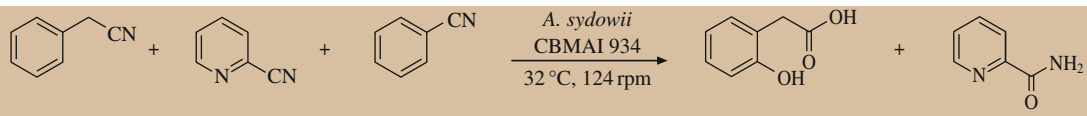


Fig. 28.38a–e GC-FID Chromatograms. **(a)** Standard of 4-chlorophenylacetonitrile **3**. **(b)** Standard of phenylacetonitrile **1**. **(c)** Standard of 2-hydroxyphenylacetic acid **1a**. **(d)** Biotransformation of 4-chlorophenylacetonitrile **3** (60.0 µL, 0.47 mmol) by *A. sydowii* CBMAI 934 in 24 h. **(e)** 4-Chlorophenylacetic acid **3a** purified by flash chromatography. Conditions of GC-FID analysis: $T_i = 100\,°C$ (1 min), $T_f = 210\,°C$ (5 min), $r = 10\,°C/min$, $t_c = 17$ min

Table 28.5 Biotransformation of phenylacetonitrile **1**, benzonitrile **6**, and 2-cyanopyridine **14** by marine fungus *A. sydowii* CBMAI 934



1 **14** **6** **1a** **14a**

Time (h)	c (%) 1	c (%) 14	c (%) 6	*c* (%) 1a	*c* (%) 14a
24	100	–	–	–	–
96[b]	–	100	100	100	–
120	–	65	22	9	4
192	–	61	–	7	32

[a] (Sect 28.2).
[b] The nitriles **6** and **14** were added (Sect. 28.2).
c = concentration of unreacted nitriles **1**, **6** and **14** determined by GC-FID analysis.
c = conversion determined by GC-FID analysis.

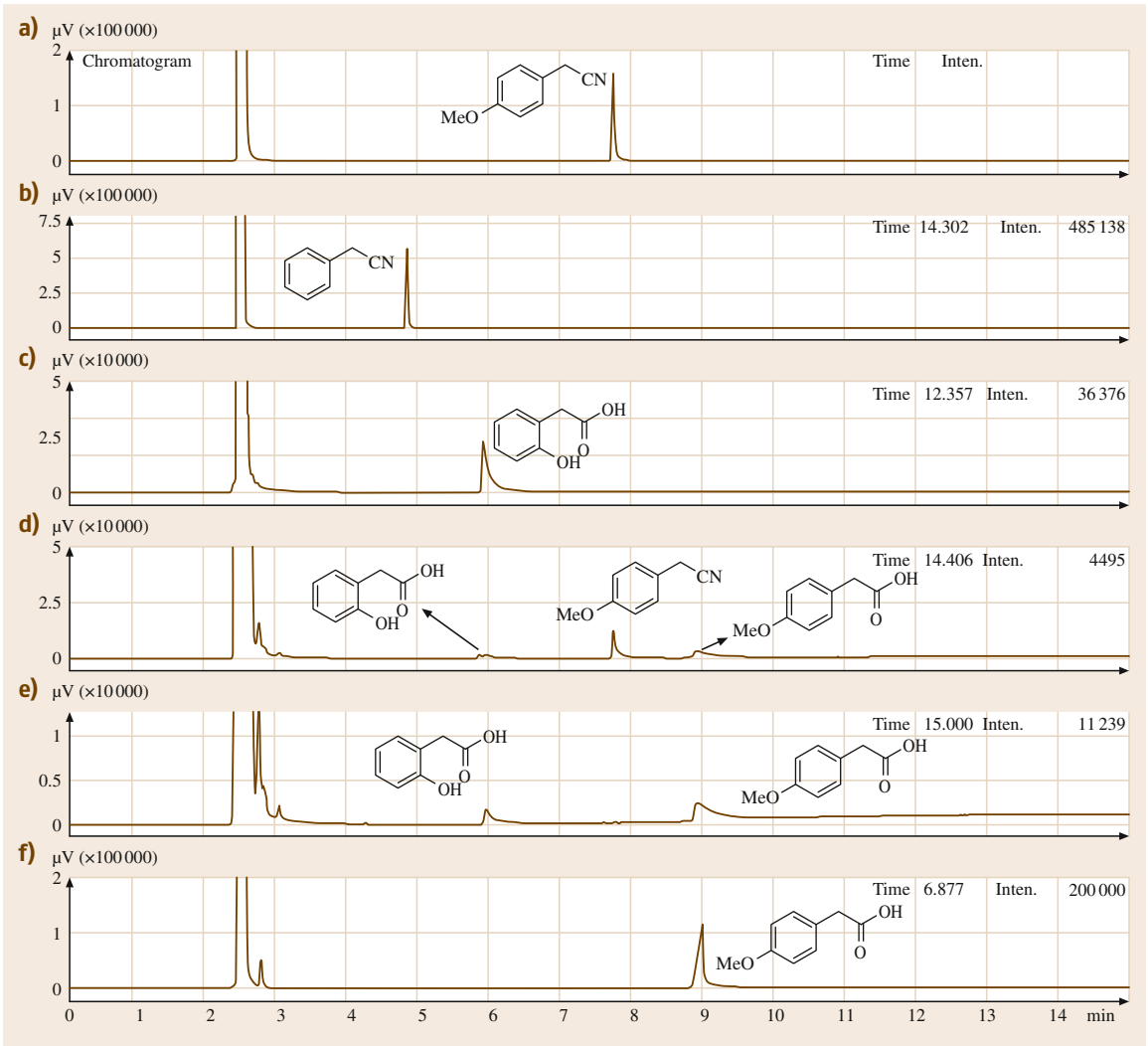


Fig. 28.39a–f GC-FID Chromatograms. **(a)** Standard of 4-methoxyphenylacetonitrile **4**. **(b)** Standard of phenylacetonitrile **1**. **(c)** Standard of 2-hydroxyphenylacetic acid **1a**. **(d)** Biotransformation of 4-methoxyphenylacetonitrile **4** (60.0 μL, 0.44 mmol) by *Aspergillus sydowii* CBMAI 934 in 24 h. **(e)** Biotransformation of 4-methoxyphenylacetonitrile **4** (60.0 μL, 0.44 mmol) by *A. sydowii* CBMAI 934 in 96 h. **(f)** 4-Methoxyphenylacetic acid **4a** purified by flash chromatography. Conditions of GC-FID analysis: $T_i = 120\,^{\circ}C$ (1 min), $T_f = 210\,^{\circ}C$ (5 min), $r = 10\,^{\circ}C/min$, $t_c = 15$ min

was converted into **4a** in 96 h (20.0, 40.0, and 60.0 μL nitrile per reaction; Table 28.3). GC-FID analyses of the reactions with nitriles **2–4** are shown in Figs. 28.37–28.39.

The carboxylic acids **2a–4a** were obtained at high conversions ($c = 100\%$), while the carboxylic acid **5a** was obtained in lower conversion. The phenylacetonitrile-induced reaction produced significant amounts of carboxylic acids **2a–5a** from nitriles **2–5**.

From the data obtained in these studies and previous work published by the authors, we conclude that the nitrile-hydrolyzing activities of marine fungus *A. sydowii* CBMAI 934 may be applied to in a variety of phenylacetonitrile derivatives. Biotransformation of organonitriles by filamentous fungi is not easily achieved, but by selective screening and induction, new biocatalysts can be obtained that provide effective reactions with excellent yields. These

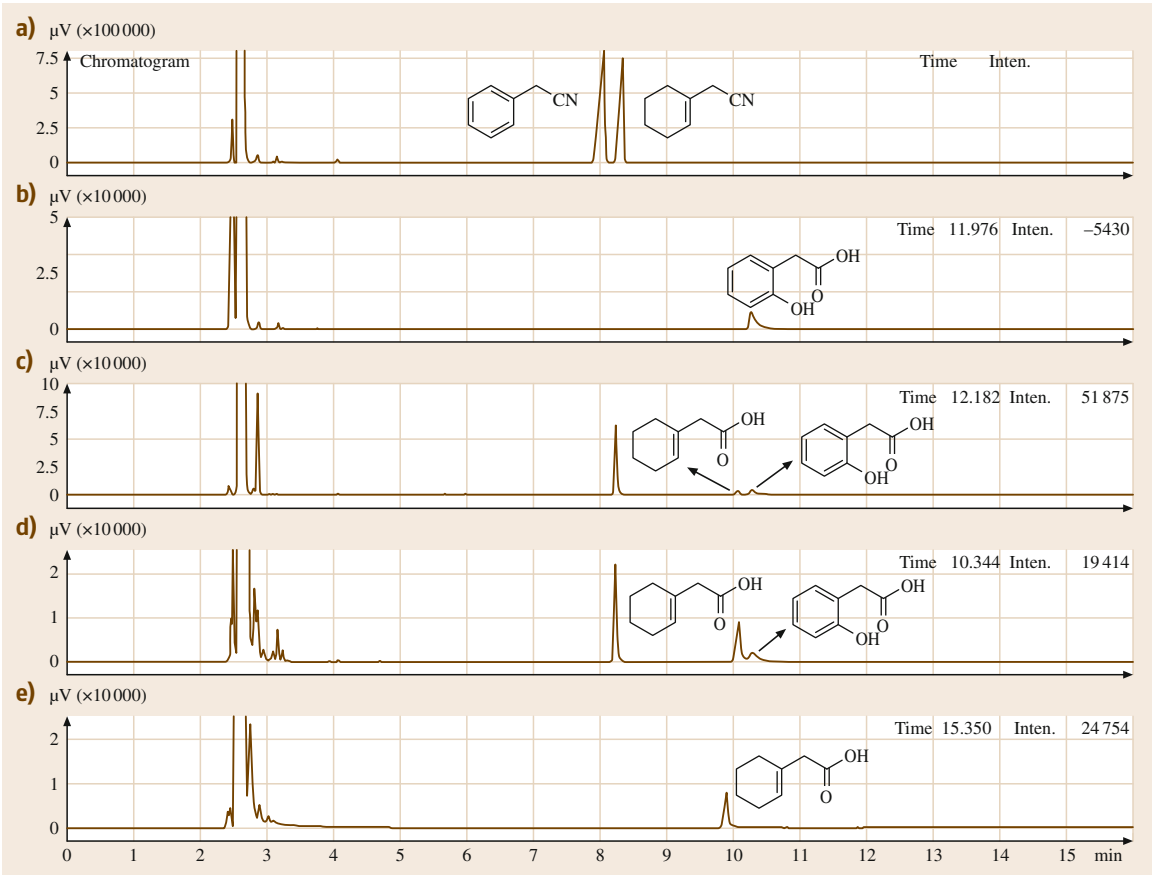


Fig. 28.40a–e GC-FID Chromatograms. **(a)** Standards of phenylacetonitrile **1** and cyclohexenylacetonitrile **5**. **(b)** Standard of 2-hydroxyphenylacetic acid **1a**. **(c)** Biotransformation of cyclohexenylacetonitrile **5** (60.0 µL, 0.48 mmol) by *A. sydowii* CBMAI 934 in 96 h without pooling the mycelial cultures (Sect. 28.2). **(d)** Biotransformation of cyclohexenyl phenylacetonitrile **5** (60.0 µL, 0.48 mmol) by *A. sydowii* CBMAI 934 in 96 h in pooled mycelial cultures (Sect. 28.2.6). **(e)** Cyclohexenylacetic acid **5a** purified by flash chromatography. Conditions of GC-FID analysis: $T_i = 100\,^{\circ}C$ (2 min), $T_f = 160\,^{\circ}C$ (2 min), $r = 5\,^{\circ}C/min$, $t_c = 16$ min

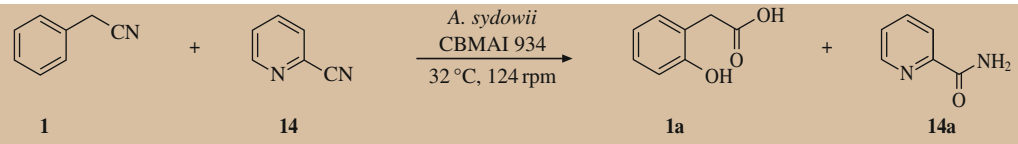
reactions are eco-friendly and sustainable, as they are carried out in an aqueous medium, at near neutral pH and mild temperatures, in the absence of oxidizing and hazardous reagents. Conversely, the chemical hydrolysis of nitriles to carboxylic acids requires severe conditions, such as strong bases, acids, and/or high temperatures, producing unwanted by-products and/or appreciable amounts of inorganic waste [28.17].

In order to widen the applicability of the methodology developed here by selection of the fungus *A. sydowii* CBMAI 934 and induction by phenylacetonitrile **1**, we decided to test another substrate containing a cyano group attached to an sp^3 carbon atom, but without an aromatic ring. For this test, cyclohexenylacetonitrile **5** was selected.

28.3.3 Biotransformation of Cyclohexenylacetonitrile 5 by the Marine Fungus *A. sydowii* CBMAI 934

Initially, the nitrile **5** was biotransformed at the concentrations of 20.0, 40.0, and 60.0 µL per reaction flask, but at low conversion rates. The best conversion of this nitrile was achieved when the three mycelial cultures were pooled in a 1 L flask and then a further 60 µL of the nitrile was added (Sect. 28.2.6; Table 28.4, Fig. 28.40). Thus, it was possible to obtain an isolated

Table 28.6 Biotransformation of phenylacetonitrile **1** and 2-cyanopyridine **14** by marine fungus *A. sydowii* CBMAI 934[a]



1 **14** **1a** **14a**

Time (h)	c (%) 1	c(%) 14	*c* (%) 1a	*c* (%) 14a
24	100	–	–	–
96[b]	–	100	100	100
192	-	19	53	28

[a] (Sect. 28.2).
[b] The nitrile **14** was added (Sect. 28.2).
c = concentration of unreacted nitriles **1** and **14** determined by GC-FID analysis.
c = conversion determined by GC-FID analysis.

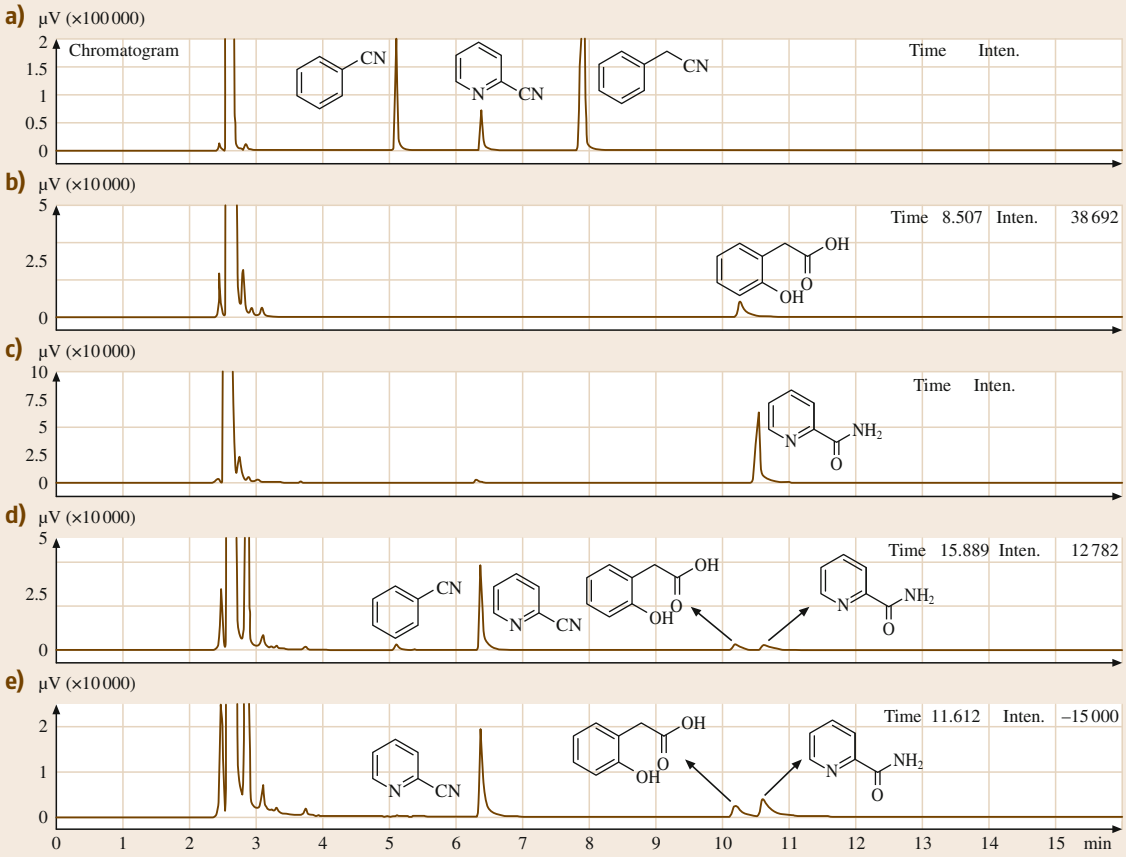


Fig. 28.41a–e GC-FID Chromatograms. **(a)** Standard of benzonitrile **6**, 2-cyanopyridine **14** and phenylacetonitrile **1**. **(b)** Standard of 2-hydroxyphenylacetic acid **1a**. **(c)** Standard of 2-pyridinecarboxamide **14a**. **(d)** Biotransformation of benzonitrile **6** (20.0 µL, 0.19 mmol) and 2-cyanopyridine **14** (20.0 µL, 0.21 mmol) by *A. sydowii* CDMAI 934 in 48 h. **(e)** Biotransformation of benzonitrile **6** (20.0 µL, 0.19 mmol) and 2-cyanopyridine **14** (20.0 µL, 0.21 mmol) by *A. sydowii* CBMAI 934 in 96 h. Conditions of GC-FID analysis: $T_i = 100\,°C$ (2 min), $T_f = 160\,°C$ (2 min), $r = 5\,°C/min$, $t_c = 16\,min$

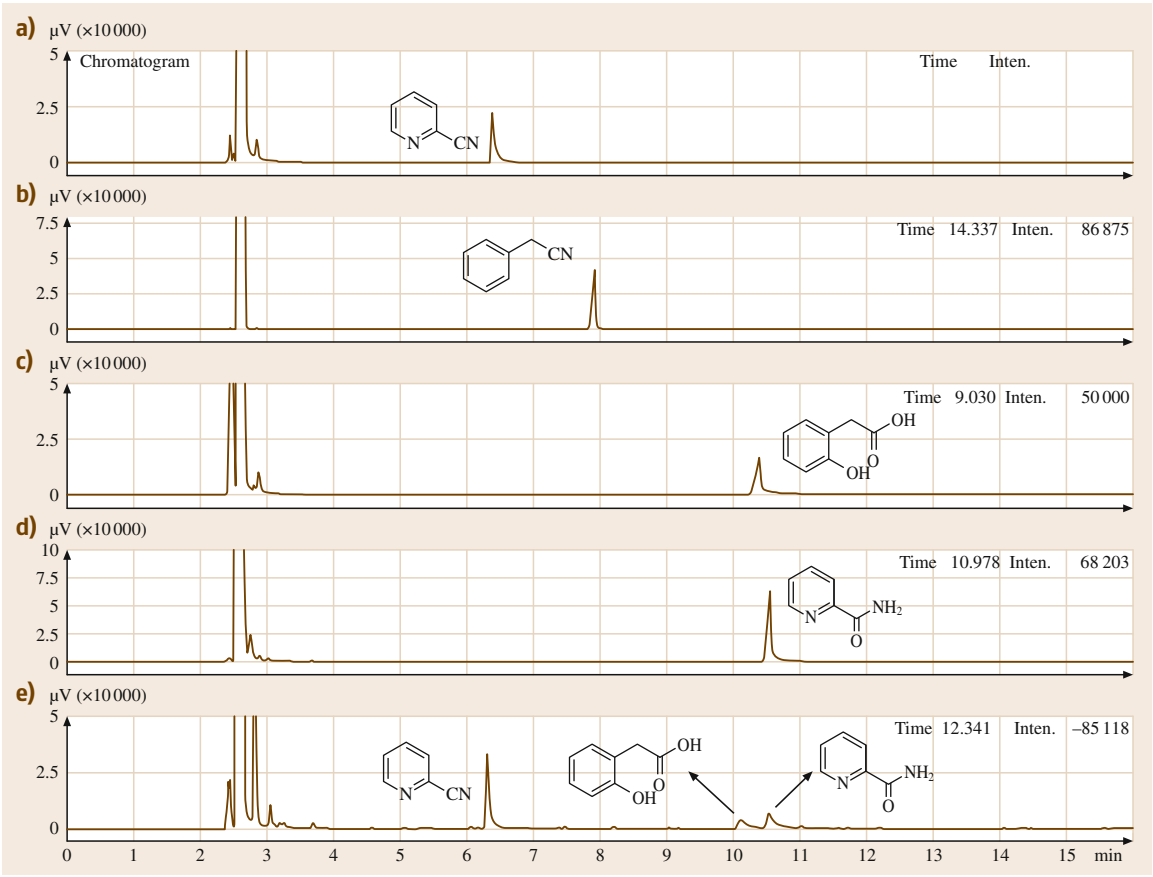


Fig. 28.42a–e GC-FID Chromatograms. **(a)** Standard of 2-cyanopyridine **14**. **(b)** Standard of phenylacetonitrile **1**. **(c)** Standard of 2-hydroxyphenylacetic acid **1a**. **(d)** Standard of 2-pyridinecarboxamide **14a**. **(e)** Biotransformation of 2-cyanopyridine **14** (20.0 µL, 0.21 mmol) by *A. sydowii* CBMAI 934 in 96 h. Conditions of GC-FID analysis: $T_i = 100\,^{\circ}C$ (2 min), $T_f = 160\,^{\circ}C$ (2 min), $r = 5\,^{\circ}C/min$, $t_c = 16$ min

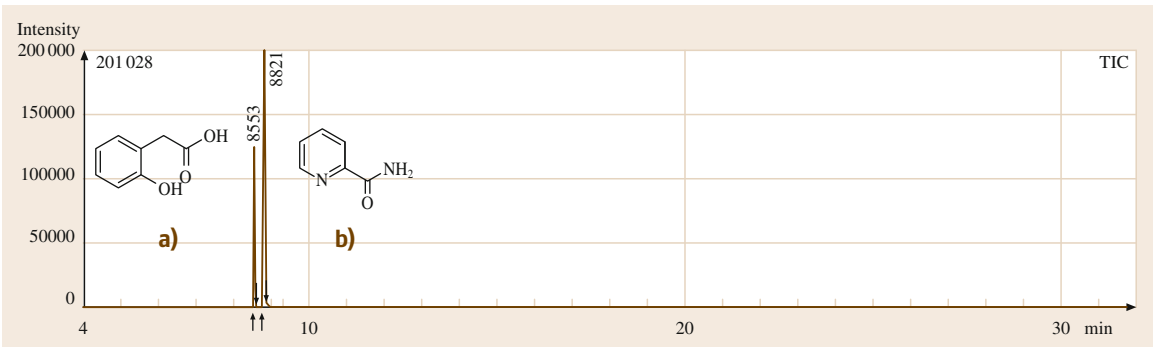


Fig. 28.43a,b GC-MS chromatogram. **(a)** Peak corresponding to biotransformation of phenylacetonitrile **1** to 2-hydroxyphenylacetic acid **1a** by *Aspergillus sydowii* CBMAI 934 in 96 h. **(b)** Peak corresponding to biotransformation of 2-cyanopyridine **14** (20.0 µL, 0.21 mmol) to 2-pyridinecarboxamide **14a** by *Aspergillus sydowii* CDMAI 934 in 96 h

yield of the biotransformed product, the cyclohexenylacetic acid **5a** (28%). We conclude that the marine fungus *A. sydowii* CBMAI 934 induced by phenylacetonitrile **1** in a mineral medium was also effective in transforming a nitrile group linked to a non-aromatic sp^3 carbon atom.

28.3.4 Biotransformation of Aromatic and Hetero(aromatic) Nitriles by the Marine Fungus *A. sydowii* CBMAI 934

To observe the behavior of the cyano group connected directly to the aromatic ring, experiments were carried out on eight aromatic nitriles: benzonitrile **6**, 2-chlorobenzonitrile **7**, 4-hydroxybenzonitrile **8**, 4-methoxybenzonitrile **9**, 3,5-dimethoxybenzonitrile **10**, 2-nitrobenzonitrile **11**, 3-nitrobenzonitrile **12**, and 4-nitrobenzonitrile **13**. In these tests, no biotransformation of any these aromatic nitriles occurred with the phenylacetonitrile-induced by fungus *A. sydowii* CBMAI 934.

In order to extend the application of the mycelium of the fungus *A. sydowii* CBMAI 934 induced by the presence of phenylacetonitrile **1**, the biotransformation reaction was performed in a mixture of benzonitrile **6** and 2-cyanopyridine **14** (Table 28.5). Surprisingly, all three substrates were biotransformed. Phenylacetonitrile **1** was converted to 2-hydroxyphenylacetic acid **1a** and 2-cyanopyridine **9** to 2-pyridinecarboxamide **14a** (Fig. 28.41). Benzonitrile **10** was possibly catalyzed to benzoic acid, but it was not possible to identify this reaction product.

Šnajdrová et al. reported that the benzonitrile **6** was the best substrate for biotransformation to its corresponding carboxylic acid; however, the benzoic acid formed was consumed by the fungus *Aspergillus niger* during the reaction [28.60]. Interestingly, benzonitrile **6** was biotransformed by *A. sydowii* CBMAI 934 in the presence of phenylacetonitrile **1** and 2-cyanopyridine **14**, whereas this biotransformation in the presence of phenylacetonitrile **1** alone was not possible.

In part, our results with the fungus *A. sydowii* CBMAI 934 corroborate studies performed by *Martínková* et al. that confirmed that 2-cyanopyridine **14** may act as a universal inducer in biotransformations of aromatic nitriles by filamentous fungi [28.30]. In our case, both phenylacetonitrile **1** and 2-cyanopyridine **14** may have acted as inducers in the biotransformation of benzonitrile **6**.

Another reaction was carried out on 2-cyanopyridine **14**, in the absence of benzonitrile **6**, which confirmed that phenylacetonitrile **1** induced the biotransformation of 2-cyanopyridine **14** (Fig. 28.42, Table 28.6).

The biotransformation of 2-cyanopyridine **14** was confirmed by comparative analysis with the synthetic standard in GC-FID (Figs. 28.41–28.42) and by analysis in GC-MS (Fig. 28.43).

Additionally, in a reaction carried out with the fungus *A. sydowii* CBMAI 934, it was previously grown on agar plates for 8 days in the presence of 2-cyanopyridine **14** (5.0 µL, 0.052 mmol per plate) which restricted growth on the plate. However, without phenylacetonitrile **1** the biotransformation of the 2-cyanopyridine **14** in liquid medium was not observed. The 2-cyanopyridine **14** remained in the reaction medium.

Thus, the phenylacetonitrile **1** was essential to the biotransformation of 2-cyanopyridine **14** to 2-pyridinecarboxamide **14a** and, under in these conditions the marine fungus *A. sydowii* CBMAI 934 demonstrated its ability to hydrolyze, of hetero(aromatic) nitriles.

Thus, we conclude that the selective biotransformation by fungus *A. sydowii* CBMAI 934 induced with phenylacetonitrile **1** may be used to hydrolyze arylaliphatic **1-4**, aliphatic **5**, and hetero(aromatic) **14** nitriles.

28.4 Conclusion

By screening 12 strains of marine fungi on solid mineral medium supplemented with glucose and phenylacetonitrile **1**, as the only nitrogen source, it was possible to select 8 of these strains, all of which showed good growth in liquid mineral medium in the presence of 20.0, 40.0, and 60.0 µL phenylacetonitrile **1**. In control experiments (without phenylacetonitrile) there was no microbial growth, demonstrating the ability of phenylacetonitrile **1** to induce the growth of these microorganisms, acting as the sole nitrogen source.

The phenylacetonitrile **1** was biotransformed to 2-hydroxyphenylacetic acid **1a**, showing that the fungi selected had the potential to biotransform derivatives of phenylacetonitrile **1**.

The direct biotransformation of nitriles **2**–**5** to the corresponding carboxylic acids **2a**–**5a** suggests the en-

zymatic route of the biotransformation of phenylacetonitrile **1**. First came the hydrolysis of the nitrile group and later, hydroxylation (insertion of hydroxyl in the *ortho* position of the aromatic ring), because the corresponding carboxylic acids **2a**–**5a** were produced only by hydrolysis of the cyano group. The biotransformation of nitriles is not trivial, and here we describe an efficient method for the production of phenylacetic acid derivatives under mild and environmentally-friendly conditions.

The induced biotransformation of specific nitrile groups by whole mycelia of the marine fungus *A. sydowii* CBMAI 934 was highly effective, using an eco-friendly methodology. The enzymes produced by this marine fungus showed substrate specificities typical of arylaliphatic and aliphatic nitrilases and hereto (aromatic) nitrile hydratases. The enzymes present in this catalyst system of the fungus *A. sydowii* CBMAI 934 exhibited activity only on addition of the inducer, indicating that these enzymes are constructive. The biotransformation of nitriles **1–5**, containing a cyano group attached to an sp^3 carbon atom, to the corresponding carboxylic acids **1a–5a** was achieved with good yields. The marine fungus *A. sydowii* CBMAI 934 is thus a promising biocatalyst for the preparation of important arylaliphatic and aliphatic carboxylic acids.

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29. Drugs and Leads from the Ocean Through Biotechnology

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The ocean's uniqueness represents an excellent source of resources and inspiration of diverse marine natural products with novel chemical entities and leads for the development of pharmaceutical agents. During the last 10 years, interest in research and development by the scientific community in this niche have continued as evidenced from joint ventures between academics, mainly partnered with industry. Drugs of marine origin that have now been approved represent excellent and novel alternatives for the following therapies and disease treatments. For neuropathic pain, a synthetic version of omega-conotoxin, produced by the tropical marine *Conus* snail is already on the pharmaceutical market. The ecteinascidins (ET-743) are marine alkaloid, anticancer agents isolated from *Ecteinascidia turbinate*, a colonial tunicate found in the Caribbean and Mediterranean Sea, and are successfully used for soft tissue sarcoma and relapsed ovarian cancer. The synthetic pyrimidine nucleoside Cytarabine, was originally isolated from the Caribbean sponge *Tethya crypta*, and approved as a chemotherapeutic to treat different forms of leukemia (e.g., meningeal leukemia, lymphocytic leukemia). From this marine sponge, also the synthetic purine nucleoside analog, Vidarabine, was developed as an antiviral drug against the herpes simplex virus and the varicella-zoster virus for the treatment of epithelial keratitis, viz, ophthalmic applications. In 2012, squalamine was been awarded Fast Track designation by the US Food and Drug Administration (FDA) for the potential treatment of the wet form of macular degeneration. These successful advances have had to overcome difficulties characteristic of marine natural products to become drugs, viz, sustainable sources, structural complexity, and toxicity, the same as the marine molecules in preclinical and clinical trials. In this chapter, the current advances and modern approaches of marine natural products and respective bioactive metabolites with potential and real functions as drugs leads are succinctly reviewed and particularly exemplified. An important aspect of this chapter is the analysis of marine natural products in relation to new paradigms and particularities of other disciplines, such as genomics, combinatorial chemistry, biosynthesis, and functional foods.

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29.1 Overview and Current Status

29.1.1 The Marine Environment, a Unique and Prolific Source of Bioactive Natural Products

A natural product can be defined as a compound that is present in or produced by living systems in nature and is not artificial or man-made [29.1]. Natural products are often structurally complex compounds that possess a well-defined spatial orientation. Their activity due to chemical compounds has evolved to interact efficiently with their biological targets and provides important clues for drug discovery. Many of these compounds exert their bioactive properties as secondary metabolites, in ecological, defensive, antifeedant, attractant, and pheromone roles. In nature, as in the marine environment, every natural product is believed to have emerged as a result of exerted pressure on the first living organisms from the sea, more than 3500 million years ago, under a context of evolution which enables marine organisms to survive in an extremely competitive milieu, such as extreme temperatures, changes in salinity and pressure, among others [29.2]. In this way, molecules acquired biochemical specialization and diverse metabolic roles and functions, as is now known. All these characteristics and properties have created conditions with a high degree of chemical diversity and a rich source of biologically active natural products found in marine environments, which are of remarkable novelty [29.3] as medicines. Expectations are high with respect to the ocean as the most chemically and biologically rich environment on the planet [29.4] is also the last frontier, unexplored and unexploited. A great number of these biologically active molecules from marine natural products has developed roles as biological receptors in a co-evolution process and allow interaction with human proteins, expressed lately as drug leads. The marine environment is characterized by physical and chemical properties that are markedly different from those of the terrestrial environment [29.5]. For instance, marine natural products have higher molecular weights than their synthetic counterparts and are characterized as having fewer nitrogen, halogen, or sulfur atoms, and a larger proportion of oxygen atoms, and are sterically complex (more bridgehead atoms, rings, and chiral centers) [29.6]. As a consequence, the development and evolution of biosynthetic pathways displayed specialized molecules, as defense, deterrents, ferhormones, and for hunting strategies. This wide range of bioactivities produced by compounds of marine organisms emphasizes the great potential of compounds for biomedical applications, which has encouraged further large-scale programs of bioprospection and systematic screening of marine organisms. Recent technological and scientific advances in the sciences have led to a multitude of discoveries of carbon skeletons and molecules hitherto unseen on land [29.4] and to contribute to the discovery of new functionalities for common targets of human diseases, and consequently new drugs from the marine environment.

Considering that unexplored marine organisms harbor the widest biological and chemical diversity, the possibilities to discover novel structures (to date, more than 21 855 compounds have been discovered) with novel modes of action, are high [29.7]. The uniqueness of marine natural products lends them to applications as preclinical and clinical lead compounds for the treatment of the most important challenge to human diseases, viz, cancer and other important neurodegenerative diseases. Presently, marine natural products and their metabolites are recognized as biologically active leads for therapeutic agents.

The field of marine natural products is now close to 50 years old. Thousands of natural products have been described through academic and industrial screening efforts for pharmaceutical candidates, but with limited success in certain targets, such as cancer, pain, or inflammation [29.8]. The natural products platform has attracted the interest of the pharmaceutical industry because of the low number of new developments in biopharmaceuticals on the market and the increasing incidence of recurrent diseases. The need for new drugs to face current and rare diseases, viz, new classes of antibiotics [29.9], many cancers, and chronic and neurodegenerative diseases is still lacking effective treatments, mainly for vulnerable populations in developing countries, which are rich in their potential of natural products. In spite of these evident needs, the number of drugs from the sea represents only a discrete number of marine-derived compounds that have been approved for the treatment of human disease, such as: Vidarabine (Ara-A) and cytarabine (Ara-C), which were derived and inspired by nucleosides found in marine sponges, ziconotide (Prialt1), a synthetic version of omega-conotoxin, produced by a tropical marine cone snail, and approved for the treatment of chronic pain; trabectedin (Yondelis) found in a tunicate was approved as an anticancer compound in the European Union.

All these successful examples represent an important effort by joint ventures of pharmaceutical companies with scientists and government, as succinctly described below.

An unfair comparison with conventional drugs on the market with a long history of research and development, in some cases discourages primary initiatives and high expectations for drugs from the sea. However, the best is yet to come, if we consider all the experience gained during these almost 50 years, which in some way is reflected in the approved products and in the impressive pipeline of marine-derived compounds in clinical and preclinical trials, suggesting that several more marine drugs, should soon be approved for pharmaceutical use [29.10]. In some way, as part of a relatively recent area, viz, marine biotechnology, and the integrated efforts of other disciplines may contribute to new developments, and on the focus on deep sea micro and macroorganisms as sources of natural products and of novel bioactivities. Actual progress in genomics, metagenomics, manipulation of biosynthetic pathways, and combinatory chemistry are promising new discoveries of pharmaceutical lead drugs. The integration, with other areas and applications of biotechnology will contribute to the development of this important area of marine biotechnology.

In this review, recent developments, mainly during the last decade, of marine natural products and the respective metabolites, are emphasized, considering their interesting biological properties and actual developments as approved marine-derived drugs and promissory compounds. The high degree of innovations per se from marine natural products, have led and will lead to a resurgence of new drugs and pharmaceutical applications for new and recurrent challenges of modern human diseases.

An important aspect of this chapter is the analysis of marine natural products in relation with other disciplines, such as genomics, combinatorial chemistry, metabolic pathways, and functional foods. Some are cases of success, others offer great potential application in other areas of biotechnology and the bioindustry.

29.2 Approved Marine Drugs as Pharmaceuticals

A high number of marine-derived chemical entities with biological activity in different therapeutic settings has been discovered during the last couple of decades. Advances and proven evidence of a positive therapeutic index indicates a favorable tolerability profile in the different clinical settings assessed. In addition, a number of approaches incorporating target-based discovery models are yielding to the identification of novelties with therapeutic potential in a number of diseases. Finally, selected interactions with other thematic fields are expected to emerge in the near future, and to lead to new developments in genome mining, biosynthetic pathways of gene holders and functional foods, and to contribute to changing the way that marine-derived drugs are discovered, thus making a significant contribution to welfare and cure of human diseases.

There are actually several excellent reviews on marine natural products and the respective pharmacology [29.10, 11]; we intend to succinctly emphasize fundamental and particular issues that will contribute to the integration and advancement of this innovative discipline and the respective drug pipeline in the context of biotechnology. It is our conviction that these initiatives will rapidly develop and expand in different approaches of human interest over the next decade.

29.2.1 ω-Conotoxin MVIIA: An Analgesic for Neuropathic Pain

The ω-conotoxins are poisons produced by piscivorous cone snails, originally isolated from *Conus magus* by *Olivera* and coworkers in 1979 [29.2]. These toxins are highly selective antagonists of N-type voltage-gated calcium channels (VGCCs)voltage-gated calcium channels [29.12, 13]. The venom of this gastropod mollusc, is a combination of 100–200 peptides (length from 9–100 amino acids), which exert inhibitory effects on the neuromuscular system of their prey [29.14, 15]. ω-Conotoxin MVIIA is a linear 25 amino acid, polycationic peptide containing six cysteine residues linked by three disulphide bridges that stabilize its well-defined three-dimensional structure [29.11] (Fig. 29.1). Compared to difficulties of chemical synthesis in other marine natural products, the complete synthesis of this peptide was successfully concluded in 1987 [29.12]. Subsequent studies on this toxin led to the identification that N-type voltage-sensitive calcium channels (NVSCCs) were its target site. The pharmacological activity of conus-derived toxins is currently used for its classification, viz, α-conotoxins are antagonists of both muscle and neuronal-type nicotinic acetyl-

choline receptors, while μ-conotoxins inhibit skeletal muscle voltage-gated Na^+ channels [29.16]. Both (α- and μ-conotoxins) produce paralysis through blocking neuromuscular transmission in vertebrates, which limits the clinical application of these conotoxins. The ω-conotoxins, are recognized as inhibitors of ion of VGCCs in an isoform-dependent manner, a property that explains the exerted role of paralysis in the prey. The synthetic equivalent of ω-conotoxin MVIIA from conus is commercially known as Ziconotide, or SNX-111 and is considered as the first venom-derived drug of VGCC antagonists. It is used as an analgesic in neuropathic pain with clinical applications [29.17–19]. Analgesia is provided by this toxin via binding to N-type voltage-sensitive calcium channels in the spinal cord. The fact that ziconotide does not easily cross the blood–brain barrier because of its poor tissue penetration as well as its hypotensive effect, promoted the development of the intrathecal adapted methods for its delivery. Thus, a special delivery strategy via an intrathecal catheter or by an external microinfusion device [29.10, 11, 20] has led to improved patient treatability and acceptability of this venom toxin as a pharmaceutical. Studies conducted on post Phase III clinical trials corroborate the benefiting effect of intrathecal ziconotide in living systems. Strikingly, ziconotide is 1000 times more potent than morphine and unlike opiates does not produce tolerance [29.21]. Another ω-conotoxin, CVID from *Conus catus*, in spite of assessed as the most potent of all N-type VGCC peptide blockers, finally exhibited the Achilles tendon problematic of marine toxins: the severe side effects in clinical trials [29.22–24]. Small molecules derived from ω-conotoxins, Z160, have actually advanced through Phase II clinical trials aimed to increase oral bioavailability [29.20, 24], and are intolerant of or refractory to other treatments (systemic analgesics, adjunctive therapies or IT morphine). The European Commission approved ziconotide for the treatment of severe, chronic pain in patients, specifically requiring intrathecal analgesia [29.11, 25]. Actually, Ziconotide is commercialized under the trade name of Prialt by pharmaceutical companies in USA and the European Union.

29.2.2 Ecteinascidins: A Therapy for Soft Tissue Sarcoma

Ecteinascidins (ET-743) are marine alkaloid, anticancer agents isolated from *Ecteinascidia turbinate*, a colonial tunicate found in the Caribbean and Mediterranean Sea [29.26]. ET-743 and its N-demethylated analog ET-729 are lactone members. Their structural features are a distinctive feature of the ecteinascidins [29.11]. Structurally, they are conformed of three fused tetrahydroisoquinoline rings connected to the base structure by a thioether bridge, giving rise to a ten-membered lactone, which is characteristic of ecteinascidins [29.26, 27]. These alkaloids are produced by semisynthesis after controlled fermentation of the marine bacterium *Pseudomonas fluorescens*, which leads to ET-743 from cyanosafracin B [29.28]. ET-743 also is named trabectedin or goes under the trade name Yondelis (Fig. 29.1).

The covalent binding of trabectedin to the deoxyribonucleic acid (DNA) minor groove has been used to explain its mechanism of action [29.29]. Trabectedin interacts with binding proteins of the nucleotide excision repair (NER) system [29.30, 31] to exert its cytotoxic activity [29.10] and induces an apoptotic effect by arrest at the G2/M stage of the cell cycle of tumors; a condition that seems independent of p53. Studies with ET-743 have shown dose limiting toxicities that include neutropenia and thrombocytopenia, and transaminase elevation [29.32]. Analysis of the effects of trabectedin in vivo and in vitro systems have evidenced positive results in the control of soft tissue sarcoma [29.33], and it has been approved in the European Union for the treatment of refractory ovarian cancer.

29.2.3 Cytarabine to Treat Meningeal Leukemia; Vidarabine, An Ophthalmic, Non-Corticosteroid Antiviral

The synthetic pyrimidine nucleoside Cytarabine was developed from the nucleoside spongothymidine, originally isolated from the Caribbean sponge *Tethya crypta*, the first-derived product of which was named Ara-C, arabinosylcytosine, or cytosine arabinoside (Fig. 29.1). Cytarabine is a specific cytotoxic compound that inhibits DNA polymerase and DNA synthesis by competition for deoxycitidine triphosphate, once converted to cytosine arabinoside triphosphate. In treatment programs it is also named antimetabolites; it main role is slowing or stopping the growth of cancer cells in human body. Vidarabine is the named assigned to the synthetic purine nucleoside, arabinofuranosyl adenine, or adenine arabinoside (Ara-A) developed from

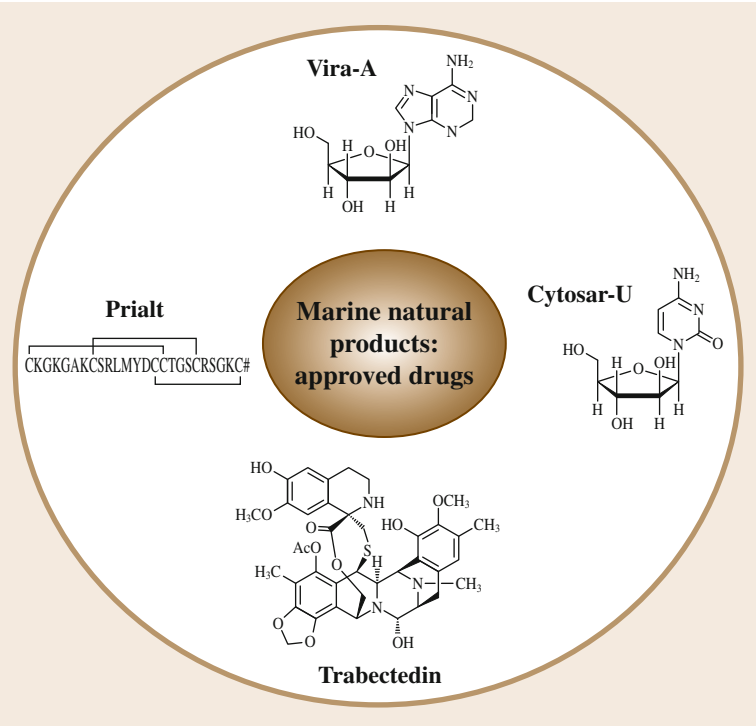

Fig. 29.1 Chemical structures of marine natural products or derivatives thereof approved by the FDA (modified after [29.10, 11])

spongouridine and isolated also from the sponge *Tethya crypta* [29.34].

Cytarabine (Ara-C) and Vidarabine (Ara-A), were among the first FDA-approved marine-derived drugs, as an anticancer drug in 1969 and as an antiviral agent in 1976, respectively [29.35]. The importance of Cytarabine and Vidarabine on preclinical and clinical antiviral pharmacology can be seen from the 4183 and 14 239 publications, respectively, in the peer-reviewed literature, upon a perusal search of their status (March 2013) in The USA National Center for Biotechnology Information-PubMed. According to the FDA, Cytarabine is recommended for the treatment of certain classes of leukemia affecting the white blood cells, mainly acute myeloid leukemia, acute lymphocytic leukemia, and chronic myelogenous leukemia [29.36, 37], and can be used in combination with chemotherapeutic drugs or alone to treat meningeal leukemia [29.10, 37]. A liposomal scheme of administration of cytarabine (Depocyt1) is recommended for intrathecal lymphomatous meningitis. Commercial presentation of Cytarabine is designed to be administered intravenously by injection, subcutaneously, or intrathecally [29.38]. On the other hand, Vidarabine has been applied to eye, which infection of the herpes simplex virus by corticosteroids alone is not recommended. One presentation of Vidarabine was designed under the trade name VIRA-A, which is also recommended for the treatment of keratoconjunctivitis, epithelial keratitis caused by the herpes simplex virus, and superficial keratitis caused by the herpes simplex virus [29.39]. *Mayer* et al. [29.10] mentioned that this drug has been discontinued by the FDA in the US market. A series of side effects derived from Cytarabine, like any similar medicine for such types of diseases, are: fever, sore throat, ongoing cough and congestion, or other signs of infection; unusual bleeding or bruising, and bloody vomit. Vidarabine (Vira-A1) received FDA approval in 1976.

Among the common side effects that could occur with the use of Vira-A1 as medication, are: burning, stinging, pain, irritation, redness, blurred vision, and sensitivity to light. Both cytarabine (Cytosar-U1) and liposomal cytarabine (Depocyt1) are marketed and have been approved for use as an antitumor agents [29.33]. Cytarabine 5′-monophosphate (CAS 7075-11-8) has been commercialized by a company in California for research purposes only and not for diagnostic or therapeutic use. In 2013, the current price for 10 mg was $US 240.

29.2.4 Marine Natural Products in Advanced Clinical Trials

Aplidine

Aplidine is a marine depsipeptide that was first reported in 1991 [29.40] by *Rinehar* and *Lithgow-Bertelloni* from *Aplidium albicans*, a tunicate that thrives in the Mediterranean Sea (Fig. 29.2). It is characterized by high antitumor activity [29.26] and has been shown to induce apoptosis by interruption of the cell cycle at the G1 and G2/M phases of leukemia cell lines, as a way to induce p53-independent apoptosis [29.41]. Its chemical structure is very similar to that of didemnin B, the only difference being that the lactate residue in didemnin B is present in the oxidized pyruvate form. In some way, the accumulated experience of more than 20 years in its closely related compound, didemnin B from the tropical *Tridemnum solidum* has contributed to the development of aplidine in advanced clinical trials. These peptides are obtained by chemical synthesis structured of six amino acid subunits as the main backbone and three amino acids in the side chain: (R)-N-Me-Leu linked to the Thr and piruvil-(L)-Pro. The aplidine named plitidepsin has demonstrated efficacy in two different Phase II clinical trials in refractory multiple myeloma and T cell lymphoma [29.42, 43]. In human leukemia, aplidin inhibits the vascular endothelial growth factor (VEGF) secretion and autocrine VEGF-VEGFR-1 stimulation [29.42–44], in principle, induced perturbations by Aplidin in cell cycle proceeds without any effect on cycling-dependent kinases [29.41]. Aplidine has also shown preclinical activity in vitro on haematological and solid tumor cell lines. This effect might contribute to the antineoplastic activity of aplidine. One bottleneck in the yield of peptides from Aplidine is the lack of a feasible production system. The supply is currently produced by total synthesis of these peptides from constituent amino acids in an iterative multistep process. It is believed that the future use of this compound will depend on the sustainable supply, which at present is limiting its production. In 2003, Aplidine was granted the status of orphan drug by the European Medicines Agency for treating lymphoblastic leukemia.

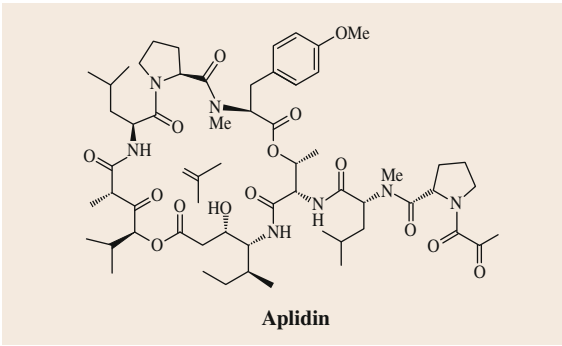

Fig. 29.2 Chemical structure of aplidin

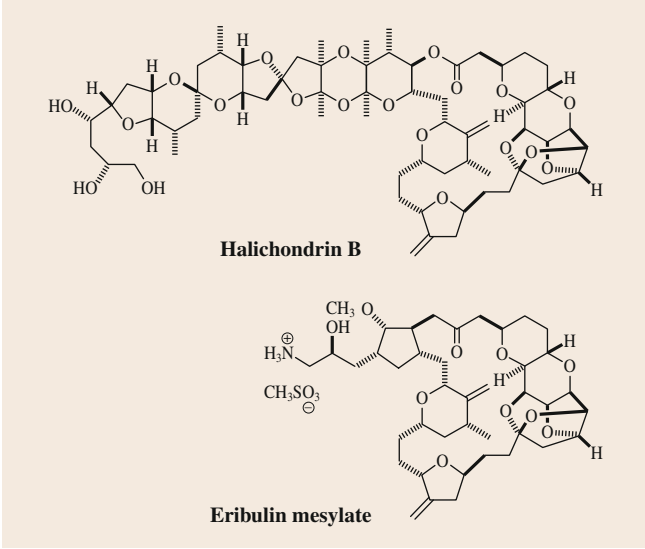

Fig. 29.3 Chemical structure of halichondrin B

Halichondrin B

Halichondrin B, also known as eribulin mesylate (E 7389), is a polyether isolated from the Japanese sea sponge *Halichondriaokadai*, which was discovered in 1986 by *Uemura* and co-workers [29.45, 46]. This macrolide (Fig. 29.3) is a member of a new class of synthetic cytotoxic agents that binds to the vinca domain of tubulin and inhibits its polymerization, which is considered as a new treatment option for metastatic breast cancer or locally advanced breast cancer [29.47]. Hence, this cytotoxic halichondrin, exhibits broad antiproliferative activity against tumor cells by binding to tubulin and arresting the cell cycle at mitosis [29.48], and leading to cell death by apoptosis [29.49]. These natural products display pharmaceutical benefits, including water solubility and chemical stability [29.50]. Clinical results from Phase II trials of eribulin mesylate as a monotherapy for refractory breast cancer were reported in 2006 [29.51]. Eribulin mesylate has been approved for the treatment of metastatic breast cancer in patients who have received at least two prior chemotherapeutic agents. The adverse effects of eribulin, which

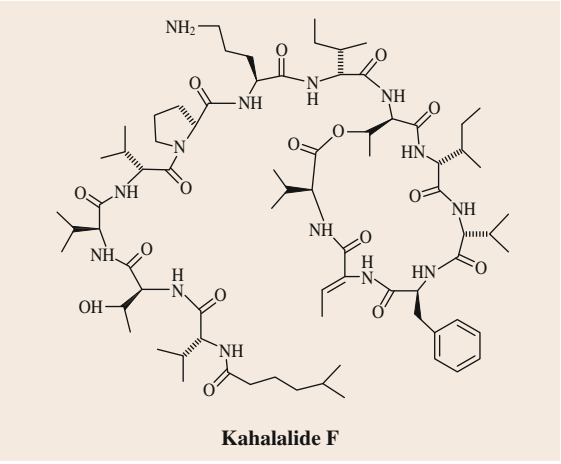

Fig. 29.4 Chemical structure of KF

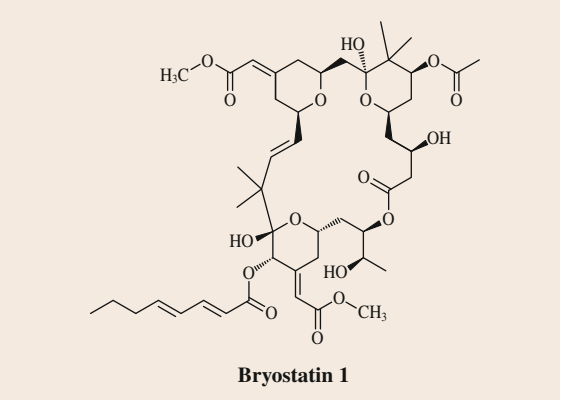

Fig. 29.5 Chemical structure of Bryostatin 1

consist mainly of neutropenia and fatigue, have been associated with a low incidence of peripheral neuropathy. In United States and in Europe, eribulin mesylate is currently under Phase III clinical trials [29.33].

Kahalalide F (KF)

Kahalalide F (KF) is a desipeptide isolated by *Scheuer* [29.52] from the hervivorous marine mollusk sacoglossan (sea slug), *Elysia rufescens* and their algal diet of *Bryopsis pennata*. It seems that both organisms use this cyclic tridecapeptide KF as a chemical defense against predation [29.52]. Even when the mechanism of action of KF (Fig. 29.4) still deserves to be fully elucidated, it seems that it is active mainly at the lysosomal level and induces vacuolization [29.53]. Hence, KF actively secrets lysosomal proteins, such as prostate cells. Induction of cell death of human prostate cell lines and breast cancer cell lines by KF takes place by oncosis [29.54]. In several human cell lines sensitive to KF, breast, vulval, hepatic, and colon carcinomas have exhibited a necrotic-like effect [29.55]. The inhibitor effect of KF on the tyrosine kinase and the phosphatidyl inositol 3-kinase-akt signaling pathway, were identified as determinants of its citotoxicity [29.56]. The apparent positive therapeutic index of KF on advanced solid tumors, melanoma, non-small cell lung cancer (NSCLC), and hepatocellular carcinoma [29.57] have promoted this compound for Phase II clinical trials.

Bryostatin 1

The bryozoan *Bugula neritina* was obtained from the Gulf of Mexico in 1968. It exhibits remarkable activity against murine P338 lymphocytic leukaemia cells [29.58]. As a Lactone, Bryostatin 1 (Fig. 29.5) is a complex molecule with 11 stereocenters and a unique polyacetate carbon backbone [29.59]. Hence, subsequent characterization of an important number of bryostatin homologs and analogs was carried out based in differences in the molecular structures of these compounds, such as their substitution at C7 and C20 by different acyloxy substituents [29.10, 11, 60]. Bryostatin 1 demonstrated potent in vitro activity against the P388 lymphocytic leukaemia cell and also other different bioactivities, such as modulation of the immune system [29.61], induction of cell differentiation [29.62], and synergistic interactions with other anticancer agents such as ara-C, paclitaxel, tamoxifen, auristatin PE, dolastatin, vincristine, doxorubicin, and prednisone [29.11, 63, 64]. This lactone has been the subject of an important number of Phase I and II clinical trials for myeloid leukaemia, lymphocytic leukaemia, melanoma, non-Hodgkin's lymphoma and NSCLC, metastatic myeloma, relapsed lymphoma, and chronic lymphocytic leukaemia [29.65]. The toxicity of this natural product is expressed as myalgia by muscular vasoconstriction, a condition originating from impaired oxidative metabolism and proton efflux from muscle cells [29.66].

Squalamine

Squalamine (Fig. 29.6) is a water-soluble aminosteroid derived from the internal organs (primarily the liver) of the dogfish shark *Squalus acanthias*. This cationic steroid is characterized by a condensation of an anionic bile salt intermediate with spermidine. As an aminosteroid, it displays strong antimicrobial action [29.67, 68] and has great potential for the treatment of some human viruses. As a broad spectrum antibiotic,

squalamine exhibits potent bactericidal activity against both Gram-negative and Gram-positive bacteria, and induces osmotic lysis of protozoa. It was established that it may be useful in the treatment in a topical eye drop formulation, diseases characteristic of elderly people, and diseases related to vision disorders (macular degeneration) [29.69, 70]. Squalamine has been shown to interrupt and reverse the process of angiogenesis. Squalamine is not a protein, instead, it is the first known example of a class of compounds called aminosterols, which the body readily uses to carry out its normal anti-angiogenesis function. By using different types of mouse cancer, squalamine was found to inhibit angiogenesis and to stop the growth of tumors. Nevertheless, squalamine was found to intensify the therapeutic effect of paclitaxel and carboplatin, inhibiting some growth factors, for instance VEGF, and causing a decrease in the amount of blood vessels around the tumor and apoptosis of tumor cells. Pharmacological investigations of squalamine are at clinical Phase I and II (pulmonary and ovarian cancers) [29.70]. Recently, Ohr pharmaceuticals Inc. announced that its squalamine eye drops have been awarded Fast Track acceptation by the US FDA for the wet form of macular degeneration.

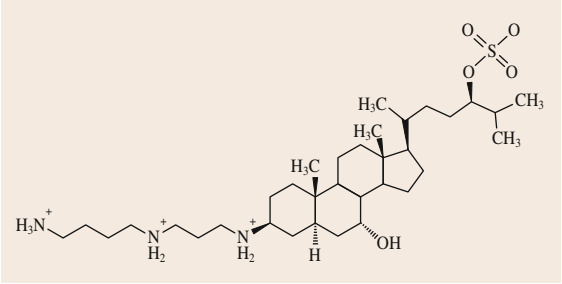

Fig. 29.6 Structure of Squalamine, an aminosterol isolated from the stomach and liver of the spiny dogfish, *Squalus acanthus*

29.3 Marine Natural Products – Overcoming Hurdles

Marine natural products face several challenges, which need to be overcome before they can enter advanced clinical trials. Apart of their inherent particularities, viz, biosynthesis and supply, target identification, toxicity, and complex structure, their role as secondary metabolites in marine organisms needs to overcome hurdles of survival in a competitive saline environment, which is quite different of that on earth. These properties of marine natural products may contribute to explain aspects of their toxicity and secondary effects in humans, which still limit its acceptance in advanced clinical trials.

29.4 *Quo Vadis*? Marine Natural Products and Clinical Trials

The potential of marine natural products to become new pharmaceuticals raises the question: *Quo vadis*? Even when these molecules seem highly promising, the aforementioned obstacles still need to be overcome in order to achieve successful clinical trials; a situation that invites us to ponder on Latin phrase *Quo Vadis*, or *Where are you going?*. During the next decade, these promissory metabolites of the global marine pharmaceutical pipeline could be part of advanced clinical trials, as in the case of marine natural products that have already acceded to the drug market. Particular features of many of these promising compounds are still waiting to be approved (Table 29.1). In 1981, the compound didemnin B, originally isolated from the Caribbean tunicate *Tridemnum solidum* [29.71] attracted interest due to its antiviral and cytotoxic activity exhibited in in vitro tests when applied in mice leukemia cell lines, as well as a potential antitumor, antiviral, and immunosuppressive agent. In principle, didemnin B binds to palmitoyl protein thioesterase and interferes in the protein synthesis in target cells [29.72]. Because of its high toxicity and secondary effects it was taken out of clinical trials at the end of the 1990s. Probably an aspect of didemnin B that is considered to be of less importance is that the accumulated experience in the management of this compound has helped to address particularities in clinical trials of other marine-derived products. In 1994, the tripeptide antimitotic *Hemiasterlin* (HTI-286/E7974) was isolated from the marine sponge *Hemiasterellaminor* by *Kashman* and co-workers [29.73]. The chemical synthesis allowed them to obtain derived analogs from substitutions of the NH 2-terminal amino acid and yielded in vitro analogs with high potency, exhibiting resistance to p-glycoprotein and positive pharmaceutical properties. The results obtained from Phase I studies showed dose-limiting toxicities, viz, neutropenia or febrile neu-

Table 29.1 Marine natural products and inspired compounds in clinical trials and their collected source, chemical class, and bioactivity (after [29.10, 70])

Metabolites	Marine organism	Chemical class	Bioactivity
Aplidine	*Aplidium albicans*	Cyclic depsipeptide	Cancer/ antitumor
Bryostatin 1	*Bugula neritina*	Polyketide	Cancer/ Alzheimer's
Kahalalide F	*Elysia rufescens*	Cyclic peptide	Lysosomes antitubuin
Salinosporamide A	*Salinispora tropica/ arenicola*	Lactam/ Lactone derivative	Proteosomal inhibitor
Pseudopterosins	*Pseudopterogorgia elisabethae*	Diterpene glycoside	Wound healing

tropenia, with other adverse events including fatigue, constipation, nausea, and vomiting [29.74]. The potent cytotoxicity of hemiasterlin is due to the induction of mitotic arrest in the metaphase with cellular dynamics similar to those of known tubulin binders, such as the chemotherapeutics paclitaxel or vinblastine. Unfortunately, Phase II trials have been interrupted. Nevertheless, high antitumor activity obtained with mice studies applying HTI-286 and in refractory prostate cancer [29.75] make hemiasterlin highly promising.

29.4.1 (C)-Discodermolide

In 1990, *Gunasekera* and co-workers [29.76] isolated (+)-Discodermolide from the sponge *Discodermia dissoluta* in Florida, USA. Discodermolide is a drug that functions as an immunosuppressant [29.77, 78] and induces G2/M phase cell-cycle arrest in lymphoid and non-lymphoid cells [29.79]. The cytotoxicity of (+)-discodermolide cause cell-cycle arrest by mitosis [29.80, 81] and an important alteration at the level of microtubules [29.82]. At present, Phase I trials with (+)-discodermolide has been discontinued as a consequence of unsafe efficacy and toxicity results. However, potential remains for its use in combination drug therapy. Dolastatin 10 is the name assigned to a series of cytotoxic peptides that were isolated from the sea hare *Dolabella auricularia* found in the Western Indian Ocean in 1972 [29.83]. In spite of its extremely low yields (90 mg of Dolastatin 10 per of *D. auricularia*), dolastatin 10 exhibited outstanding in vitro cytotoxic activity against cancer cells, specifically against murine lymphocytic leukaemia cells. Dolastatin 10 is a potent antimitotic agent that inhibits microtubule assembly and tubulin polymerization.It has demonstrated in vitro inhibitory activity against various human cancer cell lines, including melanoma, sarcoma, and ovarian cancer cells [29.85]; it entered several Phase I and II clinical trials. Because of the negative results of the Phase II clinical trials, dolastatin 10 has been withdrawn from antitumor clinical trials [29.86]. Nevertheless, the new synthetic derivative TZT-1027 [29.87] is currently being evaluated in Phase I clinical trials in Japan, Europe, and the US [29.87, 88]. In 1990, the cyanobacteria *Nostoc* spp. (ATCC 53789) was used to isolate a group of tubulin-binding compounds named *Cryptophyincs* by Schwartz and co-workers (Fig. 29.7). A Phase II study involving patients that had previously been treated with platinum-containing chemotherapy agents was unsatisfactory, and cryptophycin-52 was withdrawn from clinical trials. More recently, the analogs cryptophycin-309 and 249 have been studied in preclinical trials as anticancer drugs and seem promising for the next level of clinical trials [29.89]. Salinosporamide A or Marizomib (Fig. 29.11) are natural product discovered and derived from the marine *actinomycetes Salinispora tropica* in [29.90], and from *Salinispora arenicola* respectively. This marine strain contains the particular and characteristic β-lactone and exerts a potent inhibition of proteasome through a particular process of acylation of N-terminal Thr1O$^{\gamma}$ residue [29.91–93]. The commercial production of salinosporamide, marizomib, involves a peculiar process of saline fermentation using *Salinispora tropica* strain NPS21184 [29.90, 91]. This compound has completed Phase I clinical trials and has been shown to be effective against solid tumors and multiple myeloma, as well as demonstrating promissory bioactivity for use in combination with chemotherapeutics.

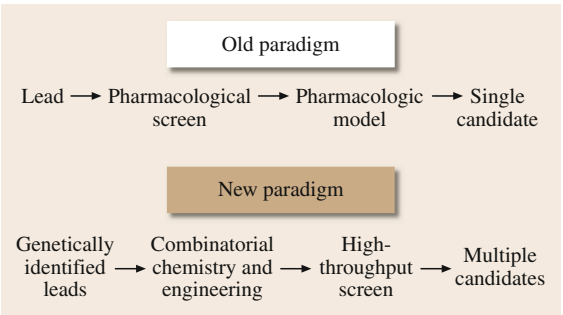

Fig. 29.7 Old and the new paradigms for drug discovery (modified after [29.84])

29.5 Marine Natural Products: New and Recurrent Challenges

29.5.1 Facing Supply Obstacles: Advances and Strategies

Marine natural products have yielded actual and promissory novel molecules with biological activity and an opportunity for the discovery of drugs. Besides the achievements reached so far, marine natural products face many difficulties. Indeed, the relation of bioactive molecules to biomass is not always enough to ensure an adequate supply for pursuing drug development based on a natural product. Bioprospecting is a key issue in this context of using natural marine compounds and at the same time protecting the source organism and its habitat from overexploitation. In some ways, a balance between sustainability and conservation is desired when collecting target marine organisms as natural product source material. However, the development of preclinical in vivo evaluation assessment of marine natural products has been hampered by a lack of material. Inventive solutions to the supply problem of these and other marine natural products with therapeutic properties may ultimately determine their future use, even if clinical outcomes are favorable [29.11]. Indeed, the supply issue concerning obtaining drugs from marine natural products and sustainable production, is still is an obstacle to be overcome and is one of the limiting and recurrent challenges for most pharmacologically active marine natural products, which can only be isolated in minute yields [29.94] (Table 29.2). The limited supply or large-scale exploitation can destroy the environment, and the complexity of the molecular structures in many cases makes the synthesis unfeasible. Currently, the methods of production include: large-scale collections, aquaculture, chemical synthesis, fermentation, and genomics (Table 29.3). The selection of the proper method to obtain any marine natural product will depend on the characteristics or complexity of the compounds of interest and their source [29.86].

Table 29.2 Limiting factors of marine derived therapeutics drugs (after [29.2])

Potential limiting factors of marine therapeutics drugs
Sustainable supply of clinical and industrial activities
Preclinical pharmacokinetics, bioavailability and half life
Pharmacogenetics, metabolic pathways
Physiological dose/therapeutics index
Toxicities, formation of possible metabolites

Among the available strategies biotechnology is an option for the production of bryostatins through the mariculture of the bryozoan *Bugula neritina* (ET-743) and the tunicate *Ecteinascidia turbinate*. However, again, this strategy is not feasible due to the costs associated with the destruction of culture facilities and stocks by storms and oceanographic phenomena or by diseases, which make production predictability unsure and unsafe. Total synthesis has been successfully established for halichondrin B, ET-743, bryostatins, and others, but in many cases has resulted in being economically infeasible due to the complexity of the molecular structures and the low yields of the final products achieved. For example, 1 t of the sponge *Lissodendoryx* sp. yields 300 mg of halichondrin analogs [29.95]. Contrary to this, the peptide ω-conotoxin MVIIA (SNX-111) from the marine mollusk *Conus magus*, known under the generic name of Ziconotide, can be obtained through synthesis in virtually unlimited amounts [29.16, 94].

When chemical synthesis is not feasible, and given the limited availability and regulation of marine resources, it is clear that product supply could never be completed successfully by these methods [29.94, 96]. In *E. turbinate*, nearly 1 t (wet weight) needs to be harvested in order to obtain approximately 1 g of ET-743 [29.97]. Another option is partial synthesis from precursors that are biotechnologically available, as in the case of ET-743, obtained through 21-step synthesis from the marine bacterium *Pseudomonas fluorescens*. However, a low overall yield of 1.4% was obtained because the starting material was generated by fermentation [29.86], a finding that exemplifies the process developed by a Spanish pharmaceutical company. Some bioactive compounds can be produced by

Table 29.3 Strategies to supply marine-derived therapeutics for clinical and industrial development (after [29.2, 10])

Potential limiting factors of marine therapeutics drugs
Natural habitats, controlled use of natural resources
Hemisynthesis: use or related compounds as starter followed by an industrial synthetic process
Aquaculture (on land) and mariculture by farming in natural mililieu
Synthesis
Fermentation
Genomics/metagenomics

microorganisms that invertebrates take up from the food chain, such as in the case of bryostatin 1, which is actually produced by a symbiotic microorganism living in the host bryozoans *B. neritina* [29.98, 99]. Hence, new methods for the development of marine natural products could diversify and facilitate production. It is hoped that synthesis and fermentation simultaneously with biotechnology may be the major methods for the development of marine natural products to reach industrial production.

29.5.2 Marine Natural Products: Paradigms, Genomics, and Biosynthesis Exemplified

The sustainable biotechnological production of pharmacologically important metabolites from marine organisms may be improved by genomics and with cloning and expression of the respective key biosynthetic gene clusters. A combination of the available biotechnological strategies must definitely be considered due the complexity of the structure and the novelty of marine natural products. Particular and more orientated approaches will open up new avenues for biotechnological production of drugs or drug candidates from the sea. In some ways, many compounds from marine organisms do not receive the required consideration that they deserve from pharmaceutical companies because of concerns about a sustainable, economic supply of the compounds [29.9]. The traditional process of drug discovery and delivery has been undertaken largely through the effects of a natural product or a derived synthetic agent on a living system (physiological process or pathological state) and a process of testing in vivo, as well as chemical synthesis. Thus, discoveries were achieved by a one molecule at a time process. The new paradigms in drug discovery are dominated by genomic-based approaches which are expected to lead to multiple new targets [29.84], which could represent a significant increase of approximately thousands of targets in the pharmacognosia of natural products (Fig. 29.7).

These approaches coupled and integrated with the tools of combinatorial chemistry, high-throughput screening, and bioinformatics would yield not just one clinical drug candidate, but rather up to hundreds of new candidates (Fig. 29.8).

The advanced and accelerated drop in the cost of DNA sequencing by new technologies makes it feasible for the highest performance and most economic approach to explore the biosynthetic potential of marine organisms and possibly to directly sequence their genomes. As many of these gene products are entirely novel, their activity cannot be inferred from a comparison to known protein databases, thus a functional metagenomics approach has the ability to identify novel genes on the basis of phenotypes, which lend themselves to high-throughput screens [29.100].

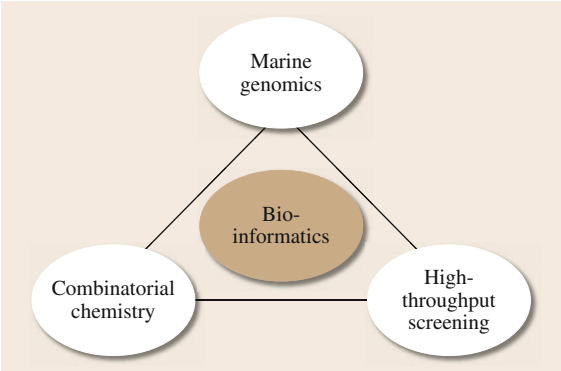

Fig. 29.8 A new model of drug discovery by coupling and integration of genomics, combinatorial chemistry, and high-throughput screening with bioinformatics (modified after [29.84])

Basically, functional metagenomics rely on screening DNA library clones for a phenotype, and the genes are recognizable by their function rather than by their sequence, while metagenomics only compares sequence data from sequences deposited in databases. When the genes of interest do not require any sequence analysis, this represents a great advantage for directly identifying new classes of genes for both known and indeed novel functions (Fig. 29.9). An environmental sample, e.g., seawater or marine invertebrate, is collected and then THE total community DNA is extracted from the sample.

The isolated DNA is then used to generate a metagenomic library using a suitable cloning vector. This library is then transferred to a suitable host strain, usually *Escherichia coli*, and individual clones can then be screened for the presence of enzymatic or other bioactivities encoded by the environmental DNA fragment. When coupled with a robust and high-throughput screen, this method is an extremely effective way of isolating novel enzymes from otherwise inaccessible microbes [29.100], as exemplified in Fig. 29.10. Advances in DNA sequencing and bioinformatic technologies make it possible to rapidly identify the gene cluster of bioactive compounds and in silico predict their chemical structure based on genomics informa-

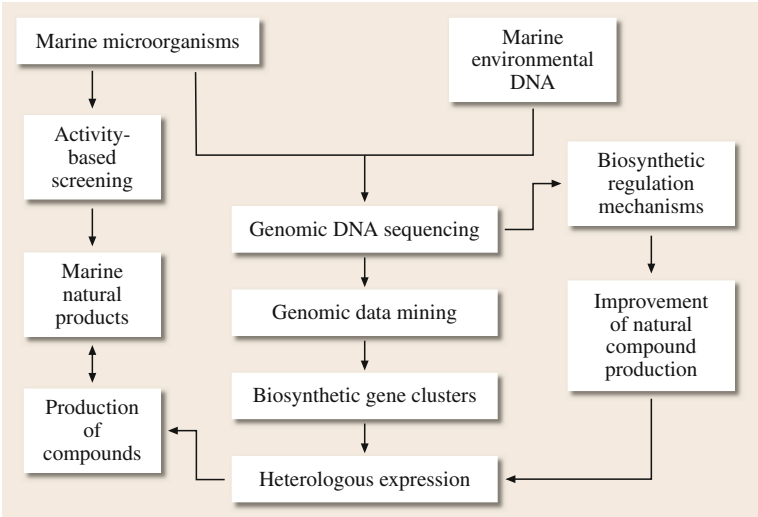

Fig. 29.9 Sequential steps of combined genome mining with activity-based screening for the discovery, generation, and production of marine natural products (modified after [29.101])

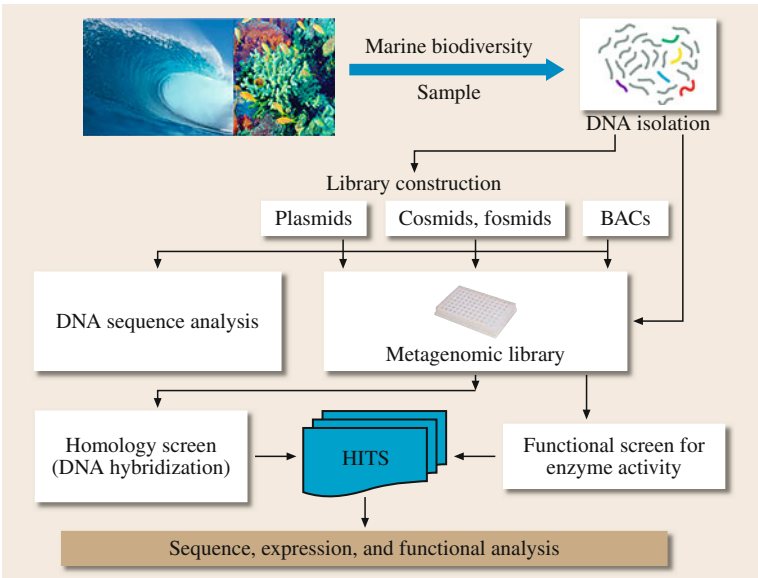

Fig. 29.10 Schematized diagram of the steps of functional metagenomics for the discovery of enzymes from samples of marine biodiversity, (after [29.100])

tion. These structural predictions can be used to guide compound purification and structure confirmation, and to identify new chemical entities.

In the case of *Salinispora tropica*, the compounds produced include the potent proteasome inhibitor salinosporamide A, the unprecedented halogenated macrolide sporolides A and B lymphostin, and salinilactam. Knowing the genomic sequence is the prerequisite to understanding and harnessing the diversity of the natural product producers. The genome sequence of the first seawater-requiring marine actinomycete, *Salinispora tropica*, was reported as containing large size of genes (516 kb) dedicated to polyketide synthase polyketide synthase (PKS) and/or non-ribosomal peptide synthetase (NRPS) biosynthesis. These megasynthases are responsible for many active natural product and are basic tools for combinational biosynthesis [29.101]. A truncated biosynthetic gene cluster was identified in the draft genome of *S. pacifica*, which is related to the 41 kb gene cluster in *S. tropica* for salinosporamide A biosynthesis, but the gene coding for the enzymes in the chloroethyl malonyl-CoA pathway

are absent in *S. pacifica*. From these results salinosporamide K was isolated, which structurally resembles salinosporamide A. The structures of salinilactam A and salinosporamide K described above are presented in Fig. 29.11; these were discovered by genome mining by [29.102].

Major advances in the biosynthesis of marine natural products have occurred during the last decade as a result of the integrated efforts in modern analytical technologies for the understanding of marine natural product biosynthesis at the genetic, enzymatic, and small-molecule natural product levels [29.103]. Amongst the vast array of marine natural products, the isoprenoids are one of the more commonly reported and discovered to date. In biosynthetic studies isoprenoids (C25, C30, C40) [29.104] represent the group of compounds of major distribution in the marine environment. Biosynthetic studies of early metabolic pathways in these molecules have been elucidated because of the differences between the mevalonate (MVL) versus the non-mevalonate 1-deoxy-xylulose-5-phosphate (DXP) route. Until quite recently, it was thought that isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the monomeric units in terpenoid biosynthesis, were made exclusively by the mevalonate pathway [29.104]. After the pioneering work of [29.105], it was demonstrated that there is an alternative non-mevalonate pathway for the formation of IPP and the isomeric DMAPP [29.106, 107] in the carotenogenesis of microalga, the richest source of natural β-carotene. This carotenoid plays an important role in cancer prevention and treatment as the major source of pro-vitamin A.

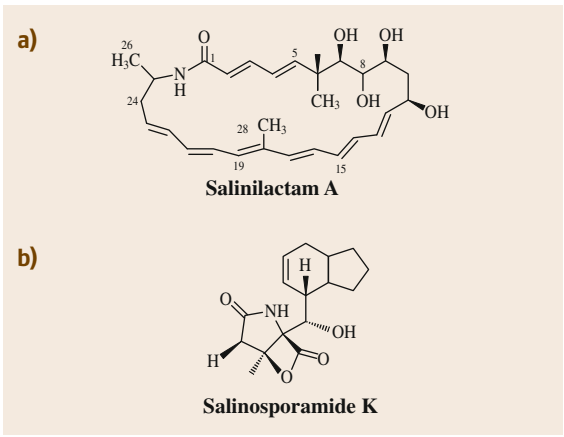

Fig. 29.11a,b Structures of salinilactam A **(a)** and salinosporamide K **(b)** discovered by comparative genomics (after [29.101])

29.5.3 Marine Natural Products at the Crossroad Between Functional Foods and Pharma

Functional food is known to play an important role in reducing health risks and improving health quality. Marine natural products have been recognized as rich sources of structurally diverse biologically active compounds and as functional ingredients in diets with health beneficial effects [29.108]. The relationship between nutrition and health has led to the concept of *functional foods* or *pharmaceutical foods*, which is a practical and new approach to achieving optimal health and possibly reducing the risk of disease. Natural products with bioactive properties that are consumed in a normal diet for enhanced health or reduced risk of disease are named functional foods according to the Food and Agriculture Organization of the United Nations (FAO. These products exhibit properties and benefits for the use in medicines, for safety and economical status reasons, and have fewer side effects than current drugs being prescribed. As is generally accepted, these metabolites have low potency as bioactive compounds when compared to pharmaceutical drugs, but since they are ingested regularly and in significant amounts as part of the diet, they may exhibit positive long-term physiological effects [29.109]. Marine natural products with functional ingredients include omega-3-oils, carotenoids, phycobiliproteins, complex polysaccharides, many bioactive metabolites and many other derivatives from alkaloids, terpenoids, and polyketides with different pharmacological properties such as cytotoxic, antitumor, anti-inflammatory, antioxidant, and therapeutic pain relievers [29.5], among others. Recently, the design of prebiotics (dietary ingredients that stimulate the growth or activity of beneficial bacteria in the gut) targeted at specific probiotics are expected to promote the general physiology and health of consumers, an aspect of great value in the emerging field of *symbiotics* (i. e., combinations of probiotics and prebiotics with enhanced health benefits). In the case of non-digestible oligosaccharides (prebiotics), a wide range of such compounds, including fructo-galacto and xylo-oligosaccharides, which specific functions have been poorly studied on target organisms. Also, the metabolism of oligosaccharides by probiotics may lead to the development of prebiotics

with specific functional properties and their associated health outcomes. Soluble polysaccharides from marine bacteria and algae are promissory for human dietary fiber and as possible prebiotic compounds. The need to incorporate functional natural products and ingredients in foods in living systems, animals and human, is a challenge to overcome. In 2006, *Ochoa* and *Olmos-Soto* [29.110] assessed functional feed supplemented with vegetable protein-carbohydrates and a *Bacillus* strain (GRAS, generally recognized as safe) as probiotic due to the high enzyme level production and secretion of antimicrobial peptides in a shrimp model (*Litopenaeus vannamei*). In this approach, the enzymatic action produced by the bacteria and the shrimp enzyme activity enhances carbohydrate digestion and assimilation and, induced a healthy condition in *L. vannamei*. Similar results were obtained when applying this biotechnology to edible fish such as Tilapia (*Orechromisaureus*) [29.111].

Moreover, natural products as well as primary and secondary metabolites from marine sources have been reported to promote various functionalities like growth enhancers, immune stimulation, antioxidants, antistress, ferhormones, and antimicrobial properties in finfish and shrimp larviculture, due to active principles such as alkaloids, carotenoids, pigments, phenolics, terpenoids, steroids, and essential oils. Functional metabolites like carotenoids are bioactive ingredients with physiological or health benefits beyond basic nutritional functions in organisms. In the case of carotenoids as bioactive molecules in shrimp, the functionality resides, to a large degree, on their conversion to retinoids. These molecules are derived from vitamin A and are involved in the activation of hormonal nuclear receptors and play a prominent role in many developmental processes, including embryonic development and differentiation of various cell types. Studies on the bioactive roles of carotenoids and retinoids in shrimp *Litopenaeus vannamei* have been undertaken [29.112, 113]. According to the excellent results obtained, authors emphasized the positive effect of these metabolites as bioactive ingredients and hormone-like compounds in shrimp maturation and reproductive performance. The anticancer properties of carotenoids via shrimp food could improve the health of consumers and accomplish the required doses for these non-produced, de novo metabolites. Innovative technologies will help to understand the relation of the functions of human genes in relation to disease, and in particular the extent to which nutrition and food components can influence health promotion and disease prevention, and to approach therapeutic pharmacology through food. World population, at all levels, is demanding natural medicines as healthier foods with special properties, mainly acting as passive immune protective and therapeutic agents, in some ways, as was the intention of the Greek philosopher Aristotle, over 2000 years ago, in his phrase: *Let food be your medicine – let your medicine be your food.*

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30. Biocatalysts from *Aplysia*: Sweet Spot in Enzymatic Carbohydrate Synthesis

Antonio Trincone

Speaking in general terms, glycosylation is considered to be an important method for the structural modification of compounds with useful biological activities. Glycosylation allows conversion of lipophilic compounds into hydrophilic ones, thus improving their pharmacokinetic properties or giving access to new drug delivery systems (prodrugs). Examples include synthetic carbohydrate-based polymers used as coating agents and as biomedical materials, or novel dietary carbohydrates introduced as food additives. Finally, chromophoric oligosaccharides are of widespread interest for the kinetic analysis of hydrolytic activities and to characterize the mode of action of particular enzymes (i. e., exo or endoglycosidases). Hence carbohydrate-based compounds have proved to be a valuable tool in different fields of applicative interest, such as clinical, biological, and food chemistry. Different enzymes acting on these molecules are commercially available, but a greater interest would entail a search for biocatalysts with new catalytic characteristics. The marine environment has been shown to be a very interesting source for new glycosyl hydrolases both for hydrolytic and synthetic applications. *Aplysia* is a genus of sea hares belonging to the family *Aplysiidae*, containing different species of organisms. *Aplysia fasciata* and *Aplysia kurodai* are two examples; they are herbivorous animals and eat a variety of red, green, or brown algae and have been revealed to be potent producers of a library of glycoside hydrolases applied in the synthesis and hydrolysis of glycosidic bonds. In this chapter, examples of applications of such enzymes will be discussed.

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30.1 Biocatalysis, Glycosylation and Marine Enzymes

In the field of biocatalysis the need for new biocatalysts is a current topic that involves a series of different points. Particular attention must be paid to several characteristics of enzymes if they are to be used on an industrial scale, and different key properties are recognized for this aspect. Broad substrate specificity, which generally characterizes extracellular fungal enzymes and is useful for the general application of a biocatalyst, may not be strictly requested in fields such as the production of fine chemicals and pharmaceuticals or the hydrolysis of specific linkages for analytical purposes, where very selective biocatalysts ensure fidelity towards very specific linkages and good yields. Substrate specificity and affinity somehow play leader roles among other catalytic characteristics. As an evolved property the specificity of a biocatalyst is linked to the

metabolic function and strictly related to the natural source and environment.

In comparison with chemical glycosylation, including methods that are generally based on harsh conditions or necessitate toxic (heavy metals) catalysts, enzymatic routes are particularly useful for accessing new products or for the modification of complex natural products such as carbohydrates [30.1]. Actually, enzymes may represent an imperative choice in fields related to the food domain or cosmetics in which adopting synthetic chemistry is sometimes not acceptable at all.

As sources of biocatalysts alternative to microorganisms, like plants or animals, almonds and pig and horse livers furnished few but relevant examples [30.2]. Carbohydrates are important molecules in various technological domains. In the field of polymer chemistry, for example, synthetic carbohydrate-containing polymers have a wide range of applications in medical biotechnology as coating agents or molecular recognition biomedical materials. Molecular networks presenting as hydrogels may find application as biomaterials because of their high water content, homogeneity, stability, and expected nontoxicity [30.3]. In food technology, during the last decade, a number of novel dietary carbohydrates produced by enzymatic synthesis have been introduced [30.4]. Resulting from advances in fine chemicals production, the easy synthesis of chromophoric oligosaccharides (such as nitrophenyl and 4-methylumbelliferyl glycosides and others) of defined structure is of widespread interest because these products are valuable tools for the kinetic analysis of hydrolytic activities and for characterization of the action of new enzymes (i. e., exo or endoglycosidases). Finally, in cosmetics the prodrug action of enzymatically glycosylated natural lipophilic antioxidants is nowadays often considered. Thus, glycosylation is considered to be an important method for the structural modification of compounds with useful biological activities. It allows conversion of lipophilic compounds into hydrophilic ones, improving pharmacokinetic properties and producing more effective drug delivery systems (prodrugs) [30.5].

The marine ecosystem is an almost absolutely new and unexplored source of biological material and can be also a surprising source of enzymes carrying new and interesting catalytic activities to be applied in biocatalysis [30.6]. Marine sources are represented by marine microorganisms, including extremophiles and marine plants or animals. Marine microorganisms may be used for industrial production of many enzymes [30.7]; moreover, although not numerous, examples of different enzymes from marine plants and animals are also reported in the literature [30.8]. Marine enzymes are characterized by well-known habitat-related features such as salt tolerance, hyperthermostability, barophilicity, and cold adaptivity . In addition, their novel chemical and stereochemical characteristics have increased the interest of biocatalysis practitioners from academia and research. Marine (micro)organisms should be considered as an important source of biocatalysts for the degradation of complex polysaccharides. Herbivorous marine molluscs such as abalone, sea hares, and small snails possessing various kinds of polysaccharide-degrading enzymes could efficiently depolymerize alginate, mannan, laminarin, and cellulose. These enzymes play important roles in the metabolism of the organism digesting polymeric materials to oligosaccharides and monosaccharides as carbohydrate nutrients. However, these molecules (poly and oligosaccharides) are known to exhibit various therapeutical functions and are recognized as potential raw materials for biofuels [30.9].

Different interesting results related to the presence of glycosyl hydrolases have been reported for *Aplysia fasciata* and *Aplysia kurodai* in the last decade. Both species have been revealed to be potent producers of a library of glycoside hydrolases applied in the synthesis and hydrolysis of glycosidic bonds. In this chapter, examples of applications of such enzymes will be discussed.

30.2 Biocatalytic Methodologies to Access Glycosides and Oligosaccharides

30.2.1 Enzymes

Nowadays there exists a copious amount of information concerning the synthesis and transformation of complex saccharides in living organisms. Glycosidic linkage is by far the most common chemical bond joining a carbohydrate to another molecule. It is one the most important chemical characteristic of a saccharide, although diverse functional groups can be found in carbohydrate chains (ester, sulphate, etc.) in various positions, which

can alter or modulate biological functions. Enzymes responsible for the synthesis of glycosidic linkage have been recognized as transglycosylases and named glycosyltransferases, specifying the glycosyl donor and the reaction product. These enzymes transfer sugar moieties from the activated donor to specific acceptors, forming a specific glycosidic bond, and are responsible in vivo for the synthesis of most cell-surface glycoconjugates, using eight common sugar nucleotides as activated donors (Leloir pathway). Sugar phosphates can act as donors for other glycosyltransferases (non-Leloir pathway).

Glycoside hydrolases (glycosidases), represent another widespread group of enzymes involved in the carbohydrate metabolism, being responsible for the hydrolysis of glycosidic linkages; these enzymes can act as exo or endoglycosidases and are involved in a series of biological events of important nature, such as energy uptake in processes inherent in cell wall metabolism, in glycan processing during in vivo glycoprotein synthesis, etc. Glycosidases and endoglycosidases are enzymes that are currently leading to new expectations in research and development due not only to their natural capability to act on the internal part of an oligosaccharide chain, but also to the possibility of transferring a whole carbohydrate block to a new acceptor, thus enabling easy reconstruction of complex glycoconjugates (e.g., glycopeptides, bioactive oligosaccharides, etc.). In addition, the profitable use of the hydrolytic capabilities of glycosyl hydrolases for mass preparation of oligosaccharides from natural marine or terrestrial polymers should be mentioned here [30.10].

The stereochemistry of the mechanisms of glycoside hydrolases, analyzed more than 50 years ago [30.11], allowed the classification of glycoside hydrolases in inverting and retaining enzymes in accord to the anomeric configuration found in the product with respect to that in the starting substrate. Very recently, it has become clear that other mechanisms are evolved, such as the one based on elimination [30.12].

In the inverting glycoside hydrolases, the glycosidic substrate, a water molecule, and two carboxylic acid groups represent the key elements of the chemical machinery within the active site of the enzyme. The two carboxyl groups acting as general acid and general base are distant; between them there is a (10.5 Å) value double that of retaining enzymes. This creates room for the substrate and a water molecule to access the active site. Hydrolysis occurs via a single displacement mechanism that involves an oxocarbenium ion-like transition state. The product has the opposite configuration at the anomeric center with respect to the substrate. If an inverting enzyme catalyzes a transglycosylation reaction transferring the sugar on an acceptor other than water, the product of this reaction would be the inverted anomer of the starting glycoside. This transglycosylating activity is never found with these enzymes; in fact, they would have to be anomerically indiscriminate to possess such activity. However, inverting enzymes catalyze oligosaccharide synthesis via reversal of the hydrolysis reaction; in the presence of a free sugar and alcohol acceptor, back reaction can produce a glycoside (see below).

A reaction characterized by a double displacement mechanism is operative in retaining glycoside hydrolases; two sequential steps can be distinguished in this case. In the first, the substrate binds the active site, then a nucleophilic group (aspartic acid, glutamic acid, or histidine) attacks the anomeric carbon atom. Protonation of aglycone oxygen by an acid proton, with concomitant glycosidic bond cleavage and ester bond formation, leads to the formation of the covalent ester glucosyl-enzyme intermediate, characterized by anomeric inversion at C1. In the second step, a hydroxyl group from a water molecule (in the case of the hydrolysis reaction) or from a different residue (for transglycosylation reactions) attacks the ester bond of the intermediate with re-inversion of configuration of the anomeric carbon. As a result, the final product has the same anomeric configuration as the initial substrate. The degree of bond cleavage may significantly vary in the transition states for each step, according to nature of the enzyme and substrate structure [30.10]. The retaining mechanism for α and β-glycosidases appears to have similarities and differences, as, for example, the complementary stereochemical configuration at the glycosyl enzyme intermediate. Speaking in general terms, evidence for ring distortions in transition states is collected for different biocatalysts. The distortion is driven by interactions between the enzyme and the substrates and greatly assists bond cleavage, allowing in-line attack of the nucleophile at the anomeric center. It can also help the substrate to acquire a conformation closer to that of the oxocarbenium transition state. Inhibition of these enzymes through the action of compounds mimicking the structure of the transition states is considered a major area for the development of new therapeutic agents. A collection of detailed studies for the elucidation of the role of single amino acids in the active sites, which could be propaedeutic for genetically tailored production of synthetic enzymes (glycosynthases, see below), can be found in literature [30.13].

A new four-step mechanism for the hydrolysis of glycosidic bonds involving oxidation-elimination-addition-reduction reactions has been reported for family 4 [30.14] of glycoside hydrolases. Members of this family require divalent metal ion and a NAD^+ cofactor for activity. Also, α-1,4-glucan lyases, cleaving glycosidic linkages in starch and glycogen, and polysaccharide lyases which are involved in the degradation of glycosaminoglycans and pectin, belong to the class of enzymes that employ elimination chemistry.

30.2.2 *Natural* and Engineered Methods for Synthesis

Two general protocols are in use for the utilization of glycoside hydrolases in the synthesis of glycosidic linkage: the reverse hydrolysis procedure and the kinetic approach. The former, starting with free monosaccharide and an acceptor, is reported as an efficient and cost-effective methodology for just a few enzymes and as a poor-yielding procedure for others [30.15]. The alternative kinetic approach is based on the use of a glycosyl donor which produces an intermediate glycosyl enzyme. In presence of a nucleophile other than water, this ester intermediate is resolved into products that as new glycosides could still be substrates for the enzyme, hence the reaction must be carefully monitored to achieve good yield. Both these systems could be considered unnatural due to the natural hydrolytic nature of glycosyl hydrolases. However, they can be called *natural* in comparison to the ad hoc produced mutant enzymes, known as glycosynthases, introduced several years ago as an elegant approach to solve the back-hydrolysis problem encountered using wild type biocatalysts. This topic has been extensively reviewed, and readers are referred to reports on this technology [30.16–18]. Synthesis of oligosaccharides by this engineered methodology can always be considered as an emerging alternative for a possible scale-up of oligosaccharide production for each single example of a newly isolated biocatalyst transformed in the glycosynthase version.

A stereochemical outcome of a glycosidase-based reaction includes natural absolute anomeric selectivity derived from the chemistry of the reaction mechanism. However, regioselectivity and other aspects are dependent on the nature of the enzyme towards the donor(s) and product(s) formed. Depending on the structure of the active site, the biocatalyst can accommodate certain sugar acceptor(s) and promote regiospecific formation of glycosidic linkages. The impressive variety of available glycosidases clearly indicates that the potential biodiversity of these enzymes (and also of the possibly derived glycosynthases) is still largely unexplored and that potential applications of these biocatalysts will increase in the near future starting from the knowledge of wild-type biodiversity.

30.3 The Marine Ecosystem as a Source for New Glycoside Hydrolases

The importance of the marine environment as a source of different and interesting glycosyl hydrolases can be found in the literature as overall results of several research projects conducted in different nations in the world from the beginning of the 1960s. Attention was focused mainly on the hydrolytic selectivity of these enzymes, since efforts of the structural identification of carbohydrate molecules then faced the complexity of oligosaccharide structures, before the flourishing development of instrumental analysis that started in the 1980s. Various potent activities were found in marine organisms such as *Charonia lampas*, *Turbo cornutus*, and others [30.19–21]. However, the interest in these enzymes also led to a raised interest in the study of phylogenetic position and feeding habits of various molluscs [30.22]. The sea still continues to furnish glycosyl hydrolases, which can be used in different fields: (i) to solve the structural complexity of new oligosaccharides obtained purely by improved chromatographic techniques, (ii) to help in the treatment of waste material for the recovery of important value-added molecules, and (iii) to allow synthesis of glycosidic bonds.

The distribution of fucoidan hydrolases and other glycosidases, for example, was only recently investigated in 33 species of marine invertebrates [30.23], although the presence of cellulases has been assessed in *Littorina* sp. [30.24] and in *Telescopium telescopium* [30.25] for a long time. It is of interest to note that both marine microorganisms and invertebrates such as *Haliotis*, *Mizyhopecten yessoensis*, and sea urchins all contain fucoidan hydrolases and other important glycosyl hydrolases [30.26–29]. Fucoidan hydrolases are important enzymes for structural identification of com-

plex structures of fucoidan, a sulphated polysaccharide from brown algae exhibiting biological activity in mammalian systems, which is very promising for human therapeutics[30.30].

An α-L-fucosidase from the liver of the marine gastropod *Charonia lampas* was purified to homogeneity [30.31]. Substrate-specificity studies using a number of fucosylated oligosaccharides of the lacto-N and lacto-N-neo series and a synthetic disaccharide confirmed that the enzyme catalyzes the hydrolysis of a broad range of fucosidic linkages. *Rhodothermus marimus* ATCC 43812, a thermophilic bacterium isolated from marine hot springs, possesses hydrolytic activities for depolymerizing substrates such as carob-galactomannan [30.32]. The Antarctic marine ecosystem has also been the source for the cold-adapted α-galactosidase as found in *Pseudoalteromonas* sp. 22b [30.33]. An updated overall analysis of the literature can be found in different articles, and readers are referred to [30.7–9] and references cited therein.

Different aspects should be considered for marine glycosidases. In the first place, scientific attention has been focused on biocatalysts from marine extremophiles because of the stability and novelty of catalytic properties that these proteins can offer and the simplicity in which they can be expressed. Marine glycosidases, as resulting from our analysis, are well placed in the expanding field of glycobiology. Among the examples, the marine α-galactosidase from *Pseudoalteromonas* [30.34], which is capable of removing immunodeterminant sugar residues and the sialidase from a tentatively assigned *Pseudomonas* used for the preparation of GM1 from a mixture of polysialogangliosides [30.35], are two exciting cases. It is outside the scope of this section to list each glycosyl hydrolase isolated from sea organisms. Above, only few examples are mentioned while in the following sections remarkable examples restricted to sea hares belonging to the family *Aplysiidae* will be reported.

Aplysia is a genus of sea hares belonging to the family *Aplysiidae* to which *Aplysia fasciata*, *Poiret* 1798, and *Aplysia kurodai* belong; these molluscs are very common. They are herbivorous and eat a variety of red, green, or brown algae and eelgrass [30.36]. Animals belonging to the *Aplysiidae* family of sea hares are often quite large and have a rounded body shape with long rhinophores on their heads. The Californian black sea hare, *Aplysia vaccaria*, could be considered the largest living shell-less gastropod (20—75 cm, weighing well over 2 kg). They are cosmopolitan and found in temperate and tropical seas where they inhabit shallow coastal areas or sheltered bays with vegetation. The genus *Aplysia* has proved useful as a model in neurobiology for the study of electrical synapses, which mediate the release of clouds of ink [30.37].

30.4 Glycoside Hydrolases Present in the Genus *Aplysia*

Various interesting results related to the presence of glycosyl hydrolases have been reported on *Aplysia fasciata* and *Aplysia kurodai* in the last decade. Both species have been revealed to be potent producers of a library of glycoside hydrolases applied in the synthesis and hydrolysis of the glycosidic bond. In this section we analyze the results obtained in the isolation and applications of biocatalysts from *A. fasciata* and *Aplysia kurodai* with emphasis on products, reaction conditions, yields, and regioselectivity.

The author's group started a general survey of glycosidase activities present in the mollusc *Aplysia fasciata* several years ago [30.38]. Three main parts of the animal were analyzed: (i) the mantle and other external parts, including oral tentacles, rhinophores, eyes, foot, and parapodia; (ii) the hepatopancreas; and (iii) other visceral mass, including the digestive, excretory, blood-vascular, and reproductive systems. Many glycosyde hydrolase activities were assayed by using chromophoric substrates of different sugars (fucose, galactose, glucose, mannose, *N*-acetyl glucosamine, and xylose), and interesting results were obtained. The extracts from hepatopancreas and from visceral mass resulted rich in glycoside hydrolases; their activities were in the range found in other marine molluscs [30.39]. By contrast, the extract of external parts did not hydrolyze any of the substrates tested. Interestingly, the β-D-galactosidase and the β-D-glucosidase enzymes from hepatopancreas and visceral mass, respectively, were present in large amounts. An interesting α-mannosidase was also noticed.

The ability of α-glucosidases to perform transglucosylation reactions is a relevant issue from the biotechnological point of view (in the food industry, for the production of glycoconjugates, etc.).

Products of various molecular structures, from simple alkyl glucosides to different complex oligosaccharides used in industrial applications, are enzymatically synthesized by the use of α-glucosidases of bacterial, fungal, animal, and vegetal origin [30.1]. The β-D-glucosidase activity was the most abundant glycosyl hydrolase activity found in the visceral mass homogenate of *Aplysia fasciata*. The purified enzyme gave only one band of 69 kDa on SDS-PAGE (SDS: sodium dodecyl sulphate, PAGE: polyacrylamide gel electrophoresis), while the molecular mass of the native enzyme was 255 kDa, indicating that this protein is a homotetramer. It showed a pH optimum at 5.8, and the optimum temperature at this pH was in the range 36–44 °C; pH 5.8 and 34 °C were identified as the best conditions for performing both hydrolytic and transglycosylation experiments [30.40]. Organic solvent resistance was also of interest in view of the low solubility of aryl substrates that can be used as donors and for conceivable use of hydrophobic acceptors in transglycosylation reactions. Enzymatic resistance to organic solvents was studied at 10% concentration. A good resistance of the enzyme to dimethyl sulfoxide (DMSO, 80% residual activity after 24 h), high sensitivity to acetonitrile and dimethylformamide (DMF, total loss of activity after few minutes), and an intermediate resistance to acetone (50% residual activity after 24 h) were recorded. The study of substrate specificity started using starch, amylopectin, amylose, isomaltose, panose, pullulan, and saccharose were not hydrolyzed by this enzyme, although a feeble reaction using trehalose was observed. Moreover, no enzymatic hydration of glucal (with formation of 2-deoxyglucose) was detectable. Nitrophenyl glucoside (*p*NP-α-D-Glc) and the α-maltoside derivative (*p*NP-α-D-Glc-(1,4)-α-D-Glc) were highly hydrolyzed, while the β-form (*p*NP-β-D-Glc-(1,4)-α-D-Glc) was hydrolyzed to a lesser extent. The α-glucosidase from *Aplysia fasciata* was very active on the α-1,4 glucosidic linkage, as it was assessed by measuring the kinetic parameters for the hydrolysis of maltose (V_{max} 115 U mg^{-1}, K_M 5.70 mM, k_{cat} 489 s^{-1}), *p*NP-α-D-Glc (k_M 0.26 mM, k_{cat} 163 s^{-1}), and *p*NP-β-D-Glc-(1,4)-α-D-Glc (k_M 2.06 mM, k_{cat} 75.9 s^{-1}). The α-1,6 glucosidic linkage was only poorly hydrolyzed as it was noticed that the specific activity for maltose was 40 times higher than that for isomaltose (V_{max} 2.9 U mg^{-1} by using 22 mM substrate). According to the Michaelis constants for *p*NP-α-D-Glc and maltose, it could be suggested that the subsite +1 of this enzyme has a higher affinity for the aryl group than for glucosyl residue. Although the k_{cat} value for maltose was three times higher than that for *p*NP-α-D-Glc, the catalytic efficiency showed that in vitro the former is the preferred substrate for this enzyme ($k_{cat}/k_M = 627$ mM^{-1} s^{-1}). A still good catalytic efficiency was observed in the hydrolysis of *p*NP-β-D-Glc-(1,4)-α-Glc (k_{cat}/K_M 36.8 mM^{-1} s^{-1}), while no hydrolytic activity was detected when the pure enzyme was incubated in the presence of *p*NP-β-D-Gal or *p*NP-β-D-Man. This overall picture of substrate specificity, together with the fact that this enzyme is not able to hydrate glucal, suggested that the enzyme from *Aplysia fasciata* may belong to family I type α-glucosidases [30.41], corresponding to family 13 of glycoside hydrolases (GH), however sequence similarity studies are necessary to establish this.

Because maltose was a very good substrate for this marine α-glucosidase, its bioconversion was studied in detail (Fig. 30.1). The time course for the bioconversion of maltose was studied using 93 mM maltose and 18 µg of pure protein per ml. Aliquots were withdrawn at different time intervals and analyzed by HPAEC-PAD (High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection) using a CarboPacTM PA1 analytical column (Dionex). In these conditions, within the first hour of reaction ca. 60% of maltose was consumed, forming maltotriose and panose; the concentration of maltotriose decreased to a very low value after 360 min, and the concentration of the regioisomer panose increased to a plateau (after ca. 180 min), reaching a value of ca. 8 g L^{-1} in

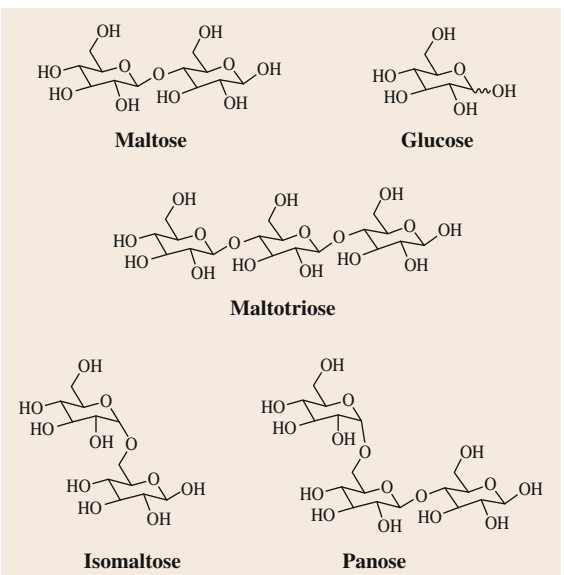

Fig. 30.1 Bioconversion of maltose

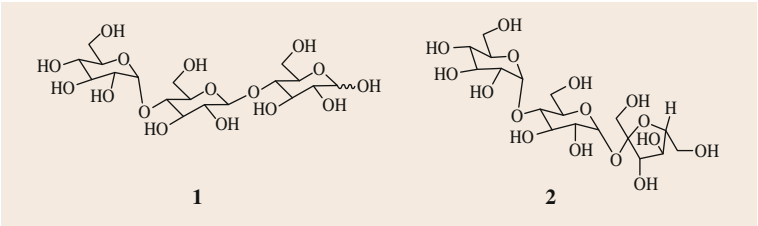

Fig. 30.2 Trisaccharides formed by transglycosylation from cellobiose and sucrose using α-glucosidase of the marine mollusc *Aplysia fasciata*

the reaction mixture. The disaccharide was identified as isomaltose (6-O-α-D-glucopyranosyl-D-glucose) by comparison of ^{1}H and ^{13}C NMR spectra of the acetylated derivative with an authentic standard sample. COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), and ^{1}H–^{13}C NMR correlation spectra of the acetylated derivative of the trisaccharide showed unambiguously the panose structure; the tetra and pentasaccharidic nature of the remaining products was established both by their R_fs in and by mass spectroscopy (ESI-MS, electrospray ionization positive ions) of acetylated derivatives. It led to the formation of several products, the most abundant being the trisaccharide panose and the disaccharide isomaltose whose structures were determined by nuclear magnetic resonance (NMR) spectroscopy. Maltotriose was also a good substrate with a pattern of products similar to that observed using maltose: maltotetra up to maltohexaose were found in the early stages of reaction but panose and isomaltose were later identified as end products also in this reaction. Using maltoheptaose the enzyme performed a reaction which is in agreement with the reaction of maltose [30.40].

Transglycosylation reactions are possible using the aryl substrate *p*NP-α-D-Glc or maltose as donors. Interestingly, polyglycosylation was often observed. When maltose was the donor and *p*NP-β-D-Glc was the acceptor, *p*NP-β-D-Glc-(1,4)-α-D-Glc was the sole product in the early stages of reaction (30 min), then the isomaltoside derivative, *p*NP-β-D-Glc-(1,6)-α-D-Glc, was also formed and it accumulated up to the end when it became the most abundant isomer with a yield of ca. 15–20%. Using *p*-nitrophenyl β-cellobioside (*p*NP-β-D-Glc-(1,4)-β-D-Glc) as acceptor; the formation of a trisaccharidic product was observed while both α and β-anomers of *p*-nitrophenyl galactopyranoside were not glycosylated in significative amounts. The preferential enzymatic formation of α-1,4 linkages (Fig. 30.1) in the early stages of reaction and the accumulation of α-1,6 products were confirmed by time course experiments. The body of these results suggested an intramolecular arrangement from maltotriose for the production of panose from maltose, instead of the alternative intermolecular direct α-1,6 glucosylation of maltose itself. Furthermore, as is indicated by the absence of higher molecular weight (MW) products in the maltoheptaose reaction, a possible molecular limit in the acceptor site of the enzyme is conceivable [30.40].

Reactions with different acceptors such as cellobiose and saccharose (Fig. 30.2), pyridoxine (Fig. 30.3), naringin and other antioxidants (Fig. 30.4), and 9-fluorenone derivatives (Fig. 30.5) were studied in detail.

The most abundant (90% at 25% yield, ca. 4 g L^{-1}) product, **1** (Fig. 30.2), from cellobiose was recognized as the α-1,4 glucosyl derivative; however, other minor isomers were also present. Also sucrose was α-glucosylated at position 4 of glucose unit forming erlose (4G-α-D-glucosyl sucrose), **2** (Fig. 30.2). Compounds of type **1**, (Fig. 30.2), α-glucosyl cellobioses, have recently been produced from sucrose donor and cellobiose acceptor by alternansucrase [30.42], while type **2** (Fig. 30.2) products, glucosyl sucroses, were also synthesized enzymatically by α-glucosidase from spinach [30.43] and other enzymes.

Glucosylation of pyridoxine by purified α-glucosidase from *Aplysia fasciata* gave the products shown in Fig. 30.3 [30.44]. The reaction was conducted adding pyridoxine portionwise (keeping its concentration at 7.8 mg mL^{-1} as the highest level) to a 1 M maltose solution containing 15.5 μg of enzyme $mmol^{-1}$ of maltose, it was possible to add four aliquots of pyridoxine (during 55 h total reaction time) that were well consumed (81% conversion), forming monoglucosidic **1** and **2** (molar yield 40%, 72 mM,

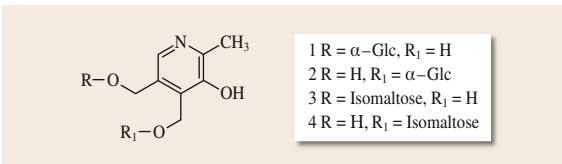

Fig. 30.3 Pyridoxine glucosides obtained by transglycosylation using the α-glucosidase from *Aplysia fasciata*

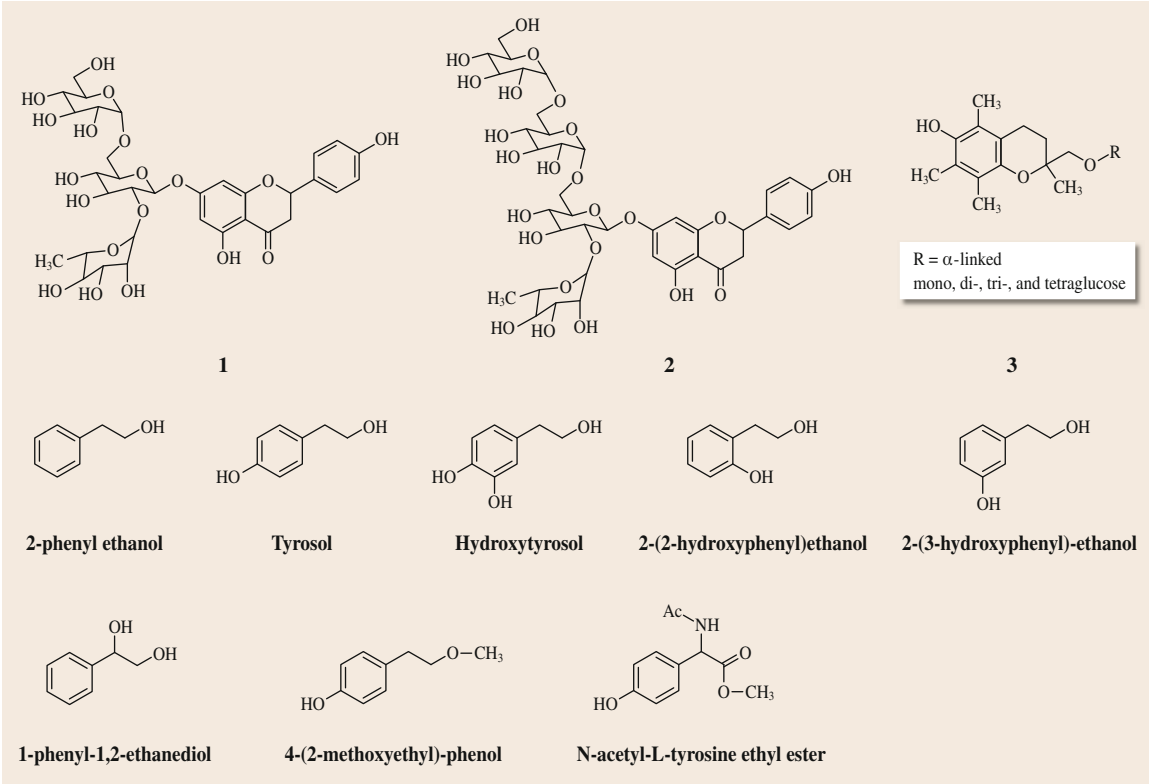

Fig. 30.4 Products formed by naringin glycosylation and substrates tested for the study of chemoselection, using α-glucosidase from *Aplysia fasciata*

24 g L^{-1}) and diglucosidic **3** and **4** (molar yield 41%, 71 mM, 35 g L^{-1}) and also traces of triglucosidic derivatives of pyridoxine. Glucosides of pyridoxine are more stable against light and heat with respect to the aglycone, and these glucosides and other derivatives are important molecules not only from a nutritional but also from a pharmaceutical perspective [30.45]. Two monoglucosides of pyridoxine are synthesized in different proportion, with **1** (Fig. 30.3) being the most abundant (75%). The disaccharide mixture is almost totally (95%) composed by compound **3** (Fig. 30.3), and it has been also found that the observed selectivity is entirely expressed only during the two transglycosylation events and it is not due to a possible differential hydrolysis of regioisomeric products **3** and **4** [30.44].

The enzymatic modification of naringin and other related compounds in citrus is of current biotechnological interest for food and pharmaceutical industries, both for the efficient and food-compatible reduction of the bitter taste of juice and for the modification of pharmacological activities of the molecule. Debittering grapefruit juices has been achieved by different techniques, including transglycosylation reaction. Glucosylation of naringin by marine α-D-glucosidase from *Aplysia fasciata* is a very efficient process in terms of regioselectivity and yield of reaction. The reaction can be conveniently conducted in a wide range of naringin content, and almost complete conversion of naringin can be obtained. Suspensions of naringin can be used up to 90 mg mL^{-1} initial acceptor concentration. In different experiments it was demonstrated that the enzyme was still active after 48 h in the presence of this high amount of acceptor and that one of the diastereomers of the naringin is preferred by the enzyme from *Aplysia fasciata* during glucosylation/deglucosylation enzymatic steps. One out of eight possible mono-glucosylated derivatives was obtained, **1** (Fig. 30.4). Further functionalization took place on the new α-glucose moiety at the C-6 specific position just added, forming only isomaltosyl derivative **2** (Fig. 30.4). Remarkably, most of the naringin naturally occurring in fresh grapefruit juice can be directly modified to form the two products shown in Fig. 30.4 [30.46].

The synthesis of interesting and potentially useful α-oligoglucosides of 2-hydroxymethyl-2,5,7,8 tetramethylchroman-6-ol, **3**, Fig. 30.4 can be effectively achieved by using the polyglycosylating α-glucosidase from the marine mollusc *Aplysia fasciata*. The conversion of the acceptor is almost total at moderate concentration of maltose (150 mM), at a molar excess of 30. At the beginning of the enzymatic reaction (20% conversion achieved in 1 h), only (S)-diastereomer of the glucoside of **3** (R = α-D-Glc) Fig. 30.4 was obtained. This is an intriguing result in that chemoenzymatic routes to enantiopure (R) and (S)-2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol are of current interest [30.47]. At the end of the polyglycosylation reaction, a number of oligoglucosides possessing different structures were observed in the reaction mixture. The percentage composition of the reaction mixture at 24 h was as follows: monosaccharide, 47%; two disaccharides, 42%; three trisaccharides, 9.4%; and a tetrasaccharide, 1.6%. The isomaltoside, the most abundant disaccharide produced, showed a radical scavenging activity similar to that of the aglycone **3** (R=H) Fig. 30.4, so that it can be proposed as a new hydrosoluble antioxidant agent. The green-based methodology by which it is synthesized allows the inclusion of various technological fields (cosmetics, the food industry, etc.) for exploitation. Furthermore, an intriguing correlation between the nature of the interglycosidic linkage and scavenging activity was noticed both in disaccharides and in trisaccharides. This structure–activity relationship deserves further investigation and the synthesis of pure diastereomeric material will be of fundamental importance [30.48].

An efficient enzymatic two-step procedure for the synthesis of hydroxytyrosol and tyrosol α-glucosidic derivatives based on α-glucosidase from *Aplysia fasciata* and a commercial tyrosinase from mushrooms, was described very recently [30.49]. Considering the entire double enzymatic process for the formation of these glycosidic derivatives of hydroxytyrosol from tyrosol, it was possible to glycosylate regioselectively only the alcoholic primary position for producing hydroxytyrosol mono and disaccharidic derivatives, with a reaction total yield which was of ca. 20% for each product obtained at final concentrations of 9.35 and 10.8 g L^{-1} of reaction, respectively. Their antioxidant activity was evaluated by a DPPH test. However, in the first part of this work, the ability of α-D-glucosidase from *Aplysia fasciata* to glycosylate an array of naturally occurring phenolic compounds with alcoholic and/or phenolic hydroxyl groups in their molecular skeleton was analyzed and the regio and chemoselectivity in their transglycosylation processes were qualitatively examined. The interesting results are briefly summarized here: 2-phenylethanol, tyrosol, and similar compounds such as 2-(2-hydroxyphenyl)-ethanol, 2-(3-hydroxyphenyl)-ethanol, hydroxytyrosol, (±)-1-phenyl-1,2-ethanediol, *R*(-)- and *S*(+)-1-phenyl-1,2-ethanediol, 4-(2-methoxyethyl)-phenol, *N*-acetyl-L-tyrosine ethyl ester monohydrate (Fig. 30.4) were all tested as acceptors in analytical scale transglycosylation reactions, and a different qualitative distribution of products at 4, 24, and 48 h was recorded. Accumulation of mono and disaccharidic derivatives was noticed for almost all acceptors up to 24 h. In many cases, at early reaction times, glycosylations were not chemoselective with the involvement of phenolic and alcoholic hydroxyls, but after 24 h, a prevalent accumulation of products glycosylated at the alcoholic position was observed, as in the case of hydroxytyrosol, tyrosol, and its regioisomers.

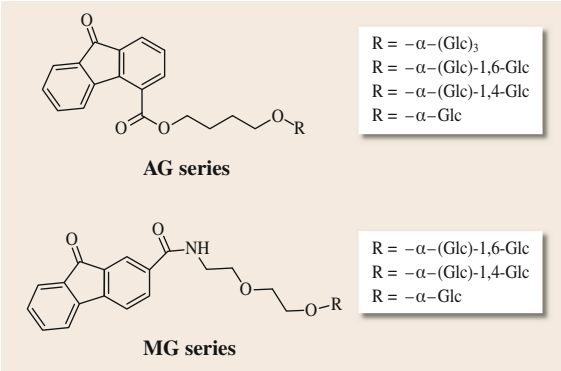

Fig. 30.5 Glucosylated products formed by glucosylation of 9-fluorenone derivatives. Carboxyhydroxyesters, indicated by AG and carboxyhydroxamides indicated by MG

For the protected derivative of tyrosol, 4-(2-methoxyethyl)phenol, disaccharidic products were still present at 24 h, and this result is in line with the characteristics of this enzyme (see above), while monoglucosides of 2-phenylethanol and *N*-acetyl-L-tyrosine ethyl ester monohydrate were partially or totally hydrolyzed, respectively. Racemic 1-phenyl-1,2-ethanediol and their pure enantiomers were polyglycosylated and partially (racemic and *R*(-)-substrate) or totally (*S*(+)-substrate) hydrolyzed after 24 h.

Compounds of pharmaceutical interest were also synthesized by α-D-glucosidase from *Aplysia fasciata*. Two new series of compounds were prepared and pharmacologically explored in the framework of research on

the antiviral and immunomodulatory activity of tilorone congeners: 9-fluorenone carboxyhydroxyesters (AG series Fig. 30.5) and 9-fluorenone carboxyhydroxamides (MG series Fig. 30.5). The α-glucosidase provided several α-*O*-glucosides of both model acceptors. The easy biocatalytic access to these polyglucosylated derivatives is very interesting for the quick screening of the pharmaceutical profiles of these drugs as modified by carbohydrate(s) moieties with respect to their parental aglycones [30.50].

The homogenate from the hepatopancreas of *Aplysia fasciata* contained a β-galactosidase activity, which after purification was shown to be a homodimer with a molecular mass of 164 kDa as estimated by gel-filtration and 78 kDa by SDS-PAGE [30.51]. β-D-Galactosidase activity measured as a function of pH revealed that the activity increases going from pH 5.5–2.3 and drastically drops at lower pH. At pH 3.7 a maximum activity around 75 °C was observed. The enzyme completely retains its activity after 23 h incubation at pH 4.7 in K-acetate buffer, while it retains 81% of its activity when incubated at pH 3.9 in Na-citrate buffer for 23 h. At pH 2.8 in glycine-HCl, the half-life time was 0.6 h. At 50 °C in K-acetate buffer pH 4.5 the half-life was 15 h. This enzyme is highly specific for *p*NP-β-D-galactopyranoside (*p*NP-β-Gal) and is completely inactive on other substrates; it is also able to hydrolyze the glycosidic linkage present in lactose, although to a much lesser extent than those of *p*NP-β-Gal and *o*NP-β-Gal. In transgalactosylation reactions this enzyme showed interesting characteristics. Due to the specificity of the acceptor site of most galactosidases for compounds with phenyl groups [30.52], the yields obtained in the reactions using free or methyl derivative of xylose and methyl β-galactopyranoside and D-galactose are interestingly high. The uncommon β-1,3 selectivity is also of interest; using free xylose or its β-allyl or methyl derivative, the β-1,3 isomer was always selectively formed. The same result was obtained by using methyl β-D-galactopyranoside and glucal. With β-aryl linked aglycones for both xylose and galactose, this β-1,3 selectivity is again expressed, although it is lost with α-anomers. However the influence of aryl groups as aglycones is not limited to the regioselectivity of reaction but also to the yield as shown when comparing the results of the reactions using the p-nitrophenyl and benzyl xylopyranosides [30.53]. o-Nitrophenyl β-D-galactopyranoside (*o*NP-β-Gal) was used as glycosyl donor in reactions with 5 equivalents of nucleoside acceptors (uridine, fluorouridine, thymidine, adenosine, cytidine, 5-chlorocytosine arabinoside, and 3′azido-3′-deoxythymidine) in the presence of this enzyme. The regioselectivity was extremely high, in all reactions only the product of galactosylation in 5′ position of the nucleoside was observed. Reaction yields were satisfactory in most cases, and very high for uridine derivatives. In particular, 5′-*O*-β-galactosyl-5-fluorouridine, the galactosylated derivative of the anticancer drug fluorouridine, was synthesized with a 60% yield, and 5′-*O*-β-galactosyl-3′-azido-3′-deoxythymidine, the derivative of the anti-HIV drug, was obtained in 43% yield [30.54]. This was the first report dealing with a glycoside hydrolase used for the modification of nucleosides with such convenient yields. Nucleoside analogs play an important role in antiviral and anticancer therapy. In addition, it has been found that many natural antibiotics possessing significant antitumor and antiviral activities have the structure of a nucleoside connected to oligosaccharides [30.55].

There are at least three main points stating the importance of enzymes acting on mannose-based carbohydrates. The first is that the β-mannopyranoside linkage is one of the most difficult glycosidic bonds to synthesize using chemical strategies [30.56], because the steric and polar effects favor the formation of α-anomer. Hence, alternative biological methodologies based on β-mannosidases are very attractive. However, natural examples of new β-mannosidase are needed also for the advancement of conformational studies of the enzymatic transition state and for the possibility of genetic manipulation of such enzymes to produce efficient synthetic catalysts (mannosynthase) [30.57]. As a third point it could be stated that β-mannosidases and β-mannanases are of interest for the reutilization of waste materials. In this context, both the degradation of β-1,4-mannan to simple sugars and the direct β-mannosidase-catalyzed synthesis of alkyl β-D-mannosides from mannobiose by β-mannosidase are possible [30.58]. β-D-Mannosidase activity was identified in the hepatopancreas of *Aplysia fasciata* and with higher specific activity in the visceral mass homogenate [30.38]. The apparent molecular mass for the purified protein was 229 kDa, while the molecular mass as determined by SDS-PAGE was 130 kDa, indicating that the purified enzyme is a homodimer. It showed an optimal activity at pH 4.5 and 45 °C [30.59]. At optimal pH the enzyme showed a half-life of 23 h. The enzyme retained most of its activity (82%) after 24 h incubation in the presence of 10% DMSO, while in the presence of other co-solvents the activity quickly dropped down (a half-life of 1 h was estimated in the presence of 10% acetonitrile, while in

acetone the half-life was 8.5 h). The K_M and V_{max} values for pNP-β-Man were determined to be 2.4 mM and 50.3 U mg^{-1}, respectively. From the body of these and other results, β-D-mannosidase from *Aplysia fasciata* was judged to possess a very good catalytic efficiency, in fact the k_{cat}/K_M obtained is 4800 min^{-1} mM^{-1}, which is higher than those of analogous enzymes from other species. This enzyme is also extremely specific for *p*NP-β-Man, a useful and needed characteristic when using a glycosydase for structural determination purposes. Self-transfer of a β-mannosyl unit was observed when *p*NP-β-man is treated with the β-D-mannosidase, in fact the most abundant disaccharidic product obtained (10–15% yield) possessed the β-1,4 intermannosydic linkage as established by the analysis of mono and two-dimensional NMR spectra of products (*p*NP-β-Man-(1,4)-β-Man). The transmannosylation was also studied using *p*NP-β-Man as donor and *o*-nitrophenyl α-D-2-deoxy-*N*-acetyl glucopyranoside as heteroacceptor, the molar ratio used was 1 : 3. Two regioisomers (12% global yield) due to the β-mannosylation of the heteroacceptor were formed in 85 : 15 ratio (by high-performance liquid chromatography, HPLC). The products were the β-1,4 and the β-1,6 derivatives as assessed by two-dimensional NMR spectroscopy.

As stated above, the production of mannooligosaccharides from mannan is based on endo-β-1,4-mannanase (EC 3.2.1.78) as biocatalyst. β-mannanases have already been established as useful biocatalysts in various industrial processes [30.60]. *Aplysia kurodai* was considered to use two mannan-degrading enzymes, namely a β-mannanase and a β-mannosidase, for the complete depolymerization of mannan from seaweeds for nutritional purposes [30.61]. The β-mannanase acted on the linear structure of mannans, producing heterooligosaccharide residues. It could degrade mannooligosaccharides larger than mannotetraose; from M5 (mannopentaose) it produced M3 and M2, and from M6 it produced M3, suggesting that the size of substrate binding site is similar to the size of mannnopentaose-mannohexaose, and splitting of the central mannosidic linkage of the mannohexaose unit occurred [30.62]. β-mannosidase and β-mannanase acted on linear mannan, degrading it to mannose in a more efficient manner [30.61]. Unfortunately, no results have been reported on these interesting enzymes about their possible transmannosylation properties on heteroacceptors. A bacterial expression system for this β-mannanase was reported to acquire information about the primary structure and other characteristic properties. The recombinant protein showed a broad pH optimum in the acidic range, as did native protein; however, heat stability was lower, indicating that the stability is derived from an appropriate folding and/or some posttranslational modifications in *Aplysia* cells [30.63].

Aplysia kurodai also contains two alginate lyase isozymes [30.64] that could be useful in the determination of fine structures of natural polymers and in the bio-production of functional monosaccharides and oligosaccharides from alginate. To improve the knowledge of primary structure and catalytic residues of marine alginate lyases, cloning complementary DNA (cDNA) encoding an alginate lyase from *Aplysia kurodai* and assessing catalytically important residues by site-directed mutagenesis were also described [30.65].

Two β-1,3-glucanases with the molecular masses of 36 kDa and 33 kDa were also isolated from the digestive fluid of *Aplysia kurodai*. They act as endolytic (EC 3.2.1.6, AkLam36) and exolytic enzymes (EC 3.2.1.58, AkLam33) degrading laminarin and related oligosaccharides to laminaritriose, laminaribiose, and glucose in the first case, while producing glucose directly from polymer laminarin in the second case. Although both these catalysts acted on laminaribiose, they were capable of degrading the disaccharide via transglycosylation reaction with laminaritriose. Both enzymes belong to the glycosyl hydrolase family 16 like other molluscan β-1,3-glucanases. According to the authors it is still unclear whether the transglycosylation is a physiologically important event in the metabolism since the transglycosylation reaction requires a relatively high concentration of laminaribiose [30.66].

Other enzymes related to chitin could be expected to be present in organisms of the *Aplysia* species. It was, in fact, long known in the literature that chitosan-based radulae and jaws of the opisthobranch *Aplysia sp*. give very strong chitosan color tests [30.67].

30.5 Other Enzymatic Activities of Interest Present in *Aplysia* sp.

Other examples of interesting enzymes have been reported in the literature on *Aplysia* sp. This marine organism, in fact, has proved to be useful as a model in neurobiology and in other scientific sectors. Some of the enzymes cited in the present section may be of interest for biocatalyst practitioners and it appears to be

worth noting them, although they are not related to the carbohydrate chemistry but to protein and lipid fields.

A large number of *Aplysia* neuropeptides were identified and some of these biomolecules show remarkable sequence similarities to mammalian neuropeptides. Enzymes acting specifically on these substrates could be of interest for their specificity in the biocatalytic field as well in other physiological studies [30.68, 69].

Lipoxygenases are also identified in *Aplysia*. These enzymes act on lipids and are of interest for the production of bioactive molecules both in vivo and in vitro. Arachidonic acid is converted to (8*R*)-hydroperoxyeicosa-5,9,11,14-tetraenoic acid (8-HPETE) during incubations with homogenates of the central nervous system of the marine mollusc, *Aplysia californica*. The product can be then reduced to the corresponding hydroxy acid or enzymatically converted to other metabolites [30.70]. In this field, enzymes of marine origin are characterized by regio and stereoselective output of reactions different from those found in terrestrial counterparts. From the synthetic chemist's point of view asymmetric oxygenation reaction of unnatural substrates has a tremendous potential [30.71].

30.6 Conclusion

Enzymatic routes are particularly useful to access new products or for the modification of complex natural products such as carbohydrates, which are very important molecules in various technological domains. Herbivorous marine molluscs possessing various kind of polysaccharide-degrading enzymes could efficiently act on these molecules. These enzymes play important roles in the metabolism of the organism digesting polymeric materials to oligosaccharides and monosaccharides as carbohydrate nutrients. They can also be used in a diverse manner for biocatalyzed synthesis of the glycosidic bond. Different interesting results related to the presence of glycosyl hydrolases have been reported in the literature on *Aplysia fasciata* and *Aplysia kurodai*. The examples have been judged to be of interest to the biocatalysis community. In *A. fasciata* β-D-galactosidase and α-D-glucosidase enzymes are present in large amounts, and an interesting β-mannosidase has also been noticed; in *A. kurodai* different enzymes acting on polysaccharides were evidenced.

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31. Antimicrobial Peptides from Marine Organisms

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Antimicrobial peptides (AMP), also known as host defense peptides or alarmins, are among the first lines of defense against infection in many organisms. Marine organisms have proved to be a rich source of AMPs, and several uniquely structured marine AMPs have been isolated using biochemical, in silico and genetic approaches. Hemolymph is the main source of AMPs in marine invertebrates, although other tissues may also contain these peptides. Based on their three-dimensional structure, AMPs may be classified into peptides with α-helix structures, peptides with β-sheets and cysteine residues, peptides enriched for modified and rare amino acids, and peptides with ring structure amino acids. This chapter describes AMPs of each structure, in terms of their antibacterial, antiviral, antifungal, insecticidal, nematicidal, and immunomodulatory functions. It is hoped that the diversity of marine AMPs may facilitate the identification of effective substitutes to existing antibiotics, thereby helping to minimize the development of antibiotic-resistant pathogen strains in response to the continuous application of a single antibiotic therapy.

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31.1 Marine Antimicrobial Peptides

Antimicrobial peptides (AMP) are short amino acid chain molecules involved in the first line of defense against invading pathogens. AMPs are synthesized by the ribosomal machinery as pro-peptides, consisting of an N-terminal signal sequence, a pro-sequence, and a C-terminal sequence with antimicrobial activity once it is cleaved from the rest of the pro-peptide.

To date, nearly 3000 AMPs have been isolated from organisms as diverse as plants, mammals, fish (marine, fresh water and aquaculture), and insects and other invertebrates. Marine organisms produce numerous AMPs to combat specific and large invading pathogens in an aquatic environment. Marine organisms are relatively understudied, and only a presumed fraction of marine AMPs has been identified. As such, it is likely that with further research we will isolate a considerable number of AMPs from marine sources. In a similar manner to the development of drug resistance, pathogens evolve to evade host AMPs. By targeting pathogens with functionally similar, yet structurally unique marine AMPs, it may be possible to prevent the evolution of resistant strains. The growing interest in the study of marine AMPs has led to the discovery of peptides similar to mammalian equivalents, but with unique differences. This chapter briefly describes the methods employed in the isolation of marine AMPs, before describing the antimicrobial functions and other applications of structurally grouped AMPs.

31.2 Isolation of Antimicrobial Peptides

Biochemical, bioinformatic, and genetic approaches have been used to identify and isolate AMPs. A biochemical approach involving solid phase extraction, followed by reverse phase high-performance liquid chromatography (HPLC) and size exclusion chromatography, was utilized to isolate a 2.7 kDa AMP from *Penaeus vannamei*, and 7.9 kDa and 8.3 kDa AMPs from *Penaeus stylirostris* [31.1].

The samples were acidified to a pH of up to 4.0 and then neutralized, before being stepwise eluted with 5, 40, and 80% aceto-nitrile water and subsequently lyopolized. Then fractions from reverse phase HPLC were collected separately, and the antimicrobial activity of the fractions were determined; active fractions were then further purified by size exclusion chromatography. Bioinformatics has been used to predict AMPs by in silico analysis; by comparing a candidate sequence to those of AMPs in an available database, it has been possible to identify AMPs like CsHepcidin from the half-smooth tongue sole, *Cynoglossus semilaevis* [31.2]. Finally, a genetic approach involving analysis of the transcriptome from shrimp *Litopenaeus stylirostris* that survived *Vibrio penaeicidae* infection was used to identify the AMP Ls-stylicin [31.3].

31.3 Characterization and Functions of AMP

Nuclear magnetic resonance (NMR) spectroscopy has been used to reveal the three-dimensional structure of AMPs [31.4]. Based on their structure, AMPs can be divided into five groups: peptides with α-helix structures, peptides with β-sheets and cysteines, peptides enriched for regular amino acids, peptides enriched for modified and rare amino acids, and ring structured amino acid peptides (which may result in a cyclic structure) [31.5]. Most marine AMPs of the β-sheet class exhibit antimicrobial function against Gram-positive and Gram-negative bacteria, and a few exhibit antiviral and antifungal activities. The α-helix AMPs are well known for their antimicrobial function at very low concentrations, but this comes with the drawback of high cytotoxicity. Cyclic AMPs form a ring structure and possess insecticidal, nematicidal, antitumor, and immunomodulatory functions. The following paragraph describes the properties of marine AMP subgroups.

Fig. 31.1 Structure of the cecropin family antimicrobial peptide

31.3.1 Alpha Helical AMPs

Cecropin (Fig. 31.1) was the first AMP of this group to be studied [31.6]. The AMPs styelin A–E (Fig. 31.2), clavanin, clavaspirin, and dicynthaurin obtained from tunicates show antibacterial activity, as they interact with lipid bilayers and thus target the membrane [31.7–11]. Clavanin (Fig. 31.3) kills *Micrococcus flavus* by permeabilizing its cytoplasmic membrane [31.12]. Stylins from *Styela clava* are phenylalanine-rich peptides with antimicrobial activity against Gram-negative and Gram-positive marine bacteria, human bacterial pathogens, *Psychrobacter immobilis*, and *Planococcus citreus* [31.7]. Styelin A and B possess strong antimicrobial activity against marine bacteria, *Psychrobacter immobilis* and *Planococcus citreus* [31.7]. Stylin C, D, and E exhibit bactericidal ability at acidic pH and high salinity. Styelin D is effective against methicillin-resistant *Staphylococcus aureus* (MRSA). Clavaspirin (Fig. 31.4a) is effective at acidic pH, at which it permeabilizes the outer and inner membranes of *Escherichia coli*, lyses liposomes and erythrocytes, and has anti-MRSA activity [31.10]. Dicynthaurin (Fig. 31.4b) acts intracellularly within the phagosome and exhibits antimicrobial activity against the Gram-positive bacteria *Micrococcus luteus*, *Staphylococcus aureus*, and *Listeria monocytogenes*, and the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* [31.11]. Two AMPs isolated from the tunicate *Halocynthia papillosa*, halocyntin (Fig. 31.5a)

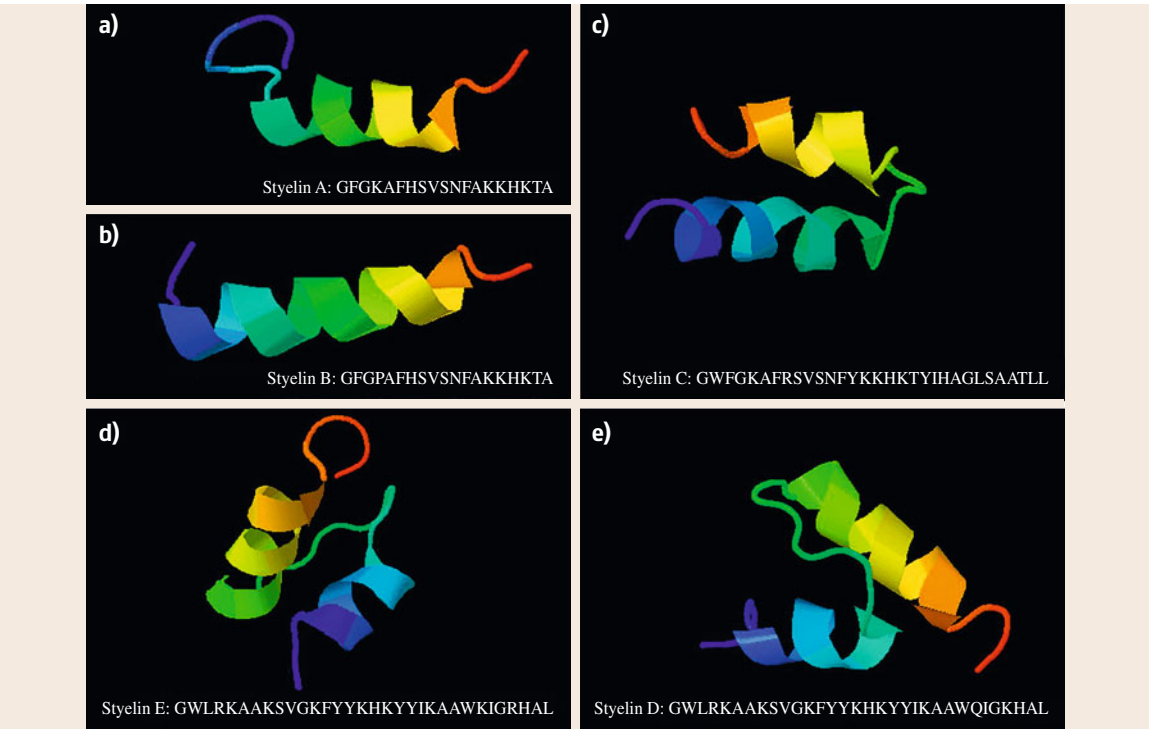

Fig. 31.2a–e Amino acid sequence and structure of the mature styelins. **(a)** Styelin A, **(b)** styelin B, **(c)** styelin C, **(d)** styelin D, and **(e)** styelin E

and papillosin (Fig. 31.5b) have two α-helical structures and exhibit antibacterial activity against Gram-positive and Gram-negative bacteria [31.13]. Ci-MAM-A (Fig. 31.5c) and Ci-PAP-A (Fig. 31.5d) are AMPs isolated from *Ciona intestinalis*; these peptides are active at high salt concentrations, and kill Gram-negative and Gram-positive bacteria by permeabilizing their cytoplasmic membranes [31.14]. Ci-MAM-A is not cytolytic to mammalian erythrocytes, but is toxic to several human and marine pathogens, as well as the yeast *Candida albicans* [31.15]. Hedistin (Fig. 31.6a), isolated from *Nereis diversicolor*, is a helix-turn-helix moiety AMP with C-terminal-amidation and bromotryptophan residues; hedistin shows activity against a large spectrum of bacteria, including MRSA and *Vibrio alginolyticus* [31.16]. Hedistin expressed in circulating natural killer (NK) cells possibly plays an important role in immunity [31.16]. Centrocin (Fig. 31.6b–c) is an AMP from *Strongylocentrotus droebachiensis*, with an alpha helix heterodimeric structure. It consists of a heavy chain with 30 amino acids and a light chain with 12 amino acids; the heavy chain has activity against bacteria, fungi, and yeasts [31.17].

31.3.2 Beta Sheet/Cysteine-Rich AMPs

Cysteine-rich peptides, such as hepcidin (Fig. 31.7), are able to form disulphide bonds. Penaeidins derived from shrimp possess a proline-rich domain at their N-terminus, and a cysteine-rich domain stabilized by three conserved disulfide bonds at their C-terminus [31.18]. Sb-BDef1 is the big defensin AMP of *Scapharca broughtonii*; it has a β-sheet strand and a highly amphipathic hydrophobic N-terminal structure, and is involved in the immune response to Gram-negative microbial infections [31.19]. The big defensin (Fig. 31.8) of *Tachyplesus tridentatus* has both antibacterial activity and anti-fungal activity against the filamentous fungi *Candida albicans* [31.20]. AiBD, a big defensin peptide in *Argopecten irradians*, possesses an α-helix and β-sheet, and is effective against bacterial and fungal infections [31.21]. The antifungal polypeptides HCt1, 2, 3 are hemocyanin C-terminal region peptides with β-sheets isolated from *Penaeus vannamei* and *Penaeus stylirostri* [31.22]. The BDS-I and II peptides (isolated from *Anemonia sulcata*) have a compact disulfide core and a stranded antiparallel β-sheet structure; they

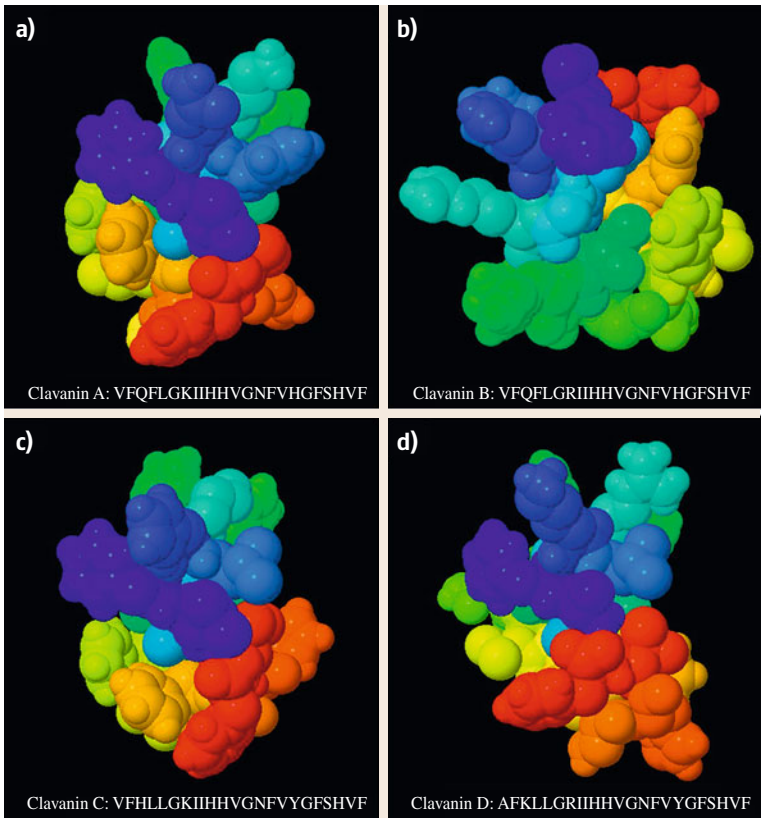

Fig. 31.3a–d Amino acid sequence and structure of the histidine rich mature clavanins from sea squirts and tunicates. **(a)** Clavanin A, **(b)** clavanin B, **(c)** clavanin C, and **(d)** clavanin D

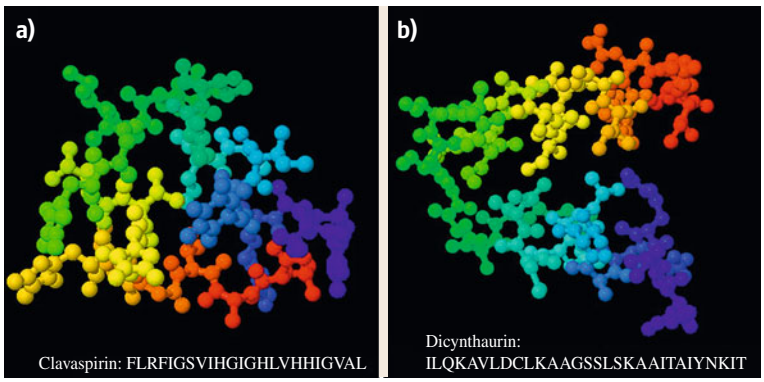

Fig. 31.4a,b Amino acid sequence and ball-and-stick structure of the mature **(a)** clavaspirin and **(b)** dicynthaurin

exhibit antiviral and antihypertensive functions, and modulate Kv3 potassium and sodium channels [31.23].

The AMP Cg-Def (isolated from *Crassostrea gigas*) possesses a cysteine-stabilized α-β motif structure, and displays antibacterial and antifungal activity [31.24]. The crustins observed in *Litopenaeus* spp. have a conserved cysteine pattern of C1(Xn)C2(Xn)C3(X5)C4(X5)C5C6(X3-5)C7(X5)C8 (where *X* is any amino acid) [31.25]. Crustin Cm-1 of *Carcinus maenas* is stable at high temperatures up to about 100 °C [31.26]. Crustin Cm1 may be used to clear bacterial pathogens that are able to survive at high temperatures. Crustin Pm1-7 binds to the cell wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA), and induces bacterial agglutination and inner membrane permeabilization [31.27]. The penaei-

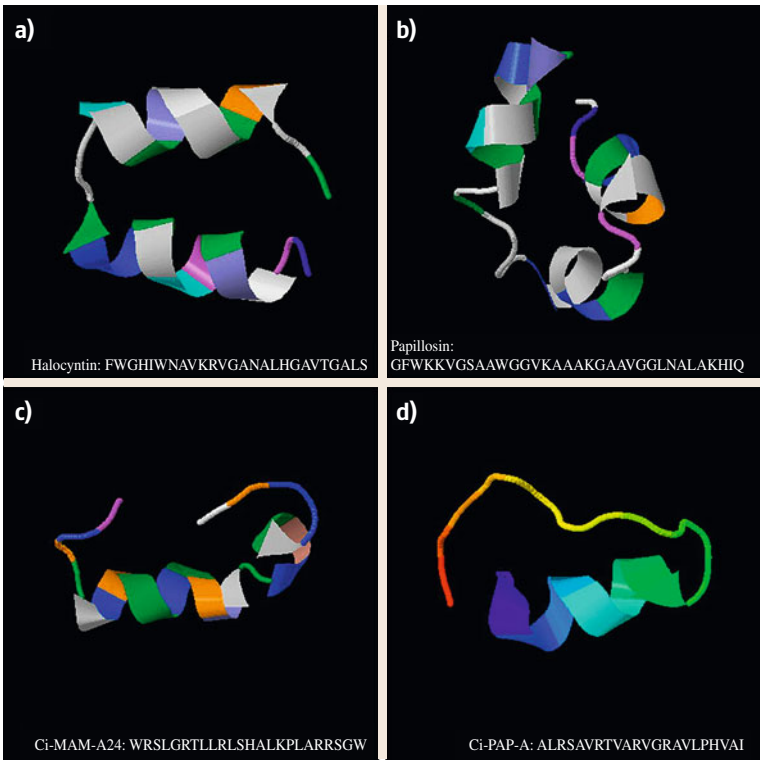

Fig. 31.5a–d Amino acid sequence and structure of the mature **(a)** halocyntin, **(b)** papillosin, **(c)** Ci-MAM-A24, and **(d)** Ci-PAP-A

dins (Fig. 31.9a) from the Hemolymph of the Pacific white shrimp contain a conserved Pro-Arg-Pro motif in the N-terminus and a cysteine-rich C-terminus [31.28]. Penaeidins possess chitin-binding activities, and bacteriostatic and bactericidal activity against *Aerococcus viridians* and *Vibrio alginolyticus*. Penaeidin-5 of the black tiger shrimp *Penaeus monodon* inhibits the growth of the filamentous fungi *Fusarium pisi* and *Fusarium oxysporum* [31.29]. The proline, cysteine, and arginine-enriched AMPs arasin-1 (spider crab, Fig. 31.9b), tachyplesins (horseshoe crabs, Fig. 31.9c), polyphemusins (American horseshoe crab, *Limulus polyphemus*, Fig. 31.9d), and callinectin (blue crab) have antibacterial activity against *Corynebacterium glutamicum* and several Gram-positive bacteria [31.30]. The arasin-like peptide of *Scylla paramamosain* has antibacterial activity against a range of Gram-negative and Gram-positive species, including the crustacean pathogens *Aerococcus viridans*, *Vibrio harveyi*, and *Vibrio anguillarum* [31.31]. Another AMP in the same species, Gastrin-releasing peptide, Scylla paramamosain (GRPSp), contains two cysteine residues at the C-terminus and two glycine-rich repeats (GGYG and GYGG); GRPSp has antibacterial activity specific to Gram-positive strains [31.31]. Tachyplesin I is a 17-residue β-sheet peptide that forms a complex with bacterial lipopolysaccharide and functions against Gram-positive and Gram-negative bacteria [31.32]. Tachyplesin III of *Tachyplesus gigas* shows strong antimicrobial activity, reducing endotoxin and tumor necrosis factor (TNF)-alpha concentrations in *Escherichia coli*-infected mouse models. Mytilins (Fig. 31.10a) destroy bacterial pathogens by membrane pore formation [31.33]. Myticin C of *Mytilus galloprovincialis* is a chemokine/cytokine-like molecule of marine invertebrates [31.34]. Mytilin B, C, and D all demonstrate strong antifungal activity against *Fusarium oxysporum* [31.35], and myticin B also exhibits antimicrobial activity against *Escherichia coli* D31 [31.36]. Dolabellanin-B2 (Fig. 31.10b) is an AMP with antibacterial and fungicidal activity, isolated from *Dolabella auricularia*; it contains a β-sheet and two disulfide bonds, and its methionine residues may be oxidized to form methionine sulfoxides [31.37]. Strongylocins of *Strongylocentrotus droebachiensis* have a β-defensin structure with a novel cysteine pattern; they are potent against *Staphylococcus aureus* and a range of other bacteria [31.38]. The AMP aurelin has

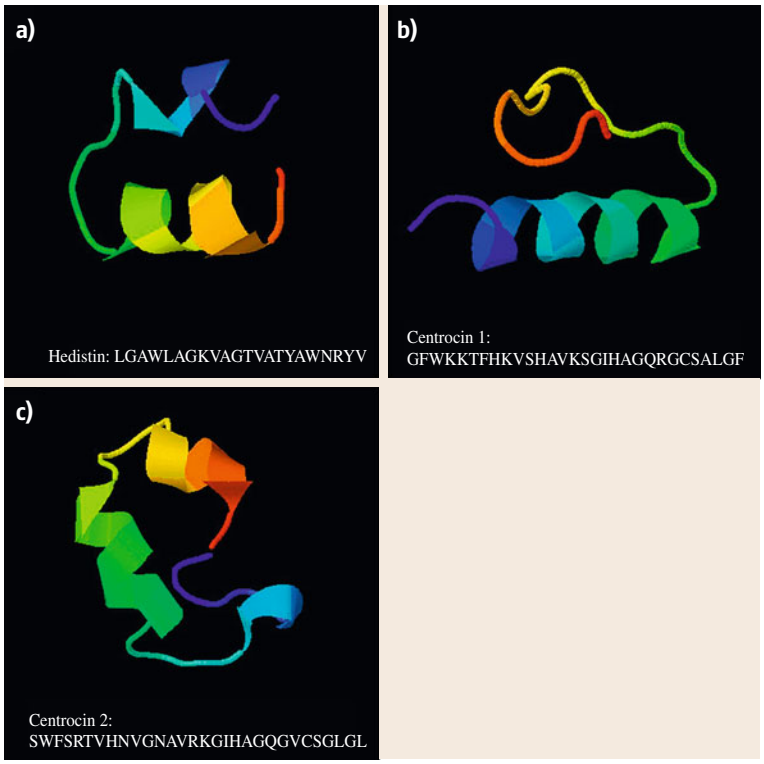

Fig. 31.6a–c Amino acid sequence and structure of the mature **(a)** hedistin from marine annelids, and **(b)** centrocin 1, **(c)** centrocin 2 from green sea urchin

β-sheet structure and partial similarity with defensins and K^+ channel-blocking toxins of sea anemones; it is toxic to a wide range of bacteria [31.39].

31.3.3 AMPs with Coil Structure and Modified Amino Acids

Plicatamide (Fig. 31.11a) is a modified octapeptide, the C-terminus of which is the oxidatively decarboxylated delta-DOPA (decarboxy (E)-alpha,beta-dehydro-3,4-dihydroxyphenylalanine) [31.40, 41]. Halocidin (Fig. 31.11b) of *Halocynthia aurantium* is a dimer, in which the two subunits are linked covalently by a single cystine disulfide bond [31.42]. Halocyamine A and B, purified from the solitary ascidian *Halocynthia roretzi*, are tetrapeptides with the structures L-histidyl-L-6,7-dihydroxyphenylalanylglycyl-6-bromo-8,9-didehydotryptamine and L-threonyl-L-6,7-dihydroxyphenylalanyl-L-histidyl-6-bromo-8,9-didehydrotryptamine, respectively [31.43]. Crustins contain a whey acidic protein (WAP) domain, characterized by a four-disulfide core (4-DSC) structure [31.25]. A 6.5 kDa AMP isolated from *Carcinus maenas* shares sequence similarity with bactenecin 7 (Fig. 31.11c), and has antibacterial activity against a range of Gram-negative and Gram-positive bacteria [31.44]. Carcinin, a cysteine and glycine-rich WAP domain-containing AMP has antimicrobial activity at NaCl concentrations of up to 500 mM [31.26]. Another WAP-domain containing AMP, homarin, has bacteriostatic activity, as well as protozoastatic and protozoacidal activity against the parasites *Mesanophrys chesapeakensis* and *Anophryoides haemophila* [31.45]. Ls-Stylicin 1 (Fig. 31.11d), an AMP with a proline-rich N-terminus and 13 cysteine residues in its C-terminus, was demonstrated to have strong antifungal activity against *Fusarium oxysporum*, and to agglutinate *Vibrio penaeicidae* in vitro [31.3]. Hyastatin, an AMP isolated from the spider crab has an N-terminus enriched in glycine residues, a short proline/arginine-rich region, and a C-terminus that contains 6 Cys residues, and is presumably amidated in the mature peptide [31.46]. Hyastatin has N-terminal chitin binding activity, and antimicrobial activity against yeasts, and Gram-positive and Gram-negative bacteria [31.46]. The AMPs tachycitin and tachystatin of *Tachypleus tridentatus* are single chain proteins containing five disulfide bonds with no N-linked sugar; they exhibit

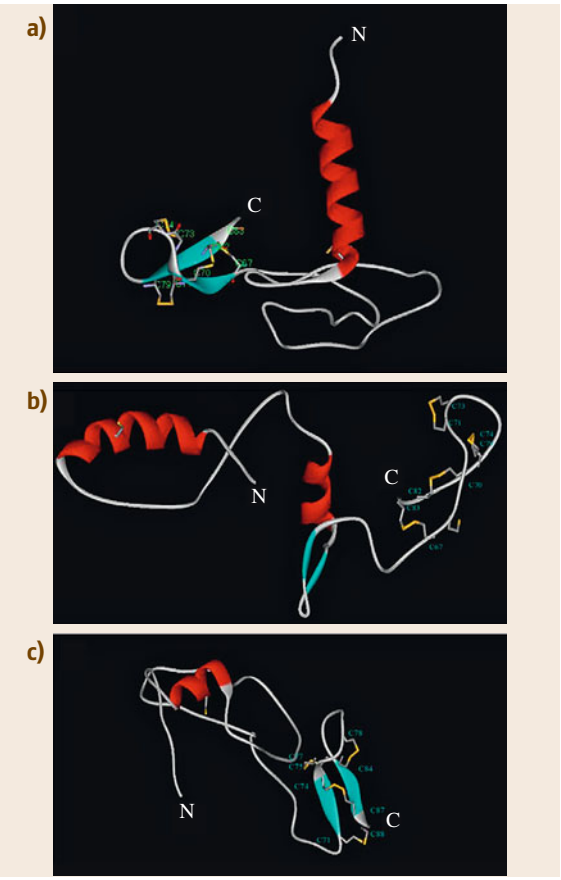

Fig. 31.7a–c Structure of beta sheet antimicrobial peptides **(a)** tilapia hepcidin (TH)1-5. **(b)** TH2-2 and **(c)** TH2-3

antibacterial and antifungal activity, and also possess bacterial agglutinating properties [31.47]. Tachycitin and big defensin act synergistically in their antimicrobial activities. The N-terminus of tachycitin shows sequence homology to chitin-binding regions found in antifungal chitin-binding peptides, chitin-binding lectins, and chitinases [31.47]. Tachycitin binds with chitin, but not to the polysaccharides cellulose, mannan, xylan, or laminarin [31.47]. Scygonadin is an anionic, hydrophobic peptide with antimicrobial activity against *Staphylococcus aureus* and other Gram-positive as well as Gram-negative species [31.48]. The *Sebastes schlegeli* antibacterial protein (SSAP), another AMP of *Tachypleus tridentatus*, is an antibacterial anionic peptide, containing an α-helix, β-sheet, and cysteine residues [31.49]. Perinerin of *Perinereis aibuhitensis Grube* has bactericidal and fungicidal activity, and an α-helix and β-sheet structure with two potassium channel toxic motifs [31.50]. Theromyzin, an anionic linear peptide isolated from *Theromyzon tessulatum*, was the first invertebrate anionic AMP observed to have bacteriostatic activity [31.51]. Astacidin 1 and 2, of *Pacifastacus leniusculus*, are proline and arginine-rich peptides with an amidated C-terminus and bactericidal activity [31.52, 53].

31.3.4 Ring Structured AMPs

Arenicin 1 and 2, bactericidal and fungicidal AMPs isolated from *Arenicola marina*, have β-hairpin structures, and each isoform has a single disulfide bond (Cys3–Cys20) forming an 18-residue ring [31.54]. The cyclic antifungal peptides aciculitin A–C of *Aciculites orientalis* effectively inhibit the growth of *Candida albicans* [31.55]. Jasplakinolide (also known as jaspamide) is a cyclic depsipeptide and polyketide of *Jaspis johnstoni*, with insecticidal activity against *Heliothis virescens* and fungicidal activity against *Candida albicans*, and the ability to inhibit breast cancer cell growth [31.56]. Calyxamides A–B, isolated from *Discodermia calyx*, are cyclic peptides containing 5-hydroxytryptophan and thiazole moieties; they are cytotoxic to P388 murine leukemia cells [31.57]. Polydiscamide B–D are depsipeptides of *Ircinia* sp. that act as sensory neuron-specific G protein coupled receptor agonists [31.58]. Polydiscamide A is a depsipeptide isolated from *Discodermia* sp., with lactonized-threonine residues in its C terminus; it inhibits growth of the human lung cancer A549 cell line. Phoriospongins A (1) and B (2), isolated from *Phoriospongia* sp. and *Callyspongia bilamellata*, have a depsipeptide structure and nematicidal function [31.59]. Finally, cyclolithistide A is a cyclodepsipeptide of *Theonella swinhoei* with fungicidal activity [31.60].

31.4 Future Directions in Marine AMP Applications

The major advantage of AMPs is their small size. They can be synthesized under lab conditions in a short time, to exacting specifications. The sequences of mature peptides were obtained from antimicrobial

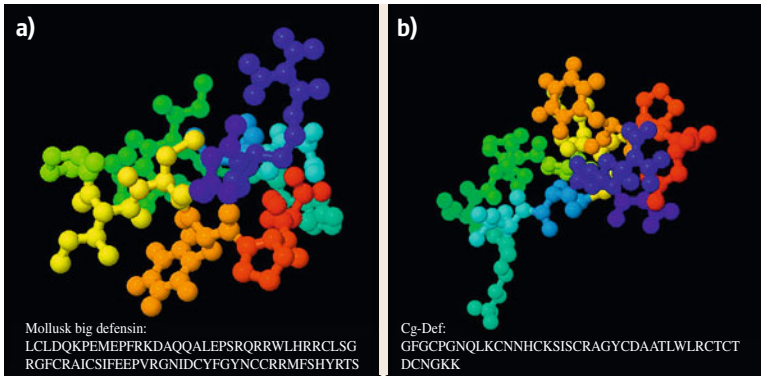

Fig. 31.8a,b Amino acid sequence and ball-and-stick structure of the mature **(a)** Mollusk big defensin and **(b)** oyster defensin (Cg-Def)

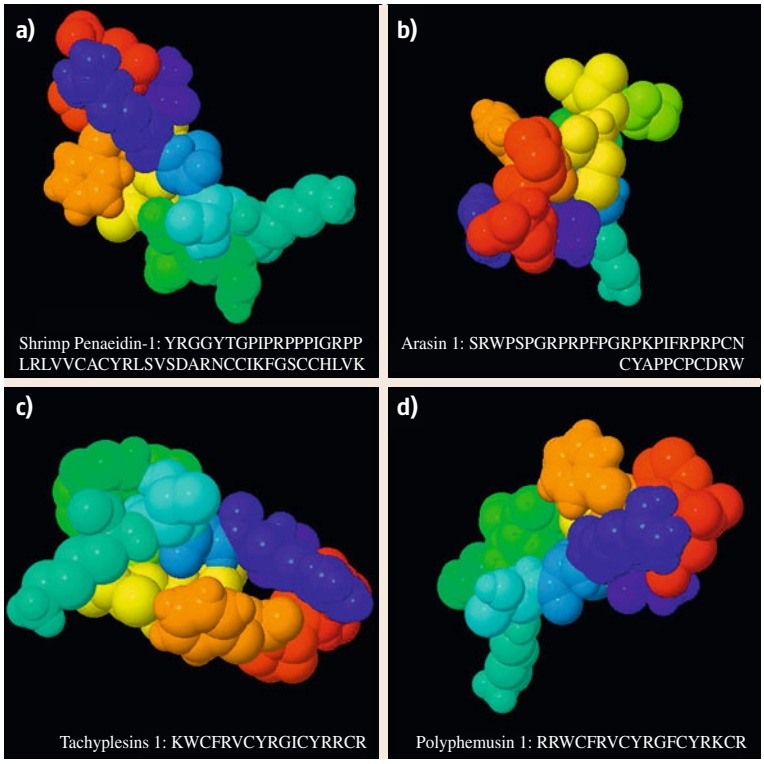

Fig. 31.9a–d Amino acid sequence and sphere structure of the mature **(a)** Shrimp penaeidin-1, **(b)** arasin, **(c)** horseshoe crab tachyplesin, and **(d)** polyphemusin

database [31.61], and the structure were predicted with Mobyle@RPBS [31.62]. This chapter mainly emphasized the antimicrobial function and other applications of AMPs isolated from marine organisms. As fish AMPs are extensively discussed elsewhere, we chose to focus on AMPs from marine invertebrates. At the time of writing, nearly 80 AMPs had been isolated from marine invertebrates, most of which exhibit antibacterial activity, and a few of which exhibit antiviral and/or antifungal activity. In addition, AMPs with insecticidal, nematicidal, tumor suppression, potassium channel blocking, and immunomodulatory activity have also been reported. Certain AMPs are undergoing clinical trials as potential treatments for skin infections. Such ongoing exploitation of marine AMPs through clinically-oriented studies can be expected to provide novel therapeutic agents in the near future. The present review highlights that marine organisms are a source of unique AMPs, and these AMPs may eventually become effective substitutes for several antibiotics.

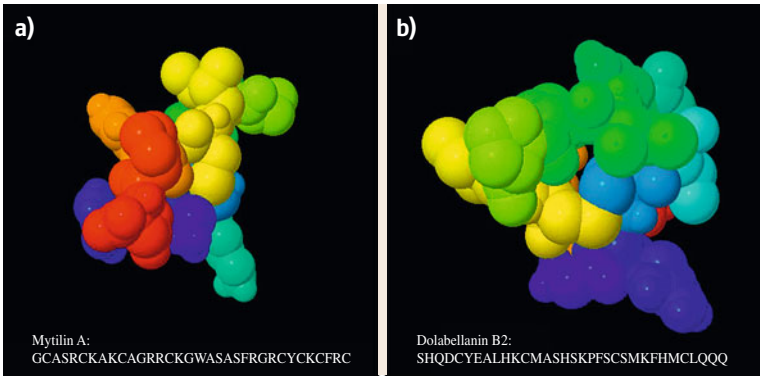

Fig. 31.10a,b Amino acid sequence and sphere structure of the mature **(a)** mytilin A and **(b)** dolabellanin B2

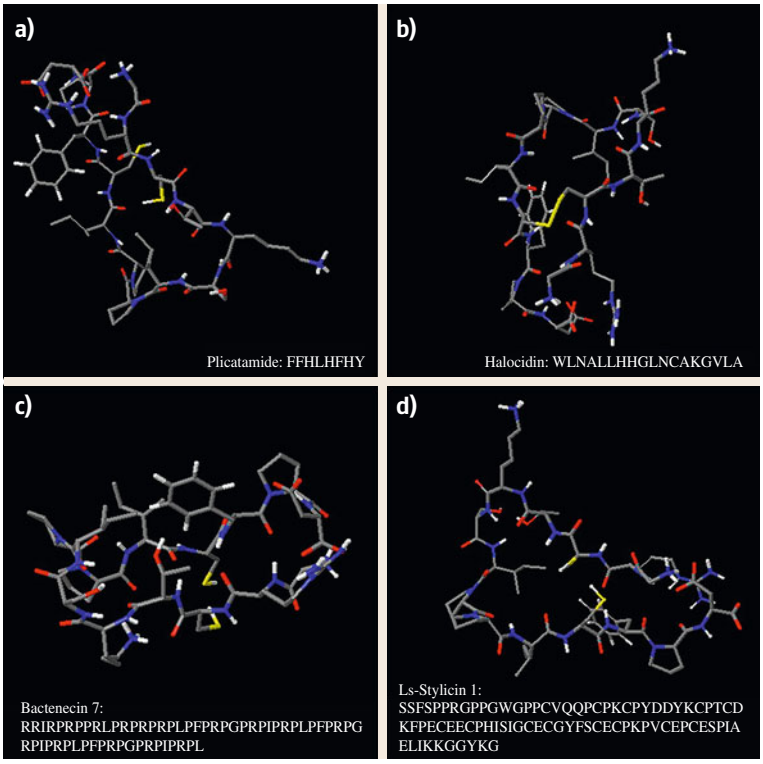

Fig. 31.11a–d Amino acid sequence and wire form structure of the mature **(a)** plicatamide, **(b)** halocidin, **(c)** bactenecin 7, and **(d)** Ls-Stylicin 1

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32. Marine-Derived Fungal Metabolites

Sherif S. Ebada, Peter Proksch

Marine-derived metabolites continue to be a prolific source for bioactive natural products with a high tendency to become drug candidates. Recently, eribulin mesylate (E7389), a synthetic derivative of halichondrin B, was approved by the US FDA (Food and Drug Administration) for the treatment of breast cancer metastases, going under the trade name Halaven. Some other marine-derived pharmaceuticals have likewise already been approved and launched onto the market, including Yondelis (trabectedin), Prialt (ziconotide), Retrovir (zidovudine), Cytosar (cytarabine, Ara-C), and Vira-A (vidarabine, Ara-A). Research interests in marine natural products have increased in the last few decades, in particular with respect to compounds isolated from marine-derived fungi. In this chapter, a historical background of marine-derived pharmaceuticals and fungal metabolites will be presented, including potential classes of bioactive chemical compounds. In addition, a perspective on the sustainability of marine-derived fungi with regard to a continuous supply of bioactive compounds for market needs rather than a pursuit of other strategies such as aquafarming will be discussed.

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32.1 Overview

Natural products continue to be a gold mine of lead structures for pharmaceuticals used for the treatment of various human diseases. More than two-thirds of active drug ingredients are either natural products or derivatives thereof [32.1]. In spite of the great advances in medicine that have been achieved, there are still no cures for some of the most deadly and crippling ailments, such as AIDS, several types of cancer, and many viral and fungal diseases. In addition, the emergence of resistant strains of previously curable illnesses, e.g., strains of tuberculosis, malaria, and the Ebola virus towards standard drug therapy has made new therapeutic regimens necessary as much as ever to save and improve human lives.

Marine ecosystems, occupying about 70% of the planet's surface area, represent an unexploited source of potential pharmaceuticals. These compounds have proved to be a pipeline of secondary metabolites featuring unprecedented chemical scaffolds and unique pharmacological properties which have encouraged natural product chemists during the last 60 years to explore marine organisms [32.2, 3]. Several marine natural products have succeeded in reaching the pharmaceutical market or are in clinical trials for the treatment of serious human disorders ranging from severe pain and microbial infections to immune diseases and cancer [32.4]. Moreover, a huge number of marine natural products have been reported

to have the potential for the development of drugs or drug leads from various marine invertebrates including sponges, ascidians, and soft corals. However, the sustainable and sufficient supply of these substances to meet the demands of clinical trials and the future market needs has been problematic and opposed the development of further marine-inspired pharmaceuticals.

Alternative production procedures and strategies, such as total and/or partial chemical synthesis, or the production of these natural compounds through microbial fermentation of endosymbionts associated with the marine host organism have been developed and in some cases they have successfully overcome the supply problems [32.5]. Due to the aforementioned conditions, relatively few marine secondary metabolites have entered preclinical or clinical trials compared to the huge number of natural products reported from marine organisms with potential bioactivities [32.3, 4].

In this chapter, we will briefly survey the history of marine-inspired pharmaceuticals and discuss marine-derived fungal metabolites according to chemical classes. In addition, a future overview of marine-derived fungi is presented as an alternative to provide a sustainable supply of lead compounds replacing other production methods, such as aquafarming which mostly failed to yield significant results contrary to earlier expectations.

32.2 Drug Screening from Marine Organisms

In this section, we will provide in brief a historical background of marine-inspired pharmaceuticals and those marine natural products that have successfully reached preclinical or clinical trial phases.

32.2.1 US FDA-Approved Marine-Inspired Pharmaceuticals

Bergman and *Feeney* succeeded in the early 1950s to identify hitherto unprecedented nucleosides from the marine sponge *Tethya crypta* [32.6, 7], which inspired the first marine-derived pharmaceuticals Ara-C (cytarabine, Cytosar, Fig. 32.1) and Ara-A (vidarabine, Vira-A). Both were approved by the FDA as anticancer and antiviral drugs, respectively [32.8]. However, the latter has been recently withdrawn from US market by an executive FDA decision in 2001, probably due to its diminished therapeutic window compared to other antivirals [32.3].

In 1987, zidovudine was the first reverse transcriptase (RT) antagonist approved by the FDA for the treatment of human immunodeficiency virus (HIV)/aquired immuno deficiency syndrom (AIDS) under the trade name Retrovir (Fig. 32.1) [32.9]. Zidovudine is structurally a 3′-azido, 3′-deoxy derivative of 2′-deoxythymidine, which was first reported from the starfish *Acanthaster planci* in 1980 [32.10, 11]. In the mid-1990s, other derivatives were also developed and approved by the FDA, such as lamivudine (Epivir) and stavudine (Zerit), which were approved as RT antagonists for HIV/AIDS and the hepatitis B virus (HBV) [32.12, 13].

Afterwards, it took about a decade to introduce the next marine-based pharmaceutical, ziconotide, which received FDA approval in December 2004. Ziconotide is the synthetic form of the naturally-occurring peptide ω-conotoxin MVIIA, which was first isolated from the Pacific piscivorous marine cone snail *Conus magus* by Olivera and co-workers in 1979 [32.14]. It took more than two decades of research and development to get ziconotide launched, under the brand name Prialt, as the first marine-inspired pharmaceutical for the specific indication of severe chronic pain (Fig. 32.1) [32.15].

Later, in July 2007, trabectedin, the synthetic form of ecteinascidin-743 isolated from the marine tunicate *Ecteinascidia turbinata*, was approved by the Committee for the Evaluation of Medicinal Products from the European Medicines Agency (EMEA) for the treatment of refractory soft tissue sarcoma and ovarian cancer [32.16]. To fulfill the supply needs during the clinical trial phases and the market needs thereafter, different strategies were evaluated, such as aquaculture and chemical synthesis [32.17, 18]. However, no promising outcomes have been achieved from either of them, but a breakthrough took place when PharmaMar, the license holder of trabectedin (ET-743), managed to develop a large-scale semisynthetic protocol starting with cyanosafracin B, an antibiotic produced on multikilogram scale by *Pseudomonas fluorescens* [32.19].

Recently, on November 15th, 2010, eribulin mesylate (E7389), a fully synthetic ketone analog of the polyether marine macrolide halichondrin B first isolated from the marine sponge *Halichondria okadai* [32.20, 21], was approved by the FDA for use

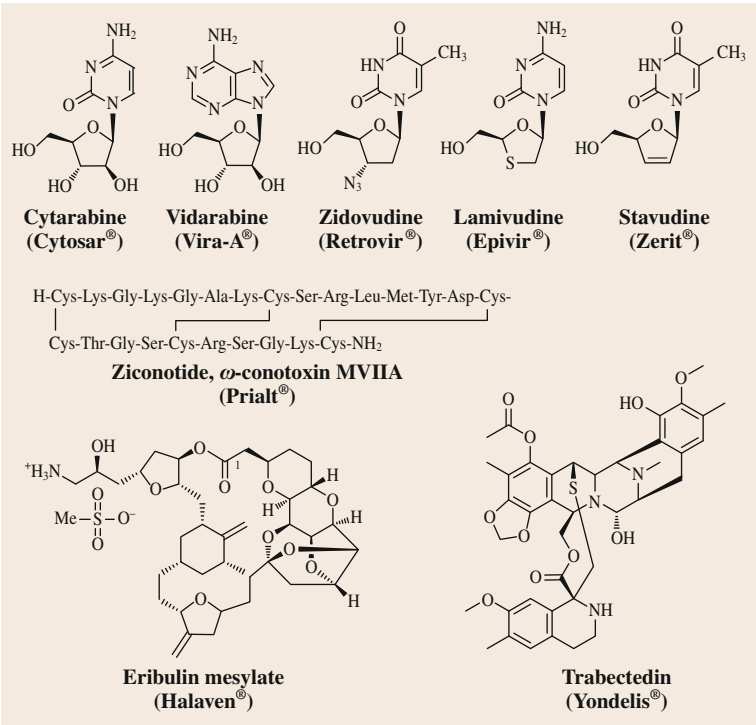

Fig. 32.1 Chemical structure of US FDA-approved marine inspired pharmaceuticals

with patients with metastatic breast cancer who have received at least two prior chemotherapy regimens for late-stage disease, including both anthracycline and taxane-based ones. Eribulin mesylate is currently marketed by Eisai pharmaceuticals under the trade name Halaven (Fig. 32.1).

32.2.2 Marine Pharmaceuticals in Phase III Clinical Trials

Soblidotin (TZT-1027, Fig. 32.2) is a synthetic derivative of the cytotoxic peptide dolastatin 10, originally isolated from the sea hare *Dolabella auricularia* [32.22]. Soblidotin revealed potent antimitotic activity through inhibiting microtubule assembly and tubulin polymerization by binding to tubulin at a site close to the vinca binding sites [32.23, 24]. Currently, TZT-1027 is under phase III clinical trials for advanced or metastatic lung cancer and non-small cell lung cancer (NSCLC).

Brentuximab vedotin (SGN-35, Fig. 32.2) is an antibody-drug conjugate consisting of the chimeric monoclonal antibody brentuximab, targeting the cell-membrane protein CD30, and linked to three to five units of the antimitotic agent monomethyl auristatin E (MMAE). The antibody portion of the drug conjugate attaches to CD30 on the surface of malignant cells, delivering MMAE, which affords therapeutic activity [32.25, 26]. In August 2011, the FDA approved brentuximab vendotin for the treatment of relapsed systemic anaplastic large cell lymphoma (ALCL) and refractory Hodgkin's lymphoma under the trade name Adcertis [32.27].

Plitidepsin (aplidine, Aplidin, Fig. 32.2) is a dihydro-derivative of the marine-derived peptide didemnin B. Plitidepsin and didemnin B were isolated by the Rinehart research group from two different tunicate genera, namely *Aplidium albicans* [32.28] and *Tridemnum solidum* [32.29], respectively. This notion supports the assumption that these metabolites are, in fact, produced by a common symbiont. Didemnin B revealed promising activity during Phases I and II clinical trials in patients with advanced pretreated cancers. These trials have now been discontinued due to the emergence of serious adverse effects, including severe fatigue [32.30] and anaphylaxis [32.31]. Therefore, plitidepsin represented an alternative which was further supported by the results of preclinical in vitro and in vivo studies. Clinically, plitidepsin exhibited preliminary efficacy in two different Phase II

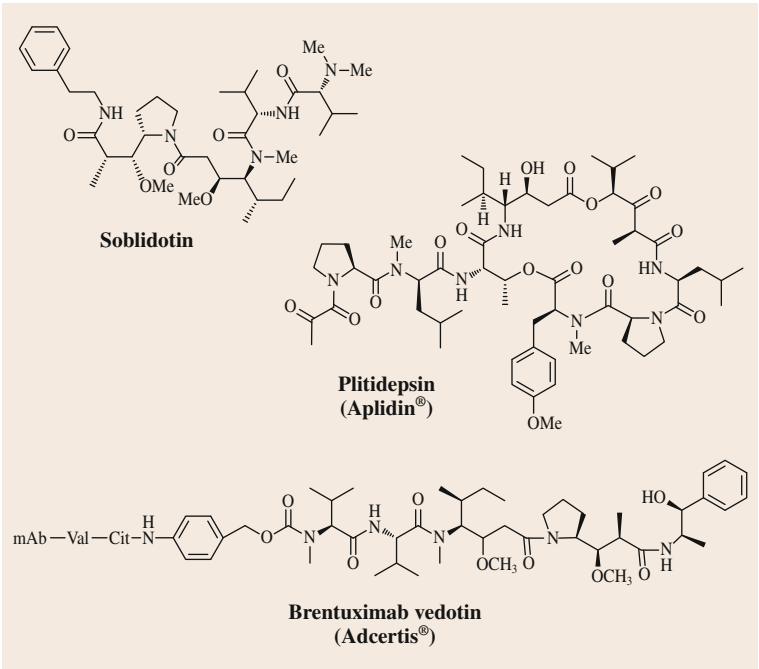

Fig. 32.2 Chemical structure of marine pharmaceuticals in Phase III clinical trials

trials against relapsing multiple myeloma and T cell lymphoma [32.32]. Currently, plitidepsin is undergoing Phase III clinical trials in spite of being accompanied by some adverse effects, including muscular toxicity, transient increase in transaminases, fatigue, diarrhoea, and cutaneous rash [32.33]; however, but no severe bone marrow toxicity was detected under plitidepsin therapeutic regimens.

32.2.3 Marine Pharmaceuticals in Phase II Clinical Trials

3-(2,4-dimethoxybenzylidene)-anabaseine (DMXBA, GTS-21, Fig. 32.3) is a synthetic derivative of anabaseine, a marine alkaloid isolated from several species of marine worms of the phylum Nemertea [32.34]. Structurally, DMXBA is a 3-(2,4-dimethoxybenzylidene)-anabaseine, which displays a selective $\alpha 7$ nicotinic acetylcholine receptor agonistic activity. DMXBA was found to improve cognitive functions [32.35]. GTS-21 has successfully completed Phase I clinical studies revealing significant improvement of cognitive functions in healthy young males [32.36] and schizophrenics [32.37]. A Phase II clinical trial using DMXBA for schizophrenics has been conducted and it has disclosed improvement of cognitive functions compared to the placebo, but only with high DMXBA doses [32.38].

Plinabulin (NPI-2358, Fig. 32.3) is a synthetic analog of halimide, a diketopiperazine alkaloid produced by the marine-derived fungus *Aspergillus* sp. CNC-139 isolated from the alga *Halimeda lacrimosa* [32.3]. Plinabulin acts as a vascular disrupting agent (VDA) through inhibiting tubulin polymerization resulting in selective collapse of tumor endothelial vasculature, in addition to its direct apoptotic effects [32.39, 40]. In Phase I clinical trial on patients with solid tumors or lymphomas, plinabulin revealed a significant reduction in tumor vasculature at doses $\geq 13.5\,\mathrm{mg\,m^2}$ and was tolerated up to $30\,\mathrm{mg\,m^2}$ [32.3]. Based on the principle that VDAs can augment chemotherapeutics and antiangiogenic agents, plinabulin is currently undergoing Phase II clinical trials [32.3].

Elisidepsin (PM02734, Irvalec, Fig. 32.3) is a marine-derived peptide of the kahalalide family, namely kahalalide F first reported from seasonal collections of the marine sacoglossan *Elysia rufescens* and the green alga *Bryopsis* sp. [32.41]. In preclinical in vitro studies, elisidepsin exhibited potent antineoplastic activity against a wide range of tested human tumor cell lines. Elisidepsin is currently in Phase II clinical trials with preliminary evidence of antitumor activity and with a favorable therapeutic index [32.42].

Zalypsis (PM1004, Fig. 32.3) is a synthetic deoxyribonucleic acid (DNA)-binding alkaloid related

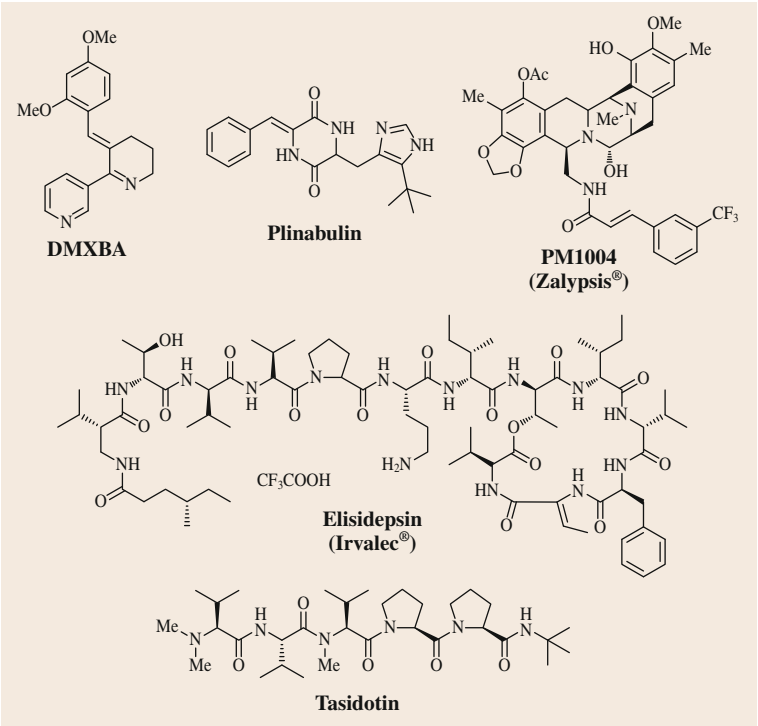

Fig. 32.3 Chemical structure of marine pharmaceuticals in Phase II clinical trials

to renieramycins and jorumycins, first isolated from the marine sponges *Reniera* sp. [32.43, 44] and the Pacific marine nudibranch *Jorunna funebris* [32.45]. Mechanistically, PM1004 acts through breaking the DNA helix by binding to guanines in selected DNA triplets, which leads to freezing cancer cells in DNA synthesis (S) phase and inducing apoptosis [32.46]. Zalypsis was promising in preclinical in vitro studies with significant antitumor activity against breast, prostate, and renal cancers. The main adverse effects that accompanied Zalypsis in Phase I clinical trials were hematological abnormalities and/or elevated transaminases, but these were transient and temporary [32.46].

Tasidotin (synthadotin, ILX-651, Fig. 32.3) is a synthetic analog of dolastatin 15 originally isolated from the sea hare *Dolabella auricularia* [32.47]. A detailed mechanistic investigation of tasidotin disclosed a potent inhibition of tubulin assembly. The active metabolite is probably the pentapeptide formed by hydrolysis of the *C*-terminal amide bond [32.48, 49]. ILX-651 has undergone Phase II clinical trials and the results showed that it was orally active and well tolerated but the efficacy was not good enough for it to proceed as a single agent therapy [32.3].

32.2.4 Marine Pharmaceuticals in Phase I Clinical Trials

Salinosporamide A (marizomib, NPI-0052, Fig. 32.4) is a rare β-lactone produced by the marine actinomycete *Salinospora tropica* recovered from deep-sea sediment [32.50, 51]. Mechanistically, marizomib exhibited a potent selective inhibition of p26 proteasome by acylation of the *N*-terminal Thr1O^{γ} residue followed by chloride displacement [32.52] resembling the marketed proteasome inhibitor bortezomib (Velcade, Millennium/Janssen-Cilag) that is approved for treatment of multiple myeloma. Moreover, salinosporamide A was able to overcome resistance to bortezomib in patients with refractory myeloma [32.53]. Salinosporamide A has successfully completed Phase I clinical trials for treatment of multiple myeloma, lymphoma, leukemia, and solid tumors [32.51, 54].

Hemiasterlins (Fig. 32.4) are antimitotic tripeptides isolated from marine sponges of different genera [32.55–57]. Hemiasterlins displayed significant in vitro cytotoxicity with IC_{50} values in the nanomolar range, which was attributed to the inhibition of the cell cycle in the mitosis (M) phase [32.56, 58]. Structure–activity relationship (SAR) studies revealed

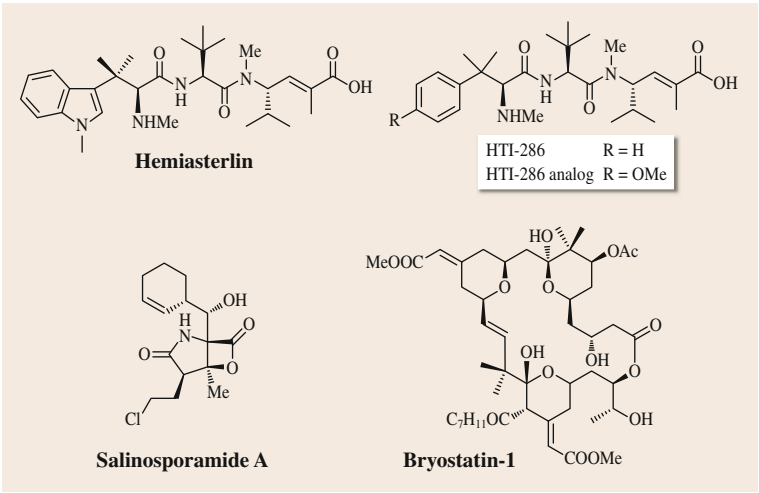

Fig. 32.4 Chemical structure of marine pharmaceuticals in Phase I clinical trials

that HTI-286, a simpler synthetic derivative of hemiasterlin possessing a phenyl moiety instead of the abrine (*N*-methyltryptophan) residue, is more potent than hemiasterlin [32.59], whereas the analog of HTI-286 with a *para*-methoxy functionality on the aromatic ring was even more potent [32.57]. HTI-286 inhibited tumor progression in preclinical studies with mice [32.60], which encouraged starting Phase I clinical trials in patients with advanced solid tumors. Results were disappointing, with no significant response accompanied with toxicities, including neutropenia, nausea, alopecia, and pain [32.61]. Therefore, Phase II studies of HTI-286 were postponed. However, the compound is still an interesting candidate, particularly after recent results illustrating its high antitumor activity against refractory and resistant prostate cancers [32.62].

Bryostatin 1 (NSC 339555, Fig. 32.4) is a 26-membered macrocyclic lactone with a hitherto unprecedented polyacetate carbon skeleton, which was first reported form the bryozoan *Bugula neritina* [32.63]. Bryostatin 1 is the parent compound for a family of 19 additional derivatives differing in the presence of acyloxy substituents [32.64]. The limited biological abundance of bryostatin 1 from its natural source was a major obstacle hindering its progression into preclinical and clinical trials. Moreover, the total synthesis of bryostatin 1 was not successful in offering an economically feasible supply of the compound; hence, the major interest was directed toward aquaculture of the bryozoans to obtain sufficient supply of bryostatin 1 from its natural source [32.65]. In Phase I clinical study against acute leukemia and B-cell malignancy, bryostatin 1 revealed encouraging results as an adjuvant therapy with Cytosar (cytarabine, Ara-C) [32.66] and Oncovin (vincristine) [32.67], respectively. As a single therapy, bryostatin 1 did not yield promising results in either Phase I or Phase II clinical trials; however, it is still of interest to be tested as a co-therapy with other chemotherapeutic agents such as Taxol (paclitaxel) against advanced gastroesophageal adenocarcinoma rather than using paclitaxel alone [32.68].

32.2.5 Marine Pharmaceuticals in the Preclinical Phase

In preclinical studies, a huge number of marine natural products have been introduced during the last two decades; these can be grouped according to their main pharmacological activities, including antitumor, antimicrobial, anti-inflammatory, anticoagulant, and antiplasmodial activities, whereas other compounds target the cardiovascular, immune, or nervous systems [32.69, 70]. The current status of marine natural products in preclinical studies is promising, and they seem to be potential sources for new pharmaceuticals that will enrich pharmacopeia for treatment and/or management of serious human diseases.

Some marine natural products showed interesting activities during the in vitro and in vivo preclinical evaluation, which suggested that they are potential drug candidates for entering clinical trial phases. These compounds include, for example, manzamine A (antimalarial, antituberculosis, anti-HIV), lasonolides (antifungal), jaspamides and geodiamolides (antitumor), and azumamides and psammaplin A (histone deacetylase inhibitors) [32.71].

It is nowadays assumed for many secondary metabolites originally isolated from macroorganisms that endosymbiotic microorganisms, including fungi and bacteria, are the real producers. This is highlighted by the isolation of paclitaxel (Taxol) from an endophytic fungus *Taxomyces andreanae* obtained from the inner bark of *Taxus brevifolia* [32.72], the production of camptothecin (Campto) by the endophytic fungus *Entrophospora infrequens* isolated from *Nothapodytes foetida* [32.73], and the production of trabectedin (Yondelis) through a semisynthetic protocol starting with cyanosafracin B, through fermentation of *Pseudomonas fluroscens* on multikilogram scale [32.19]. Therefore, there has been a sharp increase in research interests with regard to the exploration of secondary metabolites produced by endosymbiotic microorganisms such as fungi and bacteria, aiming at establishing a sustainable supply of bioactive natural products that can be further developed into pharmaceuticals through industrial fermentation or semisynthetic synthesis.

In the next part, we will survey recent reports on bioactive secondary metabolites from marine-derived fungi from 2010–2012 classified into chemical classes of compounds with particular attention placed on their structures and reported pharmacological activities.

32.3 Marine Organic Compounds

32.3.1 Alkaloids

Xylarin (**1**, Fig. 32.5), a [11]cytochalasin derivative, was purified from the mycelial extract of a marine-derived species of the genus *Xylaria* isolated from the gorgonian sea fan *Annella* sp. collected near the Similan Islands (Thailand) [32.74]. Structurally, xylarin (**1**) features a highly substituted perhydroisoindole moiety to which an octaketide macrocyclic ring derived from a head-to-tail condensation of eight acetate units affording an unbranched C_{16}-polyketide (PKS) moiety is linked. Antimicrobial activity assay of the crude extract revealed only weak antibacterial activity against *Staphylococcus aureus*, whereas the extract was inactive against methicillin-resistant Staphylococcus aureus (MRSA) [32.74].

Another species of the same fungal genus, obtained from marine sediment collected in the South China Sea, produced two new cytochalasins (**2** and **3**) together with four other known analogs (**4**–**7**) [32.75]. All isolated compounds were tested for their in vitro cytotoxicity against three different human tumor cell lines MCF-7 (breast), SF-268 (central nervous system, CNS), and NCI-H460 (lung) using cisplatin as a positive control [32.75]. Among the compounds tested, 21-*O*-deacetylcytochalasin Q (**3**) was most active against SF-268 cells ($IC_{50} = 44.3\,\mu M$), while cytochalasin D **7**) revealed the best activity against MCF-7 cells and NCI-H460 ($IC_{50} = 18.6$ and $14.4\,\mu M$, respectively). Moreover, cytochalasin Q (**4**) and 19,20-epoxycytochalasin (**5**) were active against NCI-H460 cells with IC_{50} values of 19.7 and $22.6\,\mu M$, respectively. However, 18-deoxycytochalasin Q (**2**) and 21-*O*-deacetyl-19,20-epoxycytochalasin Q (**6**) showed only moderate activity or were inactive against the cell lines tested [32.75].

Seven new dihydroquinolin-2-one-containing alkaloids, trivially named aflaquinolones A–G (**8**–**14**), were isolated from two different marine fungal isolates of the genus *Aspergillus* NRRL58570 and SF-5044 collected in Hawaii and Korea, respectively [32.76]. In addition, several known compounds belonging to aspochalasins and curvularins were also isolated from the Hawaiian isolate. An absolute configuration of aflaquinolone B (**9**) was established based on a nuclear Overhauser and exchange spectroscopy (NOESY) spectrum, a comparison of calculated and experimental electronic circular dichroism (ECD) data, and analysis of its Mosher's ester derivative [32.76]. All isolated aflaquinolones, except for **11**, were subjected to a cytotoxicity assay against four different human tumor cell lines, however, no significant activity was reported for the compounds tested at concentrations up to $80\,\mu M$ [32.76].

From the mycelial extract of the marine-derived fungus *Aspergillus fumigatus*, isolated from a Japanese zoanthid *Zoanthus* sp., two new indole alkaloids, 2-(3,3-dimethyl-prop-1-ene)-costaclavine (**15**) and 2-(3,3-dimethylprop-1-ene)-epicostaclavine (**16**), together with the known compounds costaclavine and fumgaclavines A and C, were obtained [32.77]. Relative and absolute configurations of **15** and **16** were determined based on NOESY, ^1H–^1H coupling constants, and CD (circular dichroism) spectra. Compounds **15** and **16** revealed weak cytotoxicity against mouse leukemia P388 cells [32.77].

Five unusual alkaloids (**17**–**21**) were purified from the culture broth of the endophytic fungus *Fusarium*

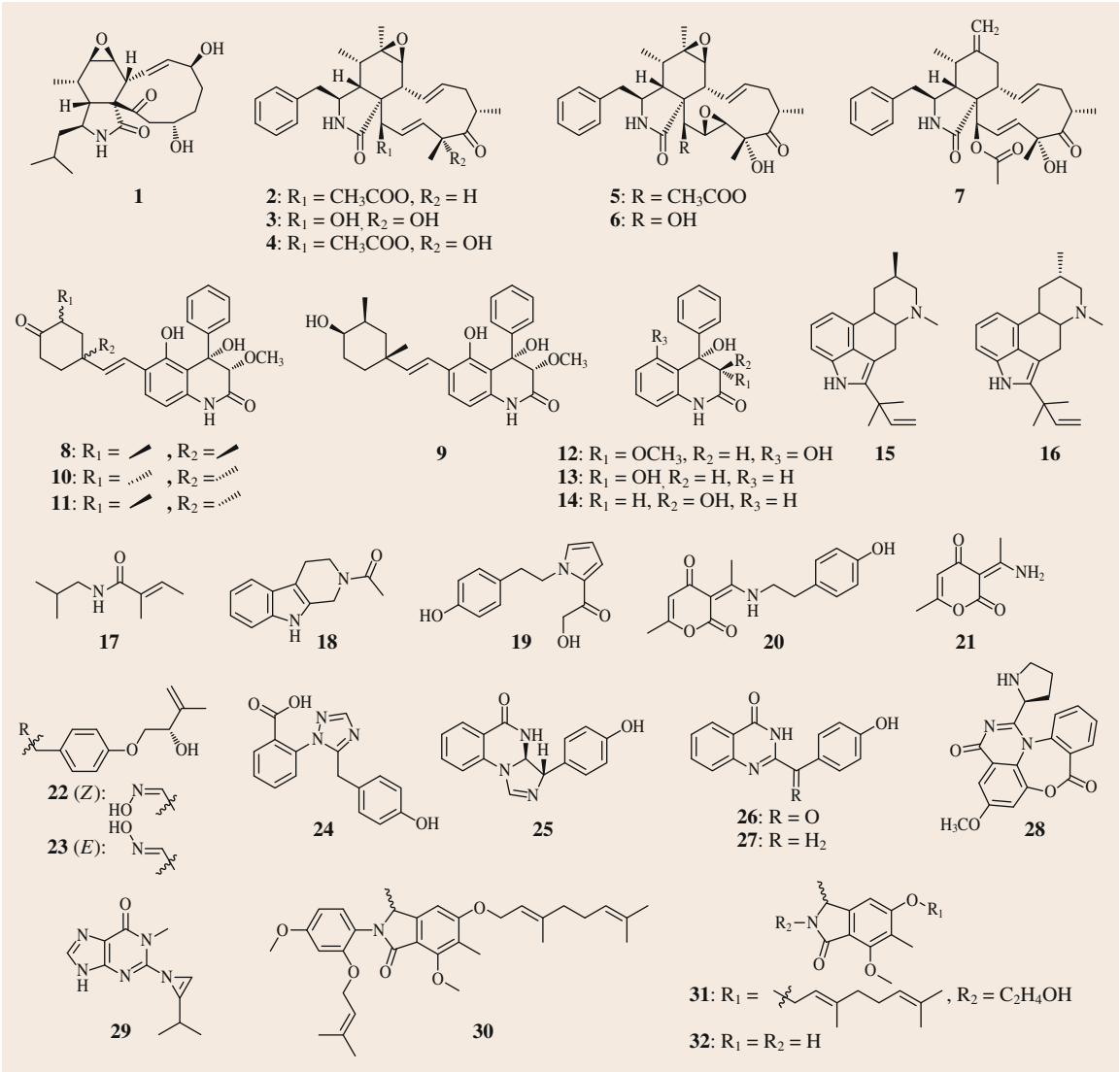

Fig. 32.5 Chemical structures of compounds **1**–**32**

incarnatum (HKI0504), isolated from the mangrove plant *Aegiceras corniculatum* [32.78]. In the antiproliferative activity assay, only 2-acetyl-1,2,3,4-tetrahydro-β-carboline (**18**), fusamine (**20**), and 3-(1-aminoethylidene)-6-methyl-2*H*-pyran-2,4(3*H*)-dione (**21**) exhibited weak cytotoxic activities against human umbilical vascular endothelial cells (HUVEC), human cervix carcinoma cells (HeLa), and human chronic myeloid leukemia cells (K562) with IC_{50} values of 41.1, 23.3, and 9.0 μM, respectively [32.78].

Two putative tyrosine-derived metabolites, stachylines A (**22**) and B (**23**), together with two *O*-prenylated derivatives, stachylines C and D, were purified from the fungal extract of *Stachylidium* sp., a marine-derived fungus isolated from the Australian sponge *Callyspongia* cf. *C. flammea* [32.79]. Stachylines are tyrosine-derived metabolites featuring oxime moiety which is incorporated in biosynthesis of marine natural products such as bastadins and psammaplins, which may suggest a metabolic relation between the marine-de-

rived fungus and its host. The isolated stachylines were subjected to a large number of bioactivity assays including cytotoxicity, antidiabetic, antimicrobial, NF-κB inhibitory activity assay, and protein kinase inhibition assays, but no activity was recognized in any of these assays [32.79].

Penipanoid A (**24**), an unprecedented triazole carboxylic acid derivative, together with two quinazolines alkaloids, penipanoids B (**25**) and C (**26**), and a quinazoline derivative (**27**) were isolated from the marine sediment-derived fungus *Penicillium paneum* SD-44 [32.80]. The structure of **24** was confirmed by X-ray crystallography establishing the compound as the first triazole metabolite from marine sediment-derived fungi, whereas **25** featured an unusual quinazolinone structure possessing a dihydroimidazole ring system [32.80]. In the antiproliferative activity assay, penipanoid A (**24**) revealed activity against the SMMC-7721 cell line ($IC_{50} = 54.2\,\mu M$), while penipanoid D (**27**) exhibited significant activity against A-549 and BEL-7402 cell lines with IC_{50} values of 17.5 and 19.8 μM, respectively, compared to fluorouracil which displayed IC_{50} values of 13.0, 13.7, and 21.8 μM against the above three cell lines [32.80].

Aspergicin (**28**) was reported as a new alkaloid together with the previously known neoaspergillic acid from mixed culture mycelia of two epiphytic fungi from the genus *Aspergillus* isolated from a rotten fruit of the marine mangrove plant *Avicennia marina* [32.81]. Both aspergicin and neoaspergillic acid revealed significant antimicrobial activity against some Gram-positive and Gram-negative bacteria with minimum inhibitory concentrations (MICs) between 2.2 and 43.0 μM [32.81].

Acremolin (**29**), an unprecedented modified base featuring a 1*H*-azirine moiety, was purified from the culture broth of the marine-derived fungus *Acremonium strictum*, isolated from an unidentified Korean Choristida sponge [32.82]. Structurally, acremolin (**29**) is a modified guanine base attached to an isoprene unit via a 1*H*-azirine moiety. In the cytotoxicity assay, acremolin (**29**) revealed only weak activity against A549 cells with IC_{50} of 200 μM [32.82].

Together with stachylines (**23** and **24**), and marilines A–C (**30**–**32**), phthalimide derivatives reported as novel metabolites were obtained from the same extract of the marine-derived fungus *Stachylidium* sp. [32.83]. The absolute configurations of the enantiomeric congeners marilines A_1 (**30a**) and A_2 (**30$_b$**) were established by a combination of experimental circular dichroism (CD) investigations and quantum chemical CD calculations. Marilines A_1 (**30a**) and A_2 (**30$_b$**) inhibited the serine protease human leukocyte elastase (HLE) with an IC_{50} value of 0.86 μM for both enantiomers [32.83]. HLE is an enzyme considered to be a main cause of tissue damage accompanying inflammatory diseases such as chronic obstructive pulmonary disease, cystic fibrosis, and adult respiratory distress syndrome [32.84, 85]. Therefore, inhibitory activity exhibited by marilines A_1 (**30a**) and A_2 (**30$_b$**) against HLE potentiates the tendency to use them as therapeutically useful candidates for the treatment of those diseases. Mariline B (**31**) did not exhibit inhibitory activity against HLE. Marilines A_1 (**30a**), A_2 (**30$_b$**), and B (**31**) showed antiplasmodial activity against the liver stages of *Plasmodium berghei* with IC_{50} values of 6.7, 11.6, and 13.8 μM, respectively [32.83]. Mariline A_1 (**30a**) and A_2 (**30$_b$**) were assessed for their cytotoxic activity against panels of 5 and 19 cancer cell lines with mean GI_{50} values of 24.4 and 11.0 μM, respectively [32.83].

Unusual polyketide-type alkaloids, (-)-cereolactam (**33**, Fig. 32.6) and (-)-cereoaldomine (**34**) comprising a lactam ring and an iminine functionality, respectively, were produced together with the related metabolite (-)-trypethelone by the marine endophytic fungus *Coniothyrium cereale* obtained from the green alga *Enteromorpha* sp. [32.86]. Both **33** and **34** selectively inhibited the activity of HLE with IC_{50} values of 9.3 and 3.0 μM, respectively [32.86].

Auranomides A–C (**35**–**37**) are three new alkaloids that have been reported from the marine-derived fungus *Penicillium aurantiogriseum* [32.87]. Structurally, auranomides A (**35**) and B (**36**) featured a new scaffold containing a quinazoline-4-one substituted with a pyrrolidin-2-iminium moiety. In the cytotoxicity assay against human tumor cell lines, auronamide B (36) was the most potent compound with an IC_{50} value of 97 μM [32.87].

Two new epimeric alkaloids, namely tumonoic acids K (**38**) and L (**39**), were isolated together with several known compounds from the marine-derived fungus *Penicillium citrinum* [32.88]. Compounds **38** and **39** represent the first example of tumonoic acid analogs derived from a fungus [32.88]. Tumonoic acids K and L revealed weak cytotoxic activity against A-375 cell lines with IC_{50} values of 65.7 and 124.6 μM, respectively [32.88].

Fumiquinazoline K (**40**), a quinazoline-type alkaloid, was isolated from a marine strain of *Aspergillus fumigatus* KMM 4631 associated with the soft coral

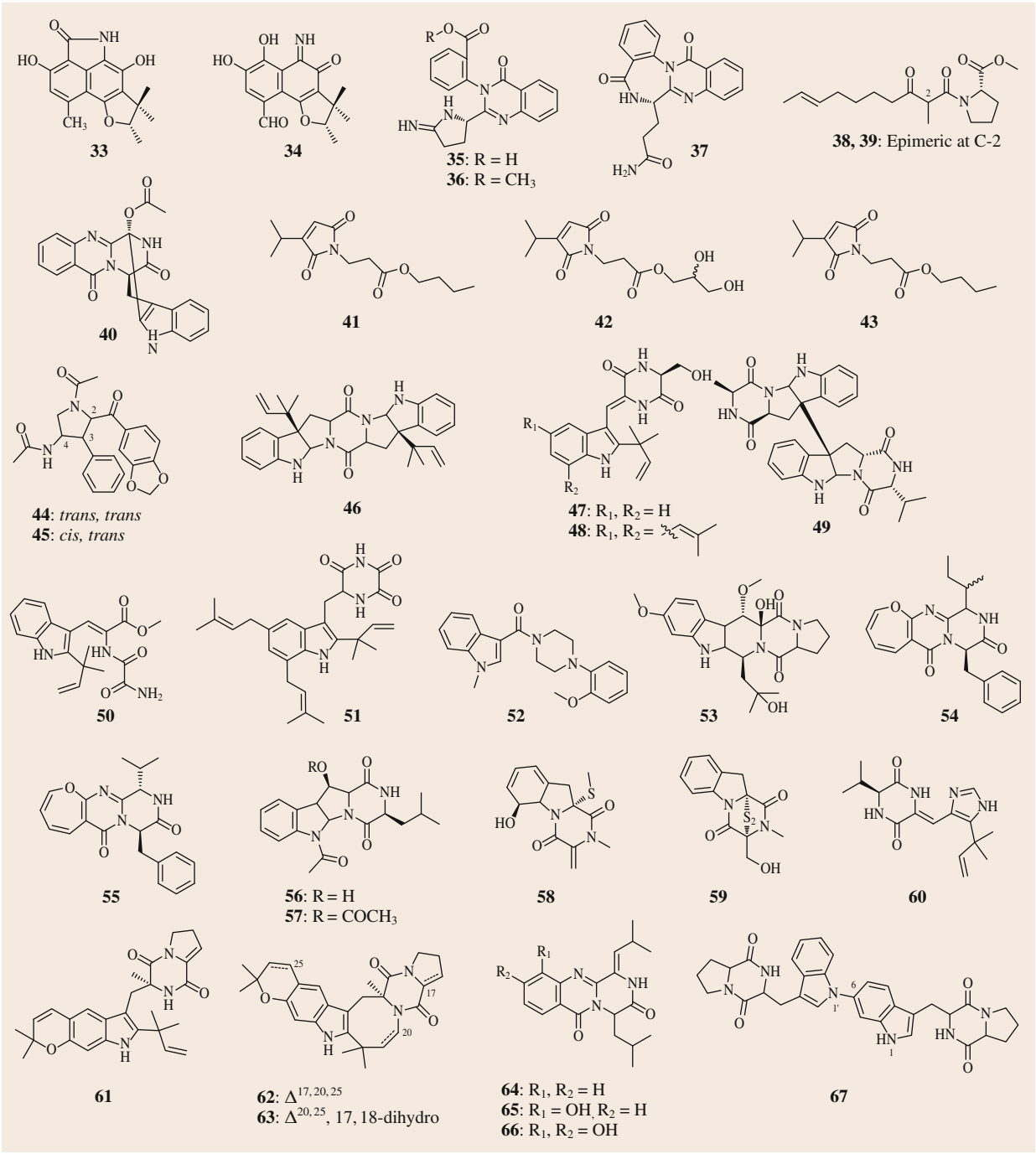

Fig. 32.6 Chemical structures of compounds **33–67**

Sinularia sp. [32.89]. Fumiquinazoline K revealed neither cytotoxic nor antibacterial activity [32.89].

Farinomaleins C–E (**41**–**43**), three new dioxopyrrole derivatives, together with one new indole metabolite were isolated from an unidentified endophytic fungus derived from the mangrove plant *Avicennia marina* [32.90]. All isolated farinomaleins were investigated for their cytotoxic and antimicrobial activities; however, none exhibited significant activities [32.90].

Aspergillamides A (**44**) and B (**45**), two new pyrrolidine alkaloids, were isolated together with the known compound aurantiamine from the marine fungus *Aspergillus ustus* derived from the Mediterranean sponge *Suberties domuncula* collected from the Adriatic Sea [32.91]. In the cytotoxicity assay against the murine lymphoma (L5178Y) cell line, neither **44** nor **45** exhibited more than 10–20% activity compared to controls [32.91].

32.3.2 Diketopiperazines

Amauromine (**46**), an indole diketopiperazine alkaloid, was isolated from the fungus *Auxanthron reticulatum* derived from the marine sponge *Ircinia variablis* [32.92]. Amauromine (**46**) was identified as the first fungal metabolite that revealed high and selective affinity for the human cannabinoid receptor CB_1 recombinantly expressed in Chinese hamster ovary (CHO) cells ($K_i = 178$ nm) [32.92]. Based on the selective antagonistic potency, **46** may be a potential candidate for further development as a lead structure for drug development.

Four further indole alkaloids, cristatumins A–D (**47**–**50**), were isolated together with six known derivatives from the endophytic fungus *Eurotium cristatum* EN-220, obtained from the marine alga *Sargassum thunbergii* [32.93]. In the antimicrobial activity assay, cristatumin A (**47**) displayed moderate activity against *Escherichia coli* and *Staphylococcus aureus*, while cristatumin B (**48**) provoked modest lethality in brine shrimp *Artemia salina* [32.93].

From the ethyl acetate (EtOAc) extract of the solid rice culture of the marine-derived endophytic fungus *Eurotium rubrum*, a new dioxopiperazine alkaloid, namely 12-demethyl-12-oxo-eurotechinulin B (**51**) was isolated together with one new anthraquinone derivative and ten other known compounds [32.94]. In the cytotoxicity assay against seven human tumor cell lines, **51** revealed only moderate activity against SMMC-7721 cells with IC_{50} of 65.5 μM, while in the antimicrobial activity assay it showed no activity [32.94].

Bioassay-guided fractionation of the mycelial extract of the marine fungal strain *Aspergillus sydowii* (SCSIO00305), obtained from the gorgonian coral *Verrucella umbraculum*, led to the isolation of two new natural products, [4-(2-methoxyphenyl)-1-piperazinyl][(1-methyl-1*H*-indol-3-yl)]-methanone (**52**) and cyclotryprostatin (**53**) together with eight known natural products [32.95]. Both **52** and **53** were subjected to a cytotoxicity (microculture tetrazolium assay, MTT), but only **52** revealed significant cytotoxic activity against human melanoma (A375) cell line with IC_{50} of 5.7 μM [32.95].

Two new oxepin-type alkaloids, protuboxepins A and B (**54** and **55**) and two diketopiperazine alkaloids, protubonines A and B (**56** and **57**), were reported from the EtOAc extract of the marine sediment-derived fungus *Aspergillus* sp. SF-5044 [32.96]. Compounds (**54**, **56**, and **57**) were subjected to a cytotoxic activity assay against five different cancer cell lines. Results revealed that only protuboxepin A (**54**) showed weak antiproliferative activity against five tested cancer cell lines, namely HL-60 (leukemia), MDA-MB-231 (breast), Hep3B (liver), 3Y1 (fibroblast), and K562 (chronic myeloid leukemia, CML) with IC_{50} values of 75, 130, 150, 180, and 250 μM, respectively [32.96].

Two new gliotoxin-related metabolites, **58** and **59**, were isolated together with other five known derivatives from the fungus *Penicillium* sp. (JMF034) obtained from deep sea sediment collected off Suruga Bay (Japan) [32.97]. The absolute configuration of the new compounds was established based on the modified Mosher's method. All isolated compounds showed significant cytotoxic activity when tested against murine leukemia cells (P388), except for bis(dethio)-10a-methylthio-3a-deoxy-3,3a-didehydrogliotoxin (**58**) which exhibited only marginal activity [32.97]. However, compounds with disulfide or tetrasulfide bonds exhibited potent inhibitory activity against histone methyltransferases (HMT) G9a and HMT Set7/9 (lysine-specific histone methyltransferases). 6-Deoxy-5a,6-didehydrogliotoxin (**59**) containing a disulfide bond showed only weak activity, which suggested that the C-6 hydroxy group interfered with the G9a inhibitory activity based on the significant activity of other metabolites lacking this hydroxyl group [32.97].

A new diketopiperazine alkaloid, pre-aurantiamine (**60**) was isolated together with two new itaconic acid derivatives from the culture broth of the fungus *Aspergillus aculeatus* CRI322-03, which was obtained from the marine sponge *Stylissa flabelliformis* collected

off the shores of Thailand [32.98]. The structure and absolute configuration of pre-aurantiamine (**60**) was established based on extensive 1-D and 2-D nuclear magnetic resonance (NMR) and X-ray crystallographic analysis.

Carneamides A–C (**61**–**63**), prenylated indole diketopiperazines, and carnequinazolines A–C (**64**–**66**), quinazoline-type alkaloids, were reported as new natural products from the marine-derived fungus *Aspergillus carneus* KMM 4638 isolated from the marine brown alga *Laminaria sachalinensis* [32.99]. Structurally, carneamides A–C (**61**–**63**) represented the first prenylated indole alkaloid possessing a 2,2-dimethyl-2,8-dihydropyrano[3,2-f]indole ring system, and in particular **62** and **63** featured a rare indoloazocine tricyclic subunit. However, carnequinazolines A–C (**64**–**66**) were identified as quinazoline alkaloids with additional oxidation and alkylation patters, whereas **64** and **65** are aryl *C*-glycosides containing glucopyranoside units of $^4C_1$ conformation [32.99].

Recently, a diketopiperazine dimer possessing a rare *N*-1 to *C*-6 linkage was purified from the culture extract of the fungus *Aspergillus taichungensis* derived from the mangrove plant *Acrostichum aureum* and it was trivially named aspergilazine A (**67**) [32.100]. The chemical structure and absolute configuration of aspergilazine A was established based on spectroscopic evaluation and on Marfey's method. In the antiviral activity assay, **67** revealed only weak activity against the influenza A (H_1N_1) virus [32.100].

32.3.3 Polyketides

Three new α-pyrone metabolites, curvulapyrone (**68**, Fig. 32.7), curvulalide (**69**), and the curvulalic acid (**70**), were identified as new compounds together with six further metabolites from the sea fan-derived fungus *Curvularia* sp. PSU-F22 [32.101]. In the antimicrobial activity assay against *Staphylococcus aureus*, MRSA, and *Microsporum gypseum*, none of the isolated compounds revealed activity at concentrations up to 200 $\mu g\,mL^{-1}$ [32.101].

Nidurufin (**71**), a new cell cycle inhibitor, was isolated from the marine sediment-derived fungus *Penicillium flavidorsum* SHK1-27 [32.102]. In the in vitro cytotoxicity assay, **71** exhibited significant antiproliferative activity against the K562 cell line (IC_{50} = 12.6 μM) by inducing cell cycle arrest at G_2/M transition [32.102].

Trichodermaquinone (**72**) and trichodermaxanthone (**73**) were isolated from the marine-derived fungus *Trichoderma aureoviride* PSU-F95 isolated from the gorgonian sea fan *Annella* sp. [32.103]. In the antimicrobial assay, neither **72** nor **73** exhibited activity against MRSA [32.103].

A new aflatoxin, aflatoxin B_{2b} (**74**), was isolated together with six known compounds from the marine fungus *Aspergillus flavus* 092 008 derived from the mangrove plant *Hibiscus tiliaceus* [32.104]. Aflatoxin B_{2b} revealed significant antimicrobial activity against *Escherichia coli*, *Bacillus subtilis*, and *Enterobacter aerogenes*, with MIC values of 22.5, 1.7, and 1.1 μM, respectively [32.104]. In addition, **74** showed cytotoxic activity against A549, K562, and L-02 cell lines, with IC_{50} values of 8.1, 2.0, and 4.2 μM, respectively [32.104]. The results disclosed that hydration and hydrogenation of the Δ^8-double bond significantly reduce the cytotoxicity of aflatoxins, while the esterification at *C*-8 increases the cytotoxicity [32.104].

Marilones A–C (**75**–**77**), three new phthalide derivatives, were isolated together with the known compound silvaticol (**78**) from the biomalt culture of *Stachylidium* sp., a marine fungus derived from the sponge *Callyspongia* sp. cf. *C. flammea* [32.105]. Marilones A (**75**) and B (**76**) featured an unusual skeleton, suggesting unique fungal biosynthetic reactions. Among the isolated compounds, marilone A (**75**) revealed antiplasmodial activity against *Plasmodium berghei* liver stages with an IC_{50} of 12.1 μM, whereas marilone B (**76**) showed selective antagonistic activity towards the serotonin receptor 5-HT_{2B} with a K_i value of 7.7 μM [32.105].

Aspergilones A (**79**) and B (**80**), two novel benzylazaphilone derivatives with an unprecedented skeleton, were isolated from the culture broth of the marine-derived fungus *Aspergillus* sp. obtained from the gorgonian *Dichotella gemmacea* [32.106]. Structurally, aspergilone B (**80**) represents a symmetrical dimer of **79** with a characteristic methylene bridge. The absolute and relative configurations of aspergilones A and B were established based on X-ray crystallography and NMR spectral analysis. Interestingly, only the monomer aspergilone A (**79**) exhibited in vitro selective cytotoxicity toward HL-60, MCF-7, and A-549 cell lines with IC_{50} values of 8.3, 72.5, and 95.85 μM, respectively [32.106]. Moreover, **79** also exhibited potent antifouling activity against the larval settlement of the barnacle *Balanus amphitrite* at EC_{50} of 20 μM [32.106].

Penicacids A–C (**81**–**83**), together with two known analogs, namely mycophenolic acid (MPA) and 4′-hydroxy-MPA, were isolated from the fungus *Penicillium* sp. SOF07 derived from a marine sediment col-

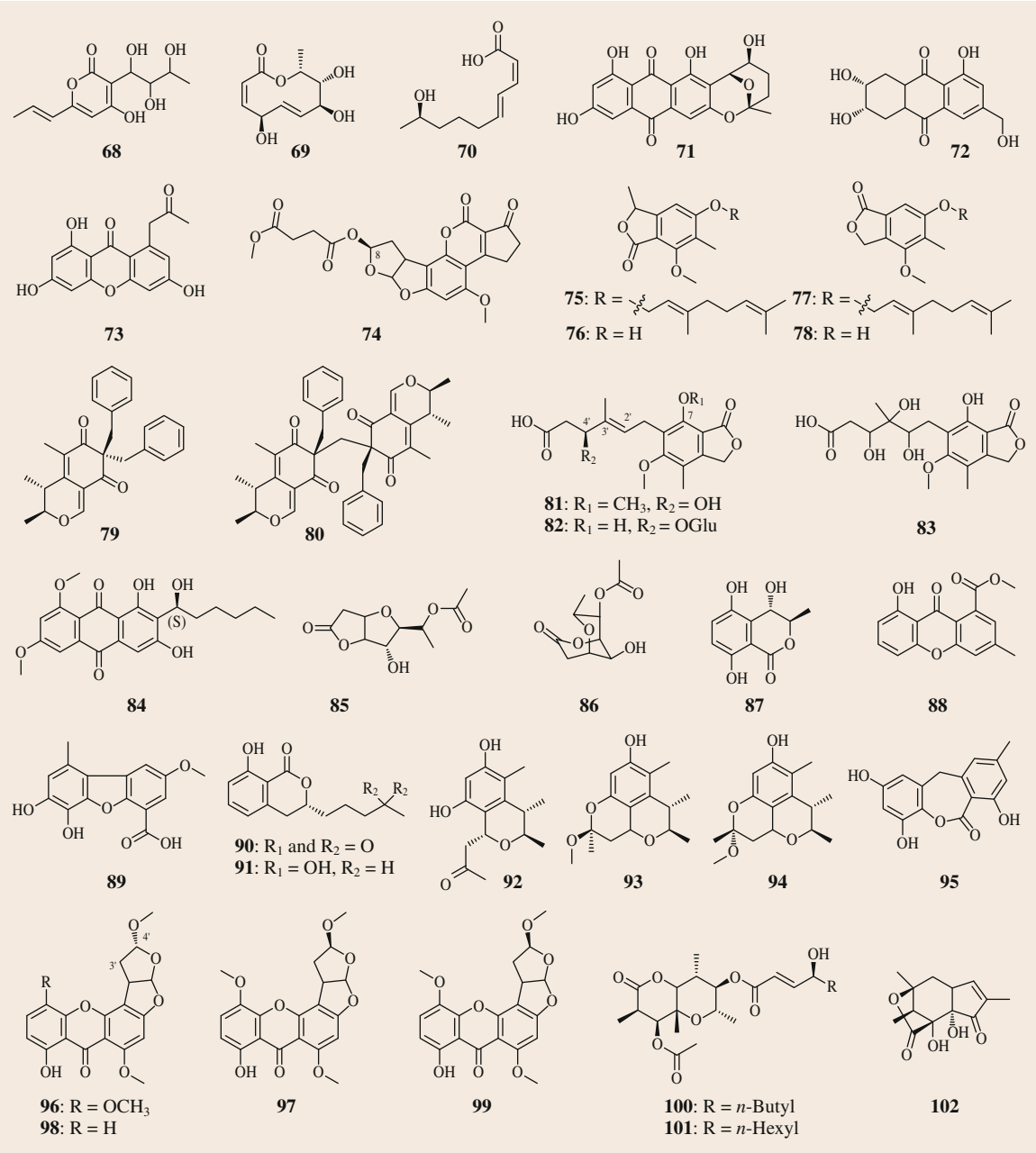

Fig. 32.7 Chemical structures of compounds **68–102**

lected in the South China Sea [32.107]. All compounds were assessed for their inhibitory activity against inosine 5′-monophosphate dehydrogenase (IMPDH), revealing IC_{50} values between 0.63 and 73 μM [32.107]. IMPDH is an essential rate-limiting enzyme controlling the guanine nucleotide pool which, in turn, controls replication, transcription, signaling, and translocation. Hydroxylation at *C*-4′, methylation at *C*-7-*OH*, or dual hydroxylation at the *C*-2′/*C*-3′ double bond of MPA diminished bioactivity, whereas glucosyl hydroxyla-

tion at *C*-4′ had no effect on the activity compared to MPA [32.107].

A new anthraquinone derivative, 6,8-di-*O*-methylaverantin (**84**), was isolated together with six known congeners from a culture of *Aspergillus versicolor* EN-7 derived from the brown alga, *Sargassum thunbergii* collected off the Qingdao coastline (China) [32.108]. Compound **84** exhibited only weak inhibition against *E. coli* but not against *S. aureus* or *Candida albicans* [32.108].

Protulactones A (**85**) and B (**86**), two polyketide-derived fungal metabolites, were been isolated from an EtOAc extract of the marine sediment-derived fungus *Aspergillus* sp. SF-5044 [32.109]. The structures of **85** and **86** were established based on NMR spectroscopy and mass spectroscopy (MS) data, along with chemical methods such as Mosher's method [32.109]. Compounds **85** and **86** represented new polyketide derivatives, possessing unique ring systems among the fungal metabolites produced by the genus *Aspergillus* [32.109].

A new isochroman, (3*R*,4*S*)-3,4-dihydro-4,5,8-trihydroxy-3-methylisocoumarin (**87**), was purified from the marine fungus *Phomopsis* sp. ZH-111 together with two known compounds [32.110]. Compound **87** accelerated the growth of subintestinal vessel plexus (SIV) branches [32.110].

A new xanthone derivative, 8-hydroxy-3-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid methyl ester (**88**), was isolated from the co-culture broth of two mangrove-derived fungi (K38 and E33) [32.111]. Primary bioassays showed that **88** possesses inhibitory activity against five microorganisms, including *Gloeasporium musae* and *Peronophthora circhoralearum* [32.111].

Porric acid D (**89**), a new dibenzofuran derivative, was obtained from the methanolic extract of a marine-derived fungus, *Alternaria* sp., isolated from the Bohai Sea [32.112]. Porric acid D exhibited mild antimicrobial activity against *S. aureus* with a MIC of $100\,\mu g\,mL^{-1}$.

Aspergillumarins A (**90**) and B (**91**), two new dihydroisocoumarin derivatives, were isolated from the marine fungus *Aspergillus* sp., derived from the Chinese mangrove *Bruguiera gymnorrhiza* [32.113]. Aspergillumarins A and B revealed weak antibacterial activity against *S. aureus* and *Bacillus subtilis* at a concentration of $50\,\mu g\,mL^{-1}$ [32.113].

Three new citrinin derivatives, penicitrinols C–E (**92–94**), were isolated along with two known compounds from *Penicillium citrinum* [32.114]. The structures of the isolated citrinin derivatives were established based on spectroscopic methods and X-ray crystallographic analyses [32.114]. In the in vitro cytotoxicity assay against P388 and HL-60 cell lines, only penicitrinols C (**92**) and E (**94**) revealed modest antiproliferative activity against HL-60 cells with IC_{50} values of 52.8 and 41.2 μM, respectively [32.114].

In addition to a new diketopiperazine (**51**), a new anthraquinone derivative, 9-dehydroxyeurotinone (**95**), was purified from the fungal strain *Eurotium rubrum*, an endophytic fungus isolated from the semi-mangrove plant *Hibiscus tiliaceus* [32.94]. Compound **95** revealed weak antibacterial activity against *E. coli* with an inhibition zone of 7.0 mm at $100\,\mu g$/disk compared to amphotericin B with an inhibition zone of 11.0 mm at $20\,\mu g$/disk [32.94]. However, in the cytotoxic activity assay, 9-dehydroxyeurotinone revealed mild but selective activity against human cholangiocarcinoma cell line SW1990 ($IC_{50} = 92.0\,\mu M$) when tested against seven tumor cell lines [32.94].

Three new sterigmatocystin derivatives, oxisterigmatocystins A–C (**96–98**), together with the known compound 5-methoxysterigmatocystin (**99**), were isolated from the deep sea-derived fungus *Aspergillus versicolor* [32.115]. All isolated sterigmatocystin derivatives were assessed for cytotoxic activity against A-549 and HL-60 cell lines, and interestingly, only **99** revealed significant cytotoxicities with IC_{50} values of 3.86 and 5.32 μM, respectively [32.115]. This result implied that the double bond between *C*-3 and *C*-4 is a crucial pharmacophoric functionality in sterigmatocystins, and the activity disappeared upon saturation [32.115].

Two new botcinin derivatives, botcinin A (**100**) and B (**101**), were isolated from the fungus *Botryotinia* sp. SF-5275 derived from an unidentified marine alga collected off Seongsan Port (Korea) [32.116]. Botcinins A and B were evaluated for their in vitro inhibitory activity against protein tyrosine phosphatase 1B (PTP1B), an insulin-antagonizing protein influencing negative regulation of the insulin pathway, which is a promising target for the treatment of diabetes and obesity. Results revealed that botcinin A (**100**) possesses higher activity than **101** with IC_{50} values of 53.6 and 340.7 μM, respectively, compared to ursolic acid that was used as a positive control ($IC_{50} = 3.1\,\mu M$) [32.116]. These data disclosed that the hexahydropyrano[3,2,b]-pyran-2(3*H*)-one moiety is an essential structural feature required for activity.

Acremostrictin (**102**), a highly oxygenated tricyclic lactone of an unprecedented skeletal class, was isolated from the culture broth of *Acremonium strictum*, a marine fungus derived from a Korean Choris-

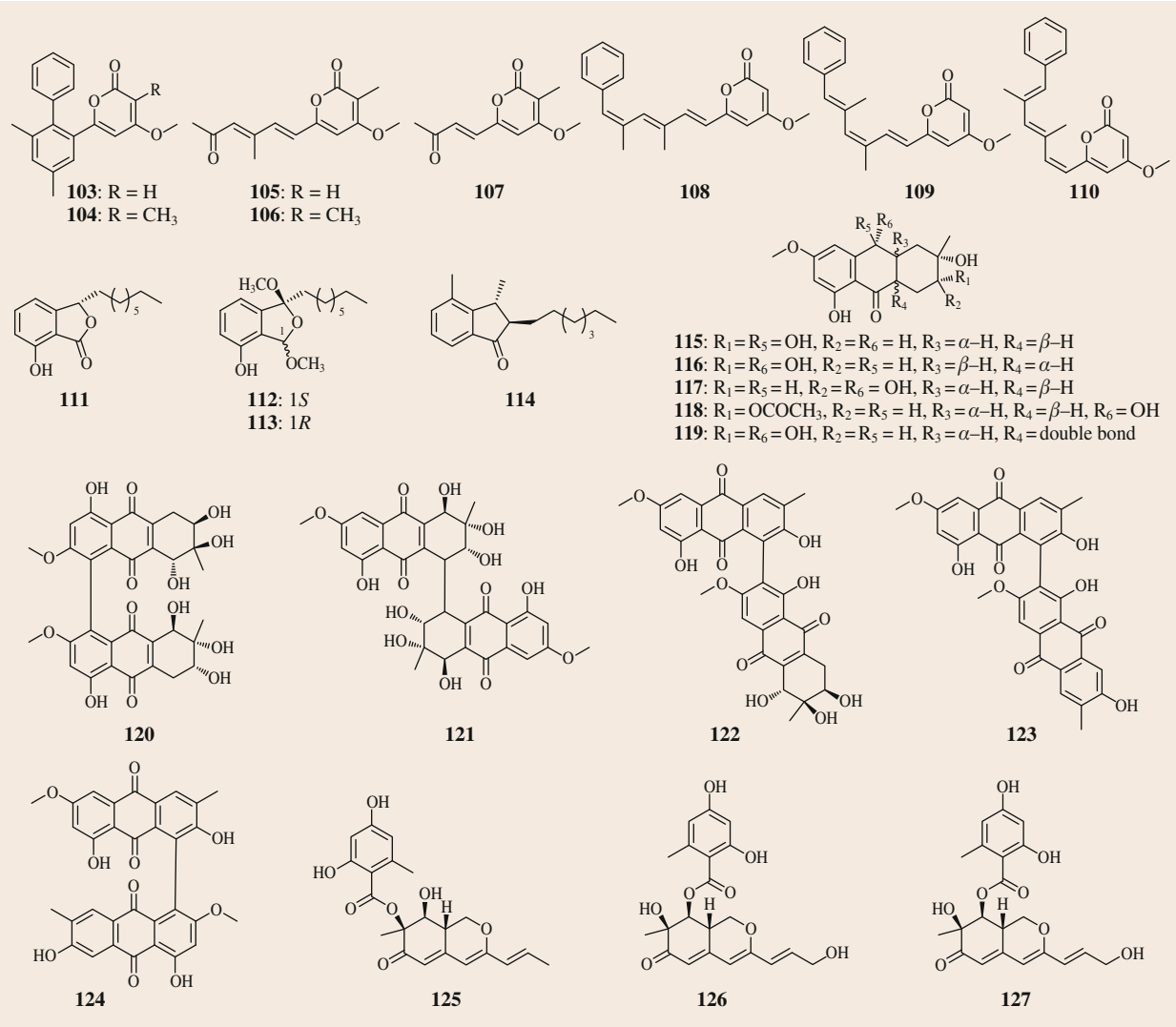

Fig. 32.8 Chemical structures of compounds **103**–**127**

tida sponge [32.117]. The structure of acremostrictin (**102**) was established based on combined spectroscopic and X-ray crystallographic analyses. In the antimicrobial activity assay, **102** revealed moderate activity with MIC values between 50 and 200 μM against *Micrococcus luteus* (IFO 12708), *Salmonella typhimurium* (ATCC 14028), and *Proteus vulgaris* (ATCC 3851) [32.117].

Eight new α-pyrone derivatives, namely nigerapyrones A–E (**103**–**110**, Fig. 32.8), were isolated from the endophytic fungus *Aspergillus niger* MA-132, derived from the Chinese mangrove plant *Avicennia marina* [32.118]. All isolated compounds were assessed for their cytotoxic and antimicrobial activity against a panel of eight tumor cell lines, two bacteria, and four plant-pathogenic fungi. Among the compounds tested, only compounds **104**, **106**, and **107** showed moderate to weak cytotoxicity against some of the tested tumor cell lines with IC_{50} values between 38 and 120 μM [32.118].

Paecilocins A–D (**111**–**114**), four new polyketide derivatives, were isolated from the fungus *Paecilomyces variotii*, derived from the jellyfish *Nemopilema nomurai* [32.119]. In the antimicrobial activity assay,

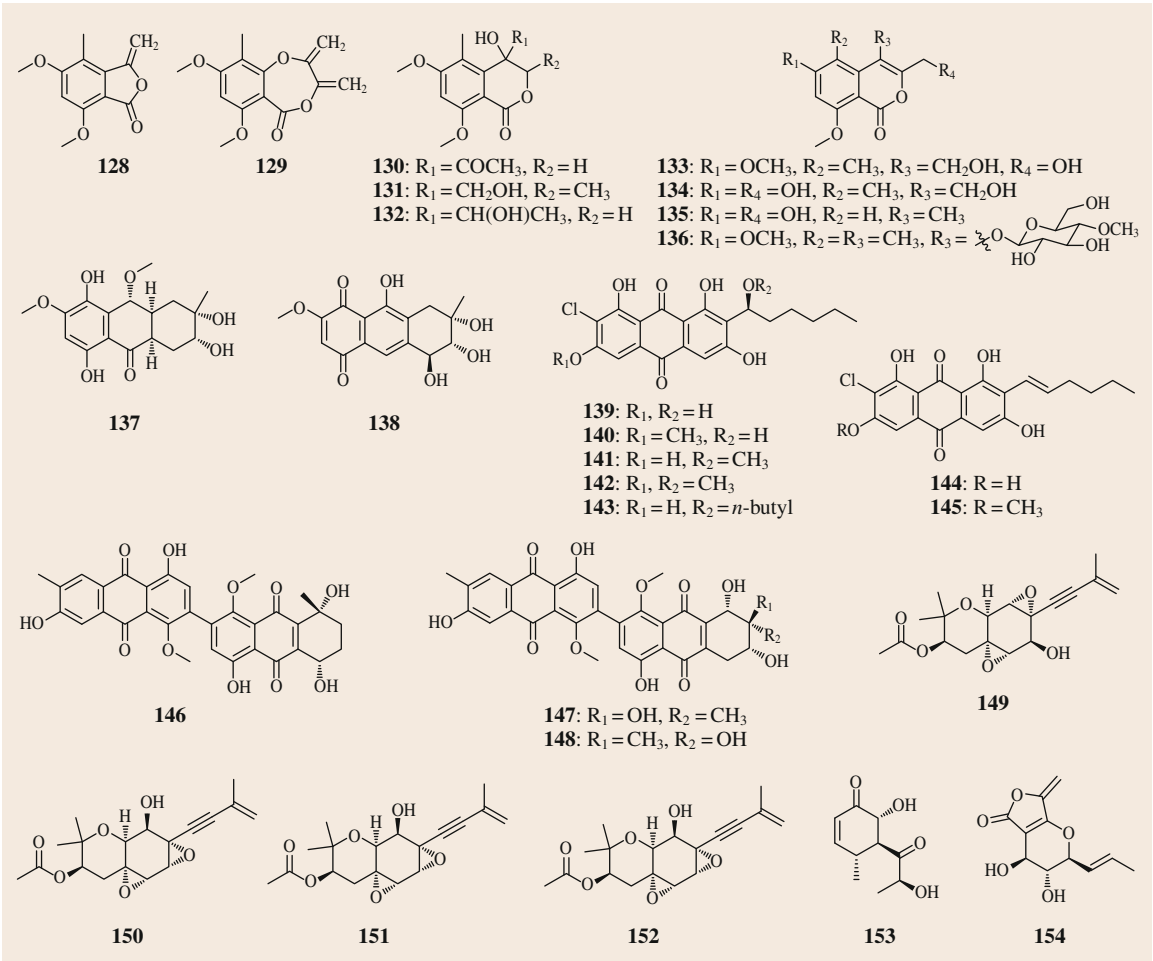

Fig. 32.9 Chemical structures of compounds **128**–**154**

paecilocins A–D revealed inhibitory activity against pathogenic bacteria including MRSA 3089 and multidrug-resistant *Vibrio parahemolyticus* 7001 with MIC values between 20 and 130 μM [32.119].

Five new hydroanthraquinone derivatives, tetrahydroaltersolanols C–F (**115**–**119**) and five new alterporriol-type anthranoid dimers, alterporriols N–R (**12**–**124**), were isolated along with seven known congeners from the culture broth of the fungus *Alternaria* sp. ZJ-2008003 obtained from the Chinese soft coral *Sarcophyton* sp. [32.120]. Structures and relative configurations of the isolated compounds were established based on extensive NMR spectroscopic analyses and single-crystal X-ray crystallography. Tetrahydroaltersolanol C (**115**) and alterporriol Q (**123**) exhibited antiviral activity against the porcine reproductive and respiratory syndrome virus (PRRSV) with IC_{50} values of 65 and 39 μM, respectively [32.120]. Alterporriol P (**122**) showed antiproliferative activity against PC-3 and HCT-116 cell lines, with IC_{50} values of 6.4 and 8.6 μM, respectively [32.120].

New hydrogenated azaphilones, pinophilins A (**125**) and B (**126**), together with Sch 725680 (**127**) were isolated from the fungal cultures of *Penicillium pinophilum* Hedgock derived from a seaweed [32.121]. Compounds **125**–**127** selectively inhibited the activities of mammalian DNA polymerases but not the activities of four X-family pols. Pinophilin A (**125**) revealed the highest activity with IC_{50} values of 48.6–55.6 μM through noncompetitive inhibition of both α and κ activities [32.121]. Interestingly, compounds **125**–**127** suppressed cell proliferation in five human cancer cell

lines, but had no effect on the viability of normal human cell lines [32.121].

Acremonide (**128**, Fig. 32.9), one new phthalide derivative and eight new isocoumarin derivatives, acremonones A–H (**129–136**) together with ten known compounds were obtained from *Acremonium* sp. PSU-MA70 isolated from a branch of the mangrove *Rhizophora apiculata* [32.122]. In the antifungal activity assay, some of the isolated compounds revealed only moderate antifungal activity with MIC values of 32 $\mu g\,mL^{-1}$ when tested against *Candida albicans* and *C. neoformans*, whereas against the latter the activity was lower [32.122].

Two new hydroanthraquinone analogs, 4a-*epi*-9α-methoxydihydrodeoxybostrycin (**137**) and 10-deoxybostrycin (**138**), together with seven known anthraquinone congeners were isolated from the fungus *Nigrospora* sp. derived from an unidentified sea anemone [32.123]. All isolated compounds were evaluated in vitro for their antibacterial activity against a panel of nine different bacterial strains. Among the isolated compounds, 10-deoxybostrycin (**138**) and 3-acetyl-4-deoxybostrycin revealed the most promising activities against *Bacillus subtilis* and *B. cereus* with IC_{50} values of 62.5 and 48.8 nM, respectively compared to ciprofloxacin as a positive control with IC_{50} values of 31.2 and 1250 nM, respectively [32.123].

Chemical investigation of the cultural extract of the marine-derived fungus *Aspergillus* sp. SCSIO F063 afforded seven new chlorinated anthraquinones (**139–145**) related to averantin [32.124]. Interestingly, the chlorinated anthraquinones were obtained upon using sea salt-containing potato dextrose broth with the addition of sodium bromide to the broth. In the cytotoxicity (MTT) assay, 6-*O*-methyl-7-chloroaveratin (**140**) displayed antiproliferative activity against three human tumor cell lines, SF-268, MCF-7, and NCI-H460, with IC_{50} values of 7.11, 6.64, and 7.42 μM, respectively [32.124].

Alterporriols K–M (**146–148**), three bianthraquinone derivatives, were reported for the first time from extracts of the endophytic fungus *Alternaria* sp. ZJ9-6B, isolated from the mangrove *Aegiceras corniculatum* collected in the South China Sea [32.125]. Compounds **146–148** were identified as the first alterporriols with a *C*-2-*C*-2 linkage. Alterporriols K and L revealed moderate cytotoxic activities towards MDA-MB-435 and MCF-7 cells with IC_{50} values between 13.1 and 29.1 μM [32.125].

Oxirapentyns B–D (**149–151**) and one known oxirapentyn A (**152**) were isolated from the lipophilic extract of the marine-derived fungus *Isaria felina* KMM4639 [32.126]. The absolute configuration of **149** was established to be 2*R*, 4*S*, 5*S*, 6*S*, 7*R*, 8*S*, 9*S* based on both the modified Mosher's method and X-ray analysis together with NOESY data [32.126]. In the cytotoxicity assay, oxirapentyn A (**152**) exhibited moderate activities against T-47D, SK-Mel-5, and SK-Mel-28 cell lines with IC_{50} values of 25, 19, and 17 μM, respectively [32.126].

Two new polyketides, arthropsadiol C (**153**) and massarilactone H (**154**), together with six known derivatives were isolated from the marine-derived fungus *Phoma herbarum* [32.127]. Some of the isolated compounds revealed moderate neuraminidase inhibitory activity with IC_{50} values between 4.15, and 9.16 μM [32.127].

Sorbiterrin A (**155**, Fig. 32.10), a novel sorbicillinoid featuring an hitherto unprecedented skeleton, was isolated from the marine sediment-derived fungus *Penicillium terrestre* [32.128]. The structure including its absolute configuration was elucidated based on NMR, MS data, and time-dependent density functional theory (TDDFT) CD calculations. Sorbiterrin A showed a moderate acetylcholinesterase (AChE) inhibitory effect with IC_{50} of 70 μM [32.128].

Two novel chlorinated sorbicillinoids, namely chloctanspirones A (**156**) and B (**157**), featuring an unprecedented bicyclo[2.2.2]octane-2-spiro cyclohexane skeleton, together with two precursors terrestrols K and L were isolated from a marine sediment-derived fungus *Penicillium terrestre* [32.129]. In the in vitro cytotoxicity assay, **156** revealed antiproliferative activity against both HL-60 and A-549 cells with IC_{50} values of 9.2 and 39.7 μM, respectively, while **157** showed only activity against HL-60 cells (IC_{50} = 37.8 μM) [32.129].

Five new polyketide derivatives including two benzopyrones (**158** and **159**), one isochroman (**160**), and two anthraquinone-citrinin derivatives (**161** and **162**) were isolated from the sea fan-derived fungus *Penicillium citrinum* PSU-F51 [32.130]. In the antimicrobial activity assay, compound **161** revealed moderate activity against both *S. aureus* and MRSA with equal MIC values of 30.8 μM [32.130].

Seven new phenalenone derivatives (**163–169**) were obtained from the fungus *Coniothyrium cereale* derived from the green alga *Enteromorpha* sp. collected from the Baltic Sea [32.131]. Conioscleroderolide (**167**) showed antimicrobial activity toward *S. aureus* SG 511 with a MIC value of 24 μM, which was suggested to

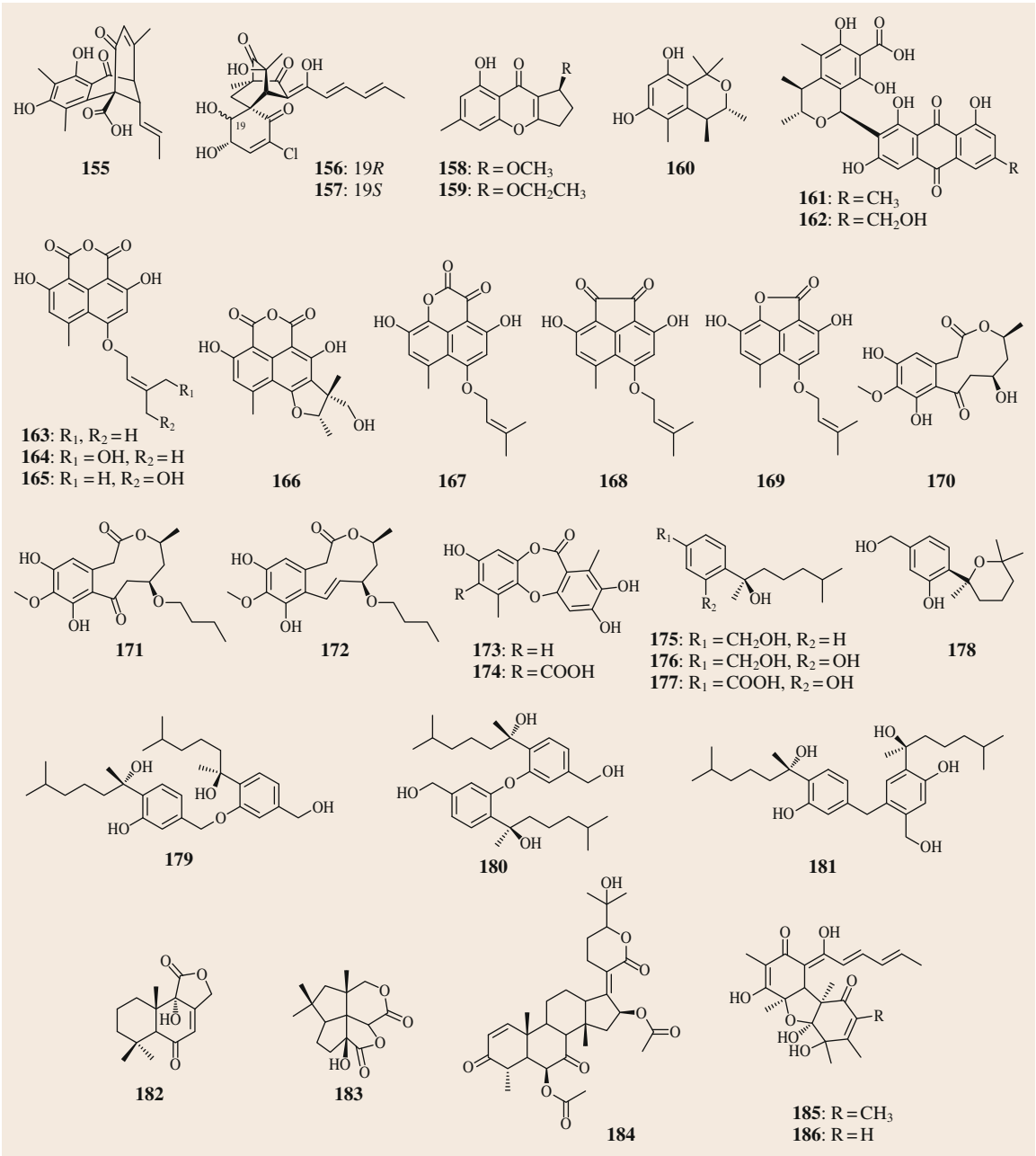

Fig. 32.10 Chemical structures of compounds **155**–**186**

be due to the diketo-lactone ring [32.131]. Moreover, in the agar diffusion assay against *Mycobacterium phlei*, considerable inhibition zones were observed for compounds **164**, **166**, and **169**, whereas **163** and **167** showed potent cytotoxic effects toward human leukocyte elastase (HLE) with IC_{50} values of 7.2 and 13.3 μM, respectively [32.131].

Five new polyketides, xestodecalactones D–F (**170**–**172**), corynesidone C (**173**), and 6-(3′-hydroxybutyl)-7-*O*-methylspinochrome B (**186**), were isolated from

the EtOAc extract of the endophytic fungus *Corynespora cassiicola*, isolated from the inner leaf tissues of the Chinese mangrove medicinal plant *Laguncularia racemosa* [32.132]. Absolute configurations of **170**–**172** were established by TDDFT ECD calculations of their solution conformers, proving that they belong to the (11*S*) series of xestodecalactones, opposite to the (11*R*) configurations of the known xestodecalactones A–C [32.132]. Compounds **170**–**174** were subjected to antibacterial, antiproliferative, antifungal, and antitrypanosomal activities. None of them proved to be active in any of these cellular screens. However, in a biochemical protein kinase activity assay using 16 different human protein kinases, only corynesidone B and 6-(3′-hydroxybutyl)-7-*O*-methylspinochrome B (**174**) exhibited significant activities against several kinases with IC_{50} values in the low micromolar range [32.132].

32.3.4 Terpenes

Aspergiterpenoid A (**175**), (−)-sydonol (**176**), (−)-sydonic acid (**177**), and (−)-5-(hydroxymethyl)-2(2′,6′,6′-trimethyltetrahydro-2*H*-pyran-2-yl)phenol (**178**), four new bisabolane-type sesquiterpenoids, were isolated from the fermentation broth of a marine-derived fungus *Aspergillus* sp. derived from the sponge *Xestospongia testudinaria* [32.133]. All isolated compounds revealed selective antibacterial activity toward eight bacterial strains with MIC values between 1.25 and 20.0 μM [32.133]. In the antifouling assay, only **178** inhibited larval settlement of the barnacle *Balamus amphitrite* at 100 μM concentration [32.133].

Another isolate of the fungus *Aspergillus* sp. was obtained from the same marine sponge *X. testudinaria* collected in the South China Sea and afforded three new phenolic bisabolane sesquiterpenoid dimers, namely disydonols A–C (**179**–**181**) [32.134]. The isolated compounds were evaluated for their cytotoxic activity against HepG-2 and Caski human tumor cell lines using MTT assay. Results revealed that only disydonol A (**179**) and C (**181**) exhibited potent cytotoxic activities toward these two cell lines with IC_{50} values between 6.0 and 26.0 μM, while disydonol B (**180**) was found to be relatively noncytotoxic (IC_{50} > 200 μM) [32.134].

In addition to prenylated indole alkaloids, carneamides A–C (**61**–**63**, Fig. 32.6) and quinazoline derivatives, carnequinazolines A–C (**64**–**66**), a new drimane sesquiterpenoid, 9α-hydroxy-5α-drim-7-ene-6-one-11,12-olide (**182**, Fig. 32.10), was isolated from the fungus *Aspergillus carneus* derived from the brown alga *Laminaria sachalinensis* [32.99]. Structurally, **182** was identified as a drimane sesquiterpenoid featuring a γ-lactone ring and enone group in its molecule and biologically it revealed no significant activity in either cytotoxic or antimicrobial assays [32.99].

Asperaculin A (**183**), an unprecedented sesquiterpenoid featuring a novel [5,5,5,6]fenestrane ring system, was isolated from the fungus *Aspergillus aculeatus* CRI323-04 isolated from the marine sponge *Xestospongia testudinaria* [32.135]. Asperaculin A (**183**) did not exhibit cytotoxic activity (at 50 μg mL^{-1}) against HepG2, MOLT-3, A549, and HuCCA-1 cancer cell lines [32.135].

In addition to the alkaloid fumiquinazoline K (**40**, Fig. 32.6), a new nordammarane triterpenoid, 6β, 16β-diacetoxy-25-hydroxy-3,7-dioxo-29-nordammara-1,17(20)-dien-21,24-lactone (**184**, Fig. 32.10), was isolated from the marine fungus *Aspergillus fumigatus* KMM-4631 associated with the soft coral *Sinularia* sp. [32.89]. Compound **184** did not exhibit activity in either cytotoxic or antimicrobial activity assays [32.89].

A novel antioxidant agent, JBIR-124 (**185**), was purified from the marine sponge-derived fungus *Penicillium citrinum* [32.136]. Structurally, **185** is identical to the known antioxidant agent JBIR-59 (**186**) and it differs only in the presence of additional methyl group at *C*-2. In the radical scavenging (2,2-Diphenyl-1-picryl-hydrazyl, DPPH) assay, JBIR-124 (**185**) and JBIR-59 (**186**) exhibited potent antioxidant activities with IC_{50} values of 30 and 25 μM, respectively, compared to α-tocopherol as a positive control (IC_{50} = 9 μM) [32.136].

Penicisteroids A (**187**, Fig. 32.11) and B (**188**), two polyoxygenated steroids, were reported as new metabolites from the endophytic fungus *Penicillium chrysogenum* QEN-24S isolated from an unidentified species of the red algal genus *Laurencia* [32.137]. Penicisteroid A (**187**) featured tetrahydroxy and *C*-16-acetoxy functionalities and exhibited potent antifungal and selective cytotoxic activities compared to penicisteroid B (**188**) and anicequol (**189**) [32.137]. These differences in bioactivity illustrated that the hydroxyl group at *C*-6 in the B ring is likely to be an important structural feature for the antifungal activity, whereas the moderate activity of **187** and **189** illustrated that one or more substitutions of hydroxyl group at the B ring could contribute to the antifungal activity. Moreover, the hydroxyl group at *C*-6 in the B ring proved to be essential for the cytotoxicity as in penicisteroid A (**187**) compared to **188** and **189** [32.137].

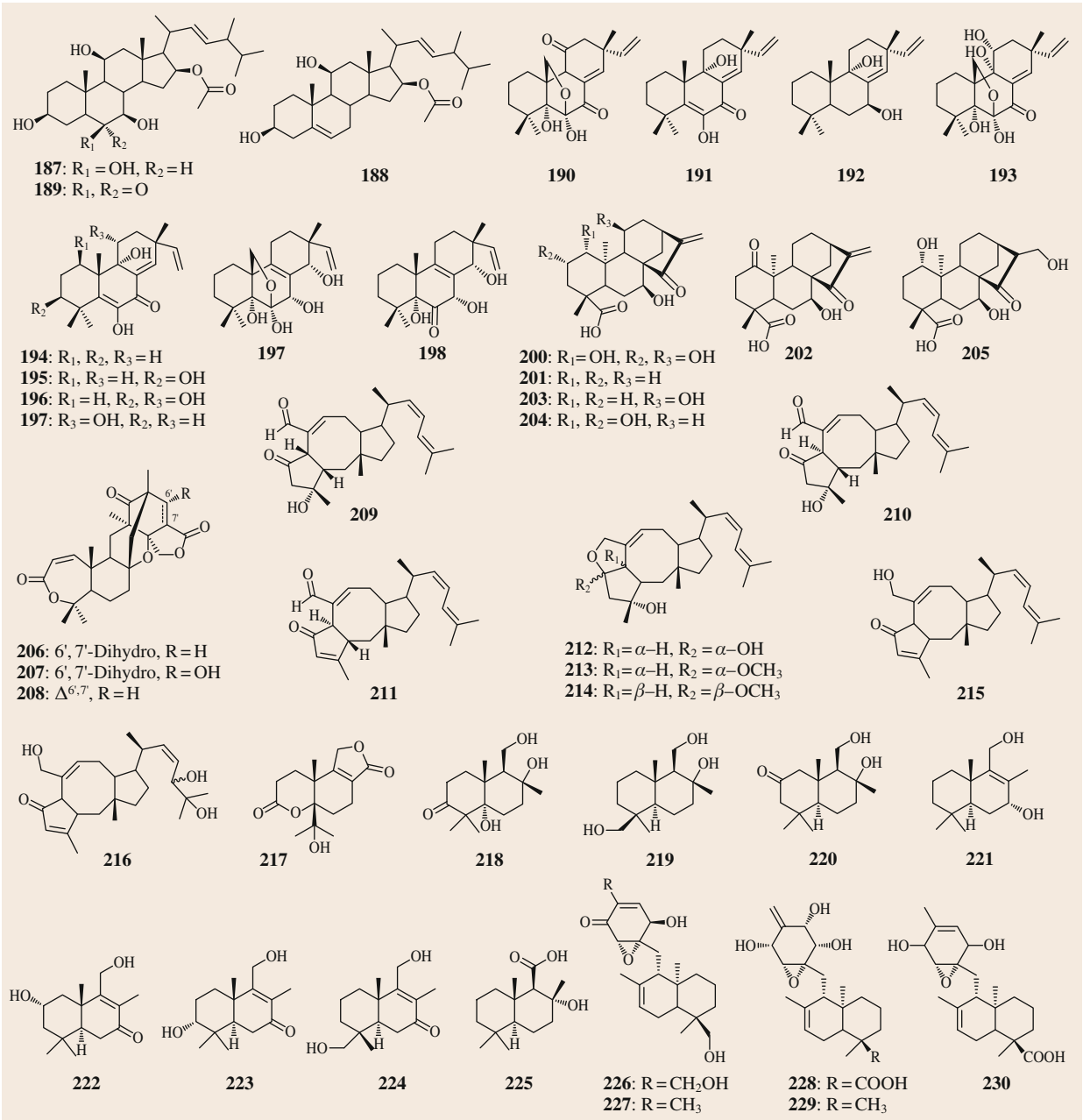

Fig. 32.11 Chemical structures of compounds **187**–**230**

Three new pimarane diterpenes, aspergilones A–C (**190**–**192**), together with the known compound **193**, were isolated from the marine-derived fungus *Epicoccum* sp. HS-1 associated with *Apostichopus japonicus* [32.138]. Absolute configurations of compounds (**190**–**192**) were determined by CD spectroscopy [32.138]. In the cytotoxicity (MTT) assay, compounds **190** and **193** revealed potent activities against KB and KBv200 cell lines with IC_{50} values of 10.0, 6.4 μM, and 11.2 and 18.0 μM, respectively [32.138].

Scopararanes C–G (**194**–**198**), five new oxygenated pimarane diterpenes, were isolated from the culture of a marine sediment-derived fungus *Eutypella scoparia* FS26 obtained from the South China Sea [32.139]. All isolated scopararanes (**194**–**198**) together with the known metabolite, libertellenone A (**199**), were subjected to cytotoxicity (MTT) assay against three human tumor cell lines, namely SF-268 (glioma), MCF-7 (breast), and NCI-H460 (NSCLC). Scopararanes C (**194**) and D (**195**) possessed moderate cytotoxic activities against MCF-7 with IC_{50} values of 35.9 and 25.6 μM, respectively. However, libertellenone A (**199**) displayed variable activities with IC_{50} values between 12.0 and 40.2 μM, whereas the other remaining scopararanes E–G (**196**–**198**) displayed weak cytotoxic activities against the MCF-7 cell line with IC_{50} values of more than 70 μM [32.139]. Conclusively, the structure–activity relationship study of compounds **194**–**199** revealed that the carbonyl group at *C*-7, the olefinic bond between *C*-8 and *C*-14, and the furan ring formed of *C*-20 methyl, *C*-6, *C*-5, and *C*-10, in addition to the number and positions of hydroxyl groups, could be important structural features influencing the cytotoxic activities [32.139].

Geopyxins A–E (**200**–**205**), new *ent*-kaurane diterpenoids, were isolated from endolichenic fungal strains of the genus *Geopyxis* [32.140]. The structures and absolute configurations of geopyxins were established based on their spectroscopic data and conducting the modified Mosher's ester method [32.140]. Methylated and acetylated derivatives of geopyxins were also prepared and all were assessed for their cytotoxic and heat-shock induction activities. Results revealed that geopyxin B (**201**), and the methylated derivatives of all geopyxins except geopyxin E (**215**), exhibited cytotoxic activities in the low micromolar range against five tumor cell lines, whereas the acetylated derivatives were found to activate the heat-shock response at similar concentrations [32.140]. Based on these findings, the preliminary structure-activity motif was deduced to be the electrophilic α,β-unsaturated ketone carbonyl functionality, but this was not sufficient for both cytotoxicity and heat-shock activation [32.140].

Insuetolides A–C (**206**–**208**), three novel meroterpenoids, together with other drimane sesquiterpenes were isolated from the fungus *Aspergillus insuetus* (OY-207), derived from the Mediterranean sponge *Psammocinia* sp. [32.141]. Insuetolides featured a hitherto unprecedented carbon skeleton derived from the cyclization of farnesyl and 3,5-dimethylorsellinic acid. Insuteloide A (**206**) exhibited weak antifungal activity toward *Neurospora crassa* with a MIC value of 140 μM, while insuetolide C (**208**) exhibited mild cytotoxicity towards MOLT-4 human leukemia cells [32.141].

Three new sesterterpenes, namely ophiobolin K (**209**), its 6-epimer (**210**), and 6-*epi*-ophiobolin G (**211**), were isolated from the marine-derived fungus *Emericella variecolor* [32.142]. Ophiobolins (**209**–**211**) inhibited biofilm formation of *Mycobacterium smegmatis* with MICs of 4.1−65 μM; however these compounds did not show antimicrobial activity at the same concentrations [32.142]. Moreover, ophiobolin K (**209**) inhibited also the biofilm formation of *M. bovis* BCG and enables restoring the antimicrobial activity of isoniazid against *M. smegmatis* through inhibiting biofilm formation [32.142].

In addition to aspergillamides A (**44**) and B (**45**, Fig. 32.6), five new ophiobolin-type sesterterpenoids (**212**–**216**, Fig. 32.11) were isolated from the marine-derived fungus *Aspergillus ustus* derived from the marine sponge *Suberites domuncula* [32.91]. None of the isolated sesterterpenoids revealed cytotoxic activity against L5178Y cells at a concentration up to 10 $\mu g\,mL^{-1}$ [32.91].

Diaporol A (**217**), possessing a unique tricyclic lactone ring, together with eight further new drimane sesquiterpenoids, diaporols B–I (**218**–**225**) were purified from the culture medium of the endophytic fungal strain *Diaporthe* sp., derived from the Chinese mangrove *Rhizophora stylosa* [32.143]. The absolute configurations of compounds **217**–**221** were established based on single-crystal X-ray crystallography [32.143]. In the cytotoxicity assay, no compound revealed significant activity against the cell lines tested at concentrations up to 20 μM [32.143].

Three new epoxyphomalins C–E (**228**–**230**), together with the parent congeners **226** and **227**, were isolated from *Paraconiothyrium* sp. fermentation extracts [32.144]. The cytotoxic activities of epoxyphomalins A–E (**226**–**230**) were investigated toward a panel of 36 human tumor cell lines. Results disclosed that **229** possesses selective cytotoxic activities toward PC3M (prostate) and BXF1218L (bladder) cancer cell lines with IC_{50} values of 0.72 and 1.43 μM, respectively [32.144]. Moreover, the inhibitory activity of epoxyphomalin A (**226**) and B (**227**) toward chymotrypsin-, caspase-, and trypsin-like activity of purified 20S proteasomes indicated that their cytotoxic effects are mediated through potent inhibition of the 20S proteasome [32.144].

Three new tetranorlabdane diterpenoids, asperolides A–C (**231**–**233**, Fig. 32.12) and five related

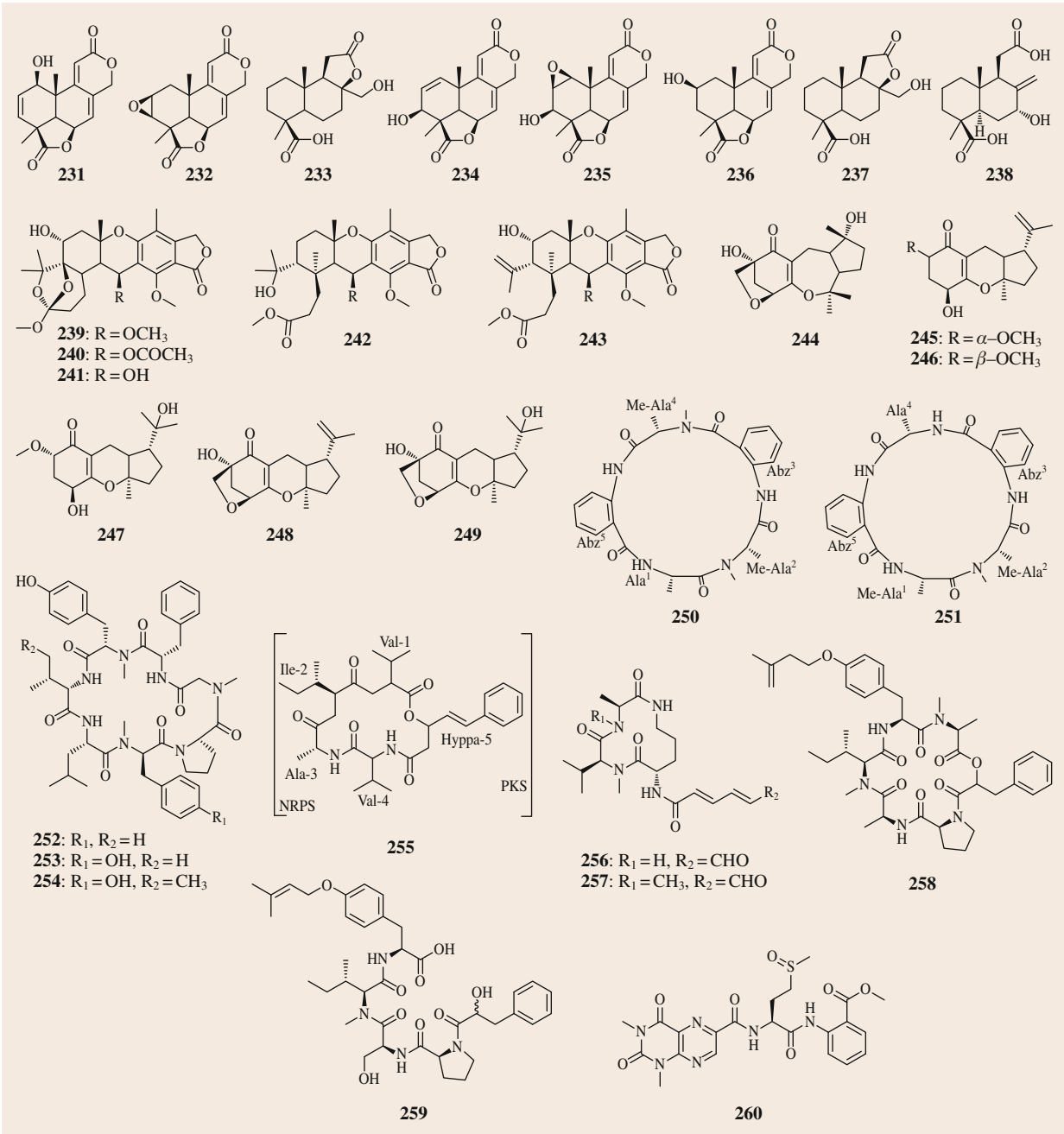

Fig. 32.12 Chemical structures of compounds **231**–**260**

derivatives (**234**–**238**) were obtained from a culture extract of *Aspergillus wentii* EN-48, an endophytic fungus isolated from an unidentified marine brown algal species of the genus *Sargassum* [32.145]. The absolute configurations of asperolide A (**231**) and wentilactone (**236**) were established based on X-ray crystallographic analysis [32.145]. All isolated compounds were assessed for their cytotoxic activities against seven differ-

ent human tumor cell lines, but only wentilactone (**236**) exhibited a potent activity among the compounds tested ($IC_{50} = 17\,\mu M$) [32.145]. However, in the antimicrobial activity assay, compound **234** revealed a considerable antifungal activity against *C. albicans* with a MIC value of 56 μM [32.145].

Chemical investigation of the crude extract of the fungus *Aspergillus* sp. obtained from the Mediterranean sponge *Tethya aurantium* afforded five new meroterpenoids, austalides M–Q (**239**–**243**), together with nine known compounds [32.146]. Absolute configurations of **239** and **242** were established by TDDFT ECD calculations, allowing the assignment of the absolute configuration of analogs **240**, **241**, and **243** [32.146]. All isolated austalides were assessed for their cytotoxic activity toward L5178Y cells using the MTT assay; however, they exhibited only weak or no activity in this assay [32.146].

Four new meroterpenes, guignardones F–I (**244**–**247**), together with two known guignardones A (**248**) and B (**249**), were obtained from the endophytic fungus A1 isolated from the mangrove plant *Scyphiphora hydrophyllacea* [32.147]. Chemical structures and relative configurations of the isolated compounds were determined based on spectroscopic data and single-crystal X-ray crystallography [32.147]. In the antibacterial activity assay against MRSA, only guignardones I (**247**) and B (**249**) exhibited inhibition zones of 9.0 and 8.0 mm at 65 μM, respectively [32.147].

32.3.5 Marine Peptides and Proteins

Versicotides A (**250**) and B (**251**), cyclic pentapeptides containing an L-alanine residue, two anthranthilic acid (2-aminobenzoic acid) residues, and two *N*-methyl-L-alanine residues were isolated from the marine sediment-derived fungus *Aspergillus versicolor* ZLN-60 [32.148]. The chemical structures of versicotides A and B were established on the basis of chemical and spectroscopic analyses. In the cytotoxicity (MTT) assays, neither versicotide A (**250**) nor versicotide B (**251**) exhibited antitumor activity [32.148].

Cordyheptapeptides C–E (**252**–**254**), three new cycloheptapeptides, were purified from the culture extract of the marine sediment-derived fungus *Acremonium persicinum* SCSIO115 [32.149]. The absolute configurations of the amino acid residues were determined by single-crystal X-ray diffraction, Marfey's method, and chiral-phase high performance liquid chromatography (HPLC) analysis. In the antiproliferative activity assay, only cordyheptapeptides C (**252**) and E (**254**) revealed cytotoxic activities toward SF-268, MCF-7, and NCI-H460 tumor cell lines with IC_{50} values ranging between 2.5−12.1 μM [32.149].

EGM-556 (**255**), a cyclodepsipeptide of hybrid biosynthetic origin of both polyketide (PKS) and nonribosomal peptide (NRPS) syntheses, was prepared through implementing the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) to turn on its biosynthesis in the Floridian marine sediment-derived fungus *Microascus* sp. [32.150]. EGM-556 (**255**) represents an interesting example for a fungal metabolite produced through induction of silent biosynthetic genes activate by means of epigenetic manipulation [32.150].

Two new tripeptides, namely pre-sclerotiotide F (**256**) and sclerotiotide F (**257**), were isolated from a marine sediment-derived fungus *Aspergillus insulicola*, along with five known compounds [32.151]. Both compounds **256** and **257** were inactive at 100 μM for in vitro cytotoxicity (MTT) assay against PC3 (prostate), MCF-7 (breast), and RAW (murine macrophage) cancer cell lines [32.151]. Moreover, this pair of tripeptides did not illustrate any inhibitory activity on NF-κB or iNOS pathways when tested in the presence of LPS [32.151].

Pullularins E (**258**) and F (**259**), two new peptides, were isolated from the endophytic fungus *Bionectria ochroleuca*, derived from the mangrove plant *Sonneratia caseolaris* collected from the island of Hainan (China) [32.152]. The chemical structures and absolute configurations of amino acids were determined by HPLC analysis of acid hydrolysates using Marfey's method [32.152]. In the cytotoxicity assay, pullularins E (**258**) and F (**259**) revealed moderate inhibitory activity toward the L5178Y cell line with IC_{50} values of 7.0 and $> 14.0\,\mu M$ [32.152].

Penilumamide (**260**), a novel lumazine peptide, was obtained from the fungal strain *Penicillium* sp. CNL-338, isolated from the red alga *Laurencia* sp. collected in the Bahamas Islands [32.153]. The chemical structure of penilumamide revealed a lumazine system with L-methionine sulfoxide and anthranilic acid ester [32.153]. Penilumamide (**260**) was assessed for its cytotoxicity, antibacterial and antifungal activities, and its influence on the Ca^{2+} levels in neuroendocrine cells (PC12); however, it revealed no activity in any of the aforementioned bioassays [32.153].

32.4 Conclusions

Marine-derived fungi continue to be a prolific source of new and novel bioactive secondary metabolites. Some examples of bioactive marine natural products such as bryostatins obtained from macroorganisms are implicated by endosymbiotic microorganisms as true producers. This notion has provoked marine natural product chemists and pharmacologists to explore marine-associated fungi for interesting chemical entities that can be developed into drug candidates. The principle of implementing marine-derived fungi to produce bioactive secondary metabolites has resolved the main obstacle facing the development of marine-inspired pharmaceuticals, which is that yields are limited, even through aquaculturing efforts. However, industrial fermentation of marine-derived fungi on a large scale will in future enable the mass production of bioactive compounds, which can be optimized to face market needs in the case of the development of a marine-inspired pharmaceutical.

The high productivity of marine-derived fungi has attracted natural product chemists to invest more research effort in this area, aiming to find a new or novel entity of bioactive natural product(s) that can be used as milestones for developing new pharmaceuticals for the treatment of serious ailments.

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33. Marine Dinoflagellate-Associated Human Poisoning

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Seafood poisoning in humans is caused by consumption of toxin-containing seafood that is contaminated with marine dinoflagellates. This has been a concern for many years. There are a number of dinoflagellate species that produce strong neurotoxins, which are often associated with the phenomenon called *red tide*. Outbreaks of red tide are caused by harmful algal blooms (HABs). HABs are not a new phenomenon, with written references dating back to biblical times. The most common type of HAB is referred to as a red tide because the bloom discolors the water, making it appear red. Humans eating seafood from infested areas during dinoflagellate bloom can become poisoned. With respect to the contaminants of toxic dinoflagellates in seafood, there are two main types of poisoning in humans. The terms *fish* and *shellfish* are associated with these illnesses because the toxins are concentrated in fish and shellfish that ingest the harmful dinoflagellates. According to the species of toxigenic dinoflagellates the poisoning syndromes have been given the names paralytic (PSP), diarrhetic (DSP), neurotoxic (NSP), and azaspiracid shellfish poisoning (AZP). Another human illness, ciguatera fish poisoning (CFP) is caused by the ciguatoxins produced by dinoflagellates that attach to surfaces in many coral reef communities [33.1]. Besides these well-known poisoning types, several new poisoning syndromes resulting from newly appearing dinoflagellate toxins, such as yessotoxin (YTX) and palytoxin (PTX), have been reported and characterized recently, and this has increased global public concerns regarding dinoflagellates associated with humans poisoning.

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33.1 Preface

Dinoflagellates are an important group of phytoplankton in marine and freshwater. Dinoflagellates are considered to be protists, under the phylum *Dinoflagellata* (*Dinophyta*) and class *Dinophyceae* [33.2]. They move in marine and fresh water using two tail-like structures called flagella. Dinoflagellates are microalgae, mostly unicellular, eukaryotic algae inhabiting freshwater and marine waters [33.3]. They have the characteristics of both plants and animals. They are known to botanists as *Pyrrophyta*, while to zoologists they are *Mastigophora*. They may be photosynthetic or non-photosynthetic; about half the species fall into each category. The photosynthetic dinoflagellates are second only to diatoms as primary producers in coastal wa-

ters. Dinoflagellates mostly fall prey to small animals, such as small crustaceans and fish larvae, and to other protists, including other dinoflagellates. Dinoflagellates are common and widespread. As they are so plentiful, they cover a large size range and fulfill so many different ecological *roles*. They are a very important source of food for many other marine organisms. Both nontoxic and toxin-producing species of dinoflagellates are present. Algae include *cyanobacteria*, *dinoflagellates*, *diatoms*, *raphidophytes, haptophytes*, and various other species, many of which produce potent toxins. The primary sources of toxins have been identified as dinoflagellates, bacteria, and blue-green algae. About 75–80% of toxic phytoplankton species are dinoflagellates [33.4]. The high abundance blooms of these toxic phytoplankton species are named harmful algal blooms (HABs). HABs causing red discoloration of water are commonly known as *red tides* [33.5, 6]. It is known that certain HAB species can produce potent toxins that impact human health through the consumption of contaminated shellfish, coral reef fish, and finfish, or through water or aerosol exposure [33.7]. Around the world, marine algal toxins cause more than 60 000 poisoning events annually with an associated mortality rate of 1.5%. It is reported that algal toxins result in more than 50 000–500 000 intoxication incidents per year, with an overall mortality rate of 1.5% on a global basis [33.8]. Poisoning caused by the ingestion of toxin-containing foods has been a part of mankind's existence and concern for centuries. Most harmful species have a restricted distribution pattern but some harmful species have a worldwide distribution [33.5, 6].

Dinoflagellates are the major group producing toxins in the marine ecosystem that impact humans [33.7, 9]. Under specific circumstances more than 40 species of dinoflagellates and diatoms are known to produce phycotoxins (marine toxins) [33.10]. Dinoflagellates are responsible for producing a wide variety of toxins that accumulate through marine food chains. The biogenetic process by which the toxins accumulate in fish and shellfish was explained by the food chain. Marine biotoxins, usually produced by phytoplanktons during harmful algal blooms, are some of the most potent toxins in the world and are extremely dangerous. For some toxins, doses at a microgram per kilogram level are more than sufficient to kill a human. When enough toxins are accumulated in fish or shellfish, small amounts of cooked or raw tissue can kill a human. For example, recorded harmful algal blooms have produced enough PSP toxin in mussels that the consumption of one or two small mussels could killed a normal, healthy adult human. While some toxins are very potent, i. e., only small amounts are required to produce illness or to bring death, other less potent toxins may accumulate to such high levels that they can still cause harm. Some dinoflagellates produce compounds that cause death only when they are extracted and injected into laboratory animals, usually mice.

In the past few decades, extensive studies have been devoted to the toxicology and pharmacology of dinoflagellate toxins [33.11]. Dinoflagellate toxins are structurally and functionally diverse, and many show unique biological activities. Phycotoxins can accumulate in various marine species such as fish, crabs, or filter feeding bivalves (shellfish) such as mussels, oysters, scallops, and clams. In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. However, when substantial amounts of contaminated shellfish are consumed by humans, this may cause severe intoxication of the consumer. The human intoxications known so far have been associated with mollusc consumption. The toxic manifestation that develops after ingestion of seafood contaminated with toxic dinoflagellates is best known of all due to their most violent form of poisoning.

This chapter concentrates on the dinoflagellate species found in marine waters, whose toxins can cause human poisoning. The major seafood poisoning syndromes caused by toxins have been identified to come from the dinoflagellates: paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrheic shellfish poisoning (DSP), and ciguatera fish poisoning (CFP). Besides these well-known types of poisoning, several new poisoning syndromes resulting from newly appearing dinoflagellate toxins, such as AZAs, YTX, and PTX have been reported and characterized recently (Table 33.1), and this has increased global public concerns regarding dinoflagellate associated toxins. Dinoflagellate toxins can be functionally categorized as neurotoxins and hepatotoxins, according to their clinical symptoms.

33.2 Historical Perspective

Dinoflagellates have been found in the fossil records for millions of years. As early as 77 A.D. the Greek naturalist, *Pliny*, in a book of notes on his observations wrote, *There are sudden fires in the waters*, most likely referring to what we now recognize as dinoflagellate bioluminescence [33.12]. In 1753, the first modern dinoflagellates were described by Henry Baker as *Animalcules which cause the sparkling light in sea water*, and named by Otto Friedrich Müller in 1773. In 1773 O.F. Muller became the first man to use the newly invented microscope on a dinoflagellate. In the 1830s, the German microscopist Christian Gottfried Ehrenberg examined many water and plankton samples and proposed several dinoflagellate genera that are still used today, including *Peridinium, Prorocentrum*, and *Dinophysis*. Dinoflagellates remained mysterious until F. Stein published his dinoflagellate monographs in 1878–1883. To this day, many genera carry the name originally coined by Stein in his famous monographs. In the last 10 years, the lists of identified dinoflagellates have exploded. The number of identified species has doubled – and our understanding of the vital roles that they play has exploded with it. Harmful algal blooms are not new phenomena; there are written references dating back to biblical times. These blooms have been recorded since the Spanish explorations in the late sixteenth century. The term red tide is now part of common language and is associated with *Gymnodinium breve* blooms that have occurred along Florida's western shelf since the 1950s. There have been restrictions on the consumption of shellfish in North America by European settlers and Red Indians [33.13]. It has been 40 years since the first recorded toxic bloom of *Pyrodinium bahamense* occurred in Papua New Guinea in 1972. Subsequently, this species has increased in importance as a paralytic shellfish poisoning toxin (PSTs) producer in several regions of the world, especially in the Indo-West Pacific [33.14].

Poisoning caused by the ingestion of toxin-containing seafood has been part of mankind's existence and concern for centuries. Ciguatera poisoning has been known since the sixteenth century and appears to be the most commonly occurring risk associated with seafood [33.15]. The recorded outbreaks of ciguatera, both in the New Hebrides aboard the vessel of the Portuguese explorer Pedro Fernandez de Queiros in 1606 and in 1774 aboard Captain Cook's Resolution, were probably two of the earliest vivid accounts of the illness. In 1866 in Cuba, Mr. Poey reported intoxication due to the consumption of a gastropod (*Livona pica*), which was known locally as *cigua*. This way the name ciguatera was introduced. While the name has its origin in the West Indies, the phenomenon was observed and recorded in the Indian and Pacific Oceans as early as the sixteenth century. *Gambierdiscus toxicus*, a dinoflagellate, was discovered in the Gambier Islands (French Polynesia) during an epidemic of ciguatera in 1976. There were many proposed theories on the occurrence of poisonous shellfish during the eighteenth and nineteenth centuries [33.16]. Illness related to *tainted shellfish* was documented in 1880 with dead birds and killed fish in Tampa [33.17].

Intoxication after consumption of shellfish is a syndrome that has been known for centuries, the most common being the PSP. It is caused by a group of toxins, saxitoxins (STXs) and derivates, produced by dinoflagellates of the genera *Alexandrium*, *Gymnodium*, and *Pyrodinium*. First reports of PSP intoxication dates back to 1920 in California, USA when at least six people were reported to have died from it [33.18].

Until the 1970s, PSP toxins had only been detected in European, North American, and Japanese waters. *Walker* was the first to record NSP in 1880 on the West Coast of Florida. It was not until the 1950s that the relationship between red tide events and NSP was better understood and researched [33.19, 20]. The first documented human intoxication caused by DSP toxins was in The Netherlands in 1961 [33.21]. DSP is a relatively recent and new type of shellfish poisoning, which was first discovered in Japan [33.22]. In 1995, the first intoxication due to AZP was reported when at least eight people got ill in The Netherlands after consumption of mussels imported from Ireland. AZP was first reported in The Netherlands but later spread in Europe [33.23]. It is a newly identified marine toxin disease. The first recorded observation on algal blooms in Indian waters was by *Hornell* in 1908 [33.24]. The first record of PSP was made in 1981 from the coastal areas of Tamilnadu, Karnataka, and Maharashtra in India [33.25]. In 1981, PSP resulted in the death of 3 persons and the hospitalization of 85 people due to consumption of affected mussel *Meretrix casta* in Tamil Nadu [33.26]. Until now, the understanding of the ecology and oceanography of these species, and how they affect other organisms, including people, continues to be a challenge for researchers.

33.3 Marine Dinoflagellates

The latest estimates suggest a total of 2294 living dinoflagellate species, which include marine, freshwater, and parasitic dinoflagellates [33.27]. Of the 2000 living species, more than 1700 are marine and about 220 are from freshwater [33.28]. Among them extant species have been described, approximately only half of which are photosynthetic. They include autotrophs, mixotrophs, and grazers. These species possess chloroplasts with chlorophyll a and c pigments. Other species are heterotrophic and can absorb small particles or other cells. The other pigments they contain are carotenes and various xanthines, including peridinin, a unique xanthine pigment specific to this group of organisms. Photosynthesizing dinoflagellates accumulate starch as a food reserve. The remaining obligate heterotrophs can have a diverse range of nutritional lifestyles, including saprophytic, parasitic, phagotrophic, or carnivorous forms [33.27, 28]. This dual animal–plant behavior within the group has led to their being classified under both the zoological and botanical nomenclature, but they are now generally classified under botanical rules. They can have complex life cycles but, for most species, the major life stage is a free-living flagellated form. They form an important component of aquatic phytoplankton, but there are also many benthic species [33.29].

Several aspects of the behavior, physiology, and ecology of dinoflagellates are notable and can be highlighted, such as: swimming behavior, bioluminescence, heterotrophy, symbiosis, and toxicity.

33.3.1 Swimming Behavior

Free-living dinoflagellates live in most wet environments, but in the open ocean they are extremely numerous, especially in more tropical waters and when concentrations of nutrients are high. As motile cells, dinoflagellates are capable of directed swimming behavior in response to a variety of parameters. These include chemotaxis, phototaxis, and geotaxis, for which movement is controlled by chemical stimuli, light, or gravity, respectively. It has long been observed that many dinoflagellates do not move randomly through the water column but instead aggregate at specific depths that can vary with the time of day. This vertical migration has proven to be a highly complex process that varies between species and with environmental or nutritional conditions [33.30].

33.3.2 Heterotrophy

About half of the extant dinoflagellates lack a plastid or pigments to carry out photosynthesis [33.31]. Most naked heterotrophic dinoflagellates have flexible cell walls that allow them to engulf living cells and particles (phagotrophy), which can then be seen inside the colorless dinoflagellate. The phagotrophic dinoflagellate *Gyrodinium fusus* has been shown to be an important grazer of *Azadinium* cf. *spinosum* during the first bloom, suggesting that its grazing activity could have acted as a modeling factor in population levels of its prey. There are also many parasitic forms, but in most cases they are very difficult to recognize as dinoflagellates. Some species will parasitize other organisms, such as zooplankton and other protists, filamentous algae, or fish. Roughly 5% are parasitic on aquatic organisms.

33.3.3 Symbiosis

Some dinoflagellates (zooxanthellae) are capable of forming symbioses with a phylogenetically wide range of marine protists and invertebrate animals. Energy generated by zooxanthellae through photosynthesis is taken up by the host (a reef coral). The energy from the zooxanthellae can amount to more than the daily requirement of the host. It is thought that the evolution of the association between dinoflagellates and corals may have been instrumental in the development of the coral reef ecosystem and might be essential for its maintenance. Within the dinoflagellate lineage, at least seven genera from four orders are found in symbiotic associations [33.32]. The hosts in dinoflagellate associations with other organisms include foraminifera, radiolarians, flatworms, anemones, jellyfish, and even bivalve mollusks. Some dinoflagellates may themselves possess endosymbionts.

33.3.4 Bioluminescence

The spectacular display of blue sparkling light seen as waves break on beaches or as a boat passes through the water in the night is called bioluminescence. The Dinoflagellata are sometimes called Pyrrophyta, meaning *fire plants*. This is because some species are capable of bioluminescence, in which chemicals made by the organism produce light in a chemical reaction. The phenomenon was first noted in the genus *Noctiluca*,

which resulted in its name (*night light*), but the reaction is now known to occur in several marine species. Many organisms in the ocean emit such light, although dinoflagellates are the only photosynthetic organisms capable of this behavior [33.33]. It is widely accepted that dinoflagellates account for much of the planktonic bioluminescence in the ocean [33.34]. The dinoflagellates begin to glow, which is often seen in the waves of the ocean on a moonless night, but will brighten considerably when disturbed, such as in the wake of a ship.

33.3.5 Toxicity

As they are biochemically diverse, they vary in photosynthetic pigments and toxin production ability. The taxonomy of harmful dinoflagellates was reviewed by *Taylor* et al. [33.35]. A number of dinoflagellate species are known to produce potent neurotoxins, which are often associated with the phenomena commonly called red tide. These outbreaks are now called HABs. Documentation of HABs has expanded greatly over the last few decades, and at present, nearly every country with marine waters is known to be affected by these blooms [33.36]. HAB toxins can affect humans, other mammals, seabirds, fish and many other animals and organisms. One major category of impact occurs when toxic species are filtered from the water as food by shellfish, which then accumulate the algal toxins to levels that can be lethal to humans or other consumers.

33.4 Algal Blooms and Red Tide Dinoflagellates

Of the 5000 marine algal species, 300 have a high proliferation rate, resulting in high dense algal clouds called blooms. Algal bloom is due to species that quite innocently cause coloring of seawater. It is their photosynthetic pigments that can tint the water during blooms. These blooms may discolor the sea to various shades of brown, orange, purple, yellow, or red. Red tide gets its name from the phenomenon by which pigmented phytoplankton reproduce to such a high concentration that it turns the water red or dark brown. This can be misleading because nontoxic algae can also cause this to happen. With the hope of clarifying this, the scientific community has uses the term HAB for toxic red tides. Dinoflagellate blooms (red tides) are regular seasonal phenomena occurring in some part of the world. Blooms of toxic algae are known as HABs. The most common type of HAB is referred to as a red Tide because the bloom discolors the water, making it appear red. However, HABs may also be yellow, orange, brown, green, white, or pink, depending on which one of the three primary types of phytoplankton is responsible for the problem; dinoflagellate, diatoms, or blue-green algae.

Dinoflagellates are perhaps best known as causing HABs. Although the dinoflagellates responsible for most red tides are comparatively simple organisms, some have the ability to synthesize potent toxins as by-products of metabolism. Among the most effective poisons known, these toxins may affect nearby marine life if ingested, or may even indirectly poison humans through the food chain. The detrimental effects of HAB can range from cell and tissue damage to organism mortality, and can be caused by a number of mechanisms, including toxin production, predation, particle irritation, induced starvation, and localized anoxic conditions. As a result, a bloom may affect many living organisms of the coastal ecosystem, from zooplankton to fish larvae to people. Other harmful algal blooms produce toxins with no identifiable effects on humans but devastating impacts on coastal living resources. For example, the flagellate *Heterosigma akashiwo* is thought to produce an ichthyotoxin that kills fish [33.37], resulting in significant threats to penned fish in mariculture operations. Dinoflagellate blooms can also cause massive killing of fish and shellfish, often causing significant economic damage.

At least 90 species of marine microalgae are known to produce toxins. Of these species, 70 are dinoflagellates [33.38]. The effects of HABs on aquatic organisms have been reviewed by [33.39]. Red tides occur in all marine regions with a temperate or warmer climate. Sometimes the dinoflagellates involved with red tides synthesize toxic chemicals. *Red tide* is due to certain species of dinoflagellates that contain neurotoxins. Toxic dinoflagellates (Pyrrhophyta, Desmophyceae, and Dinophyceae), for example, numbered 22 known species in 1984 [33.40], but increased to 59 species a little more than a decade later [33.41]. Blooms of dinoflagellates produce red tides, which injure marine life. Dinoflagellates' algal blooms create red tides, which can release strong neurotoxins, such as STX, which can be ingested by shellfish and passed on to humans who eat the infected shellfish. These toxins have been responsible for incidents of wide-scale death

of sea-life and are increasingly responsible for human intoxication.

However, no color is visible in other harmful species, such as the chlorophyll-free dinoflagellate *Pfiesteria piscicida*, several *Dinophysis* species, and a benthic one (*Gambierdiscus*) that grow on the surfaces of larger macroalgae in tropical waters. Both *Pfiesteria* and *Dinophysis* also impart toxicity at very low densities, generally less than 1000 cells L^{-1} [33.42, 43].

Unfortunately, a small number of species produce potent neurotoxins that can be transferred through the food web where they affect and even kill higher forms of life such as zooplankton, shellfish, fish, birds, marine mammals, and even humans that feed either directly or indirectly on them. Algal bloom is due to toxin-producing species, so that the poison is concentrated via the food chain and may reach humans.

Marine toxins can also affect local ecosystems by poisoning animals. Health effects range from cell damage to organism mortality through such mechanisms as toxin production and localized conditions of low oxygen. Under some conditions, so much oxygen is consumed to support the decomposition of dead algal biomass that anoxic conditions develop. This can cause severe stress or mortality in a wide range of organisms that are intolerant to low-oxygen conditions.

Genera that are commonly associated with poisonous red tides are *Alexandrium*, *Dinophysis*, and *Ptychodiscus*. The algal poisons can accumulate in marine organisms that feed by filtering large volumes of water, for example, shellfish such as clams, oysters, and mussels. If these shellfish are collected while they are significantly contaminated by red tide toxins, they can poison the human beings who eat them. Some toxins, such as that from *Ptychodiscus brevis*, the organism that causes Florida red tides, are airborne and can cause throat and nose irritations.

33.5 Toxigenic Dinoflagellate-Associated Human Poisoning

The possible presence of natural toxins in fish and shellfish has been known for a long time. The most important marine phycotoxins are shellfish toxins and finfish-ciguatoxins. Most of these toxins are produced by species of naturally occurring marine dinoflagellates (phytoplankton). A proportion of the toxic phytoplankton has a reddish-brown pigmentation, giving rise to calling algal blooms red tides. Visible red tides may contain from 20 000 to > 50 000 algal cells mL^{-1} concentrations, and as low as 200 cells mL^{-1} may produce toxic shellfish. Some dinoflagellates produce toxins that become concentrated in the bodies of organisms higher in the food chain, such as fish and shellfish. Marine animals such as oysters, crustacea, and different types of fish may eat the toxic dinoflagellates storing the toxins. The toxins are accumulated in the digestive gland of the shellfish (hepatopancreas) and do not affect the shellfish themselves. They often accumulate in shellfish or fish, and when these are eaten by humans they cause diseases. People are exposed principally to the toxins produced by harmful dinoflagellates through the consumption of contaminated seafood products. During dinoflagellate blooms humans eating seafood from infested areas can be poisoned. This can lead to serious poisoning.

Based on contaminants of toxic dinoflagellates in seafood there are two main types of human poisoning. The terms *fish* and *shellfish* are associated with these illnesses because the toxins concentrate in the fish and shellfish that ingest the harmful dinoflagellates. Fish and shellfish poisoning occur worldwide. According to the species of toxigenic dinoflagellates the poisoning syndromes have been given the names paralytic, diarrhetic, neurotoxic, and azaspiracid shellfish poisoning. A fifth human illness, CFP is caused by ciguatoxins produced by dinoflagellates that attach to surfaces in many coral reef communities [33.1]. Broadly these can be classified as shown below (see also Table 33.1).

Those caused by eating fish, namely: CFP. Those caused by eating shellfish, until now, seven groups of syndromes have been distinguished, namely:

1. Paralytic shellfish poisoning (PSP)
2. Neurotoxic shellfish poisoning (NSP)
3. Diarrheic shellfish poisoning (DSP)
4. Azaspiracid shellfish poisoning (AZP)
5. Palytoxins poisoning (PTX)
6. Yessotoxins poisoning (YTX).

Amnestic shellfish poisoning (ASP) is also a related poisoning affecting humans, which was recently discovered but will not be discussed in this chapter because it is caused by marine red algae and is tracked to certain benthic diatoms (Bacillariophyceae) of the genus *Chondria* and diatoms of the *Pseudonitzschia f. multiseries* but not by a dinoflagellate [33.44–47].

Table 33.1 Marine dinoflagellates – biotoxins and the associated poisoning in humans [33.35, 48]

Poisoning in humans	Causative dinoflagellates (examples)	Usual transvector(s)	Distribution	Main toxins	Action target	Major references
Paralytic shellfish poisoning (PSP)	*Alexandrium acatenella, A. andersonii, A. catenella, A. cohorticula, A. fundyense, A. fraterculus, A. leei, A. minutum, A. monilatum, A. tamarense, A. ostenfeldii, A. pseudogonyaulax, A. tamiyavanichii, Gymnodinium catenatum, Lingulodinium polyedrum, Pyrodinium bahamense var. compressum, Cochlodinium catenatum, C. polykrikoides*	Clams, mussels, oysters, cockles, gastropods, scallops, whelks, lobsters, copepods, crabs, fish	Temperate areas worldwide Cosmopolitan (Northwest, West, Northeast, Florida)	Saxitoxins (STXs), Gonyautoxins	Voltage-gated sodium channel 1	[33.49–52]
Diarrhetic shellfish poisoning (DSP)	*Dinophysis acuta, D. acuminata, D. caudata, D. fortii,D. norvegica, D. mitra, D. rotundata, D. sacculus, D. fortii, D. miles, D. norvegica, D. tripos, Prorocentrum. arenarium, P. balticum, P. belizeanum, P. concavum, P. faustiae, P. hoffmannianum, P. lima, P. maculosum, P. mexicanum, P. micans, P. minimum, P. ruetzlerianum, P. arenarium, P. belizeanum, P. cassubicum, P. concavum, P. faustiae, P. hoffmannianum, P. maculosum, P. reticulatum, Coolia sp. Protoperidium oceanicum, P. pellucidum, Phalacroma rotundatum*	Mussels, scallops, clams, Gastropods	Europe, Japan, Cold and warm-temperate Atlantic, Pacific, Indo-Pacific (Canada, Northeast?)	Okadaic acid, dinophysis toxins (DTXs), yessotoxins (YTXs) and pectenotoxins (PTXs)	Inhibitors of protein phosphatases 1A and 2A. They are possibly carcinogenic	[33.53]
Ciguatera fish poisoning (CFP)	*Gambierdiscus toxicus, Prorocentrum micans, P. lima, P. concavum, P. hoffmannianum, P. mexicanum, P. rhathytum, Gymnodinium sanguineum, Gonyaulax polyedra, G. polygramma Ostreopsis heptagona, O. lenticularis, O. mascarenensis, O. ovata, O. siamensis Amphidinium sp., Coolia monotis*	Fish, snail, shrimps, crabs	Tropical coral reefs (Southeast, Hawaii, Puerto Rico)	Ciguatoxins (CTXs), maitotoxins (MTXs), palytoxin, gambierol	Voltage-gated sodium channel 5 Voltage-gated calcium channel	[33.53–55]
Neurotoxic shellfish poisoning (NSP)	*Karenia brevis, K. papilonacea, K. sellformis, K. bicuneiformis, K. Concordia, Procentrum borbonicum?, Gymnodinium breve, G. catenatum, G. mikimotoi, G. pulchellum, G. veneficum Gyrodinium galatheanum*	Oyster, clams, mussels, cockles, whelks	Gulf of Mexico, southern USA coast, New Zealand subtropical/warm temperate Gulf Coast, eastern Florida, North Carolina	Brevetoxins (PbTxs)	Voltage-gated sodium channel 5	[33.56, 57]

33.5.1 Fish Poisoning in Humans

Fish constitute almost half the number of vertebrate on earth, and approximately 22 000 species of fish are contained in some 50 orders and 445 families. People love to eat marine and freshwater fish. However, like other organisms, some tropical reef fish also ingest some toxic dinoflagellates. The toxins become progressively concentrated as they move up the food chain from small fish to large fish that eat them, and reach particularly high concentrations in large predatory tropical reef fish. Barracuda are commonly associated with ciguatoxin poisoning, but eating grouper, sea bass, snapper, mullet, and a number of other fish that live in tropi-

Table 33.1 (continued)

Poisoning in humans	Causative dinoflagellates (examples)	Usual transvector(s)	Distribution	Main toxins	Action target	Major references
Azaspiracid shellfish poisoning (AZP)	*Protoperidinium crassipes*	Mussels, oysters	Europe, Ireland, UK, Norway, France, Portugal, Northern Africa (Morocco), South America (Chile) and the USA	Azaspiracids (AZAs)	Voltage-gated calcium channel	[33.58–62]
Palytoxin poisoning	*Ostreopsis siamensis*	Crabs, sea urchin	Brazil, Mediterranean Sea (Italy, Spain, Greece, and France)	Palytoxins (PTX)	Na^+-K^+ ATPase	[33.63–66]
Yessotoxin poisoning	*Protoceratium reticulatum*, *Lingulodinium polyedrum* and *Gonyaulax spinifera*	Scallops, mussels	Italy, Norway, and Portugal	Yessotoxins (YTXs)	Voltage-gated calcium/ sodium channel?	[33.67, 68]

cal oceans have also caused the disease. People may be poisoned and have a specific syndrome by eating these contaminated fish: CFP.

Ciguatera Fish Poisoning (CFP)

Ciguatera fish poisoning (CFP) is caused by eating contaminated tropical reef fish. CFP is considered to be the most common seafood-borne poisoning related to the consumption of finfish. It is most commonly associated with larger reef-dwelling fish. As the concentration of toxin is highest in the viscera, the consumption of whole, ungutted fish generally has the most severe consequences. Ciguatoxic fish are not recognizable as such by means of external features. Contaminated fish smell and taste normal. Frying, boiling, deep-freezing, or smoking the fish do not decontaminate it.

Source

More than 400 fish species have been described that may contain ciguatoxin. They belong to the following families: Murenidae (Moray eels), Sphyraenidae (barracudas), Lutjanida (snappers), Serranidae (broupers), Carangidae (jacks), Acanthuridae (surgeon fish), Balistidae (trigger fish), Scaridae (parrot fish) and to a lesser extent: Belonidae (needlefish), Holocentridae (soldierfish and squirrelfish), Labridae (wrasses), Mugilidae (mullets), Mullidae (surmullets, goatfish) and Scombridae (mackerels and their allies) [33.69].

Prevalence

The last estimate of the annual global incidence of CFP range from 25 000 to 500 000. The incidence in travelers to highly endemic areas has been estimated as high as 3%. Due to tourism and the increased export of tropical fish throughout the world, more and more doctors outside endemic regions are encountering cases of ciguatera poisoning. Regular outbreaks are reported in the Caribbean, including Florida, the South Pacific Islands, and Hawaii in the North Pacific, as well as the northeast coast of Australia (Queensland, Northern Territory). In the Pacific, the severity and incidence of poisoning increase from west to east. Ciguatera outbreaks are difficult to predict, and cases of poisoning often occur in the form of an epidemic. There are probably some 10 000–50 000 cases each year, but estimates show wide variation. The average incidence in endemic regions varies from 5–50 cases per 100 000 inhabitants per year, but in some years this can reach as high as 500/100 000 in the South Pacific.

Range

Ciguatera is widespread in tropical and subtropical waters, usually between the latitudes of 35° N and 35° S; it is particularly common in the Pacific and Indian Oceans and the Caribbean Sea.

Causative Dinoflagellates and Poisonous Ingredients (Toxins)

The main causative marine dinoflagellate is *Gambierdiscus toxicus*, which originally produced maitotoxins (MTXs), the lipophilic precursors of ciguatoxin [33.70]. MTX was first isolated in 1971 from surgeon fish (*Ctenochaetus striatus*), known as *Maito* in Tahiti. Three forms of MTX, MTX-1, MTX-2, and

MTX-3 have been identified from *Gambierdiscus toxicus* [33.71]. MTX has been proved to be the most potent toxin identified on a weight basis: the lethal dose 50 (LD_{50}) of MTX in mice is less than 0.2 μg kg^{-1} (intraperitoneally) and it is at least fivefold more toxic than tetrodotoxin. These precursors are biotransformed to ciguatoxins by herbivorous fish and invertebrates grazing on *Gambierdiscus toxicus* and then accumulated in higher trophic levels [33.72]. In ciguatera poisoning, the poisonous ingredient is ciguatoxin.

Ciguatoxin was originally isolated in Hawaii in 1967. The toxins form a family of very closely related structures with a molecular weight of 941–1117 Da. There are a number of variants, depending on whether or not certain chemical groups (−H, $-CH_3$, etc.) are present. The ciguatoxins are a family of heat-stable, lipid-soluble, highly oxygenated, cyclic polyether molecules with a structural framework reminiscent of the brevetoxins [33.73–75], and more than 20 toxins may be involved in CFP [33.76]. Scaritoxin was isolated in 1976 in Tahiti from a parrotfish (*Scarus gibbus*). It is a metabolite of ciguatoxin, but dinoflagellates are also said to be able to produce the poison in in vitro culture.

Action Mechanism

Ciguatoxin and the closely-related MTX both cause symptoms by interfering with ion channels on cell membranes. Ciguatoxin opens sodium channels and MTX opens calcium channels, disrupting the signaling between nerves and muscles.

Lethal Dose

In mice, ciguatoxin is lethal at 0.45 μg kg^{-1} ip, and MTX at a dose of 0.15 μg kg^{-1} ip. Oral intake of as little as 0.1 μg ciguatoxin can cause illness in the human adult. The pathogenic dose for humans is 23–230 μg.

Regulatory Tolerances by the Food and Drug Administration (FDA)

The FDA safety levels of CFP – 0.01 ppb P-CTX-1 equivalents for Pacific ciguatoxin and 0.1 ppb C-CTX-1 equivalent for Caribbean ciguatoxin.

Incubation Period

Ciguatoxin usually causes symptoms within a few minutes to 30 h after consumption of contaminated fish, and occasionally it may take up to 6 h.

Symptoms

Since more than 20 toxins are involved they produce more than 175 ciguateric symptoms, classified into four categories: gastrointestinal, neurological, cardiovascular, and general symptoms [33.77, 78]. It should be emphasized that the symptoms of ciguatera vary in different oceans: in the Pacific Ocean neurological symptoms predominate, while in the Caribbean Sea gastrointestinal symptoms dominate due to the difference in toxin composition. The onset of GI and neurologic symptoms after the consumption of fish are the hallmarks of ciguatera poisoning. Symptoms are usually evident within 2–6 h after ingestion and usually resolve within 24 h, although a late-presenting and extended course is not uncommon. Gastrointestinal (GI) symptoms generally consist of diaphoresis, abdominal cramps, nausea, vomiting, profuse watery diarrhea, and dysuria. GI symptoms are usually reported to occur prior to neurological symptoms. Interesting, however, is that the predominance of GI or neurologic symptoms seems to vary according to regions, with GI-predominant illness seen in the Caribbean, while neurologic symptoms predominate in the Indo-Pacific region.

Neurologic symptoms tend to occur later (up to 72 h) and may persist for months. These are predominantly paresthesias, but a myriad of other sometimes bizarre neurologic symptoms may also be observed, including pruritus; the sensation of loose painful teeth; tingling in the lips, tongue, throat, and perioral tissues; metallic taste; reversal of temperature sensation; and the sensation of heat in the superficial tissues of the extremities with concomitant sensation of cold in the deeper tissues. Further neurologic symptoms can include vertigo, ataxia, visual changes, and seizures. In more severe poisoning, bradycardia with hypotension and cardiovascular collapse may occur.

Common, nonspecific symptoms include nausea, vomiting, diarrhea, cramps, excessive sweating, headache, and muscle aches. The sensation of burning or *pins-and-needles*, weakness, itching, and dizziness can occur. Patients may experience reversal of temperature sensation in their mouth (hot surfaces feeling cold and cold surfaces hot), unusual taste sensations, nightmares, or hallucinations. Symptoms of exposure include eye and respiratory irritation, headache, and gastrointestinal complaints, skin irritation, and difficulties with learning and memory [33.79].

Mortality

Ciguatera poisoning is rarely fatal. The overall death rate from ciguatera poisoning is approximately 0.1%, but varies according to the toxin dose and availability of medical care to deal with complications. Death from CFP is rare (< 1% worldwide).

Treatment

Treatment is primarily supportive. Intravenous mannitol was thought to be the most promising of the pharmacotherapy treatments; however, it has experienced a relative decline in acceptance after a randomized, double-blind trial in 2002 failed to confirm its efficacy [33.79–81]. However, with recent case reports supporting its use and a better understanding of ciguatera poisoning, many experts in the field believe that the use of mannitol for the treatment of acute ciguatera poisoning arguably deserves revisiting [33.79, 82].

33.5.2 Human Shellfish Poisoning

Toxic illness caused by shellfish has been recognized for several hundred years. Native Americans are known to have warned early settlers to avoid shellfish during the summer months, as red tide is favored by warmer weather. Molluscan shellfish are filter feeders and continually pump water through their gills where particulate matter is removed and ingested. Mussels ingest food particles of any type of 2–90 mm in size with a rate of ingestion dependent on water temperature and environment. Optimally, they can filter 2.5 L h^{-1}, extracting 98% of the available algae. Consequently, any toxin associated with the phytoplankton ingested can rapidly accumulate and hence become concentrated in the bivalve mollusk. Bivalve mollusks such as clams, scallops, oysters, and mussels that filter feed on toxic algae can accumulate large amounts of toxins in their tissues, posing a toxic threat to humans who make shellfish a part of their diet. Shellfish are usually unaffected by toxic algae themselves but can accumulate toxins in their tissue to levels that can be lethal to humans. High concentrations of marine biotoxins in these animals can cause illness amongst people who eat contaminated shellfish. Shellfish that have fed on toxic dinoflagellates retain the toxin for varying periods of time depending on the shellfish. Some clear the toxin very quickly and are only toxic during the actual bloom. Others retain the toxin for a long time, even years. The most serious threat posed to human health by marine toxins is through shellfish contamination. The consumption of these toxic shellfish by humans causes illness, with symptoms ranging from mild diarrhoea and vomiting to memory loss, paralysis, and death. Illnesses that result from marine biotoxins are not related to the manner in which food is handled or prepared, but the exposure that shellfish has to microscopic organisms while living in the marine environment.

Several forms of shellfish poisoning may occur after ingesting filter-feeding bivalve mollusks such as mussels, oysters, clams, scallops, and cockles, or crustaceans such as crabs and lobsters that accumulate potent marine toxins produced by single-celled microscopic marine algae (dinoflagellates or diatoms) associated with algal blooms or red tides. The most common medical problems resulting from marine biotoxins, are the human shellfish poisoning syndromes PSP, NSP, DSP, and AZP. These poisoning syndromes are distinctly clear and the cases can be serious (Table 33.2).

Paralytic Shellfish Poisoning (PSP)

PSP of humans is caused by the consumption of shellfish that have been contaminated with STXs. *Blooms* of neurotoxic dinoflagellates from several genera result in outbreaks of PSP, probably the most widespread of the poisoning syndromes [33.83]. PSP is not only the most common form of shellfish poisoning, but it is also the deadliest, with a mortality rate of 6% worldwide (higher in developing countries) [33.84–86]. PSP usually occurs in outbreaks and is observed most commonly in recreational diggers.

Source

Filter-feeding bivalve molluscs (e.g., oysters, mussels, clams) nonbivalve shellfish (whelks, moon snails, and dogwinkles) or tomalley of crustaceans (crabs, lobster).

Prevalence

Clinically documented PSP outbreaks with several dozen to around 200 patients have been described in Western Europe, Taiwan, and Guatemala. Cases have also been described in Massachusetts and Alaska. Between 1927 and 1985, 505 PSP cases were recorded in California, of which 32 ended fatally. In contrast, in 10 PSP epidemics in the USA between 1971 and 1977, there were no recorded fatalities. 2/116 died in an epidemic in Taiwan. In September 1997, an outbreak of PSP was reported in Vizhinjam, Kerala, resulting in the death of 7 persons and hospitalization of over 500, following consumption of the mussel, *Perna indica* [33.87]. Recently, in September 2004, an unusual nauseating smell emanating from the coastal waters was recorded in Kollam to Vizhinjam on the southwest coast of India. More than 200 persons, especially children, complained of nausea and breathlessness for a short duration due to the smell.

Table 33.2 Summary of clinical entities of human poisoning by marine dinoflagellates

Entities	CFP	PSP	NSP	DSP	ASP	PTXs	YTXs
Incubation period	24 h	30′–3 h	5′–3 h	30′–2 h	15′–38 h	In minutes to 2–4 d	Unknown
Early Symptoms	Abdominal cramps, nausea, vomiting, profuse diarrhea, dysuria	Nausea, vomiting, tingling mouth, lips, throat. Floating feeling	Nausea, vomiting, diarrhoea, abdominal pain	Nausea, vomiting, diarrhoea, abdominal pain	Nausea, vomiting, abdominal pain	Muscle cramps include fever inaction	Unknown
Mild	Paresthesias, pruritus, painful teeth, vertigo, ataxia, visual change, seizures	Paresthesia ++ Muscular weakness, ataxia, headache	Paresthesia, vertigo, ataxia, headache	Severe diarrhoea, dehydration	Diarrhoea, headache, memory problems, mutism	Haemolysis, rhabdomyolysis, ataxia, drowsiness	Unknown
Severe	Bradycardia with hypotension and cardiovascular collapse	Dysphagia, dysarthria, diplopia, paralysis	Bradycardia, convulsions, mydriasis, no paralysis	Shock	Hemiparesis, ophthalmoplegia, convulsions, hypotension, cardiac arrhythmias	Weakness of limbs followed by death	Unknown
Foremost	GI and neurologic	Paralysis	Paresthesia	Severe diarrhoea	Progressing paralysis	Respiratory failure	Unknown
Duration	1–4 weeks	2–5 d	2–3 d	3 d	1–100 d Sometimes permanent memory problems	1–2 weeks	Unknown
Mortality	0.1%	6% average	0%	0%	4%	0.1%?	Unknown
Treatment	Supportive care and treatment with mannitol	Supportive care and mechanical ventilation	Supportive care	Supportive care	Supportive care	Supportive care	Supportive care

Range

Until 1970, cases of PSP had only been reported in the Northern Hemisphere, but by 1990, PSP had spread to southern Africa, Australia, New Zealand, India, Thailand, Brunei, Sabah, the Philippines, and Papua New Guinea. Since 1990, PSP has continued to spread. PSP is the most widespread shellfish poisoning and outbreaks are occurring worldwide.

Causative Dinoflagellates and Poisonous Ingredients (Toxins)

Members of the three dinoflagellate genera *Alexandrium, Gymnodinium*, and *Pyrodinium* have been reported to be the major sources of PSP associated toxins [33.49]. STX is produced by *Alexandrium (Gonyaulax) tamarense*, *Alexandrium catenella*, *Pyrodinium bahamense*, *Gymnodinium catenatum*, and *Cochlodinium catenatum*. PSTs are the causative agents of paralytic shellfish poisoning (PSP) and are mostly associated with marine dinoflagellates (eukaryotes) and freshwater cyanobacteria. PSTs are produced in varying proportions by different dinoflagellate species and even by different isolates within a species. STX and its 57 analogs are a broad group of natural neurotoxic alkaloids, commonly known as PST. PSP is the result of ingestion of saxitoxin, a purine alkaloid. Saxitoxin takes its name from the Alaskan butter clam *Saxidomus giganteus*. STX is the poisonous molecule in these organisms that causes paralysis by blocking sodium channels necessary for muscles to contract. Saxitoxin is the most toxic and also the most well studied among the PSP associated toxins. Many derivatives of STX are known as gonyautoxins. The name refers to *Gonyaulax*, the former name of *Alexandrium* dinoflagellates. The basic chemical structure of these gonyautoxins is identical, but they are distinguished by chemical side-chains such as: $-H$, $-OSO_3$, $-CONH_2$, $-CONHSO_3$. The toxins are heat stable and water soluble. STX blocks sodium channels, which leads to paralysis. PSP tox-

ins are heat-stable and water-soluble nonproteinaceous toxins. The basic structures of PSP toxins are 3,4-propinoperhydropurine tricyclic systems. STX and its analogs can be divided into three categories: the carbamate compounds, which include STX, neosaxitoxin, and gonyautoxins 1–4; the *N*-sulfo-carbamoyl compounds, which include the C and B toxins; and finally the decarbamoyl compounds with respect to the presence or absence of 1-*N*-hydroxyl, 11-hydroxysulfate, and 21-*N*-sulfocarbamoyl substitutions, as well as epimerization at the C-11 position. In the past few decades at least 24 structurally related imidazoline guanidinium PSP derivatives have been identified and characterized from dinoflagellate species [33.88, 89]. STX and its analogs are very dangerous compounds, with possible military potential and have been listed by the Organization for the Prohibition of Chemical Weapons (OPCW) as a Schedule 1 chemical intoxicant, the manufacture, use, transfer, and reuse of which are now strictly regulated by the OPCW (Chemical Weapons Convention, September 1998, The Hague, The Netherlands).

Action Mechanism

The pharmacological action of the PSP toxins strongly resembles that of TTX. STX and several other PSP toxins block the voltage-gated sodium channel with great potency, thus slowing or abolishing the propagation of the action potential. However, they leave the potassium channel unaffected.

Lethal Dose

The lethal dose for humans is 0.1–1 mg. It is estimated that 0.5–1 mg can be fatal to humans [33.90]. There is evidence that children are more susceptible to STX than adults. The pathogenic dose for humans 0.1–2 mg. The lethal oral dose in humans is 1–4 mg (5000–20 000 mouseunits), depending on the gender and physiological condition of the patient [33.91].

Regulatory Tolerances by FDA

The FDA regulatory tolerance is 0.8 ppm STX equivalent (80 μg/100 g tissue) in all fish.

Incubation Period

Symptoms may occur within a few minutes and up to 10 h after ingestion.

Symptoms

The symptoms of PSP include a tickling sensation of the lips, mouth and tongue, numbness of the extremities, gastrointestinal problems, difficulty in breathing, and a sense of dissociation followed by complete paralysis [33.92]. In the case of serious intoxication, PSP leads to a variety of neurological symptoms culminating in respiratory arrest and cardiovascular shock or death. Paralysis is foremost here.

Mortality

Based on mortality figures from recent outbreaks, children appear to be more sensitive to the STXs of PSP than adults. The case-fatality ratio averages 6%. The death rate may be particularly high in children. If a patient survives the first 12–18 h, the prognosis is good. Muscle weakness can persist for days to weeks. In cases of severe poisoning, muscle paralysis and respiratory failure occur, and in these cases death may occur in 2–25 h. Its high mortality rate in some areas is caused by poor access to advanced life support capabilities. In an epidemic in Guatemala, the mortality rate was 26/187, whereby young children of < 6 years had a mortality rate of 50% and adults of > 18 years of 7%.

Treatment

No specific treatment exists. Victims require supportive care that may include mechanical ventilation. It is symptomatic and supportive. Since respiratory depression can develop surreptitiously, extreme vigilance should be exercised to monitor and support patients, especially during the first 12 h. The use of monoclonal neutralizing antibodies has been proposed. Endotracheal intubation and artificial respiration may be necessary for a period of several days.

Neurotoxic Shellfish Poisoning (NSP)

NSP shellfish poisoning is caused by the unarmored dinoflagellates *Gymnodinium breve* (*Ptychodiscus breve*, also called *Karenia brevis*) producing brevetoxins. The polyether brevetoxins are toxic to fish, marine mammals, birds, and humans, but not to shellfish. An unusual feature of *Gymnodinium breve* is the formation by wave action of toxic aerosols, which can lead to asthma-like symptoms in humans. Until 1992/1993, NSP was considered to be endemic to the Gulf of Mexico and the east coast of Florida, where red tides had been reported as early as 1844. NSP is the least common of the shellfish poisonings. The illness is self-limiting and resolves within several days with no lasting effects.

Sources

People can become ill after ingesting filter-feeding bivalve shellfish (e.g., oysters, mussels, clams) that concentrate the toxin and are subsequently consumed by predators, including humans.

Prevalence

The largest documented outbreak of NSP occurred in New Zealand in 1992–1993 with over 180 cases reported over a period of several weeks. Green mussel, cockles, and oysters were implicated in the New Zealand clusters [33.93–98]. Scattered reports of cases have occurred in Florida in recent years with two cases in 1995, three in 1996, two in 2001, and four in 2005 [33.99, 100]. Many reports of NSP involve a single case or small case series [33.101–104] with few large outbreaks recorded. The largest and best documented outbreak in the USA occurred in North Carolina, 48 documented cases. It began in October 1987 when a *Karenia brevis* bloom became entrained in the Gulf Stream off eastern Florida and was transported up the eastern seaboard [33.105].

Range

NSP causes health problems around the Gulf of Mexico and the Atlantic coast of the southern USA, The Caribbean, and New Zealand.

Causative Dinoflagellates and Poisonous Ingredients (Toxins)

The classic causative dinoflagellate *Gymnodinium breve* (*Ptychodiscus brevis*) is found in the Caribbean and the Gulf of Mexico, although similar species occur throughout the world. The *Gymnodinium breve* is relatively fragile and easily breaks open, releasing the endotoxins. It produces two types of lipid soluble toxins: hemolytic and neurotoxic. The neurotoxic toxins are known as brevetoxins. The major brevetoxin produced is PbTx-2; lesser amounts of PbTx-1, PbTx-3, and hemolytic components are produced. The massive fish kills are due to neurotoxin exposure, with a possible contribution of the hemolytic fraction. They disturb neuromuscular transmission. After being inhaled as aerosol they cause bronchial spasms. This may be manifested as an asthma crisis, rhinitis, sneezing, cough, or burning eyes after walking on the beach while a strong breeze splashes up water (with the toxin). With the inhalation of the aerosolized red tide toxins (especially the brevetoxins) from the sea spray, respiratory irritation and possibly other health effects have been reported in both humans and other mammals.

Action Mechanism

Brevetoxins act by disrupting the flow of Na^+ ions in nerve cells. They bind to sites near the voltage gated sodium channels, allowing an unchecked flow of Na^+ ions into or out of the cell. This disruption of ion flow within nerve cells is responsible for the neurological effects associated with NSP. Incidentally, brevetoxins have nearly the opposite effect to STXs, which bind to a different site and effectively block Na^+ ions from passing through the sodium channel (NIEHS 2000).

Lethal Dose

The pathogenic dose for humans is in the order of 42−72 mouse units. In human cases of NSP, the brevetoxin concentrations present in contaminated clams have been reported to be 30−18 µg (78−120 µg mg^{-1}).

Regulatory Tolerances by FDA

The FDA tolerances are 0.8 ppm brevetoxin-2 equivalent (20 mouse units/100 g) in clams, mussels and oysters. In the USA, legislation has been set by the FDA; the current regulatory limit is 800 µg brevetoxin-2 (PbTx-2) equivalents kg^{-1} shellfish [33.102]. At the time of writing, the European Food Safety Authority (EFSA) had not published a scientific opinion on NSP-type toxins.

Incubation Period

The time to onset is anywhere from 15 min to 12 h (mean time of 3 h).

Symptoms

Symptoms begin 1−3 h after eating the contaminated shellfish and include numbness, tingling in the mouth, arms and legs, incoordination, and gastrointestinal upset. As with ciguatera poisoning, some patients report temperature reversal. In addition, formation of toxic aerosols by wave action can produce respiratory asthma-like symptoms. They are similar to ciguatoxins in that they are sodium channel openers that cause neuroexcitatory effects. This results in neurological complaints such as paresthesia, temperature reversal, and ataxia, as well as GI symptoms such as nausea, abdominal pain, and diarrhoea. Symptoms are generally mild and self-limited. NSP presents itself as a milder gastroenteritis with neurologic symptoms compared with PSP. The symptoms of NSP include nausea, tingling and numbness of the perioral area, loss of motor control, and severe muscular pain [33.106, 107]. Paresthesia is foremost here.

Mortality

No deaths have been reported and the syndrome is less severe than ciguatera, but nevertheless debilitating.

Treatment

There is no antidote. Treatment is supportive care. In the case of inhalation: treatment of asthma attacks. Supportive care is given to help the patient feel more comfortable while recovering. Recovery normally occurs in 2–3 d.

Azaspiracid Shellfish Poisoning (AZP)

AZP is one of the more recently discovered seafood poisonings and was first reported in the Netherlands but later became a continuing problem in Europe [33.108]. It was identified following cases of severe GI illness from the consumption of contaminated mussels from Ireland, and now contamination has been confirmed throughout the western coastline of Europe. Azaspiracids (AZAs) are a group of lipophilic polyether toxins implicated in incidents of shellfish poisoning in humans. *Azadinium spinosum* has been identified as a species producing AZAs, formerly called Killary toxin-3 or KT3, reported to cause human poisoning etiologically similar to diarrheic shellfish poisoning. AZAs are accumulated by molluscs via the food web which, in turn, may cause human poisoning after consumption of contaminated shellfish.

Source

AZAs are accumulated by molluscs via the food web, which, in turn, may cause human poisoning after consumption of contaminated shellfish. The implicated toxins, AZAs, accumulate in bivalve mollusks that feed on toxic microalgae. AZAs were identified in mussels (*Mytilus galloprovincialis*), 0.24 $\mu g\,g^{-1}$, from Galicia, Spain, and scallops (*Pecten maximus*), 0.32 $\mu g\,g^{-1}$, from Brittany, France. Toxin profiles were similar to those found in the equivalent shellfish in Ireland in which AZA1 was the predominant toxin. They may accumulate in shellfish and can result in illnesses when consumed by humans.

Prevalence

Although its first discovery was thought to be in Ireland, but the search for the causative toxins, named azaspiracids, in other European countries has now led to the first discovery of these toxins in shellfish from France and Spain. Incidents of human intoxications throughout Europe, following the consumption of mussels have been attributed to AZP, particularly in northern Europe, which are produced by the small marine dinoflagellate *Azadinium spinosum*.

Range

Since then several AZP outbreaks have occurred in Ireland and by now AZAs have been detected in Ireland, UK, Norway, France, Portugal, Northern Africa (Morocco), South America (Chile), and the USA [33.109–114]. According to current EU legislation the total amount of AZAs should not exceed 160 $\mu g\,kg^{-1}$ AZA1-equivalents [33.115].

Causative Dinoflagellates and Poisonous Ingredients (Toxins)

Recently, it was discovered that AZAs are actually produced by a minute dinoflagellate [33.116]. This dinoflagellate, *Azadinium spinosum*, is smaller (12–16 μm) than any of the other toxin-producing dinoflagellates known so far. It is caused by consumption of contaminated shellfish associated with the dinoflagellate *Protoperidinium crassipes*, which can produce high intracellular concentrations of AZA1, a lipophilic, polyether toxin. To date about one dozen derivatives (AZA2 to 11) of AZA1 have been identified and characterized from *Protoperidinium crassipes* and contaminated shellfish. Until now, 24 different AZAs have been described, with AZA1, AZA2, and AZA3 as the predominant ones [33.117]. These compounds were detected in three species/strains, i. e., in North Sea isolates of *Azadinium poporum* (molecular mass: 845.5 Da), in a Korean isolate, which has been designated as *Azadinium* cf. *poporum* (molecular mass: 857.5 Da) and in *Amphidoma languida* isolated from Bantry Bay, Ireland (molecular masses: 815.5 and 829.5 Da). Toxicological studies have indicated that AZAs can induce widespread organ damage in mice and that they are probably more dangerous than previously known classes of shellfish toxins [33.110, 118]. AZAs differ significantly from other dinoflagellate toxins; they have unique structural features characterized by a trispiro assembly, an azaspiro ring fused with a 2,9-dinoxabicyclononane and a terminal carboxylic acid group.

Action Mechanism

The action mechanism of AZAs is unknown at present. Some studies indicate that AZAs might have different targets, since AZA1 and AZA2 increase $(Ca^{2+})_i$ by activation of Ca^{2+}-release from internal stores and Ca^{2+}-influx, while AZA3 induces only Ca^{2+}-influx. AZA5 does not modify intracellular Ca^{2+} homeosta-

sis. Recent investigation of the effect of AZA4 on cytosolic calcium concentration $[Ca^{2+}]_i$ in fresh human lymphocytes demonstrated that AZA4 inhibits store-operated Ca^{2+} channels (SOC channels) and Ca^{2+} influx, and that this process is reversible. It was postulated that AZA4 inhibits SOC channels by direct interaction with the channel pore, with another region of channel protein or with a closely associated regulatory protein, and it was also found that AZA4 acts through another type of Ca^{2+} channel, probably some nonselective cation channel usually activated by MTX [33.119]. AZA groups are novel inhibitors of Ca^{2+} channels, SOC, and non-SOC channels.

Lethal Dose

Recently, EFSA reviewed all available toxicity data and suggested that a safe level of AZA toxins in shellfish is below the acute reference doses (ARfD) of 30 µg AZA-1 equivalents kg^{-1} shellfish regulatory tolerances by FDA: 160 µg AZA equivalents kg^{-1}.

Regulatory Tolerances by FDA

FDA tolerances are 160 µg azaspiracid equivalents kg^{-1}.

Incubation Period

The incubation period ranges from 15 min to 38 h.

Symptoms

The symptoms of AZP include nausea, vomiting, severe diarrhoea, and stomach cramps. Neurotoxic symptoms have also been observed [33.119–121].

Mortality

To date, no deaths and no fatalities have ever been observed.

Treatment

Treatment is symptomatic and supportive.

Palytoxin (PTX) Poisoning

PTX is a large, water soluble polyalcohol first isolated from the soft coral of the genus *Palythoa*, and subsequently found in a variety of marine organisms ranging from dinoflagellates to fish, and implicated in seafood poisoning with a potential danger to public health. PTX is a polyhydroxylated compound that shows remarkable biological activity at an extremely low concentration. This toxin was first isolated from the soft coral *Palythoa toxica* and subsequently from many other organisms such as seaweeds and shellfish. PTX, a polyether marine toxin originally isolated from the zooxanthid *Polythoa toxica*, is one of the most toxic nonprotein substances known. Fatal poisonings have been linked to ingestion of PTX-contaminated seafood, and effects in humans have been associated with dermal and inhalational exposure to PTX containing organism and water.

Source

Cases of death resulting from PTX have been reported to be due to consumption of contaminated crabs in the Philippines [33.122], sea urchins in Brazil [33.123], and fish in Japan [33.124–126]. PTX has become of worldwide concern due to its potential impact on animals, including humans.

Prevalence

Results from 2008 and 2009 showed that there is a real danger of human poisoning, as these demonstrated bioaccumulation of the PTX group (PTX and ovatoxin-a) in both filter-feeding bivalve molluscs (mussels) and herbivorous echinoderms (sea urchins) [33.127]. So far in temperate areas, *Ostreopsis ovata* blooms have been reported to cause intoxications of humans by inhalation and irritations by contact [33.128].

Range

PTX is found in the Caribbean Sea, the Mediterranean Sea, the Pacific Ocean, and the Indian Ocean.

Causative Dinoflagellates and Poisonous Ingredients (Toxins)

Recently, PTX was also found in a benthic dinoflagellate, *Ostrepsis siamensis*, which caused blooms along the coast of Europe [33.129–134], leading to extensive death of edible mollusks and echinoderms [33.131, 132] and human illnesses [33.130, 131]. PTX is a large, very complex molecule with both lipophilic and hydrophilic regions, and has the longest chain of continuous carbon atoms in any known natural product. Recently several analogs, ostreocin-D (42-hydroxy-3, 26-didemethyl-9, 44-dideoxypalytoxin) and mascarenotoxins were identified in *Ostrepsis siamensis*.

Action Mechanism

Over the past few decades much effort has been devoted to define the action mechanisms of PTXs; however these have not been identified. Pharmacological

and electrophysiological studies have demonstrated that PTXs act as a haemolysin and alter the function of excitable cells. PTX selectively binds to the Na^+, K^+-ATPase with an equilibrium dissociation constant K_D of 20 pM and transforms the pump into a channel permeable to monovalent cations with a single-channel conductance of 10 pS [33.135–137]. Presently, three primary sites of action of PTXs have been postulated: PTX first opens a small conductance, nonselective cationic channel, which results in membrane depolarization, K^+ efflux and Na^+ influx. Subsequently, the membrane depolarization may open voltage-dependent Ca^{2+} channels in synaptic nerve terminals, cardiac cells, and smooth muscle cells, while Na^+ influx may load cells with Na^+ and favor Ca^{2+} uptake by the Na^+/Ca^{2+} exchanger in synaptic terminals, cardiac cells, and vascular smooth muscle cells. Then the increase of $[Ca^{2+}]$ stimulates the release of neurotransmitters by nerve terminals of histamine by mast cells and of vasoactive factors by vascular endothelial cells as a signal. It also induces contractions of striated and smooth muscle cells. Additional effects of a rise in $[Ca^{2+}]$ may be activation of phospholipase C [33.138] and phospholipase A2 [33.139]. There are reports that PTX opens an H^+ conductive pathway, which results in the activation of the Na^+/H^+ exchanger [33.140, 141]. Other investigators suggest that PTX raises $[Ca^{2+}]$ independently of the activity of voltage-dependent Ca^{2+} channels and Na^+/Ca^{2+} exchange [33.142]. The last two actions might act as the opening of H^+ specific and Ca^{2+} specific channels. Overall, PTX might possess more than one site of action in excitable cells and acts as an agonist for low conductance channels conducting Na^+/K^+, Ca^{2+} and H^+ ions.

Lethal Dose

PTX is regarded as one of the most potent toxins known so far [33.143], LD_{50} 24 h after intravenous injection varies from 0.025 µg kg^{-1} in rabbits and about the same in dogs to 0.45 µg kg^{-1} in mice, with monkeys, rats, and guinea pigs around 0.9 µg kg^{-1}. By extrapolation, a toxic dose in humans would range between 2.3 and 31.5 µg [33.144].

Regulatory Tolerances by FDA

FDA makes no recommendations and has no specific expectations with regard to controls for PTX.

Incubation Period

The incubation period ranges from minutes to 2–4 d.

Symptoms

Symptoms include fever inaction, ataxia, drowsiness, and weakness of limbs, followed by death.

Mortality

The onset of symptoms is rapid, with death occurring within minutes.

Treatment

Treatment is symptomatic and supportive therapy probably as for a coronary spasm.

Yessotoxin (YTX) Poisoning

YTXs are a group of structurally related polyether toxins produced by the dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera* [33.145]. YTX is a sulfated polyether compound originally isolated in Japan from the digestive gland of the scallop *Patinopecten yessoensis*, and is produced by phytoplanktonic microalgae of some dinoflagellate species.

Source

YTX is found in scallops and mussels of different origins [33.146, 147].

Prevalence

YTX and its analogs, which are disulfated polyether compounds, occur increasingly in seafood and are a worldwide concern due to their potential risk to human health.

Range

YTX was originally isolated from the scallop *Patinopecten yessoensis*, collected at Mutsu Bay, Japan [33.148]. Since then, YTXs have been found in Europe, South America, and New Zealand, and have become a worldwide concern due to their potential risk to human health.

Causative Dinoflagellates and Poisonous Ingredients (Toxins)

Three dinoflagellate species, *Protoceratium reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera* produce YTXs [33.146, 149]. YTX and its derivatives, 45-hydroxy YTX (45-OH-YTX), 45,46,47-trinor YTX, homo YTX, and 45-hydroxyhomo YTX [33.150, 151] are disulfated polyether lipophilic toxins [33.146]. Recently, several new YTX analogs: carboxyyessotoxin (with a COOH group on the C_{44} of YTX instead of a double bond); carboxyhomoyesso-

toxin (with a COOH group on the C_{44} of homo YTX instead of a double bond); 42,43,44,45,46,47,55-heptanor-41-oxo YTX and 42,43,44,45,46,47,55-heptanor-41-oxohomo YTX in Adriatic mussels (*Mytilus galloprovincialis*) were identified in dinoflagellates [33.152, 153]. Originally, YTXs were classified among the toxins responsible for DSP, mainly because they appear and are extracted together with the DSP toxins, okadaic acid (OA) and DTXs [33.146]. However, YTXs are proved to be not diarrheagenic compared to OA and its derivatives, the DTXs, which cause intestinal fluid accumulation or inhibition of protein phosphatase 2A.

Action Mechanism

Recently, it was demonstrated that YTX is a potent neurotoxin for neuronal cells. However, the action site and the mechanism are unknown [33.154]. YTX was observed to induce a twofold increase in cytosolic calcium in cerebellar neurons, which was prevented by the voltage-sensitive calcium channel antagonists nifedipine and verapamil. These results suggest that YTX might interact with calcium channels and/or sodium channels directly. Previous studies also showed that YTX activated nifedipine-sensitive calcium channels in human lymphocytes [33.155], and YTX was postulated to activate noncapacitative calcium entry and inhibit capacitive calcium entry by emptying internal calcium stores.

Lethal Dose

Toxicological studies indicate that acute oral administration at doses up to $10\,mg\,kg^{-1}$ YTX or repeated (7 days) oral exposure to high ($2\,mg\,kg^{-1}\,d^{-1}$) doses of the toxin causes neither mortality nor strong signs of toxicity in mice [33.156–158]. YTX causes motor discoordination in mice before death due to cerebellar cortical alterations [33.158, 159]. A histopathological study revealed that YTX provoked alterations in the Purkinje cells of the cerebellum, including cytological damage to the neuronal cell body and changes in the neurotubule and neurofilament immunoreactivity [33.159].

Regulatory Tolerances by FDA

The tolerance regulated by the FDA is 1 mg yessotoxin equivalent kg^{-1}. The FDA implemented a maximum permitted level (MPL) of 1 mg YTX equivalents kg^{-1} shellfish intended for human consumption (Directive 2002/225/EC) [33.160].

Incubation Period

The incubation period is unknown.

Symptoms

Symptoms of intoxication produced by YTX in humans are relatively unknown due to the fact that no human intoxication has been reported to date. However, it seems clear that YTX does not produce diarrhoea in humans. The scarcity of toxicological studies on YTX and its analogs, which are necessary for the assessment of its human health risks, have been hampered until now by the limited availability of the toxin.

Mortality

There is no information about the mortality rate.

Treatment

There is no known treatment.

33.6 Biotechnological Significance of Toxic Marine Dinoflagellates

As stated above, dinoflagellates are not only important marine primary producers and grazers, but also the major causative agents of HABs. Many dinoflagellates are associated with the production of various natural marine toxins. Most of these natural toxins are neurotoxins which present themselves with highly specific effects on the nervous system of animals, including humans, by interfering with nerve impulse transmission. Neurotoxins are a varied group of compounds, both chemically and pharmacologically. They vary in both chemical structure and mechanism of action, and produce a very distinct biological effect, which provides a potential application of these toxins in pharmacology and toxicology. These toxins can be extremely toxic and many of them are effective at far lower dosages than conventional chemical agents. Dinoflagellate toxins and bioactives are of increasing interest because of their commercial impact, influence on the safety of seafood, and potential medical and other applications [33.38]. The lack of sufficient quantities of toxins for investigational purposes remains a significant limitation. The production of quantities of dinoflagellate bioactives requires the ability to mass culture them. Concerns relating to bioreactor culture of gen-

erally fragile and slow-growing dinoflagellates are of important consideration. Dinoflagellate bioactives are inaccessible in large quantities, and this severely limits research in potential applications of these compounds. Some of the toxins that are available in small amounts are quite expensive. Chemical synthesis of most dinoflagellate toxins is complex and expensive. Gaining access to toxins requires the ability to mass culture dinoflagellates. These microorganisms appear to be extremely sensitive to hydrodynamic shear forces and pose new challenges in photobioreactor engineering. Bioprocess engineering studies to enable economic use of dinoflagellates as cell factories are only just beginning.

33.7 Control and Prevention

Due to the complexities, current knowledge of bloom dynamics and the secondary effects that arise from intervention procedures, its control, mitigation, and prevention are becoming difficult. Because of the large impacts of coastal HABs, the control and prevention of problem species are of major interest. Geographic expansion of known toxic species has been linked, in some cases, to introductions by ballast water transport along shipping routes [33.161, 162]. Most long-known harmful taxa appear to operate independently from human influences other than physical transport [33.40, 163]. However, government agencies are working closely with academic institutions and nonprofit organizations to mitigate the effects of HABs and to develop prevention technologies for the future.

Toxic seafood poisonings are more common in summer than in winter because dinoflagellates grow well in warmer seasons. It is estimated from cases with available data that 1 person dies every 4 years from toxic seafood poisonings. In moderate and colder regions, the occurrence of shellfish poisoning is largely limited to the warm summer months. The rule of thumb says that shellfish should not be eaten during the months in which there is no *r* (May, June, July, August in the Northern Hemisphere). This phenomenon has led to the general teaching in North America that shellfish are safe to eat only if harvested in a month containing the letter *r*. In tropical and subtropical zones, there is no seasonal dependency. Shellfish poisoning usually occurs in the form of epidemics in these regions, but outbreaks in areas remote from the coast, where poisonous shellfish have been imported are also possible.

Ciguatoxin cannot be identified by odor, taste, or appearance. It is also temperature stable, so cooking or freezing will not destroy it. Ciguatoxin cannot be eliminated by salting, drying, smoking, or marinating either. The contaminated fish can remain toxic for years, even on a nontoxic diet. Apart from the avoidance of the consumption of large predatory fish, animal screening tests are the only tools presently available to prevent intoxication. It is important to notify public health departments about even one person with marine toxin poisoning. Investigators can then try to determine if a specific restaurant, oyster bed, or fishing area has a problem. This prevents others becoming ill. Finfish or shellfish sold as bait should not be eaten. Bait products do not need to meet the same food safety regulations as seafood for human consumption.

Natural toxins are very heat stable. Normal household cooking (e.g., boiling, steaming, frying) has no or very little effect on toxin levels. The primary preventive tool for intoxication with natural toxins is the monitoring of toxin levels in algae in the harvesting areas. Based on the presence of toxins, waters can be classified and harvesting of shellfish forbidden, if levels of toxin are too high. Other elements of a control program will include (FDA, 1998):

- A requirement that containers of in-shell molluscan shellfish bear a tag that identifies the type and quality of shellfish, harvester, harvest location, and date of harvest.
- A requirement that molluscan shellfish harvesters be licensed.
- A requirement that processes the chuck molluscan shellfish or ship, repack the chucked product, and certify.
- A requirement that containers of chucked shellfish bear a label with the processor's name, address, and certification number.

Depuration and ozonation are not effective and are not used in reducing toxins in shellfish. Many countries have their own guidelines for acceptable toxin levels. If these levels are exceeded, the government will close commercial mussel and oyster banks, forbid the sale of certain seafood, and advise against the use of it. For example, for STXs the limit is set at more than 500 cells

of *Pyrodinium bahamense* per liter of sea water or more than 40 µg of STXs per 100 g of mollusc. For brevetoxin there is a guideline that only the total absence of the toxin is acceptable. A guideline such as this leads to practical problems.

Programs that monitor toxins in shellfish food supplies have proven successful in limiting shellfish poisoning in the USA, Canada, and Europe. Expanding these programs to other parts of the world would further diminish the threat of marine dinoflagellate toxins to human health. The most reliable method to measure the amount of toxins in shellfish is by mouse bioassay. PSP is associated with relatively few reported outbreaks, most likely because of the effective control programs that prevent human exposure to toxic shellfish. PSP can be a serious public health problem, as was demonstrated in Guatemala in a 1987 outbreak in which 187 cases with 26 deaths resulted from the ingestion of clam soup [33.164].

In the USA, approximately 30 cases of poisoning by marine toxins are reported each year. Because healthcare providers are not required to report these illnesses, and because many milder cases are not diagnosed or reported, the actual number of poisonings is much greater.

There is very little published literature or formal epidemiologic studies on the human health effects of the diseases, by either ingestion of NSP or inhalation of aerosolized red tide toxins causing respiratory irritation. As a nonreportable disease, NSP is highly under-reported and under-diagnosed; for example, there are no existing statistics for the incidence of NSP or aerosolized red tide toxin respiratory irritation, even in endemic areas, nor on possible chronic health effects in humans. There are no established biomarkers for either of the Florida red tide toxin-associated conditions in humans, nor have there been any formal studies published on aerosolized red tide toxin respiratory irritation surveillance monitoring. There is very little information on the appropriate treatment and prevention methodologies [33.165–167].

33.8 Discussion and Conclusion

HABs have become a global epidemic. The number of harmful algal blooms has increased over the last 40 years, especially in regions with large increases in coastal populations and overfishing. It is feared that blooms of dinoflagellates are becoming more common due to human activities that increase nutrient levels in the ocean (e.g., run-off from fertilized pastures, sewage disposal). It is also thought that the indiscriminate dumping of ballast water may allow toxic strains to spread rapidly over long distances. Dinoflagellates can move and hence can seek deeper layers for nutrients to overcome surface nutrient deficiency. This may lead to an increased number of HABs as a result of climate change when upper stratification of surface layers occur. Human shellfish poisoning may result from the presence of large numbers of toxic dinoflagellates in seawater. The species of marine dinoflagellates responsible for human illness depend on the geographical location. In the Southern Hemisphere *Pyrodinium* species will be responsible for PSP, while *Alexandrium, Gymnodinium* and *Dinophysis* are responsible for PSP, NSP, and DSP in the Northern Hemisphere. *Gonyaulax tamarensis* sometimes blooms in the North Sea, while in California *Gymnodinium catenatum* causes poisoning problems. Most clinical cases occur between late spring and early autumn, which corresponds to the bloom season of these photosynthesizing organisms.

Human poisoning by contaminated shellfish is not a pandemic global problem that poses grave health risks to humans as a whole. The typical year will see relatively few deaths or severe illnesses caused by consumption of contaminated shellfish. Nevertheless, shellfish poisoning is a persistent and annoying problem that regularly affects consumers of seafood, which is a large portion of the population in some areas. Monitoring of shellfish supplies for toxins has proved very successful in limiting human shellfish poisoning in developed countries and this monitoring should be continued and expanded to lesser-developed nations as soon as possible.

PSP, CFP, and NSP poisoning have been known for centuries, although their toxins were only recently described [33.10]. Diarrheic shellfish poisoning was recognized in the 1960s. A new human toxin syndrome, AZP came to light in the 1990s [33.168]. Of all these marine dinoflagellates-associated human poisonings, PSP poses the greatest threat to public health, and fatal cases have been reported around the world. PSP is caused by several species of dinoflagellate organ-

isms including *Alexandrium tamarense*, *Gymnodinium catenatum*, and *Pyrodinium bahamense* [33.169]. *Azadinium spinosum* has been identified as a species producer of AZAs, the marine toxins which are reported to cause human poisoning etiologically similar to diarrheic shellfish poisoning.

Many countries rely on biotoxin monitoring programs to protect public health and close harvesting areas when toxic algal blooms or toxic shellfish are detected. Because of the risks of poisoning associated with eating marine shellfish, many countries routinely monitor the toxicity of fish and shellfish using various sorts of assays. One commonly used mouse bioassay gives an indication of contamination of the fish and shellfish by marine dinoflagellate toxins. However, the mouse bioassay is increasingly being replaced by more accurate methods of determining the presence and concentration of marine toxins using analytical biochemistry. In nonindustrialized countries, particularly in rural areas, monitoring for harmful algal blooms does not routinely occur and death due to red tide toxins commonly occurs. According to *Dr. Don Anderson*, who carries out research on algal speciesat the Woods Hole Oceanographic Institute in Massachusetts:

> *There are more toxic algal species, more algal toxins, more fisheries resources affected, more food-web disruption, and more economic losses from harmful algal blooms than ever before.*

Their occurrence and persistence represent a significant and expanding threat to human health and marine resources across the nations [33.170].

Consumption of raw molluscan shellfish poses well-known risks of food poisoning, however, intoxication from finfish is not so well known. Most algal toxins associated with seafood poisoning are heat stable and are not inactivated by cooking. It is also not possible to visually distinguish toxic from nontoxic fish and shellfish. The rule of thumb is that shellfish should only be eaten during months with an *R* in them, and not during May to August.

Human poisoning arising directly or indirectly as a result of exposure to the noxious metabolites produced by marine dinoflagellates during red tides are global in occurrence. Understanding the causes of these phenomena, and mitigating and preventing their consequences are international concerns. World public health authorities must assume responsibility consider the problem properly, establish a proper diagnosis and universal therapy program, and develop specific assay kits for red tide toxins. They should also invest in the geographic localization of the wide variety of toxins, and in chemical tests capable of detecting all toxins in any suspected food source.

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34. Carotenoids, Bioactive Metabolites Derived from Seaweeds

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Health is top priorities for many people worldwide. Food diets and dietary components play a vital role in accomplishing these needs. With the increasing knowledge of health benefits associated with seaweeds and utilization of these materials has accelerated. Seaweeds represent a valuable resource of bioactive materials, in association with a healthy lifestyle including correct dietary habits and moderate physical activity. Hence, isolation and investigation of bioactive ingredients with biological activities from seaweeds have attracted great attention. Among bioactive ingredients identified from seaweeds, carotenoids have received particular attention. These carotenoids exhibit various beneficial biological activities such as antioxidant, anticancer, anti-inflammatory, anti-obesity, and anti-angiogenic activities. This chapter focuses on biological activities of seaweeds-derived carotenoids and emphasizing their potential applications in foods as well as pharmaceuticals areas.

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34.1 Seaweeds

In recent years, seaweeds have served as an important source of bioactive natural substances. Many metabolites isolated from seaweeds have shown to possess biological activities and potential health benefits. Therefore, a new trend has been emerged to isolate and identify bioactive compounds and constituents from seaweeds [34.1]. One particular interesting feature in seaweeds is their richness in carotenoids. Besides their role in photosynthetic and pigmentation effects, carotenoids have also been reported to biological activities (Table 34.1). Therefore, various carotenoids isolated from seaweeds have attracted much attention in the fields of food, cosmetic and pharmacology [34.2]. Novel extraction and separation techniques, such as supercritical CO_2 extraction, centrifugal partition chromatography and pressurized liquid method have recently been employed in the development of carotenoids derived from seaweeds [34.3].

Carotenoids are linear polyenes that function as light energy harvesters and antioxidants that inactivate reactive oxygen species (ROS) formed by exposure to light and air [34.4, 5]. Carotenoids are considered to be accessory pigments; since they augment the light-harvesting properties of seaweeds by passing on light excitation to chlorophyll [34.6]. Carotenoids can be classified into two types: carotenes, which are unsaturated hydrocarbons; and xanthophylls, which present one or more functional groups containing oxygen [34.7]. One very visible carotenoid in seaweeds is fucoxanthin; the brown pigment which colours kelps and other brown seaweeds as well as the diatoms. Fucoxanthin is one of the most abundant carotenoids contributing around 10% estimated total production of carotenoids in nature [34.8]. It has a unique structure including an unusual allenic bond and a 5,6-monoepoxide in its molecule.

34.2 Biological Activities of Carotenoids and Health Benefit Effects

34.2.1 Antioxidant Activity

Recently, there is a considerable interest in the food and pharmaceutical industries for the development of antioxidants from natural sources, such as marine flora and fauna. Seaweeds are rich sources of structurally diverse bioactive compounds with valuable antioxidant activities. Antioxidants may have a positive effect on human health as they can protect the human body against damage by ROS, which attack macromolecules such as membrane lipids, proteins and deoxyribonucleic acid (DNA), lead to many health disorders such as cancer, diabetes mellitus, aging and neurodegenerative diseases [34.16]. Furthermore, deterioration of some foods has been identified due to the oxidation of lipids or rancidity and the formation of undesirable secondary lipid peroxidation products. Lipid oxidation by ROS such as superoxide anion, hydroxyl radicals and H_2O_2 also causes a decrease in nutritional value of lipid foods, and affect their safety and appearance. Therefore, in food and pharmaceutical industry, several synthetic commercial antioxidants such as butylated hydroxyanisole [34.17], butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been used widely to retard the oxidation and peroxidation processes [34.18–20]. However, the use of these synthetic antioxidants must be under strict regulation due to potential health hazards [34.21, 22]. Hence, the search for natural antioxidants as safe alternatives is important in food and pharmaceutical industry Recently, there is a considerable interest in the food as well as pharmaceutical industry for the development of antioxidants from natural sources, such as marine flora and fauna. Among marine resources, seaweeds represent one of the richest sources of natural antioxidants [34.23].

Fucoxanthin was identified as the major antioxidant of *Hijikia fusiformis*. Although it has been previously reported that carotenoids such as zeaxanthin, β-carotene and lutein did not show 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity, fucoxanthin possess a strong radical scavenging activity. The potential involvement of fucoxanthin in radical scavenging activity may correlate to the presence of unusual double allenic bonds at C-7′ position. These findings were confirmed in a recent study carried by *Sachindra* et al. [34.24] which isolated fucoxanthin from *Undaria pinnatifida* and prepared two fucoxanthin metabolites, fucoxanthinol and halocynthiaxanthin. The antioxidant activities of these three carotenoids were assessed by DPPH, hydroxyl radical scavenging activity and singlet oxygen quenching activity. The order of scavenging activity of each carotenoid followed a pattern of fucoxanthin > fucoxanthinol > halocynthiaxanthi [34.24]. The major structural differences in these three carotenoids are the presence of an allenic bond in fucoxanthin and fucoxanthinol, suggesting that the allenic bond is responsible for the higher scavenging activity of fucoxanthin and fucoxanthinol. Therefore, fucoxanthin may have great potential for use as nutraceuticals and pharmaceuticals as a substitute for synthetic antioxidants. In addition, when fucoxanthin added to ground chicken meat at a content level of 200 mg kg^{-1} the formation of secondary oxidation products including TBA reactive substances were reduced in the same level as α-tocopherol. Oral administration of fucoxanthin has been reported to improve plasma antioxidant status and meat color in broiler chicks [34.25]. Moreover, fucoxanthin obtained from *Padina tetrastromatic* has shown higher potential to be used as antioxidant than β-carotene in modulating antioxidant enzyme in plasma and liver of retinol deficiency rat [34.17, 26]. However, the exact mechanisms of action how fucoxanthin exerts antioxidative effect in rat induced by retinol deficiency are not yet completely understood. In addition, cytoprotective effect of fucox-

Table 34.1 Biological activities of seaweeds-derived carotenoids

Carotenoids	Biological activities	Seaweeds sources
Fucoxanthin [34.9–14]	Antioxidant	*Hijikia fusiformis*, *Undaria pinnatifida*, *Fucus serratus*, *Padina tetrastromatic*
	Anticancer	*Undaria pinnatifida*
	Anti-inflammatory	*Myagropsis myagroides*
	Anti-obesity	*Undaria pinnatifida*
	Anti-angiogenic	*Undaria pinnatifida*
	Neuroprotective	*Hijikia fusiformis*
	Prevent osteoporosis	*Laminaria japonica*
	Photoprotective	*Laminaria japonica*
Siponaxanthin [34.15]	Anticancer Anti-angiogenic	*Codium fragile* *Codium fragile*
Lutein [34.3]	Antimutagenic	*Porphyra tenera*
β-carotene [34.3]	Antimutagenic	*Porphyra tenera*

anthin against ROS formation induced by H_2O_2 has been observed in vitro [34.27]. Two hydroxyl groups present in the ring structure of fucoxanthin may correlate to the inhibition of ROS formation. Several studies have indicated that the number of hydroxyl groups on the ring structure of fucoxanthin is correlated with the effects of ROS suppression.

34.2.2 Anticancer Activity

Many recent studies have been carried out to find cancer chemopreventive and/or chemotherapeutic agents from edible and natural resources such as fruits, vegetables, and terrestrial plants. Seaweeds also emerged as one of the important sources for dietary supplements and a number of them are potentially active and useful. Seaweeds have been used as a source of food and medicine and their metabolites are also well-known. The marine seaweeds have a variety of chemo-protective compounds such as carotenoids.

Exciting research studies have been published regarding carotenoids and its anticancer qualities. *Ishikawa* et al. [34.28] showed anti-adult T-cell leukemia effects of fucoxanthin and its deacetylated metabolite fucoxanthinol. The inhibitory activities of fucoxanthin and fucoxanthinol were stronger than those of β-carotene and astaxanthin [34.28]. Adult T-cell leukemia is a fatal malignancy of T lymphocytes caused by human T-cell leukemia virus type 1 infection and remains incurable. Therefore, carotenoids could be potentially useful therapeutic agents for adult T-cell leukemia patients. A recent study from Japan demonstrates that anticancer activity of fucoxanthin goes way beyond its ability to induce apoptosis. Apoptosis inducing effect of fucoxanthin on human leukemia cells (HL-60) has been reported [34.29, 30]. The apoptosis induction was associated with activation of caspase-3, -8 and -9 which can be thought of as central executioner of the apoptotic pathway [34.31]. Very recently, siphonaxanthin derived from *Codium fragile* showed more potent growth-inhibitor against HL-60 cells than fucoxanthin. The structural differences between these two carotenoids are fucoxanthin contains epoxide and an allenic bond in its structure, whereas siphonaxanthin does not contain those functional groups; however, siponaxanthin has an additional hydroxyl group. Since esterified form of siphonaxanthin showed lower inhibitory effect, suggesting that the presence of that hydroxyl group are contributed to the strong inhibitory effect of siphonaxanthin [34.32]. Meanwhile, anti-proliferative effect and apoptosis induction by fucoxanthin in human colon cancer cells (Caco-2, HT-29 and DLD-1) have also been demonstrated. Fucoxanthin remarkably reduced the viability of human colon cancer cell lines and treatment with fucoxanthin induced DNA fragmentation. Exposure to fucoxanthin decreased the level of apoptosis suppressing protein (Bcl-2), suggesting that anticancer mechanism of fucoxanthin bring through apoptosis mechanism. Apoptosis inducing effect of fucoxanthin in human prostate cancer cells (PC-3, DU 145 and LNCaP) has also been observed [34.33, 34]. Although current knowledge of relationship between the structure and apoptosis activity of the fucoxanthin is limited, some researchers suggest that conjugated double bonds and 5,6-monoepoxide are thought to be highly susceptible to acids, alkali and oxygen lead to their prooxidant actions which might cause apoptosis induction in the several cancer cells.

34.2.3 Anti-Obesity Activity

Obesity is a contributing factor in many diseases. However, many peoples are not aware of this association and do not consider obesity to be a serious health concern. A detrimental effect of obesity on reproductive system has also been demonstrated consistently. Furthermore, it is reported that media and socio-cultural continues to pressures young generations to be thin which promotes body dissatisfaction, eating disturbance, depression and negative effect in young generations. Many categories of natural and synthetic compounds which demonstrated as anti-obesity drugs have been used to reduce weight. However, synthetic anti-obesity agents are believed to have certain side effects such as unacceptable tachycardia, hypertension, improve lipid blood levels, improve glucose metabolism and disturbance of reproductive system. Hence, more scientific efforts have been dedicated to study medicinal foods that can act as anti-obesity agents. Since the excessive growth of adipose tissue in obesity has been suggested to result from adipocyte hypertrophy and the recruitment of new adipocytes from precursor cells, regulation of adipogenesis also appears to be a potential strategy for the treatment of obesity [34.35]. Fucoxanthin isolated from *Undaria pinnatifida* and fucoxanthinol have been reported to inhibit the differentiation of 3T3-L1 preadipocytes into adipocytes [34.9]. The inhibitory effect of fucoxanthin and fucoxanthinol on adipocyte differentiation might be mediated through the down-regulation of adipogenic transcription factors, such as peroxisome proliferator-activated receptor-γ. Structure suppressive effect on adipocyte differentiation has been

reported by *Okada* et al. [34.36]. In their study, they used 13 naturally occurring carotenoids found in human diet. Interestingly, carotenoids with keto group, epoxy group, hydroxyl carotenoid, epoxy-hydroxy carotenoid and keto-hydroxy carotenoid did not showed suppressive effect on adypocite differentiation. Meanwhile treatment with fucoxanthin and neoxanthin showed significant suppressive effect suggesting that allenic bond is crucial factor for the anti-obesity effect. The result of those studies leads to the hypothesis that other carotenoid with an allenic group and an additional hydroxyl group in the end may also effective in suppressing adypocite differentiation.

Another interesting studies revealed that oral treatment with fucoxanthin significantly reduced the abdominal white adipose tissue (WAT) weight of obese mice model, KK-Ay female mice and normal mice fed with a high-fat diet [34.10–12, 37]. Moreover, no reductions on normal mice fed with normal diet were found. Those results suggest that fucoxanthin specifically suppress adiposity in the obese mice. The mechanisms of action for the observed antiobesity effect of fucoxanthin were mainly mediated by the induction of uncoupling protein 1 (UCP1) in abdominal WAT. UCP1 is inner-membrane mitochondrial protein that has the ability to dissipate energy through uncoupling of oxidative phosphorylation which, instead of adenosine triphosphate (ATP) , produces heat. UCP1 is expressed exclusively in brown adipose tiss and known as a significant component of whole body energy expenditure. However, adult humans have very little BAT, making it unlikely to be a major contributor to human weight regulation. In humans, most of fat is stored in WAT which is the predominant type of adipose tissue and commonly called as *fat* in mammals [34.38]. Besides its role in energy storage, WAT is now recognized as an endocrine and active secretory organ through its production of biologically active mediators termed, adipokines [34.39]. As nutrigenomic study revealed that fucoxanthin induces UCP1 expression in WAT, fucoxanthin will be an important and attractive agent for the development of anti-obesity therapies. However, further efforts to clarify the molecular mechanisms various intracellular signaling pathways also need to be investigated in order to obtain a better understanding of the underlying UCP1 induction by fucoxanthin.

Dietary fucoxanthin has been reported to suppress insulin levels and hyperglycemia in diabetic/obese KK-Ay mice [34.12, 40, 41]. Furthermore, the release and expression levels of pro-inflammatory adipocytokines were also attenuated by fucoxanthin [34.40]. Several studies demonstrated that pro-inflammatory adipocytokines secretions are elevated through the accumulation of fat in adipocytes and causes insulin resistance in obese animal models. Hence, fucoxanthin could be used as an inhibitor in regulating pro-inflammatory adipocytokines, insulin and hyperglycemia. Furthermore, fucoxanthin may assist therapeutic treatment of diabetic diseases. Moreover, dietary intake of fucoxanthin and fucoxanthinol has been demonstrated to enhance the amount of DHA and arachidonic acid content in the liver of C57BL6J/6J normal male mice [34.42, 43]. However, further studies are needed to clarify the molecular mechanisms how fucoxanthin promotes DHA and AA synthesis in the mice liver.

34.2.4 Anti-Inflammatory Activity

Inflammation is a response by an organism to injury related to biological, chemical or physical stimuli. In acute inflammation, this response is intended to inactivate or destroy invading organisms, remove irritants, and finally set the stage for tissue repair. In chronic inflammation, however, this response is involved in multiple diseases, such as periodontal disease, colitis, arthritis, atherosclerosis, asthma, multiple sclerosis and inflammatory bowel diseases. Nowadays, much attention has been paid to the development of anti-inflammatory agents from seaweeds.

Secondary metabolites derived from seaweeds are known to have promising anti-inflammatory activities [34.13]. However, the scientific analysis of anti-inflammatory activity of seaweeds-derived carotenoids has been poorly carried out and until now only few studies were reported. For example, *Shiratori* et al. reported that anti-inflammatory effect of fucoxanthin is comparable with predinisolone, a commercially available steroidal anti-inflammatory drug [34.44]. Supporting those finding, *Heo* et al. [34.45] screened inhibitory effect of NO production from nine species of brown seaweeds and confirmed that inhibition of NO production were correlates with fucoxanthin contents. In addition, they also demonstrated anti-inflammatory effect of fucoxanthin isolated from *Myagropsis myagroides* in LPS-stimulated RAW 264.7 cells [34.46]. Fucoxanthin treatment attenuates the productions of NO and PGE_2 by inhibiting inducible NO synthase (iNOS) and cyclooxigenase-2 (COX-2) expressions. The release and expression levels of inflammatory cytokines (TNF-α, IL-6 and IL-1β) were attenuated by fucoxanthin in a dose-dependant fashion [34.45]. The anti-inflammatory activities of fucoxanthin were due to

the suppression of nuclear factor-κB (NF-κB) and the phosphorylation of mitogen activated protein kinases (MAPKs) [34.47]. Production of pro-inflammatory mediators has been continuously reported in many inflammatory tissues, along with increased expression of their messenger ribonucleic acid (mRNA) and proteins. Therefore, inhibition of pro-inflammatory mediators by carotenoids suggests its potential for the treatment of inflammatory and other related diseases.

34.2.5 Anti-Angiogenic Activity

Cancer is a serious disease with a complex pathogenesis, which threats human life greatly. The invasion and metastasis of cancer depend on the angiogenesis of tumorous stroma and the degradation of extracellular matrix. Therefore, tumor angiogenesis is a common hallmark of various cancers. Angiogenesis refers to the process of new blood vessel formation from a pre-existing vasculature that occurs under, either physiological or pathological conditions. *Sugawara* et al. [34.48] showed that fucoxanthin significantly suppressed human umbilical vein endothelial cells (HUVEC) proliferation and tube formation at more than 10 μM. Fucoxanthin effectively suppressed the differentiation of endothelial progenitor cells into endothelial cells involving new blood vessel formation. Fucoxanthin and fucoxanthinol suppressed microvessel outgrowth in vivo and ex vivo angiogenesis assay using a rat aortic ring [34.48]. Anti-angiogenic effect of siphonaxanthin derived from green seaweeds, *Codium fragile*, has been demonstrated in a recent study. Anti-angiogenic effects of siponaxanthin were comparable with fucoxanthin [34.15]. The structure similarity between fucoxanthin and siponaxanthin are the presence of hydroxy group on the 3 and 3′ position. Therefore, the presence of those hydroxyl groups might conceivably parted of their anti-angiogenesis effect.

34.2.6 Other Biological Activities

Fucoxanthin isolated from *Laminaria japonica* has been reported to suppress tyrosinase activity in UVB-irradiated (UVB: ultraviolet B radiation) guinea pig and melanogenesis in UVB-irradiated mice. Oral treatment of fucoxanthin significantly suppressed skin mRNA expression related to melanogenesis, suggesting that fucoxanthin negatively regulated melanogenesis factor at transcriptional level [34.14]. Moreover, fucoxanthin has been demonstrated to possess photoprotective properties in human fibroblast cells via inhibition of DNA damage and enhance antioxidant activity [34.49]. These studies suggest that oral administration of fucoxanthin might prevent or minimize the negative effects of ultraviolet (UV) radiation such as melanin formation.

34.3 Concluding Remarks

Collectively, carotenoids are a valuable source of bioactive compounds and could be introduced for the preparation of novel functional ingredients in food and also a good approach for the treatment or prevention of chronic diseases. Recently, much attention has been paid by the consumers toward natural bioactive compounds as functional ingredients in foods, and hence, it can be suggested that, carotenoids are an alternative source for synthetic ingredients that can contribute to consumer's well-being, by being a part of new functional foods and pharmaceuticals. Furthermore, the wide ranges of biological activities associated with seaweeds-derived carotenoids have potential to expand its health beneficial value in food, and pharmaceutical industries.

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35. Marine Bioactive Compounds from Cnidarians

Joana Rocha, Miguel Leal, Ricardo Calado

Cnidaria is a large, diverse and ecologically important phylum of marine invertebrates, which includes corals, sea fans, anemones, and jellyfishes. It contains over 11 000 species, 7500 of them belonging to the class Anthozoa. Over 3000 marine natural products have been described from this phylum alone, most of them in the twenty-first century. The present work provides an overview of some of the most promising marine bioactive compounds, from a therapeutic point of view, isolated from cnidarians since the year 2000. The order Alcyonacea (class Anthozoa) exhibits the highest number of species yielding promising compounds. Antitumor activity has been the major area of interest in the screening of cnidarian compounds, the most promising ones being terpenoids (monoterpenoids, diterpenoids, and sesquiterpenoids). Future trends and challenges for the bioprospecting of new marine bioactive compounds produced by cnidarians are also discussed, with emphasis on the sustainable production of target cnidarians biomass and the role played by symbiotic microorganisms in the synthesis of important biomolecules.

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35.1 Cnidarians

The phylum Cnidaria is a large, diverse and ecologically important group of marine invertebrates that contains over 11 000 extant species (Table 35.1) [35.1], including hydroids, jellyfish, anemones, and corals, among others (Fig. 35.1). This group of invertebrate animals is found exclusively in aquatic environments, mostly in marine ecosystems. Cnidarians have simple body forms, usually of a polyp or medusa (Fig. 35.2) [35.2]. For instance, an anemone is a single polyp, whereas corals are, in general, a colony of individual polyps, which are typically tubular and attached to a surface at their base. Both forms may occur during the life cycle of some cnidarians. All species within the phylum Cnidaria have tentacles surrounding the opening (mouth), with stinging cells in their tips that are used to capture and subdue prey. The stinging cells are coiled structures that shoot out and inject toxins via a dart-like tip [35.2].

Cnidarians are usually found in a wide geographic range: from deep waters near hydrothermal vents to polar seabeds and tropical reefs. Some cnidarian species, mostly from the class Scyphozoa (jellyfish), are pelagic and live in the water column. Cnidarians lack mechanical means to prey and have optimized their mechanisms to feed throughout evolutionary history, such as stinging cells with powerful toxins that help to disable their prey and drive off predators [35.3]. In addition to these chemical weapons present in the stinging cells, cnidarians also display other potent compounds that are useful for deterring predators and keeping competitors away [35.4–6]. For instance, tropical reefs are ecosystems with a vast biodiversity, where the substrate available for benthic cnidarian species to settle and develop is scarce [35.7]. Chemical interactions between different species are thus an important mechanism for inter-

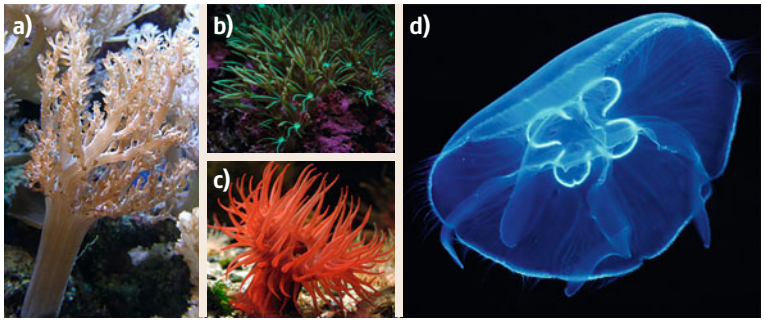

Fig. 35.1a–d Marine invertebrates from the phylum Cnidaria (all images by Ricardo Calado). **(a)** Soft coral (*Sinularia* sp.), **(b)** soft coral (*Briareum* sp.), **(c)** sea anemone (*Actinia equina* sp), **(d)** jellyfish (*Aurelia aurita*)

specific competition, which may have dramatic consequences for the organism being outcompeted in this *chemical war*. Therefore, organisms inhabiting highly biodiverse tropical areas, particularly coral reefs, have developed a large array of chemical compounds that have been the focus of recent bioprospecting efforts [35.8].

Corals form the structure and foundation of tropical coral reefs and are also important structural elements of some highly diverse deep-sea habitats. Other cnidarian species, such as anemones, are also very diverse and abundant in these tropical ecosystems [35.9]. These benthic cnidarians display a great variety of molecules that have different biological functions. The harsh chemical and physical environmental conditions of the areas inhabited by these organisms may have been important drivers for the production of a variety of molecules with unique structural features. For instance, the incidence of predation in the majority of these organisms is low due to the toxic compounds they produce to deter predators [35.10]. Other examples of biological functions of these compounds are defensive functions against pathogens, as well as against fouling organisms, herbivores, and microorganisms [35.11]. Such chemical compounds have been targeted by scientists searching for new chemical entities from the sea, usually known as marine natural products (MNP). Although more than 20 000 compounds have been discovered since the field of MNP began in the mid 1960s, only a very limited number have reached the end of the drug discovery pipeline [35.12].

Table 35.1 Classes and orders in the phylum Cnidaria (according to the classification proposed in the World Register of Marine Species (WoRMS)) [35.1]

Phylum	Class	Order
Cnidaria (≈ 11 287 species)	Anthozoa (≈ 7500 species)	Actiniaria; Zoanthidea Antipatharia; Alcyonacea Ceriantharia; Gorgonacea Corallimorpharia; Helioporacea Scleractinia; Pennatulacea
	Cubozoa (≈ 36 species)	Carybdeida; Chirodropida
	Hydrozoa (≈ 3500 species)	Anthoathecata Leptothecata; Limnomedusae Siphonophorae; Narcomedusae Actinulida; Trachymedusae
	Polypodiozoa (1 species)	Polypodiidea
	Scyphozoa (≈ 200 species)	Coronatae Rhizostomeae; Semaeostomeae
	Staurozoa (≈ 50 species)	Stauromedusae

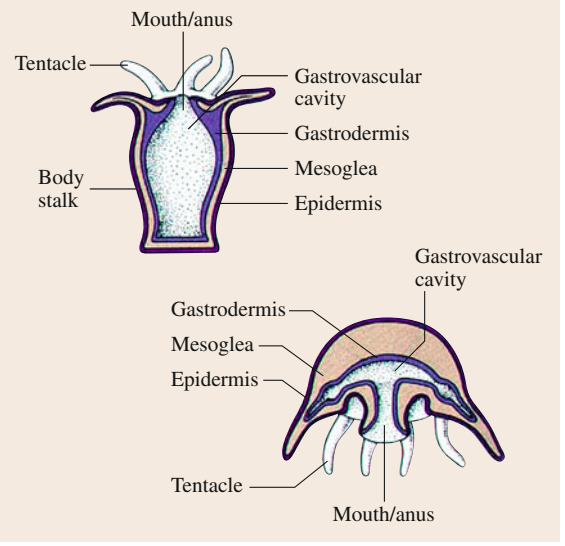

Fig. 35.2 Diagram of polyp and medusa forms of cnidarians

This chapter focuses the compounds that have been discovered from marine cnidarian species, providing a brief overview of this topic and discussing the role of biodiversity and biogeography in the chemical diversity of cnidarians. Cnidarian molecules discovered since the year 2000 that display important bioactivities and promising biotechnological applications are discussed in detail. The rationale for this approach is that the drug discovery pipeline, i. e., the time between discovering a new natural product and the commercialization of that marine drug, is a relatively long process that usually takes between 10–15 years. In this view, natural products discovered before 2000 had already had time to go through this pipeline, which means that their biotechnological potential is already fully exploited if the compound did not fail any step of the drug discovery process. Finally, we will discuss the challenges that we foresee for future research on bioactive compounds from cnidarians, as well as their biotechnological application to the industry.

35.1.1 Overview of Natural Product Discovery from Cnidarians

Research on marine natural products began in the 1950s [35.13], at a time when important breakthroughs in the taxonomy of marine animals took place [35.14]. This research field expanded during the 1970s and 1980s. It was only at the end of the 1980s and the beginning of the 1990s that an economically appealing activity started to take shape [35.6, 15]. Since the beginning of MNP, sponges (phylum Porifera) have been recognized as the most interesting group of marine invertebrates [35.16]. However, with growing bioprospecting efforts, and the screening of previously unexplored marine habitats and organisms, the biotechnological potential of other groups of marine invertebrates has also started to become appealing for researchers. The phylum Cnidaria is a large, diverse and ecologically important group of marine invertebrates, which is renowned for the ability to produce powerful toxins and venoms [35.17]. A total of 3244 marine natural products have been described from this phylum alone since 1990 (and until 2011), which shows the importance of cnidarians for marine natural product research. Since the early 1990s, the number of new compounds from marine cnidarians has been higher than the discovery of compounds from sponges [35.8], and the trend that we currently observe is still a continuous increase of natural product discovery (Fig. 35.3). This shows that bioprospecting efforts on these organisms have been continuously increasing.

Fig. 35.3 Number of new marine natural products from cnidarians discovered between 1990 and 2011

The quest for new MNP from cnidarians has benefited from a renaissance since 2005, namely due to the development of new methods in analytical technology, spectroscopy, and high-throughput screening [35.18]. It has also benefited from the failure to deliver new drug leads in significant numbers by competing technologies, such as chemical synthesis. These two different reasons may support the continuous growth of natural product discovery from cnidarians in the last decade.

Bioprospecting efforts have not been evenly distributed among cnidarian taxa. From the 3244 new compounds yielded by marine cnidarian species since 1990, 99% were discovered in within class Anthozoa. The remaining 1% is associated with species from class Hydrozoa. Anthozoans display a higher biodiversity, with a higher number of orders (Table 35.1). Nonetheless, 94% of the 3244 compounds were discovered in organisms from a single anthozoan order: the Alcyonacea. Only through the analysis of the taxonomic level below order, e.g., the family level, is it possible to observe a more even distribution of new compounds among taxa. Figure 35.4 shows the cumulative number of natural products discovered from alcyonaceans according to family level. It is important to emphasize the Alcyoniidae family due to the continuous increase of new compounds relative to other Aclyonacea families.

The overall increase of new compounds associated with different Cnidaria taxa is displayed in Table 35.2. Of the most representative families from the order Alcyonacea, only the family Briareidae showed a small decrease in the number of new compounds discovered in the last two decades. All other families represented in

Table 35.2 Number of new compounds discovered in the most representative taxa of the phylum Cnidaria in the 1990s and 2000s (after [35.8])

Taxon	New compounds in the 1990s	New compounds in the 2000s	Variation of new compounds per decade (%)
Phylum Cnidaria	1031	1773	+72%
Class Anthozoa	1017	1758	+73%
Sub-class Octocorallia	963	1715	+78%
Order Alcyonacea	934	1694	+84%
Family Alcyoniidae	293	489	+67%
Family Briareidae	158	156	−1%
Family Clavulariidae	41	150	+266%
Family Gorgoniidae	109	165	+51%
Family Nephtheidae	58	227	+291%
Family Plexauridae	97	99	+2%
Family Xeniidae	72	147	+107%

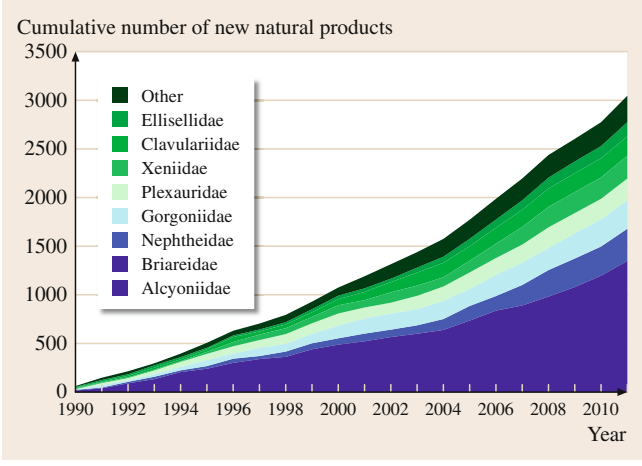

Fig. 35.4 Cumulative number of new marine natural products from cnidarians according to the taxonomical level *family*. The group *other* refers to the families Acanthogorgiidae, Anthothelidae, Coelogorgiidae, Isididae, Melithaeidae, Nidaliidae, Paragorgiidae, Paralcyoniidae, Primnoidae, Subergorgiidae, and Tubiporidae

Table 35.2 showed an increase, which in some particular cases was relatively high (e.g., families Clavulariidae and Neptheidae). These results recorded by *Leal* et al. [35.8] show that the popularity of cnidarians in bioprospecting efforts continues to increase, with large numbers of new compounds being discovered every year.

The high chemical diversity associated with cnidarians may be related to the high biodiversity displayed by this group. While about 11 000 cnidarian species are currently known [35.1], new compounds have only been recorded from 337 species (distributed over 117 genera). This means that only ≈ 3.1% of cnidarian biodiversity has yielded new chemical compounds. This does not necessarily mean that the remaining ≈ 97% of cnidarian species do not display any different compounds. Most likely, this is a result of the preference of scientists to search for new chemical entities in a relatively low number of species. For instance, the most popular species among the Alcyoniidae are *Clavularia viridis*, *Briareum excavatum*, and *Antillogorgia elisabethae* (Fig. 35.5), which have been important cnidarians in the history of MNP research [35.8]. Diversification of bioprospected species has been relatively low, as can be observed in Fig. 35.5. In this figure, we plot the number of new compounds discovered in cnidarian species since 1990 and sort that information according to the number of new compounds discovered in each species. The uneven result among bioprospected species is clearly observed. As most cnidarian species displaying a high number of new compounds inhabit tropical areas, this may suggest that bioprospecting efforts have been biased toward these particular species, probably driven by previous studies showing the high chemical diversity displayed by such taxa [35.8]. Although the assumption the all cnidarian species display similar chemical diversity is incorrect, Fig. 35.5 shows that a large number of new molecules associated to other cnidarians are yet to be unveiled. This is particularly evident if we consider that the compounds that are currently known were discovered from only ≈ 3% of total cnidarian biodiversity.

Another issue that should be noted is biodiscovery hotspots of new cnidarian compounds. Although

biogeography itself is a well-studied topic, its investigation in MNP research is still scarce. This is probably justified by the lack of precise geographical information on collection sites. However, recent studies already started to address this topic and clearly reveal bioprospecting efforts to be biased towards tropical areas [35.8, 19]. New molecules discovered from cnidarian species over the past decades have mostly resulted from bioprospecting on Asian territories close to tropical areas, particularly in Taiwan, Japan, and China. Remarkably, 50% of such new molecules discovered in cnidarians since 1990 resulted from organisms collected in the marine environment surrounding these two territories.

The compounds discovered in cnidarian species belong to various chemical groups, although the majority are terpenoids. *Leal* et al. [35.19] showed that in the last decade, 66% of the compounds discovered in cnidarians were terpenoids, which contrasts with the relatively low percentage of discovered alkaloids (10%), steroids (9%), aliphatic compounds (8%), and carbohydrates (6%). This data shows that particular chemical groups, such as terpenoids in the case of cnidarians, have been unquestionably more popular among researchers searching for new compounds. Terpenoids are secondary metabolites that are not directly involved in critical physiological processes. These compounds often play a role in interspecific and other ecological interactions. In terrestrial ecosystems, particularly in plants, terpenoids are known to be very abundant and structurally diverse [35.20]. Terpenoids are the chemical group that includes most natural products isolated so far from marine environments [35.21, 22]. This may be related to the large range of structural types that can be included in this group, which is, in part, associated with the fact that their biosynthetic unit can be rearranged and highly oxidized [35.14, 23]. Terpenoids also display a wide array of known bioactivities and biological functions [35.24–26]. Indeed, it was probably because previous studies showed that terpenoids usually display remarkable bioactivities that researchers would increase the chances for successful drug discovery and consequent patenting and commercialization by preferentially targeting molecules of this chemical group [35.27]. Several examples are already described

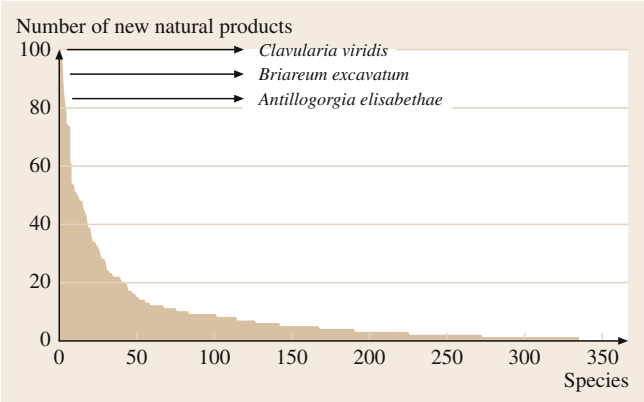

Fig. 35.5 Number of new natural products discovered per cnidarian species. Each bar corresponds to a single species (total of 337 species). The three species yielding the highest number of natural products are indicated

for the application of terpenoids in the pharmaceutical and food industry due to their potential and effectiveness as medicines and flavor enhancers [35.20, 27].

Besides bioprospecting efforts directed towards particular groups of organisms and chemical structures, researchers have also been narrowing their searches to particular molecules, with emphasis on the type and relevance of bioactivity displayed, in order to identify the most promising targets for their drug discovery pipelines [35.28]. These marine molecules display various types of biological activities, such as anticancer, anti-inflammatory, antitumor, antimalarial ones, etc. It is not surprising that over the past 40 years major advances in the discovery of marine drugs have been recorded in clinical trials for cancer [35.29]. Although there are several marine bioactive compounds in preclinical and clinical trials, only a relatively small number of molecules have reached this stage of the drug discovery pipeline. This process is very complex and encompasses several steps: target identification and validation, assay development, lead identification and optimization, predevelopment, preclinical development, clinical research phase I to phase III, regulatory approval, and phase IV (post-approval studies) [35.12, 18, 30]. The drug discovery pipeline usually takes 10–15 years from the first to the last step.

35.2 The Most Promising Marine Natural Products from Cnidaria

In this section, we overview the most promising marine bioactive compounds isolated from cnidarians in the twenty-first century. This information was assembled through the survey of the most relevant peer reviewed literature published during this period covering marine natural products [35.31–52]. Over 2000 molecules from cnidarians have been described in the twenty-first century. In order to address only those compounds displaying a high potential for industrial applications, it was decided to use the values of IC_{50} (half maximal inhibitory concentration) as guidelines. IC_{50} is a quantitative measure that indicates how much of a particular substance (inhibitor) is needed to inhibit a given biological process or component of a process by half. It is important to highlight that the NCI has renamed the IC_{50} to GI_{50} [35.53] in order to emphasize the correction for cell count at time zero in cancer cells; in this way, some results of this quantitative measure are now also presented under these directives. Additionally, ED_{50} (the median dose that produces the desired effect of a drug in half the test population) was also used to identify promising marine bioactive compounds produced by cnidarians. Only the compounds displaying an $IC_{50} \leq 10.0\,\mu g\,mL^{-1}$, or μM (except where stated otherwise), and $ED_{50} \leq 4.0\,\mu g\,mL^{-1}$ were considered in the present review, as these values are commonly used in the surveyed literature to ascertain relevant bioactivity (e.g., [35.54, 55]). In the few cases where neither IC_{50} nor ED_{50} values were described for an MNP in a study, that compound was selected to be part of the present survey only if either the authors of that study, or those citing it, clearly stated that the results recorded were highly promising for industrial applications. All species producing the compounds selected for the present topic were grouped into classes and orders of the phylum Cnidaria (Table 35.1) (according to the latest classification proposed in WoRMS) [35.1].

This approach allowed us to identify which taxonomic groups of cnidarians screened so far display the highest potential to yield new drugs or pharmacological products derived from marine bioactive compounds. Nonetheless, it is important to highlight that the identification of cnidarian species is a challenging task, and it is possible that some of the species (or even genera) referred to in the scientific literature may have been misidentified. In this way, it is of paramount importance that in future work, the authors addressing marine bioactive compounds produced by cnidarians provide a detailed description on how the target species was identified [35.28].

35.2.1 Class Anthozoa

The class Anthozoa currently includes 10 orders and over 7500 valid species (about $\frac{2}{3}$ of all known cnidarian species; Table 35.1). Within the Anthozoa class, the order Alcyonacea (soft corals and sea fans) is the one that has contributed with the highest number of promising bioactive marine compounds, although other orders, such as Actiniaria (sea anemones) and Scleractinia (hard corals), have also yielded relevant compounds [35.56–59].

The Order Alcyonacea (Soft Corals and Sea Fans)

Soft corals are generally brightly colored and rich in nutritionally important substances. However, the incidence of predation in the majority of these organisms is low due to the toxic compounds they produce to deter predators [35.60]. Several biosynthetic studies have been carried out on the metabolites of soft corals [35.61] and some of those compounds have already shown great potential for the development of new pharmaceuticals and antifoulants. Sea fans are also well-known sources of compounds exhibiting significant biological activity [35.62]. Table 35.3 summarizes the most promising compounds from the order Alcyonacea (class Anthozoa).

Soft corals are rich sources of secondary metabolites such as diterpenes, sesquiterpenes, furanoditerpenes, terpenoids, capnellene, and steroids (e.g., *Lobophytum*, *Sinularia*, *Sarcophyton* [35.141], *Capnella* [35.142], and *Dendronephthya* [35.143]), that have been shown to display HIV-inhibitory [35.73], cytotoxic [35.144, 145], anti-inflammatory [35.146, 147], anticancer [35.148, 149], and antimicrobial activity [35.150], as well as cardiac and vascular responses [35.151].

Chemical investigations on octocorals belonging to the genus *Cladiella* have resulted in a series of interesting diterpenoids. Terpenoids have been found to display complex structures and various bioactivities [35.63], especially anti-inflammatory properties. *Cladiella australis* produces an anti-inflammatory natural product, austrasulfone, which was found to

Table 35.3 The most promising compounds studied in the twenty-first century from cnidarian species in the order Alcyonacea (soft corals), class Anthozoa

Family and species	Drug class	Compound	Chemistry	Country	Reference
Alcyoniidae					
Cladiella sp.	Anti-inflammatory	Cladielloide B	Diterpenoid	TAIW	[35.63]
Cladiella sp.	Antitumor	Cladieunicellin B and E	Diterpenoid	ID	[35.64]
Cladiella sp.	Anti-inflammatory	Cladieunicellin C	Diterpenoid	ID	[35.64]
Cladiella australis	Anti-inflammatory	Austrasulfone	Sulfone	TAIW	[35.65]
Cladiella hirsuta	Anti-inflammatory	Hirsutalins B–D and H	Diterpenoid	TAIW	[35.66]
Klyxum simplex	Anti-inflammatory	Simplexin E	Diterpenoid	TAIW	[35.67]
Klyxum simplex	Antitumor	Klysimplexin B and H	Diterpenoid	TAIW	[35.68]
Klyxum simplex	Anti-inflammatory	Klysimplexin sulfoxide A–C	Diterpenoid	TAIW	[35.69]
Klyxum simplex	Anti-inflammatory	Klysimplexin J–N, R and S	Diterpenoid	TAIW	[35.70]
Klyxum molle	Anti-inflammatory	Klymollin F and G	Diterpenoid	TAIW	[35.71]
Lobophytum sp.	Antitumor	Lobophytene	Diterpenoid	VN	[35.72]
Lobophytum sp.	Anti-HIV	Lobohedleolide	Diterpenoid	PHL	[35.73]
Lobophytum sp.	Anti-HIV	(7Z)-lobohedleolide,	Diterpenoid	PHL	[35.73]
Lobophytum sp.	Anti-HIV	17-dimethylamino lobohedleolide	Diterpenoid	PHL	[35.73]
Lobophytum crassum	Anti-inflammatory	Crassumolides A and C	Terpenoid	TAIW	[35.74]
Lobophytum crassum	Antitumor	13-acetoxysarcophytoxide	Cembranoid	TAIW	[35.75]
Lobophytum crassum	Anti-inflammatory	Lobocrassin B	Cembranoid	TAIW	[35.76]
Lobophytum crassum	Antitumor	Lobocrassin B	Cembranoid	TAIW	[35.76]
Lobophytum crassum	Antitumor	Culobophylin A and B	Cembranoid	TAIW	[35.77]
Lobophytum cristagalli	Antitumor	Cembranolide diterpene	Diterpenoid	RSC	[35.78]
Lobophytum durum	Anti-inflammatory	Durumolides A–C	Terpenoid	TAIW	[35.79]
Lobophytum durum	Anti-inflammatory	Durumhemiketalolide A–C	Cembranoid	TAIW	[35.80]
Lobophytum durum	Antitumor	Durumolide P	Cembranoid	TAIW	[35.81]
Lobophytum durum	Antiviral	Durumolide Q	Cembranoid	TAIW	[35.81]
Lobophytum laevigatum	Antitumor	Lobophytosterol	Steroid	TAIW	[35.82]
Lobophytum pauciflorum	Anti-inflammatory	Lobophytone Z	Cembranoid	TAIW	[35.83]
Sarcophyton crassocaule	Antitumor	Crassocolides H–M	Cembranoid	TAIW	[35.84]
Sarcophyton crassocaule	Antitumor	Crassocolide N–P	Cembranoid	TAIW	[35.85]
Sarcophyton crassocaule	Anti-inflammatory	Sarcocrassocolide F–L	Cembranoid	TAIW	[35.86]
Sinularia sp.	Antiulcer	Sinulide	Spermine		[35.87]
Sinularia sp.	Antimicrobial	Lipids	Polyketide	RUS	[35.88]
Sinularia capillosa	Antitumor	Capilloquinol	Farnesyl quinoid	TAIW	[35.89]
Sinularia flexibilis	Antitumor	Flexilarin D	Cembranoid	TAIW	[35.90]
Sinularia flexibilis	Antifoulant	11-episinulariolide	Diterpenoid	AUS	[35.91]
Sinularia gibberosa	Anti-inflammatory	Gibberoketosterol	Steroid	TAIW	[35.92]
Sinularia querciformis	Anti-inflammatory	Querciformolide C	Terpenoid	TAIW	[35.93]
Briareidae					
Briareum sp.	Antitumor	Brialalepolide B and C	Diterpenoid	VUT	[35.94]
Briareum sp.	Anti-inflammatory	Brialalepolide B and C	Diterpenoid	VUT	[35.94]
Briareum asbestinum	Antimalarial	Briarellin D, K and L	Diterpenoid	PAN, USA	[35.95]
Briareum excavata	Anti-inflammatory	Briaexcavatin E	Diterpenoid	TAIW	[35.96]
Briareum excavata	Antitumor	Briaexcavatolides L and P	Diterpenoid	TAIW	[35.97]

Table 35.3 (continued)

Family and species	Drug class	Compound	Chemistry	Country	Reference
Clavulariidae					
Clavularia sp.	Nervous system	Stolonidiol	Diterpenoid	JPN	[35.98]
Clavularia koellikeri	Antitumor	Cembrane-type diterpenoid	Diterpenoid	JPN	[35.99]
Clavularia viridis	Antitumor	Claviridic acid	Prostanoid	TAIW	[35.100]
Clavularia viridis	Antitumor	Clavulones	Prostanoid	TAIW	[35.100]
Clavularia viridis	Antitumor	Claviridenone	Prostanoid	TAIW	[35.55]
Clavularia viridis	Antitumor	Halogenated prostanoids	Prostanoid	JPN	[35.101]
Clavularia viridis	Antitumor	Bromovulone III	Prostanoid	TAIW	[35.102, 103]
Clavularia viridis	Antitumor	Yonarasterols	Steroid	JPN	[35.102]
Clavularia viridis	Antitumor	Stoloniferone E	Steroid	TAIW	[35.55]
Clavularia viridis	Antitumor	Claviridin A-D	Prostanoid	TAIW	[35.103]
Carijoa sp.	Anti-inflammatory	carijoside A	steroid	TAIW	[35.104]
Telesto riisei	Antitumor	Punaglandins	Prostaglandin	USA	[35.105]
Ellisellidae					
Junceella fragilis	Anti-inflammatory	Frajunolides B and C	Terpenoid	TAIW	[35.106]
Junceella juncea	Antifoulant	Juncin ZII	Diterpenoid	TAIW	[35.107]
Gorgoniidae					
Antillogorgia acerosa	Antitumor	Bis(pseudopterane) amine	Dialkylamine	BHS	[35.108]
Antillogorgia bipinnata	Antituberculosis	Bipinnapterolide B	Terpenoid	USA	[35.109]
Antillogorgia bipinnata	Antimalarial	Caucanolide A and D	Diterpenoid	COL, PAN, USA	[35.110]
Antillogorgia elisabethae	Antimicrobial	Pseudopterosin X	Diterpenoid	USA	[35.111]
Antillogorgia elisabethae	Antituberculosis	Ileabethoxazole	Diterpenoid	USA	[35.112]
Antillogorgia elisabethae	Antituberculosis	Homopseudopteroxazole	Diterpenoid	USA	[35.113]
Antillogorgia elisabethae	Antituberculosis	Caribenols A and B	Terpenoid	USA	[35.114]
Antillogorgia elisabethae	Antituberculosis	Elisapterosin B	Diterpenoid	USA	[35.115]
Antillogorgia elisabethae	Antimalarial	Aberrarone	Diterpenoid	COL	[35.116]
Antillogorgia kallos	Antimalarial	Bielschowskysin	Diterpenoid	PAN, USA	[35.117]
Antillogorgia kallos	Antitumor	Bielschowskysin	Diterpenoid	PAN, USA	[35.117]
Antillogorgia rigida	Antimicrobial	Curcuphenol	Terpenoid	USA	[35.118]
Leptogorgia setacea	Antifoulant	Homarine	Pyridine	GEO	[35.119]
Leptogorgia virgulata	Antifoulant	Homarine	Pyridine	GEO	[35.119]
Leptogorgia virgulata	Antifoulant	Pukalide	Diterpenoid	USA	[35.120]
Leptogorgia virgulata	Antifoulant	Epoxypukalide	Diterpenoid	USA	[35.120]
Pseudopterogorgia sp.	Antitumor	Secosterols	Steroid	USA	[35.121]
Pseudopterogorgia sp.	Anti-inflammatory	Secosterols	Steroid	USA	[35.121]
Isididae					
Isis hippuris	Antitumor	Polyoxygenated gorgosterol (2-4)	Steroid	JPN	[35.122]
Isis hippuris	Antitumor	Polyoxygenated steroid (3)	Steroid	TAIW	[35.123]
Isis hippuris	Antitumor	Suberosenol B	Terpenoid	TAIW	[35.124]
Isis hippuris	Antitumor	Polyoxygenated steroid	Steroid	IND	[35.125, 126]
Isis hippuris	Antitumor	A –nor-hippuristanol	Steroid	TAIW	[35.127]
Isis hippuris	Antitumor	Isishippuric acid B	Steroid	TAIW	[35.127]

Table 35.3 (continued)

Family and species	Drug class	Compound	Chemistry	Country	Reference
Nephtheidae					
Dendronephthya sp.	Antifoulant	Isogosterones A–D	Steroid	JPN	[35.128]
Dendronephthya rubeola	Antitumor	Capnell-9(12)-ene-8β,10α-diol	Sesquiterpenoid	DE	[35.129, 130]
Lemnalia flava	Anti-inflammatory	Flavalin A and B	Sesquiterpenoid	TAIW	[35.125]
Nephthea chabroli	Antitumor	Chabranol	Terpenoid	TAIW	[35.126]
Nephthea erecta	Anti-inflammatory	Ergostanoids 1 and 3	Ergostanoid	TAIW	[35.131]
Paralemnalia thyrsoides	Anti-inflammatory	Paralemnolin Q and S	Sesquiterpenoid	TAIW	[35.132]
Plexauridae					
Astrogorgia sp.	Antitumor	Astrogorgol F	Secosteroid	TAIW	[35.129]
Echinogorgia pseudosassapo	Antifoulant	3β-methoxyguaian-10(14)-en-2β-ol	Sesquiterpenoid	TAIW	[35.133]
Eunicea sp.	Antimalarial	Sesquiterpenoids	Sesquiterpenoid	COL, PAN, USA	[35.130]
Eunicea sp.	Antimalarial	Dolabellane	Diterpenoid	COL	[35.134]
Eunicea fusca	Anti-inflammatory	Fuscisides	Diterpenoid	USA	[35.135]
Eunicea fusca	Anti-inflammatory	Fuscoside E	Diterpenoid	COL	[35.136]
Euplexaura flava	Anti-inflammatory	Butenolide	Lipid	JPN	[35.137]
Xeniidae					
Asterospicularia laurae	Antitumor	Asterolaurin A	Diterpenoid	TAIW	[35.138]
Cespitularia hypotentaculata	Antitumor	Cespitularin C	Diterpenoid	TAIW	[35.139]
Xenia novaebritanniae	Antibacterial	Xeniolide I	Diterpenoid	ISR	[35.140]
Xenia plicata	Antitumor	Blumiolide C	Diterpenoid	TAIW	[35.54]

exhibit potent neuroprotective effect against the 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in neuroblastoma SH-SY5Y, a human dopaminergic neuron often used for the study of Parkinson's disease. The cytotoxicity of 6-OHDA on SH-SY5Y cells was effectively and dose-dependently inhibited by pretreatment at concentrations of 10^{-3}–10 μM. The ED_{50} was 0.011 ± 0.014 μM [35.65]. Hirsutalins B-D and H, diterpenoids from *Cladiella hirsuta*, at a concentration of 10 μM, displayed significant in vitro anti-inflammatory activity in LPS (LPS)-stimulated RAW 264.7 macrophage cells by inhibiting the expression of the inducible nitric oxide synthase (iNOS), with hirsutalin B also effectively reducing the level of COX-2 (COX) protein. These compounds, in particular hirsutalin B and D, could be promising anti-inflammatory agents [35.66]. Cladielloide B and cladieunicellin C, diterpenoids from a non-identified *Cladiella*, displayed significant inhibitory effects on superoxide anion generation (IC_{50} 5.9 ± 0.7 and IC_{50} 8.1 ± 0.3 μg mL^{-1}, respectively). The first compound also showed significant inhibitory effects against elastase release (IC_{50} 6.5 ± 1.9 μg mL^{-1}) by human neutrophils at 10 μg mL^{-1} [35.63, 64]. The same species that produces cladieunicellin C also produces cladieunicellin B and E, two diterpenoids that exhibited significant cytotoxicity against human colorectal adenocarcinoma (DLD-1, IC_{50} 2.0 μg mL^{-1}) and human promyelocytic leukemia (HL-60, IC_{50} 2.7 μg mL^{-1}) cells, respectively [35.64].

Soft corals of the family Nephtheidae are known for their content of sesquiterpenes and particularly capnellenes [35.41]. Some sesquiterpenes isolated from *Capnella imbricate* [35.142, 152–154] showed anti-inflammatory activity and a dihydroxycapnellene (capnell-9(12)-ene-8β, 10α-diol) from *Dendronephthya rubeola* demonstrated antiproliferative activity against murine fibroblasts cell line (L-929, GI_{50} 6.8 μM L^{-1}) and cytotoxicity against cancer cell lines implicated in human leukemia (K-562, IC_{50} 0.7 μM) and human cervix carcinoma (HeLa, IC_{50} 7.6 μM) [35.143]. Capnell-9(12)-ene-8β, 10α-diol strongly inhibits the interaction of the oncogenic transcription factor Myc with its partner pro-

tein Max [35.155, 156], making it a therapeutically interesting compound in oncology [35.143]. *Nephthea chabroli* also produces a nor-sesquiterpene compound named chabranol, which displays moderate cytotoxicity against mouse lymphocytic leukemia cells (P-388) with an ED_{50} 1.81 $\mu g\,mL^{-1}$ [35.126]. *Nephthea erecta* produces two proteins in mediated inflammatory responses, the oxygenated ergostanoids 1 and 3. At a concentration of 10 μM, these two compounds significantly reduced the levels of the iNOS (45.8 ± 9.9 and $33.6 \pm 20.6\%$, respectively) and COX-2 protein (68.1 ± 2.3 and $10.3 \pm 6.2\%$, respectively), when compared with the control cells stimulated with lipopolysaccharides (LPS) [35.131]. Soft corals of the genus *Paralemnalia* and *Lemnalia* have been found to be rich sources of sesquiterpenoids of nardosinane-type. *Lemnalia flava* produces flavin A, a sesquiterpenoid, that showed significant in vitro anti-inflammatory activity by exhibiting concentration-dependent inhibition of LPS-induced iNOS and COX-2 protein expression (ED_{50} values toward both proteins were $4.8 \pm 0.3\,\mu g\,mL^{-1}$ ($20.5 \pm 1.3\,\mu M$) and $6.2 \pm 0.6\,\mu g\,mL^{-1}$ ($26.5 \pm 2.6\,\mu M$), respectively). This compound and flavin B also exhibited significant neuroprotective activity [35.125]. This neuroprotective activity using 6-OHDA-induced neurotoxicity in neuroblastoma SHSY5Y, a human dopaminergic neuron often used for study of Parkinson's disease, was also demonstrated for paralemnolins Q and S [35.132, 157] and 2-deoxy-7-*O*-methyllemnacarnol [35.157] from *Paralemnalia thyrsoides*. Nonetheless further investigation for their therapeutic potential against neurodegenerative diseases is suggested.

Species in the genus *Xenia* (family Xeniidae) are a rich source of diterpenoids. Xeniolides I, isolated from *Xenia novaebrittanniae* demonstrated antibacterial activity at a concentration of 1.25 $mg\,mL^{-1}$ in *Escherichia coli* ATCC and *Bacillus subtilis* [35.140]. Blumiolide C, a diterpenoid from the *Xenia blumi* (presently accepted as *Xenia plicata*), exhibited potent cytotoxicity against mouse lymphocytic leukemia (P-388, ED_{50} 0.2 $\mu g\,mL^{-1}$) and human colon adenocarcinoma (HT-29, ED_{50} 0.5 $\mu g\,mL^{-1}$) cells [35.54].

Polyoxygenated cembranoids, crassocolides H–P [35.84, 85] from *Sarcophyton crassocaule*, demonstrated cytotoxicity against cancer cell lines of human medulloblastoma (Daoy cells). Crassocolides I, M, and P were found to be more active (IC_{50} 0.8, 1.1, and 1.9 $\mu g\,mL^{-1}$, respectively). Crassocolide H and N inhibited the growth of human oral epidermoid carcinoma (KB) cells (IC_{50} 5.3 and 4.7 $\mu g\,mL^{-1}$, respectively), and crassocolide N and crassocolide L were also active against human cervical epitheloid carcinoma (HeLa) cells (IC_{50} 4.7 and 8.0 $\mu g\,mL^{-1}$, respectively) [35.84, 85]. Other cembrenoids from the same species, sarcocrassocolides F–L, were found to exhibit anti-inflammatory activities by significantly reducing the levels of iNOS protein. Furthermore, sarcocrassocolide I could also effectively reduce COX-2 expression with LPS treatment. All these compounds might be useful anti-inflammatory agents, sarcocrassocolide I being a promising anti-inflammatory lead compound [35.86]. Lobophytone Z, from *Lobophytum pauciflorum*, inhibits NO production in mouse peritoneal macrophages induced by LPS (IC_{50} 2.6 μM) [35.83]. NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological and cellular processes. Overproduction of NO is associated with various human diseases, including inflammatory and neuronal disorders [35.158]. The level of NO released may reflect the degree of inflammation and provides an indicator to assess inflammatory processes [35.83].

Lobophytum durum and *Lobophytum crassum* produce durumolides A–C [35.79], durumhemiketalolide A–C [35.80], and crassumolides A and C [35.74], with anti-inflammatory effects. They have been shown to inhibit up-regulation of the proinflammatory iNOS and COX-2 proteins in LPS-stimulated murine macrophage cells at $IC_{50} < 10\,\mu M$ [35.74, 79]. From *Lobophytum crassum*, lobocrassin B displayed significant inhibitory effects on the generation of superoxide anion and the release of elastase by human neutrophils (IC_{50} 4.8 and 4.9 $\mu g\,mL^{-1}$, respectively). Cytotoxicity of this cembrenoid toward tumor cells showed that it exhibited only modest cytotoxicity against human erythromyeloblastoid leukemia (K562), human T-cell acute lymphoblastic leukemia (CCRF-CEM), human acute lymphoblastic leukemia (Molt4), and human hepatocellular liver carcinoma (HepG2) cells [35.76].

Another example of a potential new therapeutic anticancer agent is a cembranolide diterpene from *Lobophytum cristagalli*, which has shown a potent inhibitory activity (IC_{50} 0.15 μM) [35.78] over farnesyl protein transferase (FPT), an important protein in signal transduction and regulation of cell differentiation and proliferation [35.159]). This type of FPT inhibition enhanced interest in this group of metabolites [35.141]. Other species of this genus also showed cembranoids with significant cytotoxic activity against human lung adenocarcinoma (A549, lobophytene [35.72], lobophytosterol [35.82], and 13-

acetoxysarcophytoxide [35.75]), human colon adenocarcinoma (HT-29, lobophytene [35.72]; DLD-1, culobophylin A [35.77], and HCT-116 lobophytosterol [35.82]), and human promyelocytic leukemia (HL60, lobophytosterol [35.82], and culobophylin A and B [35.77]) cell lines. The diterpenoids, lobohedleolide, (7*Z*)-lobohedleolide, and 17-dimethylaminolobohedleolide were isolated from the aqueous extract of *Lobophytum* species and exhibited moderate HIV-inhibitory activity (IC_{50} approximately $7-10\,\mu g\,mL^{-1}$) in a cell-based in vitro anti-HIV assay [35.73]. Additionally, other significant antiviral activity against human cytomegalovirus (IC_{50} of $5.2\,\mu g\,mL^{-1}$) [35.81] was described for the compound durumolide Q produced by *Lobophytum durum*.

Eunicellin-based diterpenoids are secondary metabolites often isolated from the genus *Klyxum*. *Klyxum molle* has been shown to produce molecules (e.g., klymollin F and G) with interesting bioactivities, such as anti-inflammatory agents [35.71]. *Klyxum simplex* also produces diterpene compounds, such as simplexin E, klysimplexins J–N, R, and S, and klysimplexin sulfoxide A–C, which at a concentration of $10\,\mu M$ were found to considerably reduce the levels of iNOS protein. The compounds simplexin E, klysimplexins R and S, and klysimplexin sulfoxide C could also effectively reduce the level of COX-2 protein. These results have shown that this compound significantly inhibits the accumulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated RAW 264.7 macrophage cells, being potential anti-inflammatory agents [35.67, 69, 70]. This species also produces two diterpenes, klysimplexins B and H, which exhibit moderate cytotoxicity towards human carcinoma cell lines. Klysimplexin B exhibits cytotoxicity toward human hepatocellular carcinoma (Hep G2, IC_{50} $3.0\,\mu g\,mL^{-1}$, and Hep 3B, IC_{50} $3.6\,\mu g\,mL^{-1}$), human breast carcinoma (MDA-MB-231, IC_{50} $6.9\,\mu g\,mL^{-1}$ and MCF-7, IC_{50} $3.0\,\mu g\,mL^{-1}$), human lung carcinoma (A549, IC_{50} $2.0\,\mu g\,mL^{-1}$) ,and human gingival carcinoma (Ca9-22, IC_{50} $1.8\,\mu g\,mL^{-1}$) cell lines. Metabolite klysimplexin H demonstrated cytotoxicity toward human hepatocellular carcinoma (Hep G2, IC_{50} $5.6\,\mu g\,mL^{-1}$, and Hep 3B, IC_{50} $6.9\,\mu g\,mL^{-1}$), human breast carcinoma (MDA-MB-231, IC_{50} $4.4\,\mu g\,mL^{-1}$, and MCF-7, IC_{50} $5.6\,\mu g\,mL^{-1}$), human lung carcinoma (A549, IC_{50} $2.8\,\mu g\,mL^{-1}$), and human gingival carcinoma (Ca9-22, IC_{50} $6.1\,\mu g\,mL^{-1}$) cell lines [35.68].

A tetraprenylated spermine derivative – sinulamide – has been isolated in *Sinularia* sp., which revealed an H,K-ATPase inhibitory activity. H,K-ATPase is a gastric proton pump of the stomach and is the enzyme primarily responsible for the acidification of the stomach contents. Its inhibition is a very common clinical intervention used in diseases including dyspepsia, peptic ulcer, and gastroesophageal reflux (GORD/GERD). Sinulide is a potential antiulcer drug, as it inhibits production of gastric acid by H,K-ATPase (IC_{50} $5.5\,\mu M$) [35.87]. Although it has been chemically synthesized [35.160], no clinical trials seem to have been reported. The steroid gibberoketosterol [35.92], isolated from *Sinularia gibberosa*, and the diterpenoid querciformolide C [35.93] isolated from *Sinularia querciformis*, showed significant inhibition of the up-regulation of the pro-inflammatory iNOS and COX-2 proteins in LPS-stimulated murine macrophages at a concentration of $< 10\,\mu M$ [35.92, 93]. *Paralemnalia thyrsoides* showed significant inhibition of pro-inflammatory iNOS protein expression (70% at IC_{50} $10\,\mu M$) [35.157]. *Sinularia* species produce significant bioactive molecules. Lipids from *Sinularia grandilobata* and another unspecified species of *Sinularia* possess antibacterial and antifungal activity [35.88]. The diterpene 11-episinulariolide from *Sinularia flexibilis* is an interesting antifoulant exhibiting strong algacidal properties [35.91]. This species also produces cembrenoids, named flexilarins, which evidenced cytotoxic activity in cancer cell lines. Flexilarin D exhibited potent cytotoxicity in human hepatocarcinoma (Hep2) cells with IC_{50} $0.07\,\mu g\,mL^{-1}$ and moderate cytotoxic activity against human cervical epitheloid carcinoma (HeLa, IC_{50} $0.41\,\mu g\,mL^{-1}$), human medulloblastoma (Daoy, $1.24\,\mu g\,mL^{-1}$), and human breast carcinoma (MCF-7, $1.24\,\mu g\,mL^{-1}$) cell lines [35.90]. Capilloquinol from *Sinularia capillosa* displayed cytotoxicity against P-388, with an ED_{50} of $3.8\,\mu g\,mL^{-1}$ [35.89].

Antifouling agents from natural sources are of increasing interest since the International Maritime Organization (IMO) banned the use of certain antifouling agents, such as tri-*n*-butyltin (TBT), due to the ecological impacts of these biocides in the marine environment. Several studies have demonstrated that soft corals can yield large quantities of promising antifouling metabolites [35.161, 162]. In fact, 17.95% of potential antifouling natural compounds are from cnidarians (e.g., soft corals) [35.163]. One of the most promising natural antifouling agents identified so far is an isogosterone isolated from an unspecified *Dendronephthya* [35.128]. Also 3β-methoxyguaian-10(14)-en-2β-ol, a sesquiterpene from the gorgonian *Echinogorgia pseudossapo*, was evaluated for its antilarval activity against *Am-*

phibalanus amphitrite and *Bugula neritina* larvae. The results showed that this compound had significant antilarval activity towards *Amphibalanus amphitrite* larvae with an EC50 value of 17.2 $\mu g\,mL^{-1}$ (68.2 μM), and showed 50% inhibition towards the settlement of *Bugula neritina* larvae at concentration of 25 $\mu g\,mL^{-1}$. This EC_{50} value is lower than the standard requirement of an EC_{50} of 25 $\mu g\,mL^{-1}$ established by the US Navy program as an efficacy level for natural antifoulants, indicating that 3β-methoxyguaian-10(14)-en-2β-ol is a potential natural antifouling agent [35.133].

Most of the bioactive substances from the family Clavulariidae with promising biotechnological potential are antitumor molecules from the genus *Clavularia*. Even so, *Carijoa* sp. produces a sterol glycoside, carijoside A, that displayed significant inhibitory effects on superoxide anion generation (IC_{50} 1.8 $\mu g\,mL^{-1}$) and elastase release (IC_{50} 6.8 $\mu g\,mL^{-1}$) by human neutrophils in anti-inflammatory activity testing [35.104]. As was previously mentioned, genus *Clavularia* contains promising secondary metabolites with unique structures and remarkable biological activities. Some of the species in this genus produce prostanoids (icosanoids) [35.55, 101, 103, 164–166], steroids [35.102], and diterpenoids [35.99, 167]. The bioactive marine diterpene, stolonidiol, isolated from an unidentified *Clavularia*, showed potent choline acetyltransferase (ChAT) inducible activity in primary cultured basal forebrain cells and clonal septal SN49 cells, suggesting that it may act as a potent neurotrophic factor-like agent on the cholinergic nervous system [35.98]. Cholinergic neurons in the basal forebrain innervate the cortex and hippocampus, and their function may be closely related to cognitive function and memory. The degeneration of neuronal cells in this brain region is considered to be responsible for several types of dementia, including Alzheimer's disease. One of the neurotransmitters, acetylcholine, is synthesized from acetyl coenzyme A and choline by the action of ChAT. Therefore, induction of ChAT activity in cholinergic neurons may improve the cognitive function in diseases exhibiting cholinergic deficits [35.168–170].

Prostanoids (claviridic acid) isolated from *Clavularia viridis* exhibited potent inhibitory effects on phytohemagglutinin-induced proliferation of peripheral blood mononuclear cells (PBMC, 5 $\mu g\,mL^{-1}$), as well as significant cytotoxic activity against human gastric cancer cells (AGS, IC_{50} 1.73–7.78 $\mu g\,mL^{-1}$) [35.100]. Claviridenone extracts also showed potent cytotoxicity against mouse lymphocytic leukemia (P-388) and human colon adenocarcinoma (HT-29), and exceptionally powerful cytotoxicity against human lung adenocarcinoma (A549) cells, with ED_{50} between 0.52 pg/mL and 1.22 $\mu g\,mL^{-1}$ [35.55]. Claviridins A–D exhibited potent cytotoxicity against four human cancer cell lines: Hep2 (ED_{50} 0.19–0.35 $\mu g\,mL^{-1}$), Doay (ED_{50} 0.18–0.29 $\mu g\,mL^{-1}$), colon adenocarcinoma (WiDr, ED_{50} 0.22–0.34 $\mu g\,mL^{-1}$), and HeLa (ED_{50} 0.31–0.88 $\mu g\,mL^{-1}$) [35.103]. Halogenated prostanoids also showed cytotoxic activity against human T lymphocyte leukemia cells (MOLT-4, IC_{50} 0.52 $\mu g\,mL^{-1}$), human colorectal adenocarcinoma (DLD-1, IC_{50} 0.6 $\mu g\,mL^{-1}$), and human diploid lung fibroblast (IMR-90, IC_{50} 4.5 $\mu g\,mL^{-1}$) cells [35.101]. The cyclopentenone prostanoid, bromovulone III is a promising marine natural compound for the treatment of prostate, colon, and hepatocellular carcinoma, which showed antitumor activity against human prostate (PC-3) and human colon (HT29) cancer cells at an IC_{50} of 0.5 μM [35.164], and induced apoptotic signaling in a sequential manner in Hep3B cells [35.171]. In the case of prostate cancer cells, this compound displayed an antitumor activity 30–100 times more effective than cyclopentenone prostaglandins (known to suppress tumor cell growth and to induce apoptosis in prostate cancer cells), by causing a rapid redistribution and clustering of Fas (member of the tumor necrosis factor (TNF) receptor superfamily). Apoptotic stimulation of Fas by specific ligand or antibodies caused the formation of a membrane-associated complex comprising Fas clustering) in PC-3 cells [35.172]. *Clavularia viridis* also produces steroids that show cytotoxic activity against human colorectal adenocarcinoma (DLD-1, $0.02 < IC_{50} < 50\,\mu g\,mL^{-1}$) and also against human T lymphocyte leukemia cells (MOLT-4, $0.01 < IC_{50} < 10\,\mu g\,mL^{-1}$) in the case of yonarasterols [35.102]. Additionally, stoloniferone displayed potent cytotoxicity against mouse lymphocytic leukemia (P-388), human colon adenocarcinoma (HT-29), and human lung adenocarcinoma (A549) cells [35.55]. This species produces several compounds with antitumor activity in different types of human tumors, although more in vitro studies are needed to determine which compounds are potential anticancer agents. *Clavularia koellikeri* produces diterpenoids as secondary metabolites, which display cytotoxic activity against human colorectal adenocarcinoma (DLD-1, IC_{50} 4.2 $\mu g\,mL^{-1}$) and strong growth inhibition against human T lymphocyte leukemia cells (MOLT-4, IC_{50} 0.9 $\mu g\,mL^{-1}$) [35.99].

In the genus *Cespitularia*, several interesting diterpenes of cembrane and neodolabellane skeletons have

been identified. In *Cespitularia hypotentaculata* (family Xeniidae) a significant production of diterpenoids was detected. Cespitularin C exhibited potent cytotoxicity against mouse lymphocytic leukemia (P-388, ED_{50} 0.01 $\mu g\,mL^{-1}$) and human lung adenocarcinoma (A549, ED_{50} 0.12 $\mu g\,mL^{-1}$) cells, while cespitularin E exhibited potent cytotoxicity against human lung adenocarcinoma (A549, ED_{50} 0.034 $\mu g\,mL^{-1}$) cell cultures [35.139]. A less active diterpene, Asterolaurin A, from *Asterospicularia laurae* (a species from the same family) exhibited cytotoxicity against human hepatocellular carcinoma (HepG2) cells with an IC_{50} of 8.9 μM [35.138].

Telesto riisei produces punaglandins, highly functional cyclopentadienone and cyclopentenone prostaglandins. Cyclopentenone prostaglandins have unique antineoplastic activity and are potent growth inhibitors in a variety of cultured cells. These punaglandins have been shown to inhibit P53 accumulation (a tumor suppressor protein) and ubiquitin isopeptidase activity (IC_{50} between 0.04 and 0.37 μM) (enzyme involved in protein degradation system) in vitro and in vivo [35.105]. Since these proteasome inhibitors exhibit higher antiproliferative effects than other prostaglandins [35.173], they may represent a new class of potent cancer therapeutics.

Sea fans are well-known sources of compounds exhibiting significant biological activity [35.62]. Studies on *Isis hippuris* resulted in the isolation of a series of novel metabolites such as sesquiterpenes [35.124], steroids [35.174], A-nor-hippuristanol [35.127], and isishippuric acid B [35.127]. These compounds exhibit potent cytotoxicity against cancer cell lines of human hepatocellular carcinoma (HepG2 and Hep3B, IC_{50} 0.08–4.64 $\mu g\,mL^{-1}$, and 0.10–1.46 $\mu g\,mL^{-1}$, respectively) [35.127, 175], human breast carcinoma (MCF-7, IC_{50} 0.20–4.54 $\mu g\,mL^{-1}$ and MDA-MB-231, IC_{50} 0.13–2.64 $\mu g\,mL^{-1}$) [35.175], mouse lymphocytic leukemia (P-388), human lung adenocarcinoma (A549), and human colon adenocarcinoma (HT-29) with ED_{50} of values less than 0.1 $\mu g\,mL^{-1}$ [35.127, 174] and IC_{50} of 0.1 $\mu g\,mL^{-1}$ [35.124]. Polyoxygenated steroids were also isolated from this species and showed moderate cytotoxicity against cultured NBT-T2 rat bladder epithelial cells (IC_{50} between 1.8 and 7.5 $\mu g\,mL^{-1}$) [35.122], P-388 and A549 cell lines (ED_{50} 3.2 and 3.86 $\mu g\,mL^{-1}$, respectively), and inhibitory activity against HCMV (EC_{50} 2.0 $\mu g\,mL^{-1}$) [35.123].

Species from the genus *Pseudopterogorgia* (currently accepted as *Antillogorgia* sp.) are a rich source of unusual biologically active diterpenoids, sesquiterpenes, and polyhydroxylated steroids, which exhibit diverse structures [35.109, 176, 177]. A sample of the organic extract of *Antillogorgia bipinnata* (formerly *Pseudopterogorgia bipinnata*) was included in an initial screening carried out as part of an effort in the discovery of new antimalarial agents. This extract was found to be active in inhibiting the growth of *Plasmodium falciparum* (a protozoan parasite responsible for the most severe forms of malaria). Caucanolide A and D demonstrated significant in vitro antiplasmodial activity against chloroquine-resistant *P. falciparum* W2 (IC_{50} 17 $\mu g\,mL^{-1}$ and IC_{50} 15 $\mu g\,mL^{-1}$, respectively) [35.110]. Three secosterols isolated from an unidentified gorgonian from genus *Pseudopterogorgia* inhibited human protein kinase C (PKC) α, βI, βII, γ, δ, ε, η, and ζ, with IC_{50} values in the range 12–50 μM [35.121]. PKC is a key player in cellular signal transduction and has been implicated in cancer, cardiovascular and renal disorders, immunosuppression, and autoimmune diseases such as rheumatoid arthritis [35.159]. Semisynthetic derivatives also showed a similar activity [35.121]. Promising antimicrobial substances were also reported from *Antillogorgia rigida* (formerly *Pseudopterogorgia rigida*) (e.g., curcuphenol) [35.118] and from *Antillogorgia elisabethae* (formerly *Pseudopterogorgia elisabethae*) (e.g., pseudopterosin X and Y) [35.111]. Ileabethoxazole, homopseudopteroxazole, caribenols A and B and elisapterosin B from *A. elisabethae* and bipinnapterolide B from *Antillogorgia bipinnata* inhibit *Mycobacterium tuberculosis* H37Rv at a concentration of 12.5 $\mu g\,mL^{-1}$ [35.113, 115] (for elisapterosin B and homopseudopteroxazole) and at a concentration range of 128–64 $\mu g\,mL^{-1}$ [35.112, 114, 178] (for other compounds). In fact, the inhibition of *Micobacterium tuberculosis* H37Rv is within the range recorded for rifampin [35.112]. *A. elisabethae* and *A. bipinnata* also produce antituberculosis compounds. Bielschowskysin, a naturally occurring diterpene isolated from *Antillogorgia kallos* (formerly *Pseudopterogorgia kallos*) [35.117] and aberrarone isolated from *A. elisabethae* [35.116] exhibited antiplasmodial activity (IC_{50} 10 $\mu g\,mL^{-1}$) when tested against *P. falciparum*. The first compound was also found to display strong and specific in vitro cytotoxicity against the EKVX non-small cell lung cancer ($GI_{50} < 0.01\,\mu M$) and CAKI-1 renal cancer (GI_{50} 0.51 μM) [35.117]. Bis(pseudopterane) amine from *Antillogorgia acerosa* (formerly *Pseudopterogorgia acerosa*) was found to exhibit selective activity against HCT116 (IC_{50} 4 μM)

cell lines [35.108]. Astrogorgol F, a secosteroid produced by *Astrogorgia* sp., showed significant inhibition against protein kinases IGF-1R (insulin-like growth factor receptor-1), SRC and VEGF-R2 with IC_{50} of 3.16, 2.40, and 4.95 μM, respectively. These kinases are currently regarded as very important therapeutic targets for cancer. Protein IGF-1R activates crucial signaling pathways that benefit cancer cells. Inhibition of this protein function has shown to significantly decrease cancer cell proliferation and increase sensitivity to chemotherapy and radiation treatment. Kinase VEGF-R2 plays an important role in tumor angiogenesis, and its relevance as pharmacological target for the treatment of a large variety of solid cancers has been extensively described in the literature. The potent inhibitory activity of secosteroids toward VEGF-R2 indicates that they may induce the inhibition of tumor angiogenesis. SRC family kinases play a critical role in cell adhesion, invasion, proliferation, survival, and angiogenesis during tumor development. It was reported that the three kinds of kinases (SRC, VEGF-R2, and IGF-1R) involved in the different signaling pathways are influenced by crosstalk and interaction with each other [35.129].

Fuscosides, originally isolated from *Eunicea fusca* [35.135], selectively and irreversibly inhibited leukotriene synthesis. Leukotrienes are molecules of the immune system that contribute to inflammation in asthma and allergic rhinitis and their production is usually related to histamine release [35.179]. Pharmacological studies indicated that fuscoside B inhibits the conversion of arachidonic acid (AA) to leukotriene B4 and C4 (LTB4 and LTC4) [35.135, 180] by inhibiting the 5-lipoxygenase (5-LO), in the case of LTB4 with an IC_{50} of 18 μM [35.180]. These selective inhibitors of lipoxygenase isoforms can be useful as pharmacological agents, as nutraceuticals, or as molecular tools [35.159]. Fuscoside B and E were also used in the classical experiment of acute inflammation, the TPA-induced ear edema model, which allows evaluation of the anti-inflammatory properties of some natural products. The topical application on the mouse ear edema of the of these two compounds extracts showed high inflammation inhibition levels of 80.5% and 81.5%, respectively, when compared to the activity shown by the anti-inflammatory commercial drug indomethacin (77.3%) used as reference [35.136]. A diterpenoid, dolabellane, and sesquiterpenoids metabolites isolated from *Eunicea* sp. displayed antiplasmodial activity against the malaria parasite *P. falciparum* W2 (chloroquine-resistant) strain, with IC_{50} values of 9.4 μM, and IC_{50} values ranging from 10–18 $\mu g\,mL^{-1}$, respectively [35.130, 134].

The gorgonian *Junceella fragilis* produces secondary metabolites, frajunolides B and C, with anti-inflammatory effects towards superoxide anion generation and elastase release by human neutrophils, with an $IC_{50} > 10\,\mu g\,mL^{-1}$ [35.106]. When properly stimulated, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion ($O_2^{\bullet -}$), a precursor of other reactive oxygen species (ROS), granule proteases, and bioactive lipids [35.181, 182]. The production of the superoxide anion is linked to the killing of invading microorganisms, but it can also directly or indirectly damage surrounding tissues. In contrast, neutrophil elastase is a major secreted product of stimulated neutrophils and a major contributor to the destruction of tissue in chronic inflammatory disease [35.183]. The anti-inflammatory butenolide lipide [35.184] from the gorgonian *Euplexaura flava* [35.137] can be currently synthesized, which opens the possibility of advancing into a new level of anti-inflammatory pharmaceuticals.

Some of the most interesting compounds identified so far in the on-going search for new antifouling agents have been recorded in the order Gorgonacea. Notable examples of such compounds are juncin ZII from *Junceella juncea* [35.107], homarine from *Leptogorgia virgulata* and *Leptogorgia setacea* [35.119], and pukalide and epoxypukalide so far only recorded from *Leptogorgia virgulata* [35.120].

Species of the genus *Briareum* (family Briareidae) (which commonly exhibit an incrusting appearance rather than the fan-like shape of many gorgonians) are widely abundant in Indo-Pacific and Caribbean coral reefs. These organisms have been recognized as a valuable source of bioactive compounds with novel structural features. Briarane-related natural products are a good example of such promising compounds due to their structural complexity and biological activity [35.185, 186]. Briaexcavatin E, from *Briareum excavata*, also occasionally referred to as *Briarium excavatum*, inhibited human neutrophil elastase (HNE) release with an IC_{50} between 5–10 μM [35.96]. Briaexcavatolides L and P, diterpenoids from the same species, exhibited significant cytotoxicity against mouse lymphocytic leukemia (P-388) tumor cells with ED_{50} of 0.5 [35.97] and 0.9 $\mu g\,mL^{-1}$ [35.187], respectively. Brialalepolides B and C, from a non-identified *Briarium* species, reduced the expression of COX-2 in human colon adenocarcinoma (RAW 264.7) cells, as well as in murine macrophage cells. This is significant because the metabolic products of COX-2 have

been implicated in the pathogenesis of colon cancer and other diseases. Additionally, mouse macrophages cells are used to test the effects of drugs on inflammation pathways. These data support the idea that briaranes such as brialalepolides B and C might be interesting candidates for therapeutic consideration as dual-acting cancer cell cytotoxins and inflammatory response inhibitors [35.94]. Diterpenoids produced from *Briareum polyanthes* (presently accepted as *Briareum asbestinum*), namely Briarellin D, K, and L, exhibited antimalarial activity against *P. falciparum* with an IC_{50} between 9–15 $\mu g\,mL^{-1}$ [35.95].

Other Orders

Sea anemones (order Actiniaria) are a rich source of biologically-active proteins and polypeptides. Several cytolytic toxins, neuropeptides, and protease inhibitors have been identified from this group of organisms [35.56]. In addition to several equinatoxins, potent cytolytic proteins and an inhibitor of papain-like cysteine proteinases (equistatin) were isolated from the sea anemone *Actinia equina* [35.188]. Equistatin has been shown to be a very potent inhibitor of papain and a specific inhibitor of the aspartic proteinase cathepsin D [35.189]. While papain-like cysteine proteases have been implicated in various diseases of the central nervous system, such as brain tumors, Alzheimer's disease, stroke, cerebral lesions, neurological autoimmune diseases, and certain forms of epilepsy [35.190], aspartic proteinase cathepsin D is involved in the pathogenesis of breast cancer [35.191] and possibly Alzheimer's disease [35.192]. An acylamino acid, bunodosine 391 (BDS 391) was isolated from the venom of the sea anemone, *Bunodosoma cangicum*. Intraplantar injection of BDS 391 into the hind paw of a rat induced a potent analgesic effect. This effect was not altered by naloxone (an opioid receptor antagonist), but was completely reversed by methysergide (a serotonin receptor antagonist), indicating that the effect is mediated by activation of serotonin receptors [35.193].

Cycloaplysinopsin C, a bis(indole) alkaloid isolated from *Tubastraea* sp. (order Scleractinia), was found to inhibit growth of two strains of *P. falciparum*, one chloroquine-sensitive (F32/Tanzania) and another chloroquine-resistant (FcB1/Colombia) with IC_{50} 1.48 and 1.2 $\mu g\,mL^{-1}$, respectively [35.59]. Cladocorans A and B, isolated from *Cladocora caespitosa* (order Scleractinia) [35.57], are marine sesterterpenoids that possess a γ-hydroxybutenolide moiety, which is thought to be responsible for the biological activity of these compounds. The potent anti-inflammatory activity of these natural metabolites was attributed to the inhibition of secretory phospholipase A2 (sPLA2, IC_{50} 0.8–1.9 μM). Given the general role of inflammation in diseases that include bronchial asthma and rheumatoid arthritis, the identification and development of potent inhibitors of sPLA2 continues to be of great importance for the pharmaceutical industry, with this type of metabolite being of paramount importance for future research [35.58].

A sphingolipid, 2S*,3S*,(4E,8E)-2N-[35-tetradecanoyl]-4(E),8(E)-icosadiene-1, 3-diol and a steroid (22E)-methylcholesta-5,22-diene-1a,3b,7a-triol, were isolated from the black coral *Antipathes dichotoma* (order Antipatharia) and screened for antibacterial activity against Gram-positive and Gram-negative bacteria at 1 mg/ml concentration. Data obtained showed that the sphingolipid exhibits potent activity against *Bacillus subtilis* and *Pseudomonas aeruginosa* (MIC 0.4 and 0.2 $mg\,mL^{-1}$, respectively), while the trihy droxy steroid showed potent activity against *B. subtilis* (MIC 0.6 $mg\,mL^{-1}$) [35.194].

35.2.2 Class Hydrozoa

The class Hydrozoa includes 7 orders and nearly 3500 valid species (Table 35.1), some of which are solitary while others are colonial. Among the most emblematic species are probably hydroids and the Portuguese man-o-war (*Physalia physalis*). Despite the large number of species in the class Hydrozoa, only a few have yielded interesting marine natural products in the last decade.

Immune escape plays an important role in cancer progression and, although not completely understood, it has been proposed that indoleamine 2,3-dioxygenase (IDO) plays a central role in the evasion of T-cell-mediated immune rejection [35.195]. IDO catalyzes the oxidative cleavage of the 2,3-bond of tryptophan, which is the first and rate-limiting step in the kynurenine pathway of tryptophan catabolism in mammalian cells [35.196]. The polyketides annulins A, B, and C, purified from the marine hydroid *Garveia annulata* (order Anthoathecata), potently inhibited IDO in vitro (Ki 0.12–0.69 μM) [35.197]. These annulins are more powerful than most tryptophan analogs known to be IDO inhibitors. These compounds are active at concentrations higher than $\approx 10\,\mu M$ and are, therefore, more effective than 1-methyltryptophan (Ki 6.6 μM), one of the most potent IDO inhibitors currently available [35.198]. Solandelactones C, D, and G are cyclopropyl oxylipins isolated from the hydroid *Solan-*

deria secunda (order Anthoathecata) and exhibit moderate inhibitory activity against FPT (69, 89, and 61% inhibition, respectively) at a concentration of 100 $\mu g\,mL^{-1}$ [35.199]. Note that FPT is associated with cell differentiation, and proliferation and its inhibition may be a target for novel anticancer agents (as was already mentioned above for the soft coral *Lobophytum cristagalli*).

35.2.3 Class Scyphozoa

Approximately 200 species are currently classified in the three orders of the class Scyphozoa (Table 35.1). However, in the last decade, only a single marine natural product was purified from the mesoglea of the jellyfish *Aurelia aurita* (order Semaeostomeae) and considered to be promising enough to be included in the present overview. This compound, aurelin, is a novel endogenous antibacterial peptide that exhibited activity against Gram-positive and Gram-negative bacteria. As an example, aurelin displayed an IC_{50} of 7.7 $\mu g\,mL^{-1}$ for *Escherichia coli* (Gram-negative bacteria) [35.200].

35.2.4 Other Classes

Staurozoa, Cubozoa, and Polypodiozoa are the classes with the least number of species in the phylum Cnidaria (Table 35.1). This fact may explain the current lack of data on secondary metabolites produced by these organisms. It is possible that with growing bioprospecting efforts new compounds may be revealed once these cnidarian species are screened. Cubozoa (box jellies), for example, produce some of the most harmful cnidarian toxins known to humans [35.201].

35.3 Concluding Remarks and Future Challenges

The intense pressure to find and develop more profitable molecules for all sorts of industries continues to fuel bioprospecting efforts of marine invertebrates. While the phylum Cnidaria is not the most significantly bioprospected at present, this chapter shows that some cnidarian species are promising sources of marine bioactive compounds of medical, economic, and scientific interest. Green fluorescent protein (GFP), GPF-like proteins, red fluorescent, and orange fluorescent protein (OPF) are good examples of biotechnological metabolites derived from cnidarians that are currently employed as molecular biomarkers. They were first purified from a fluorescent hydrozoan medusa [35.202] and have since been recorded in other cnidarian species [35.203–208].

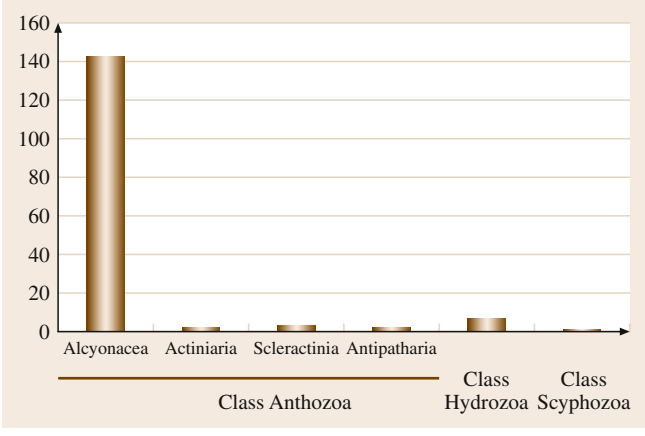

Fig. 35.6 Marine bioactive compounds with high biotechnological potential studied from the phylum Cnidaria in the twenty-first century (after [35.28])

In the present survey of the most promising bioactive marine natural products from cnidarians, only about 0.57% of extant cnidarian species are represented, with the class Anthozoa displaying by far the highest number of promising bioactive marine natural products (91%) (Fig. 35.6). This result is probably due to the fact that this class displays the highest number of species in the phylum (Table 35.1). Additionally, many anthozoans occupy marine habitats that can be readily accessed for the collection of biomass (e.g., coral reefs and intertidal regions), which facilitates bioprospecting. Of all the compounds presented in this review, 89% were detected in cnidarians collected from tropical waters (mostly from Southeast Asia and the Caribbean Sea), with the remaining 11% recorded from species that are mostly present in temperate waters (e.g., European countries and Japan).

Antitumor drugs are the main area of interest in the screening of marine natural products from cnidarians (39%, Fig. 35.7). This is not surprising, as the major financial effort for the screening of new marine compounds is made by cancer research [35.209]. Terpenoids (terpenoids, diterpenoids, sesquiterpenoids, sesterterpenoids, cembranoids) [35.14] (Fig. 35.8) are

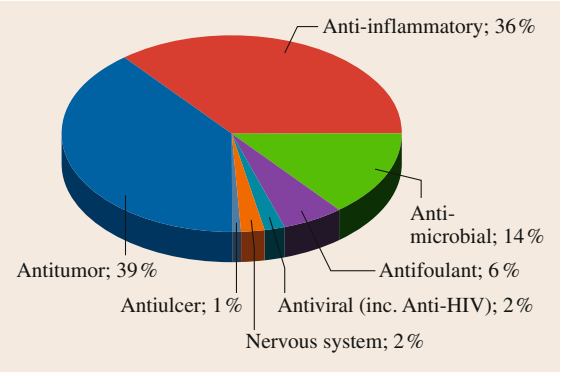

Fig. 35.7 Distribution in drug classes of marine bioactive compounds with high biotechnological potential studied from cnidarian species in the twenty-first century (after [35.28])

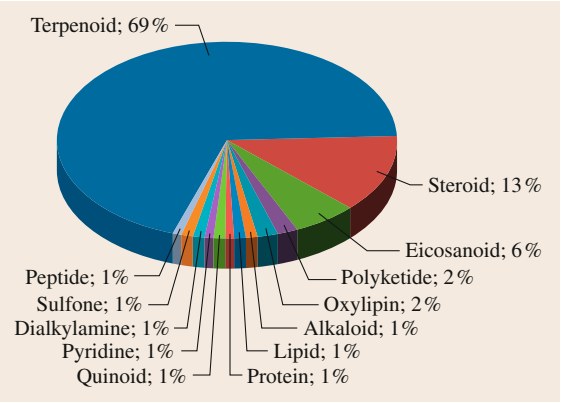

Fig. 35.8 Distribution of chemistry classes of marine bioactive compounds with high biotechnological potential studied from cnidarian species in the twenty-first century (after [35.28])

the main chemistry group in the MNPs analyzed in this survey.

Even though most pharmaceutical industries abandoned their natural product-based discovery programs over a decade ago, the lack of new compounds in their pipelines in some strategic areas (e.g., antibiotics) suggests that a renewed interest in this field is imminent. The establishment of small biotechnology companies can play a decisive role in the initial discovery of promising marine bioactive compounds, as these enterprises will work closely together with academics and governmental agencies to take the initial steps in the discovery of new chemical entities. Collaboration between private companies and public institutions can be of paramount importance for financial support in the discovery process. On the other hand, crude extracts and pure compounds produced by academic laboratories may be screened by diverse bioassays as part of broader collaboration programs, nationally and internationally, with private biotechnology companies. One challenge for universities is to devise mechanisms that protect intellectual property and simultaneously encourage partnerships with the private sector, by recognizing that the chances of a major commercial pay-off are small if drug discovery is pursued by a single institution [35.29].

The commercial use of some promising marine bioactive compounds isolated from cnidarians may still be several years away. However, new compounds other than toxins and venoms produced by members of this highly diverse group of marine invertebrates may soon be discovered in the ongoing quest for new MNP.

As with most marine organisms, the bioprospecting of cnidarians has been mostly limited to shallow habitats accessible either by foot, snorkeling, or SCUBA diving. In recent years, with the advent of deep-sea exploration it has been possible to bioprospect several unique ecosystems that had remained inaccessible to researchers [35.210, 211]. Deep-sea habitats (including hydrothermal vents and cold seeps), as well as seamounts, are commonly colonized by unique cnidarian species [35.212–214] that exhibit remarkable adaptation to extreme environments [35.215] and are promising candidates for the discovery of new MNPs [35.216]. Marine biodiversity conservation has been capturing the growing attention of nations worldwide. The growing concerns towards the conservation of marine habitats are already conditioning the bioprospecting of coral reefs, deep sea and other endangered marine habitats [35.217–219]. Even so, it is likely that in the years to come bioprospecting for new marine natural products will continue to grow and that new cnidarian groups will be targeted by researchers.

Modern screening techniques rely on the use of a significantly lower amount of biomass (micrograms) than what was required a decade ago for the discovery of new chemical compounds [35.18]. The incorporation of modern molecular biology and bioinformatics to complement the use of chemical approaches in the study of biosynthetic pathways has allowed researchers to make significant breakthroughs in the production of marine drugs [35.220]. Nonetheless, the production of such compounds at a commercial level is still a remarkable challenge. Large-scale production of a given com-

pound can be possible either through chemical synthesis or through its extraction from the source organism. Unfortunately, the first option is not always possible, as several complex molecules are simply impossible to produce or incur production costs that cannot be afforded by commercial applications [35.221, 222]. The harvest of the source animal from the wild for the extraction of a compound is invariably an unsustainable practice and rarely a long-term option [35.219]. On the other hand, the production of source organism biomass (either *in* situ or *ex* situ) has been considered as a potential alternative to the collection of wild specimens [35.223]. Additionally, the production of source organisms under controlled conditions may help to control the ecophysiological diversity promoted by environmental interactions and maximize the production of target marine molecules. Unfortunately, the culture of most target organisms has turned out to be more technically challenging and significantly more expensive than initially assumed [35.224, 225].

There is growing evidence that microorganisms associated with marine invertebrates may be the true producers of some of the MNPs isolated from these animals and may even be responsible for the variation of chemical diversity at species level [35.226–228]. The microbiome present in marine invertebrates is likely to shift geographically [35.229, 230], which may enhance the production of different secondary metabolites. Whether this is also the case of most cnidarians remains to be confirmed [35.231]. Nonetheless, it has already been recognized that one of the promising compounds recorded from the gorgonian *Antillogorgia elisabethae* is, in fact, produced by its symbiotic dinoflagellates [35.232]. In this way, it is possible that future bioprospecting efforts may shift from invertebrate hosts towards symbiotic microorganisms. Under this scenario another constraint for the commercial use of these compounds must be overcome, as the culture of symbiotic microorganisms is generally not possible using classic/standardized methodologies. Once isolated from their host symbiotic microorganisms rarely thrive in vitro or no longer produce the desired compound [35.223–228].

The early impetuous of natural product-based discovery programs by pharmaceutical industries has decreased in the twenty-first century [35.233]. Nonetheless, the lack of new compounds in some of the strategic pipelines for drug discovery impels researchers to consider the new tools available for blue biotechnology [35.234] and the importance of joining efforts with academic institutions for the early stages of marine organisms bioprospecting. New drugs derived from MNP isolated from cnidarians may still be several years away, but it is unquestionable that more chemical entities, besides toxins and venoms, will be recorded from cnidarians [35.28]. In conclusion, this diverse group of marine invertebrates is destined to play a major role in the pursuit of new drugs from the sea.

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36. Fatty Acids of Marine Sponges

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Among all organisms, thus also marine sponges, accumulate fatty acids (FAs) with a very rich chemistry and are the source of an unprecedented diverse range of chemical structures. This chapter accounts the most recent developments concerning the occurrence of different FA structures in marine sponges and their potential applications. While the first section of the chapter deals with the importance of FAs in various applications, the second section of the chapter focuses on the diversity of FAs with different structural features in marine sponges. FAs in marine sponges differ in the number of olefinic bonds, the extent of branching, the length of the hydrocarbon chain, and the number of functional groups. The last section of the chapter deals with the biomedical potential of the FAs of marine sponges including antibacterial, antifungal, antimalarial, and cytotoxic activities, etc.

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36.1 Fatty Acids – Pharmaceutical and Biomedical Importance

36.1.1 Fatty Acids – An Introduction

Fatty acids (FAs) are ubiquitous metabolites of all organisms, characterized by the presence of a carboxyl group (−COOH) at one end and a methyl group at the other end. Fatty acid molecules are usually joined together in groups of three, forming a molecule called triglyceride. As an important source of energy, FAs play a number of biological roles in the body. In the absence of glucose the body uses FAs to fuel cells to obtain energy. They are needed for the construction of cell membranes and also play a key role in protein acylation, signal transduction, growth, etc.

FAs are either saturated or unsaturated. Most FAs are straight-chain compounds with the frequent occurrence of an even number of carbon atoms. Chain lengths range from 2 to 80, but most commonly from 12 to 24. With a chain length from 2 to 6 (or 4) they are referred to as short-chain, from 8 (or 6) to 10 as medium-chain, and 12–24 as long-chain FAs. Among straight-chain FAs, the simplest are referred to as saturated FAs. When double bonds are present, FAs are said to be unsaturated; monounsaturated (MUFAs) if only one double bond is present, and polyenoic (or polyunsaturated fatty acids = PUFAs) if they have two or more double bonds generally separated by a single methylene group (methylene-interrupted unsaturation).

Various conventions are used for indicating number and position of the double bond in the structure of an unsaturated FA. For example, in the structure of palmitoleic acid (16 : 1), the end methyl carbon is known as the ω carbon or the n carbon. $\omega 7$ or (n-7) indicates a double bond on the seventh carbon counting from ω or the n carbon,

$$\begin{array}{ll} \omega & 2\ \ 3\ \ 4\ \ 5\ \ 6\ \ 7 \\ & \mathrm{CH_3CH_2CH_2CH_2CH_2CH{=}CH(CH_2)_7COOH} \\ n & \qquad 16:1\ \omega 7 \text{ or } 16:1(n\text{-}7)\,. \end{array}$$

IUPAC-IUB (International Union of Pure and Applied Chemistry and International Union of Biochemistry and Molecular Biology) Commissions have agreed to 16 : 1 (*n*-7) form of the nomenclature rather than 16 : 1 ω7 because of its convenience to biochemists with interests in fatty acid metabolism in animals. In mammals, additional double bonds are introduced only in between the existing double bond and the carboxyl carbon, yielding three series of FAs known as the ω-9, ω-6, and ω-3 families, respectively.

Some uncommon PUFAs have two adjacent double bonds separated by more than one methylene group, and they are named polymethylene-interrupted FAs. In some organisms, the structures of FAs may be more complex since they can have an odd number of carbon atoms, branched chains, or a variety of other functional groups, including acetylenic bonds, epoxy, hydroxy, or keto groups, and even ring structures (cyclopropane, cyclopropene, cyclopentene, furan, and cyclohexyl) or a coenzyme A moiety (acyl CoA).

36.1.2 Importance of Fatty Acids

Use of Fatty Acids in Cosmetics

A large variety of FA derivatives are used in cosmetics [36.1]. Stearic acid, oleic acid, lauric acid, palmitic acid and myristic acid are used in a variety of cosmetic creams, cakes, soaps, and pastes. The use of lipids and FAs in hair products is based on their ability to hold hair in place and to make the hair glossy. They also play a critical role as vehicles for the application of various drugs to the skin and as bases for pigmented decorative cosmetics. The applications of FAs in cosmetics are mostly based on the adhesive properties of the lipid/FAs [36.2].

Nutritional Importance

Food lipids enhance the palatability of foods, such as taste, texture, and create a sensation in the mouth. Dietary fats and oils provide an important source of concentrated food energy [36.3, 4]. They also provide a source of fat-soluble vitamins (A, D, E, and K) and help to facilitate the digestion and absorption of these vitamins [36.4]. The saturated FAs 12 : 0, 14 : 0, and 16 : 0 elevate the circulating levels of plasma low-density lipoprotein (LDL)-C [36.5], a major risk factor for atherosclerosis. Conversely, consumption of (*n*-6) and (*n*-3) PUFAs are associated with decreased blood pressure [36.6]. Consumption of *n*-3 PUFAs reduces the risk of cancer, cardiovascular disease, and diabetes [36.7–10].

Application of Fatty Acids in Pharmaceuticals

The majority of FAs and their derivatives are used as:

1. Processing aids such as antifoaming agents and tablet lubricants that assist in manufacturing operations.
2. Pharmaceutical components or excipients such as emulsifiers, stabilizers, solubilizers, carriers, suppository bases, coatings, adjuvants, etc.
3. Active pharmaceuticals.

Processing Aids.

In Antibiotic Manufacture. Antibiotic yields have been shown to increase markedly in the presence of specific FAs. The addition of unsaturated FAs enhances the growth and antibiotic production by streptomyces [36.11].

As Tablet Lubricants. A widely known application of FA derivatives, in particular the metallic salts of FAs, involves their use as tablet lubricants [36.12]. Magnesium, calcium, sodium and aluminium stearates, sodium myristate, and stearic acid have been employed preferentially as tablet lubricants.

As Pharmaceutical Components and Excipients. In parenterals. A number of FA derivatives, in particular those classified as nonionics, have been used in the preparation of parenterals. Polyoxyethylene sorbitan monolaurate and monooleate, sorbitan trioleate, and aluminium stearate have been used as surface active agents in the preparation of parenteral dispersions [36.13].

As Protective Coatings. Oleic and stearic acid were shown to be essential components of a coating formulation that released aspirin at pH 6.5. Ethyl esters of stearic acid and oleic acid were used for preparing protective coating for metal surfaces [36.14]. Polyethylene glycol esters of C12–C18 FAs have been employed in the preparation of medicinal capsules having delayed action. Glyceryl monostearate and glyceryl monopalmitate have been used for enteric coatings. Dimers of C6–C26 unsaturated FAs in combination with glyceryl or polyoxyethylene monostearate were found to be suitable as thin film coatings for pharmaceuticals.

In Ointment Bases. Fatty acid esters have found widespread use in ointment bases for the administration of pharmaceuticals. The diethanolamine condensates of

lauric acid have been used as an ointment base for steroidal hormones such as corticosteroids. Silicone oil, in combination with calcium salts of FAs or polyhydric alcohol esters of FAs, has been found to be an effective base for pharmaceutical ointments.

In Suppositories. Suppositories for the nasal, urethal, vaginal, and rectal insertion of local or systemic medicinals, such as steroids, hypnotics, diuretics, analgesics, sedatives, and so forth, have been prepared from FAs and their derivatives. An effective water-soluble suppository base having a high melting point range and a high percentage of medicinal release has been developed, which contains polyoxyethylene stearate, wax, water, and sodium dioctyl sulfosuccinate [36.15].

As Gastrointestinal Absorption Aids. Various FA derivatives have been used to increase the absorption of difficult absorbing substances in the gastrointestinal tract. Thus, polyoxyethylene sorbitan monooleate or monopalmitate have been used to facilitate the resorption of iron and vitamin B_{12} from the digestive canal to the blood. The ingestion of polyoxyethylene sorbiton monolaurate in combination with iron salts has resulted in an adequate haemoglobin response brought about by increased enteric iron absorption [36.16].

As Active Pharmaceuticals.

As Antimalarial, Antimycobacterial, and Antifungal Agents. The antimalarial, antimycobacterial, and antifungal properties of novel FAs are reviewed by *Carballeira* [36.17]. C18 FAs have shown antimalarial activity and oleic acid (9–18 : 1) was the most inhibitory fatty acid with an IC_{50} of 23 μg/mL [36.18]. Some methoxylated FAs derived from marine sponges have shown antimycobacterial activity [36.19]. Propylene glycol mono and diesters of C_2–C_{10} FAs have been shown to be effective antifungal agents in the treatment of epidermophytosis. *Carballeira* et al. studied the antifungal activity of acetylenic FAs [36.20].

As Bactericides, Germicides, and Nematoxic Agents. Unsaturated FAs derived from cod-liver oil were found to be an effective therapeutic treatment of pulmonary tuberculosis, which showed that its addition in amounts of 1 : 50 000 retards growth of tubercle bacilli in vitro [36.21]. Neomycin salts of FAs, such as lauric, palmitic, and stearic acid, have been shown to demonstrate remarkable germicidal properties for the reduction of transient and resident bacteria present on the human skin. The C2–C11 FAs have been shown to be effective agents in suppressing nematode parasites. Inhibitory effects of C8 and C10 saturated FAs on Gram-positive and Gram-negative organisms have also been reported [36.22].

As Spermicidal Agents. The glyceryl, ethylene glycol, propylene glycol monoesters of C10–C18 unsaturated FAs, polyethylene glycol, polypropylene glycol esters of C10–C18 unsaturated FAs, glyceryl monolactate monoesters of C10–C18 unsaturated FAs or higher FAs, a C10 unsaturated FA or higher in combination with a sulfonated FA or in combination with alkyl phenoxyethanols, polyoxyethylene glycol mixed esters of C6 or higher FAs, e.g., dodecaethylene glycol monolaurate monooleate, polyoxyethylene glycol monoesters of C6 or higher FAs and their acetylated derivatives have been recommended for use as spermicides [36.23].

36.1.3 Biological Functions of PUFAs and Their Biomedical Applications

PUFAs fulfil many structural and functional roles and are unequalled amongst FAs in the wide spectrum of biological events in which they participate. First, PUFAs are critical components of cells and organelle membranes [36.24–26]. They are crucial for regulating the architecture, dynamics, phase transitions, and permeability of membranes, and for the control of membrane-associated processes. Moreover, they regulate membrane-bound proteins such as ATPase, transport proteins, and histocompatibility complexes, and modulate interactions with extracellular components such as fatty acid-binding proteins. In addition, PUFAs also regulate the expression of certain genes, including those coding for fatty acid synthase, nitric oxide synthase, sodium channel proteins, and cholesterol-7-α-hydroxylase and, thereby, affect processes including fatty acid biosynthesis, cancer induction and cholesterol regulation [36.27] and, thus, exhibit an impact on cellular biochemical activities, transport processes, and cell-stimulus responses. They are also implicated in physiological processes, including lipid metabolism and targeting immune responses and cold adaptation by lymphocyte stimulation, and are involved in pathological conditions such as carcinogenesis and cardiovascular disease [36.24–27].

Especially, some specific *n*-3 and *n*-6 PUFAs play an important role in human and animal health. The lack of such acids in animals and humans has slowed down

growth, induced skin diseases (eczema), and stopped the normal function of kidneys and the reproductive system [36.28]. The long-chain *n*-3 PUFAs are important for the brain and retina. Learning disabilities and loss of visual activity were reported in animals consuming low levels of *n*-3 essential FAs [36.29]. These FAs have a variety of beneficial effects in protecting against atherosclerotic disease. Diets (fish oils) enriched in FAs (especially EPA (EPA) (C20 : 5) and DHA (DHA) (C22 : 6) have produced effects associated with lowered serum triacylglycerols or plasma cholesterol and blood pressure [36.30], reduced platelet aggregability (prevention of thrombosis) and platelet function [36.31], and lower incidences of coronary heart disease, diabetes, rheumatism, and immunological responses associated with psoriasis [36.32].

36.2 Sponge Fatty Acids

Marine sponges are the most primitive multicellular animals and contain many bioactive metabolites including lipids [36.33, 34]. The study of sponge lipids is as important as other secondary metabolites. In depth studies on lipids of sponges started in the 1970s [36.35] and have been stimulated because of the great diversity of FAs, which have unusual and sometimes unique structures. Investigators focus on sponge lipids mainly for an interpretation new FA structures, biological activity, sources of biologically important major PUFAs, chemotaxonomy, etc.

Some unusual FAs of sponges exhibit biological activity and they are found to be antimalarial, antimycobacterial, and antifungal agents [36.17, 36]. Various application of lipids, phospholipids, and saturated and unsaturated FAs in pharmaceutical formulations have also been reported [36.37, 38]. Sponges (Demospongiae) are a source of novel FAs; especially unusual long-chain Δ5,9 FAs, sometimes have a third double bond or a bromine atom in their structures [36.39, 40]. Many new FAs were identified in sponge lipids, like unsaturated FAs with unusual distribution of double bonds [36.41], branched chain FAs [36.42], and FAs with unusual substituents in the carbon chain, such as the cyclopropane group [36.43], the methoxy group [36.44], the acetoxy group [36.45], etc. Demosponges possess unusual membrane lipids reflecting specific enzyme systems which control biosynthetic properties such as carbon chain elongation and the introduction of distinctive double bonds. In particular, many investigations revealed the presence of unique long-chain fatty acids (LCFA, >24) in demosponges, so-called demospongic acid. Demospongic acids like 23 : 2 Δ5,9 and 24 : 2 Δ5,9 were found to be antimycobacterial agents [36.46] and (5*Z*,9*Z*)-22-methyl-5,9-tetracosadienoic acid was found to be cytotoxic to cancer cells [36.47]. These compounds provide excellent targets for chemotaxonomic analyses at the class level [36.35, 48]. Sponge classification needs to be supported by chemotaxonomic criteria, in particular regarding FA. Particular FAs appear as biomarkers for such organisms. Complete and correct information on the lipid and fatty acid composition of sponges is necessary for the elucidation of biosynthetic pathways and functional role of FAs in these archaic organisms. Many sponges are able to synthesize lipids and FAs that can be extracted; some of these are presented below.

36.2.1 Saturated Fatty Acids

Linear Saturated Fatty Acids

Marine sponges are a good source of saturated FAs. They contain both short-chain as well as long-chain saturated FAs. Linear saturated FAs from C8 : 0–C32 : 0 are identified in the lipids of marine sponges. Among them, the percentage of C16 : 0 and C18 : 0 is much higher than in other FAs in most of sponges. Marine sponges such as *Spirastrella inconstans* and *Hyattella cribriformis* contain 95.8 and 63.9% C16 : 0, respectively [36.49]. C18 : 0 is found to be dominant in *Acanthella elongata* (39.1%) and *Acanthella cavernosa* (38.57%) [36.49]. Short-chain linear saturated FA C8 : 0 was identified in marine sponges like *Plakina monolopha* Schulze, *Cinachyrella alloclada*, and *Cinachyrella kukenthali* [36.49, 50]. C10 : 0, C11 : 0, and C12 : 0 were identified in *Hymeniacidon sanguinea* [36.51]. However, there are other sponge species that have a major content of long-chain saturated FAs. For example, in *Azorica pfeifferae*, C25 : 0 is the major constituent among the saturated FAs [36.52]. A long-chain saturated FA C28 : 0 was identified from the marine sponge *Xestospongia* sp. [36.53]. C29 : 0 was identified from the marine sponge *Cinachyrella alloclada* [36.50]. Linear saturated FAs from marine sponges are presented in Table 36.1.

It is interesting to note that the percentage content of saturated FAs of a marine sponge varies based on the depth of collection and the locations from which it is collected. The same sponge species collected from two different depths have different content of saturated FAs. It is mostly observed that the sponge species collected at shallow water contain a high content of saturated FAs. *Axinella carteri* collected in shallow water has a higher content of saturated FAs than the same species collected at a greater depth [36.54]. The content of saturated FAs of a sponge species also varies based on the location of where it was collected. The marine sponge *Phycopsis* sp. collected at two different regions was found to have a different content of saturated FAs [36.55]. Long-chain saturated FAs C22 : 0–C32 : 0 are identified in sponge lipids.

Monobranched Saturated Fatty Acids

Monobranched saturated FAs (most of them being iso- and anteiso – i. e. with methyl branching on the penultimate and antepenultimate carbon atoms, respectively) were particulary abundant in the marine sponges and their content may reach 40–50% of the FA total. The content of mono branched saturated FA in *Phycopsis* sp. 1 and 2 is 44.87 and 38.83%, respectively. While a long-chain monobranched FA i. e. 27-methyl octacosanoic acid was identified in the lipid of a marine sponge *Petrosia pellasarca* [36.30], a number of other monobranched long-chain FAs i. e., i-Me-C23 : 0, br-C25 : 0, br-C26 : 0, br-C27 : 0, br-C25 : 0 were identified from a marine sponge *Cinachyrella alloclada* [36.50]. Other marine sponges like *Axinella carteri*, *Didiscus oxeata* contain rarely found long-chain mono branched saturated FAs [36.54, 64]. These acids have bacterial origin and the high content of these acids in some sponges is due to the bacterial symbionts [36.65, 66]. Monobranched saturated FAs from marine sponges are presented in Table 36.2.

Table 36.1 Linear saturated FAs from marine sponges

Linear saturated FAs	Marine sponge	Reference
C8 : 0	*Cinachyrella alloclada*	[36.50]
C9 : 0	*Phycopsis* sp.	[36.55]
C10 : 0	*Fasciospongia cavernosa*	[36.56]
C11 : 0	*Hymeniacidon sanguinea*	[36.51]
C12 : 0	*Fasciospongia cavernosa*	[36.56]
C13 : 0	*Suberites domuncula*	[36.57]
C14 : 0	*Myrmekioderma granulata*	[36.58]
C15 : 0	*Cinachyrella kukenthali*	[36.50]
C16 : 0	*Spirastrella inconstans*	[36.49]
C17 : 0	*Chondrosia reniformis*	[36.59]
C18 : 0	*Acanthella elongata*	[36.49]
C19 : 0	*Chondrosia reniformis*	[36.59]
C20 : 0	*Halichondria panicea*	[36.60]
C21 : 0	*Verongia aerophoba*	[36.61]
C22 : 0	*Azorica pfeifferae*	[36.52]
C23 : 0	*Myrmekioderma granulata*	[36.58]
C24 : 0	*Fasciospongia cavernosa*	[36.56]
C25 : 0	*Azorica pfeifferae*	[36.52]
C26 : 0	*Siphonodictyon coralliphagum*	[36.62]
C27 : 0	*Haliclona* sp.	[36.63]
C28 : 0	*Xestospongia* sp.	[36.53]
C29 : 0	*Cinachyrella alloclada*	[36.50]
C30 : 0	*Dragmaxia undata*	[36.64]
C32 : 0	*Petrosia pellasarca*	[36.30]

Table 36.2 Monobranched saturated FAs identified in marine sponges

Monobranched saturated FAs	Marine sponge	Reference
i-Me-C11 : 0 i-Me-C12 : 0	*Verongia aerophoba*	[36.61]
i-Me-C13 : 0 i-Me-C14 : 0 ai-Me-C14 : 0	*Chondrosia reniformis*	[36.59]
i-Me-C15 : 0 ai-Me-C15 : 0	*Axinyssa ambrosia*, *Didiscus oxeata*	[36.64]
i-Me-C16 : 0 ai-Me-C16 : 0	*Verongia aerophoba*, *Axinyssa ambrosia*, *Didiscus oxeata*	[36.61, 64]
i-Me-C17 : 0	*Axinyssa ambrosia*	[36.64]
i-Me-C17 : 0 ai-Me-C17 : 0	*Halichondria panicea*	[36.60]
11-Me-C18 : 0	*Chondrosia reniformis*	[36.59]
i-Me-C19 : 0 ai-Me-C19 : 0	*Halichondria panicea*	[36.60]
i-Me-C20 : 0 ai-Me-C20 : 0	*Verongia aerophoba*, *Chondrosia reniformis*	[36.59, 61]
i-Me-C21 : 0 ai-Me-C21 : 0	*Halichondria panicea*	[36.60]
i-Me-C22 : 0	*Chondrosia reniformis*	[36.59]
i-Me-C23 : 0	*Cinachyrella alloclada*	[36.50]
i-Me-C24 : 0 ai-Me-C24 : 0	*Verongia aerophoba*	[36.61]
br-C25 : 0	*Didiscus oxeata*, *Cinachyrella alloclada*	[36.50, 64]
br-C26 : 0 br-C27 : 0	*Cinachyrella alloclada*	[36.50]
27-Me-C28 : 0	*Petrosia pellasarca*	[36.30]

Polybranched Saturated Fatty Acids

Polymethyl-branched saturated FAs are often found in marine sponges. The most abundant are 4,8,12-trimethyltridecanoic acid (TMTD) and phytanic acid. It was observed that the percentage composition of C16 : 0(3,7,11,15-tetramethyl) varied from species to species of the class Demospongiae. There are species of the Demospongiae class having no or trace amounts of 3,7,11,15-tetramethyl-C16 : 0 [36.59, 61], while the content was more than 20% in *Axinella carteri* [36.54]. The *Spheciospongia vesparium*, marine sponge contains 23% of 4,8,12-trimethyltridecanoic acid [36.67]. It was suggested by *Carballeira* et al. that these two acids do not occur together in the same sponge [36.67]. However, interestingly, the two isoprenoid FAs mentioned above occurred concomitantly in the marine sponges *Halichondria magniconulosa* and *Dragmaxia undata* [36.64]. Some sponges belonging to the genus *Cinachyrella* contain both 4,8,12-TMTD and 5,9,13-trimethyltetradecanoic acid. *Hymeniacidon sanguinea*, a marine sponge collected from the Canary Island contains 3, 13-di-Me-C14 : 0 [36.51]. A rare 4,8,11-trimethyl-C16 : 0 FA was found in *Suberites domuncula* [36.57]. 18,14-di-Me-C26 : 0 was identified from *Cinachyrella alloclada* [36.50]. A list of some of the polybranched saturated FAs from marine sponges is presented in Table 36.3.

Table 36.3 Polybranched saturated FAs found in marine sponges

Polybranched saturated FAs	Marine sponge	Reference
3,7,11-tri-Me-C12 : 0	*Xestospongia muta*	[36.68]
4,8,12-tri-Me-C13 : 0	*Spheciospongia vesparium*, *Halichondria magniconulosa*, *Dragmaxia undata*	[36.64, 67]
3,13-di-Me-C14 : 0	*Hymeniacidon sanguinea*	[36.51]
10,13-di-Me-C14 : 0	*Cinachyrella kukenthali*	[36.50]
5,9,13-tri-Me-C14 : 0	*Hymeniacidon sanguinea*	[36.51]
8,10-di-Me-C16 : 0	*Chondrosia reniformis*	[36.59]
12,13-di-Me-C16 : 0	*Suberites domuncula*	[36.57]
3,7,11,15-tetramethyl-C16 : 0	*Axinella carteri*, *Halichondria magniconulosa*, *Dragmaxia undata*	[36.54, 64]
4,8,11-tri-Me-C16 : 0	*Suberites domuncula*	[36.57]
11,14-di-Me-C17 : 0	*Suberites domuncula*	[36.57]
4,13-di-Me-C17 : 0	*Suberites domuncula*	[36.57]
12,14-di-Me-C17 : 0	*Suberites domuncula*	[36.57]
12,15-di-Me-C18 : 0	*Suberites domuncula*	[36.57]
12,16-di-Me-C19 : 0	*Suberites domuncula*	[36.57]
12,17-di-Me-C20 : 0	*Suberites domuncula*	[36.57]
12,18-di-Me-C21 : 0	*Suberites domuncula*	[36.57]
12,17-di-Me-C21 : 0	*Suberites domuncula*	[36.57]
12,19-di-Me-C22 : 0	*Suberites domuncula*	[36.57]
18,14-di-Me-C26 : 0	*Cinachyrella alloclada*	[36.50]

36.2.2 Unsaturated Fatty Acids

Monounsaturated Fatty Acids

Linear MUFAs. Marine sponges are specified by great diversity of monoenic FAs (MUFAs) [36.69]. Among the monounsaturated FAs, C16 : 1 and C18 : 1 are widespread. C16 monoenic acids such as, C16 : 1(5), C16 : 1(6), C16 : 1(7), C16 : 1(8), C16 : 1(9), C16 : 1(11), and C16 : 1(18) have been identified in marine sponges, among which C16 : 1(9) is the most commonly found in sponges [36.54, 56, 58]. However, there are sponge species which are completely devoid of C16 monoenic acids [36.52]. Among C18 monoenic acids, C18 : 1(9) and C18 : 1(11) are found abundantly in marine sponges [36.69]. *Myrmekioderma granulata* contains a good amount of C18 : 1(9) acid. C18 monoenic acids such as C18 : 1(8) and C18 : 1(11) were reported from a marine sponge *Axinella carteri* [36.54]. C18 : 1(13) was identified in a marine sponge *Azorica pfeifferae*. C17 and C19 monoenic acids are rarely found in marine sponges. C19 : 1(11) was identified in some marine sponges like *Aplysina fistularis* and *Azorica pfeifferae* [36.52, 69].

Some marine sponge such as *Callyspongia fallax*, *Cinachyrella schulzei* and *Dysidea fragilis* contain C17 monoenic acids. C20 and C22 monoenic acids with double bonds at different positions have been identified in marine sponges. C20 : 1(11) monoenic acid has been found in a marine sponge *Fasciospongia cavernosa*, while the sponge *Axinella carteri* contains a C22 : 1(13) monoenic acid [36.54, 56]. Long-chain monoenic FAs with double bonds at different positions are also found in marine sponges. C23 : 1(16) was identified from *Amphimedon compressa* Pallas and *Mycale laevis* [36.70], C23 : 1(17) and C23 : 1(18) have been identified from *Microciona prolifera* and *Amphimedon compressa* Pallas, respectively [36.70, 71]. Both C24 : 1(7) and C24 : 1(9) monoenic acids occur in *Dysidea fragilis* [36.72], while C24 : 1 with

double bonds at 11 and 15 positions were identified from *Pseudaxinella* aff. *Lunaecharta* [36.73]. A C24 : 1(17) was also identified in the lipid of *Calyx podatypa* [36.74]. C25 : 1 with double bonds at 16 and 18 positions were identified from *Mycale laevis* [36.70], while C25 : 1 monoenic acid with double bonds at 17, 19, and 9 positions were reported from *Geodia gibberosa, Amphimedon compressa* Pallas and *Dysidea fragilis*, respectively [36.70, 72, 75]. 19-Hexacosenoic (26 : 1) was identified from *Cinachyrella alloclada* [36.50]. C27 : 1 monoenic acids, with double bonds at 18 and 20 positions were reported from *Cinachyrella* aff. *Schulzei*. C28 : 1 monoenic acids with double bonds at 5, 19, and 21 positions were identified from *Cinachyrella* aff. *Schulzei*, *Haliclona cinerea* and *Amphimedon compressa* Pallas [36.70, 76, 77]. 21-Triacontaenoic acid (30 : 1) and 23-triacontaenoic acid (30 : 1), two long-chain monoenic acids were identified from *Haliclona cinerea* [36.77]. A list of long-chain monoenic acids from marine sponges is presented in Table 36.4.

Branched MUFAs. Branched monoenic acids with branching at different positions have been identified from marine sponges. A number of monobranched monoenic acids such as 13-Me-C14 : 1(4), i-Me-C16 : 1(9), ai-Me-C16 : 1(6), 14-Me-C17 : 1(5), and i-Me-C17 : 1(9) have been identified from the marine sponge *Hymeniacidon sanguinea* [36.51]. Dimethyl monoenic acids like 7, 8-di-Me-C15 : 1(7) and 8,17-di-Me-C18 : 1(5) were identified from *Hymeniacidon sanguinea* and *Halichondria lutea*, respectively [36.51, 64]. 10,14-di-Me-15 : 1(6), a novel monoenic FA was reported in three marine sponges such as *Lissodendoryx pulviliformis, Tedania dirhaphis* and *Poecillastra* sp. collected from the Sea of Okhotsk [36.78]. The unique monounsaturated FA 15-Me-24 : 1(14) was observed for the first time in the sponge *Forcepia uschakowi* [36.79]. Novel monoenic acids like 2-Me-24 : 1 and 2-Me-26 : 1 along with br-C25 : 1 and br-C27 : 1 were revealed in the marine sponge *Halichondria panicea* [36.80]. The presence of long-chain monobranched monoenic acids such as 19-Me-C26 : 1(5), 19-Me-C27 : 1(5), and 19-Me-C28 : 1(5) have been reported in the marine sponge *Strongylophora durissima* [36.81]. The occurrence of 20-Me-C24 : 1(18) and 23-Me-C24 : 1(8) have also been reported in *Celtodoryx ciocalyptoides and Axinyssa ambrosia*, respectively [36.64]. A list of branched monoenic acids in marine sponges is presented in Table 36.5.

Table 36.4 Occurrence of long-chain MUFAs in marine sponges

Long-chain MUFAs	Marine sponge	Reference
16-23 : 1	*Amphimedon compressa* Pallas, *Mycale laevis*	[36.70]
17-C23 : 1	*Microciona prolifera*	[36.71]
18-C23 : 1	*Amphimedon compressa* Pallas	[36.70]
17-24 : 1	*Calyx podatypa*	[36.74]
11-C24 : 1 15-C24 : 1	*Pseudaxinella* aff. *lunaecharta*	[36.73]
7-C24 : 1 9-C24 : 1	*Dysidea fragilis*	[36.72]
19-C25 : 1	*Amphimedon compressa* Pallas	[36.70]
16- C25 : 1 18-C25 : 1	*Mycale laevis*	[36.70]
17-C25 : 1	*Geodia gibberosa*	[36.75]
9- C25 : 1	*Dysidea fragilis*	[36.72]
9- C26 : 1	*Dysidea fragilis*	[36.72]
19- C26 : 1	*Cinachyrella alloclada*, *Halichondria panicea*	[36.50, 60]
17- C26 : 1	*Pseudaxinella* aff. *Lunaecharta*, *Halichondria panicea*	[36.60, 73]
18-C27 : 1	*Cinachyrella* aff. *schulzei*	[36.76]
20-C27 : 1	*Cinachyrella* aff. *schulzei*, *Amphimedon compressa* Pallas	[36.64, 76]
5-C28 : 1	*Cinachyrella* aff. *schulzei*	[36.76]
21-C28 : 1	*Amphimedon compressa* Pallas	[36.70]
21-C30 : 1 23-C30 : 1	*Haliclona cinerea*	[36.77]
23-C30 : 1	*Trikentrion Loeve* Carter	[36.82]

Polyunsaturated Fatty Acids

Linear PUFAs. Important polyunsaturated fatty acids (PUFAs) such as linoleic acid (36.C18 : 2(9,12), *n*-6) 7-linolenic acid (36.C18 : 3(6,9,12), *n*-6), dihomo-γ-linolenic acid (36.C20 : 3(8,11,14), *n*-6), α-linolenic acid (36.C18 : 3 (9,12,15), *n*-3), eicosatetraenoic acid (36.C20 : 4 (8,11,14,17), *n*-3) arachidonic acid (36.C20 : 4 (5,8,11,14), *n*-6) EPA (36.C20 : 5 (5,8,11,14,17), *n*-3), DHA (36.C22 : 6 (4,7,10,13, 16,19), *n*-3), etc. have been identified from several marine sponges. *Oceanapia* sp. and *Callyspongia fibrosa* contain 71.3 and 65.1% of C22 : 6, respectively [36.49]. *Acanthella elongata* contains 28.59% of α-linolenic acid [36.49]. Five numbers of PUFAs such as C18 : 2, C18 : 3, C20 : 4, C20 : 5, and C22 : 4 have been reported in *Raspailia* sp. [36.49]. A high percentage of linoleic acid was reported in *Fasciospongia cavernosa* [36.56]. Linoleic acid, α-linolenic acid,

Table 36.5 Branched monoenic FAs from marine sponges

Branched monoenic FAs	Marine sponge	Reference
13-Me-C14 : 1(4)	*Hymeniacidon sanguinea*, *Suberites massa*, *Dysidea fragilis*	[36.51, 72, 83]
7, 8-di-Me-C15 : 1(7)	*Hymeniacidon sanguinea*	[36.51]
10,14-di-Me-C15 : 1(6)	*Lissodendoryx pulviliformis*, *Tedania dirhaphis*, *Poecillastra* sp.	[36.78]
7-Me-C16 : 1(8)	*Desmapsama anchorata*, *Amphimedon complanata*	[36.84, 85]
14-Me-C16 : 1(8)	*Desmapsama anchorata*	[36.84]
i-Me-C16 : 1(9)	*Hymeniacidon sanguinea*, *Verongia aerophoba*	[36.51, 61]
ai-Me-C16 : 1(6)	*Hymeniacidon sanguinea*	[36.51]
15-Me-C16 : 1(9)	*Didiscus oxeata*	[36.64]
14-Me-C17 : 1(5)	*Hymeniacidon sanguinea*	[36.51]
16-Me-C17 : 1(11)	*Dragmaxia undata*	[36.64]
i-Me-C17 : 1(9)	*Hymeniacidon sanguinea*	[36.51]
5-Me-C18 : 1(6)	*Axinyssa ambrosia*	[36.64]
8,17-di-Me-C18 : 1(5)	*Halichondria lutea*	[36.64]
20-Me-C24 : 1(18)	*Celtodoryx ciocalyptoides*	[36.64]
23-Me-C24 : 1(8)	*Axinyssa ambrosia*	[36.64]
2-Me-C24 : 1	*Halichondria panicea*	[36.80]
15-Me-C24 : 1(14)	*Forcepia uschakowi*	[36.79]
br-C25 : 1	*Halichondria panicea*	[36.60]
2-Me-C26 : 1	*Halichondria panicea*	[36.80]
17-Me-26 : 1(16)	*Lissodendoryx pulviliformis*, *Melonachora kobjakovae*, *Stelodoryx toporoki*	[36.78]
25-Me-C26 : 1	*Cinachyrella alloclada*	[36.50]
19-Me-C26 : 1(5)	*Strongylophora durissima*	[36.81]
19-Me-C27 : 1(5)	*Strongylophora durissima*	[36.81]
br-C27 : 1	*Halichondria panicea*	[36.60]
19-Me-C28 : 1(5)	*Strongylophora durissima*	[36.81]

eicosatetraenoic acid, eicosatetraenoic acid, eicosapentaenoic acid, docosatetraenoic acid, docosapentaenoic acid, and docosahexaenoic acid have been reported in the lipid composition of *Halichondria panicea* [36.60]. Linoleic acid, eicosatetraenoic acid, and eicosapentaenoic acid have been identified in the marine sponge *Verongia aerophoba* [36.61]. PUFAs such as linoleic acid, α-linolenic acid, 7-linolenic acid, and eicosatetraenoic acid along with rare PUFAs, such as C18 : 2(11,14), C18 : 2(5,11), C18 : 4(6,9,12,15), and C18 : 5(3,6,9,12,15) have been identified from the marine sponge *Hymeniacidon sanguinea* [36.51]. A rare polyenic acid i. e., C16 : 4(4,7,10,13) has been reported from the marine sponge *Callyspongia* sp. [36.86]. Two important PUFAs i. e., C22 : 6(4,7,10,13,16,19, *n*-3) and C22 : 5(7,10,13,16,19, *n*-3) have been reported in the marine sponge *Petromica ciocalyptoides* [36.64]. 7,13,16-docosatrienoic acid, a rare PUFA, has been identified from the marine sponge *Petrosia ficiformis* [36.87]. C28 : 2(9,21), a polyenic FA that is new for sponges, has been identified from *Mycale* sp. [36.78]. Very long-chain PUFAs such as 15,18,21,24-triacontatetraenoic acid (C30 : 4, *n*-6), and 15,18,21,24,27-triacontapentaenoic acid (C30 : 5, *n*-3) have been reported from the marine sponge *Cliona celata* [36.88]. 15,18,21,24-Tetratriacontatetraenoic (C34 : 4) and 19,22,25,28,31-tetratriacontapentaenoic acid (C34 : 5) have been reported from *Amphimedon compressa* and *Petrosia pellasarca*, respectively [36.30, 89]. A list of polyenic FAs from marine sponges is listed in Table 36.6.

Linear Δ5,9 FAs. Nonmethylene-interrupted dieneic FAs of various structures are found in marine sponges. In recent extensive review work on non-methylene-interrupted FAs of marine sponges and other marine invertebrate it has been reported that among all, Δ5,9 long-chain FAs are commonly found in marine sponges [36.91]. Δ5,9-FAs as with unbranched carbon chain and branched iso and anteiso structures and FAs with midbranched chains and sometimes with a third double bond occur among them the most often. Marine sponges (Demospongiae) are a good source of these FAs called demospongic acids. A number of FAs with double bonds at 5,9 positions have been reported from marine sponges. *Trikentrion loeve* and *Pseudaxinella* cf. *lunaecharta* contain 15 (25.7% of the total acid mixture) and 13 (30.4%) Δ5,9 FA, respectively [36.92]. *Axinella carteri* contains more than 40% C26 : 2(5,9) [36.54]. A marine sponge *Halichondria panicea* contains a good amount of C26 : 2(5,9). C25 : 2(5,9) is present in a good amount in the marine sponge *Azorica pfeifferae* [36.52]. C30 : 2(5,9) has been reported from the marine sponge *Petrosia pellasarca* [36.30]. A very long-chain FA

Table 36.6 Linear PUFAs from marine sponges

Linear PUFAs	Marine sponge	Reference
C16 : 2(9,12)	*Hymeniacidon sanguinea*	[36.51]
C16 : 2(7,10)	*Halichondria panicea*	[36.60]
C16 : 3(4,7,10)	*Hymeniacidon sanguinea*	[36.51]
C16 : 4 (4,7,10,13)	*Callyspongia* sp.	[36.86]
C18 : 2(9,12)	*Raspailia* sp., *Fasciospongia cavernosa*, *Halichondria panicea*, *Verongia aerophoba*, *Hymeniacidon sanguinea*	[36.49, 51, 56, 60, 61]
C18 : 2(10,13)	*Cinachyrella alloclada* uliczka	[36.90]
C18 : 2(5,11)	*Hymeniacidon sanguinea*	[36.51]
C18 : 2(11,14)	*Hymeniacidon sanguinea*	[36.51]
C18 : 3(9,12,15)	*Acanthella elongata*, *Halichondria panicea*, *Hymeniacidon sanguinea*	[36.49, 51, 60]
C18 : 3(6,9,12)	*Hymeniacidon sanguinea*	[36.51]
C18 : 4(6,9,12,15)	*Hymeniacidon sanguinea*	[36.51]
C18 : 5(3,6,9,12,15)	*Hymeniacidon sanguinea*	[36.51]
C20 : 2(11,14)	*Verongia aerophoba*, *Chondrosia reniformis*	[36.59, 61]
C20 : 2(5,11)	*Halichondria panicea*	[36.60]
C20 : 3	*Myrmekioderma granulata*	[36.58]
C20 : 4(5,8,11,14)	*Hymeniacidon sanguinea*, *Halichondria panicea*, *Verongia aerophoba*	[36.51, 60, 61]
C20 : 5(5,8,11,14,17)	*Halichondria panicea*, *Verongia aerophoba*	[36.60, 61]
C21 : 5(6,9,12,15,18)	*Halichondria panicea*	[36.60]
C22 : 3(7,13,16)	*Petrosia ficiformis*	[36.87]
C22 : 4(7,10,13,16)	*Halichondria panicea*	[36.60]
C22 : 5(7,10,13,16,19)	*Petromica ciocalyptoides*	[36.64]
C22 : 5(4,7,10,13,16)	*Halichondria panicea*	[36.60]
C22 : 6 (4,7,10,13,16,19)	*Petromica ciocalyptoides*, *Oceanapia* sp., *Callyspongia fibrosa*	[36.49, 64]
C24 : 2(4,17)	*Halichondria lutea*	[36.64]
C26 : 2(9,19)	*Halichondria panicea*	[36.60]
C28 : 2(9,21)	*Mycale* sp.	[36.78]
C30 : 2(9,23)	*Trikentrion Loeve* Carter	[36.82]
C30 : 4(15,18,21,24)	*Cliona celata*	[36.88]
C30 : 5(15,18,21,24,26)	*Cliona celata*	[36.88]
C34 : 4(15,18,21,24)	*Amphimedon compressa*	[36.89]
C34 : 5(19,22,25,28,31)	*Petrosia pellasarca*	[36.30]

C31 : 2(5,9) along with C22 : 2(5,9), C23 : 2(5,9), C24 : 2(5,9), C25 : 2(5,9), C26 : 2(5,9), C27 : 2(5,9), C28 : 2(5,9), and C31 : 2(5,9) have been reported from *Verongia aerophoba* [36.61].

C25 : 3(5,9,17) and C25 : 3(5,9,19) have been identified from *Dysidea fragilis* [36.72]. 26 : 3(5,9,19) was the main PUFA in marine sponges like *Hymeniacidon assimilis*, *Haliclona* sp., and *Mycale* sp. [36.78]. C27 : 3(5,9,19) and C27 : 3(5,9,20) have been identified from *Microciona prolifera* and *Halichondria panicea*, respectively [36.71, 93]. C28 : 3(5,9,19) was reported from *Xestospongia halichondroides* [36.94]. C28 : 3(5,9,21) was the main PUFA in sponges like *Poecillastra* sp., *Polymastia* sp., *Tedania dirhaphis*, *Lissodendoryx pulviliformis*, and *Myxilla incrustans* [36.78]. In the marine sponge *Tedania dirhaphis*, C28 : 3(5,9,21) constitutes 63.3% of the total FA content [36.95]. C28 : 3(5,9,23) was reported from the marine sponge *Aplysina fistularis* [36.96]. A new PUFA 28 : 3 Δ5,9,22 has been identified in *Stelodoryx toporoki* and *Myxilla incrustans* [36.78]. C29 : 3(5,9,22) has been identified from the marine sponge *Verongia aerophoba* [36.61]. C30 : 3(5,9,23) is the principal FA of the marine sponge *Chondrilla nucula*, which constitutes 34% of the total FA content [36.97]. A list of linear Δ5,9 FAs from marine sponges is given in Table 36.7.

Table 36.7 Linear Δ5,9 FAs from marine sponges

Δ5,9 FAs	Marine sponge	Reference
C16 : 2(5,9)	*Hymeniacidon sanguinea*, *Chondrosia reniformis*	[36.51, 59]
C17 : 2(5,9)	*Hymeniacidon sanguinea*	[36.51]
C18 : 2(5,9)	*Cinachyrella alloclada*, *Cinachyrella kukenthali*	[36.50]
C20 : 2(5,9)	*Chondrosia reniformis*	[36.59]
C22 : 2(5,9)	*Chondrosia reniformis*, *Verongia aerophoba*	[36.59, 61]
C23 : 2(5,9)	*Chondrosia reniformis*, *Verongia aerophoba*, *Azorica pfeifferae*	[36.52, 59, 61]
C24 : 2(5,9)	*Verongia aerophoba*, *Haliclona* sp.	[36.61], [36.63]
C25 : 2(5,9)	*Chondrosia reniformis*, *Verongia aerophoba*, *Phycopsis* sp.	[36.55, 59, 61]
C25 : 3(5,9,17)	*Dysidea fragilis*	[36.72]
C25 : 3(5,9,19)	*Dysidea fragilis*	[36.72]
C26 : 2(5,9)	*Halichondria panicea*, *Axinella carteri*	[36.54, 60]
C26 : 3(5,9,17)	*Halichondria panicea*	[36.60]
C26 : 3(5,9,19)	*Hymeniacidon assimilis*, *Haliclona* sp., *Mycale* sp.	[36.78]
C27 : 2(5,9)	*Cinachyrella alloclada*, *Verongia aerophoba*, *Fasciospongia cavernosa*	[36.50, 56, 61]
C27 : 3(5,9,19)	*Microciona prolifera*	[36.71]
C27 : 3(5,9,20)	*Halichondria panicea*	[36.93]
C28 : 2(5,9)	*Cinachyrella alloclada*, *Verongia aerophoba*	[36.50, 61]
C28 : 3(5,9,19)	*Xestospongia halichondroides*	[36.94]
C28 : 3(5,9,21)	*Poecillastra* sp., *Polymastia* sp., *Lissodendoryx pulviliformis*, *Myxilla incrustans*, *Tedania dirhaphis*,	[36.78, 95]
C28 : 3(5,9,22)	*Stelodoryx toporoki*, *Myxilla incrustans*	[36.78]
C28 : 3(5,9,23)	*Aplysina fistularis*	[36.96]
C29 : 2(5,9)	*Chondrilla nucula*	[36.98]
C29 : 3(5,9,22)	*Verongia aerophoba*	[36.61]
C30 : 2	*Petrosia pellasarca*	[36.30]
C30 : 3(5,9, 23)	*Chondrilla nucula*	[36.97]
C31 : 2(5,9)	*Verongia aerophoba*	[36.61]

Branched PUFAs. Branched PUFAs with various structures are reported from marine sponges. A rare branched Δ5,9 FA i. e., 15-Me-C16 : 2(5,9) has been identified from the marine sponge *Didiscus oxeata* [36.64]. Novel Δ5,9 FAs i. e., 17-Me-C19 : 2(5,9) and 18-Me-C19 : 2(5,9) were identified for the first time in the marine sponge *Pseudospongosorites suberitoides* [36.99]. 17-Me-C24 : 2(5,9) has been identified from *Chondrosia reniformis* [36.59]. Both iso-24 : 2(5,9) (30% of the total free FA) and anteiso-24 : 2(5,9) (19.5%) have been reported from the marine sponge *Geodinella robusta* [36.47]. ai–25 : 2(5,9) has been identified from a marine sponge *Haliclona* sp. [36.78]. 24-Me-C25 : 2(5,9) has been identified from *Petrosia ficiformis* [36.87]. 24-Me-25 : 2(5,9), 23-Me-25 : 2(5,9), 25-Me-26 : 2(5,9), 24-Me-C26 : 2(5,9), 25-Me-27 : 2(5,9), and Me-C28 : 2(5,9) have been reported from the marine sponge *Cinachyrella kukenthali* [36.50]. br-C27 : 2 has been identified from *Halichondria panicea* [36.60]. 22-Me-28 : 2(5,9) has also been reported from *Verongia aerophoba* [36.61]. A dimethyl Δ5,9 PUFA i. e., 22,27-di-Me-C28 : 2(5,9) has been reported from the marine sponge *Didiscus oxeata* [36.64]. A list of some branched PUFAs found in marine sponges is presented in Table 36.8.

36.2.3 Fatty Acids with Unusual Structures

Many new FAs with unusual substituents in the carbon chain, such as the cyclopropane group [36.4], the methoxy group [36.44], the acetoxy group [36.45] etc., have been reported from marine sponges.

Three novel cyclopropane FAs such as 17-Me-4,5-methylene-C18 : 0, 18-Me-4,5-methylene-C19 : 0, and 17-Me-4,5-methylene-C19 : 0 were reported for the first time from the marine sponge *Pseudospongosorites suberitoides* [36.99]. 19,20-Methylene-C26 : 0 has been identified in the sponge *Calyx niceansis* [36.101], while the sponge *Amphimedon* sp., collected in Australia, contains 10,11-methylene-C27 : 2(5,9), a topoisomerase I inhibitor with an IC_{50} = 1.21M [36.102]. 9,10-Cyclopropyl-C16 : 0 and 11,12-cyclopropyl-C18 : 0 were reported from marine sponges like *Hymeniacidon sanguinea, Chondrosia reniformis* and *Verongia aerophoba* [36.51, 59, 61].

The marine sponge *Polymastia gleneni* contains nine saturated long-chain (C22–30)-acetoxy FAs with an acetoxy group in the second position of the carbon chain [36.45]. Methoxylated FAs from marine sponges were reviewed by *Carballeira* in 2002 [36.103]. 2-Methoxy-14-methylpentadecanoic acid and the novel 2-methoxy-14-methylhexadecanoic acid have been identified in the sponge *Agelas dispar* [36.104]. 2-Methoxy-12-methyltetradecanoic acid was identified for the first

Table 36.8 Occurrence of branched PUFAs in marine sponges

Branched PUFAs	Marine sponge	Reference
15-Me-C16 : 2(5,9)	*Didiscus oxeata*	[36.64]
16-Me-C18 : 2(5,9), 17-Me-C18 : 2(5,9)	*Pseudospongosorites suberitoides*	[36.99]
17-Me-C19 : 2(5,9), 18-Me-C19 : 2(5,9)	*Pseudospongosorites suberitoides*	[36.99]
19-Me-C20 : 2(5,9)	*Pseudospongosorites suberitoides*	[36.99]
17-Me-C24 : 2(5,9)	*Chondrosia reniformis*	[36.59]
i-24 : 2(5,9), ai-24 : 2(5,9)	*Geodinella robusta*	[36.47]
ai-25 : 2(5,9)	*Haliclona* sp.	[36.78]
24-Me-C25 : 2(5,9)	*Petrosia ficiformis*, *Cinachyrella kukenthali*	[36.50, 87]
23-Me-C25 : 2(5,9)	*Cinachyrella kukenthali*	[36.50]
25-Me-C26 : 2(5,9), 24-Me-C26 : 2 (5,9)	*Cinachyrella kukenthali*, *Petrosia ficiformis*, *Petrosia hebes*, *Didiscus oxeata*	[36.50, 64, 100]
25-Me-C27 : 2(5,9)	*Cinachyrella kukenthali*	[36.50]
26-Me-C27 : 2(5,9)	*Cinachyrella alloclada* Uliczka	[36.90]
br-C27 : 2	*Halichondria panicea*	[36.60]
22-Me-C28 : 2(5,9)	*Verongia aerophoba*	[36.61]
27-Me-C28 : 2(5,9), 26-Me-C28 : 2(5,9)	*Didiscus oxeata*	[36.64]
22,27-di-Me-C28 : 2(5,9)	*Didiscus oxeata*	[36.64]

time from *Pseudospongosorites suberitoides* [36.99]. 2-Methoxylated saturated FAs such as 2-methoxytetradecanoic acid, 2-methoxypentadecanoic acid, and 2-methoxyoctadecanoic, as well as the monoenoic methoxylated FAs such as 2-methoxy-6-tetradecenoic acid, 2-methoxy-6-pentadecenoic acid, and 2-methoxy-13-methyl-6-tetradecenoic acid were identified for the first time in nature in the phospholipids from the Caribbean sponge *Callyspongia fallax* [36.105]. 2-Methoxy-C15 : 0 has been reported from the marine sponge *Petromica ciocalyptoides* [36.64]. Novel fatty acid 2-methoxy-C16 : 2(5,9), 2-methoxy-C18 : 2(5,9), 2-methoxy-C19 : 2(5,9), and 2-methoxy-C20 : 2 along with the iso-methyl-branched FAs such as 2-methoxy-15-methyl-C16 : 1(9) and 2-methoxy-15-methyl-C16 : 2(5,9) have been identified in *Erylus goffrilleri* [36.106].

2-Hydroxy-C20 : 0, 2-hydroxy-C21 : 0, 2-hydroxy-C22 : 0, 2-hydroxy-C24 : 0, 2-hydroxy-23-methyl-C24 : 0, and 2-hydroxy-C25 : 0 have been isolated from the Caribbean sponges *Verongula gigantea* and *Aplysina archeri* [36.107]. 2-Hydroxy-C22 : 0 and 2-hydroxy-C23 : 0, have been identified in the Caribbean sponge *Amphimedon compressa* and constitute 52% of the total FA content [36.89]. 2-Hydroxy-C20 : 0, 2-hydroxy-C22 : 0, 2-hydroxy-C24 : 0, and 2-hydroxy-C26 : 0 have been reported from the marine sponge *Dragmaxia undata* [36.64]. 2-Hydroxy-C24 : 0 and 2-hydroxy-C25 : 0 have been found in *Halichondria lutea* [36.64]. 2-Hydroxy-C26 : 0 has been found in the marine sponge *Axinyssa ambrosia* [36.64].

Many acetylenic FAs have been reported from marine sponges. 12-Methyloctadeca-17-ene-5-ynoic acid, 2-methoxy-12-methyloctadeca-17-ene-5-ynoic acid, and 2-methoxy-12-methyloctadeca-17-ene-5-ynoic anhydride are the new acetylenic FAs reported from marine sponges of the genus *Stelletta* [36.108]. Bioactive brominated acetylenic acids have been reported from marine sponges like *Petrosia volcano* Hoshino, *Xestospongia muta*, *Xestospongia testudinaria*, etc. [36.109–111].

Brominated $\Delta 5,9$ FAs have been reported from marine sponges. 6-Br-26 : 2(5,9) has been reported from the marine sponge *Forcepia uschakowi* [36.79]. Very long-chain brominated FAs such as 6-Br-C24 : 2(5,9), 6-Br-23-Me-C24 : 2(5,9), 6-Br-C25 : 2(5,9), and 6-Br-24-Me-C25 : 2(5,9) have been reported from *Agelas* sp. [36.112]. 6-Br-C24 : 2(5,9), 6-Br-C25 : 2(5,9), and 6-Br-C26 : 2(5,9) have been reported from *Amphimedon terpenensis* [36.113]. 6-Br-C27 : 2(5,9), a bioactive FA has been reported from the marine sponge *Xestospongia* sp. [36.114]. Branched brominated FAs such as 6-Br-24-Me-C26 : 2(5,9) and 6-Br-25-Me-C26 : 2(5,9) have been revealed in marine sponges like *Petrosia ficiformis* and *Petrosia hebes* [36.100]. 6-Br-23-Me-C24 : 2(5,9), 6-Br-C25 : 2(5,9), and 6-Br-24-Me-C25 : 2(5,9) have been reported from the marine sponge *Axinyssa ambrosia* [36.64].

36.3 Bioactive Lipids/FAs from Marine Sponges

Marine FAs are of interest for the different roles and biological properties they exhibit in the cells of marine organisms. Some of these FAs have displayed interesting biological activities. The literature survey indicates that antimicrobial activity is exhibited by many lipids of sponges [36.115–118], including FAs [36.119, 120]. *cis*-9-octadecenoic and *cis*-9,12-octadecadienoic acids [36.118, 121] have maximum antimicrobial activity. Among a series of linear-chain saturated FAs (C_2–C_{20}), myristic acid displays the strongest bactericidal activity at 0.04 mM against a highly virulent strain (Ravenel) of *Mycobacterium bovis* and an avirulent strain ($H_{37}Ra$) of *Mycobacterium tuberculosis* [36.132]. Many reports on the study of antibacterial and antifungal activity of synthetic saturated, monounsaturated FAs and PUFAs are available [36.120, 133–136]. The anti-inflammatory properties of marine lipid compositions have also been studied [36.137]. Recent findings on the antimalarial, antimycobacterial, and antifungal properties of novel FAs derived from marine sponges are well documented in an extensive review by *Carballeira* [36.17]. The antimicrobial activity of FAs derived from marine sponges was reviewed by *Abad* et al. [36.138]. Methoxylated FAs derived from

Table 36.9 Bioactive FAs from marine sponges

Bioactive FAs	Marine sponge	Bioactivity	Reference
Mixture of 23-Me-C24 : 2(5,9) and 22-Me-C24 : 2(5,9)	*Geodinella robusta*	Cytotoxicity	[36.47]
6-Br-C27 : 2(5,9)	*Xestospongia* sp.	Cytotoxicity	[36.114]
Motualevic acids A–F	*Siliquariaspongia* sp.	Antimicrobial	[36.122]
6-Bromo-icosa-3*Z*,5*E*,8*Z*,13*E*,15*E*-pentaene-11,19-diynoic acid	*Haliclona* sp.	Cytotoxicity against NBT-T2 rat bladder epithelial cells	[36.123]
Methyl-18-bromooctadeca-9*E*,17*E*-diene-7,15-diynoate	Unidentified sponge	Antimicrobial	[36.124]
6-{5-[(5*E*)-Oct-5-en-7-ynyl]thiophen-2-yl}hex-5-ynoic acid	*Paragrantia* cf. *waguensis*	Antimicrobial	[36.125]
12-Methyloctadeca-17-ene-5-ynoic acid 2-Methoxy-12 methyloctadeca-17-ene-5-ynoic acid 2-Methoxy-12-methyloctadeca-17-ene-5-ynoic anhydride	*Stelletta* sp.	Cytotoxicity against human leukemia cell-line (K562)	[36.108]
Callysponginol sulfate A	*Callyspongia truncata*	MT1-MMP Inhibitor	[36.126]
7*E*,11*E*-Tetradecadiene- 5,9-diynoic acid.	*Oceanapia* sp.	Antimicrobial	[36.127]
10,11-Methylene-C27 : 2(5,9)	*Amphimedon* sp.	DNA Topoisomerase I inhibitor	[36.102]
Brominated C18 acetylenic acids	*Petrosia volcano* Hoshino	Antifungal	[36.109]
Brominated polyacetylenic acids	*Xestospongia muta*	HIV Protease inhibitors	[36.110]
Xestospongic acid and ethyl ester of xestospongic acid	*Xestospongia testudinaria*	Antimicrobial	[36.111]
Brominated C14 PUFAs	*Oceanapia* sp.	Cytotoxicity against KB cells	[36.128]
Carticatic acids A–C	*Petrosia carticata*	Antifungal	[36.129]
Br-C27 : 3(5,9,24) Br-C28 : 3(5,9,24)	*Xestospongia* sp.	Cytotoxicity against L1210 murine leukaemia cells and KB human epidermoid carcinoma cells	[36.114]
18-Brominated C23 acetylenic acids	*Phakelia carduus*	Antibacterial	[36.130]
C30 : 3(5,9,23)	*Chondrilla nucula*	Elastase inhibitor	[36.117]
C30 : 3(5,9,21)	*Amphimedon* sp.	DNA Topoisomerase I inhibitor	[36.102]
cis C23–C26 Δ5,9 FAs	*Agelas oroides*	Enoyl reductase inhibitor Antiprotozoal activity	[36.46]
Amphimic acids A and B	*Amphimedon* sp.	DNA Topoisomerase I inhibitor	[36.131]

marine sponges have been found to have antimycobacterial activity [36.19]. Methoxylated FAs display biological activities like antibacterial, antifungal, antitumor, and antiviral activities [36.103]. 2-methoxy-5-hexadecenoic acid and 2-methoxy-6-hexadecenoic acid have shown antimicrobial activity [36.139]. The antifungal activity of FAs was also studied by *Carballeira* et al. [36.20]. The antimalarial properties of *n*-3 and *n*-6 PUFAs have been studied, where acids such as 22 : 6 (*n*-3), and 20 : 5 (*n*-3) have been found to be the most effective for the in vitro killing of intraerythrocytic forms of *Plasmodium falciparum* [36.140]. Again, consumption of *n*-3 highly unsaturated FAs, such as linolenic acid, EPA (20 : 5, *n*-3), and DHA (22 : 6, *n*-3), is correlated with a reduced risk of cancer and cardiovascular disease in clinical and animal studies [36.7, 8]. Epidemiological studies suggest that there is low prevalence of diabetes in populations with a high intake of *n*-3 PUFAs [36.9, 10]. Δ5,9 long-chain FAs of marine sponges are good topoisomerase inhibitors as well as some of them are cytotoxic to cancer cells. C27 : 2(5,9) inhibited human topoisomerase I with an IC_{50} of 0.9 μM [36.102, 131], while a mixture of 23-Me-C24 : 2(5,9) and 22-Me-C24 : 2(5,9) was cytotoxic to mouse Ehrlich carcinoma cells with ED_{50} of 1.8 μg/mL and showed a hemolytic effect on mouse erythrocytes [36.47]. A brominated Δ5,9 i. e. 6-Br-C27 : 2(5,9), showed cytotoxicity towards L1210 murine leukaemia cells (IC_{50} = 11 μg/mL) and KB human epidermoid cancer cells (IC_{50} = 18.5 μg/mL) [36.114]. A marine FA, 2-methoxy-13-methyltetradecanoic acid was cytotoxic to human chronic myelogenous leukemia K-562 (EC_{50} = 238 ţm), histiocytic lymphoma U-937 (EC_{50} = 250 mM) and promielocytic leukemia HL-60 (EC_{50} = 476 mM) in RPMI 1640 medium [36.141]. A list of some bioactive FAs from marine sponges is presented in Table 36.9.

36.4 Summary

Marine sponges are bestowed with a remarkable diversity of FAs. The first work of research on the lipid composition of marine sponges was carried out by *Litchfield* and colleagues [36.35], who studied 31 species of Demospongiae sponges. Similarly, *Bergquist* and *Lawson* and their colleagues [36.142, 143] compared the FA composition of 80 sponge species from New Zealand and Australia. *Thiel* and colleagues [36.144] also studied the FA composition of marine sponges. The composition of FAs and other lipids of marine sponges have been well documented in a series of extensive reviews by *Berge* et al. [36.69, 145]. They are the important sources of some enriched saturated and unsaturated FAs and contain biomedically important (*n*-3) or (*n*-6) PUFAs viz., linoleic acid (C18 : 2), α-linolenic acid (C18 : 3), dihomo-γ-linolenic acid (C20 : 3, *n*-6), arachidonic acid (C20 : 4), EPA (C20 : 5), etc. Further, LCFAs, important MUFAs, branched and polybranched saturated and unsaturated FAs, and FAs with unusual structures are also reported in most of the marine sponges. Some of the FAs of marine sponges are active and possess anticancer, antifungal, antibacterial, etc., properties.

These studies lead to the conclusion that the composition of different individual FAs vary greatly within the marine sponges. The FA characteristics of marine sponges can reveal the characteristic chemotaxonomic attributives of sponges in a family at genus levels and permit further interpretation of LCFA biosynthetic pathways in sponges. Further studies on the FA profile of marine sponges will lead to the discovery of new FA structures and certainly other important FAs that possess new biological activities.

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37. Marine Biotoxins

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This chapter revises current knowledge on marine biotoxins that are known nowadays, considering all the distinct groups based on chemical structure and lipophilic or hydrophilic characteristics. Diarrheic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), ciguatera fish poisoning (CFP) are some of the groups that will be reviewed in this chapter.

The recent development and application of advanced technologies from the generically defined *-omics* sciences coupled with bioinformatics platforms has been included in this chapter in order to understand the ecology and evolution of phytoplankton species and bloom dynamics. Dinoflagellate toxins are structurally and functionally diverse, and many present unique biological activities.

The literature and information regarding the biological activities and the potential application of these phycotoxins has been gathered in this book section.

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Marine biotoxins are natural compounds mainly produced by marine microalgae, dinoflagellates, and diatoms. A small percentage of algae produce toxins that can harm human beings and also pose a deadly risk. Toxins are nonproteinaceous metabolites with different and complex structures, physical properties, and specific mechanisms of action. In general, they have low molecular weight ranging from 250 to 3000 Da. Shellfish and fish may become contaminated with these phycotoxins due to accumulation through marine food webs. Risk to humans often results in gastrointestinal disease, loss of short-term memory, neurological disorders, paralytic effects, or even death. In addition, the threat of marine biotoxins is not only a major cause of concern on human health and food safety, but it is also detrimental to the explotation of marine resources

around the world and the economy of shellfish producers and associated industries, including tourism. This chapter revises current knowledge on marine biotoxins that are known nowadays, considering all the distinct groups based on chemical structure and lipophilic or hydrophilic characteristics. Diarrheic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), ciguatera fish poisoning (CFP) are some of the groups that will be reviewed in this chapter. Contamination by marine biotoxins often involves more than one group of toxins and monitoring programs typically cover a range of toxins. There has been an increased effort to develop rapid and feasible screening methods for marine biotoxins. Many methods to detect toxins and phytoplankton have several technical and, sometimes, ethical limitations and generally lack an adequate validation. Functional assays, rapid screening tests, generally based on immunological methods and molecular biology techniques, among others are attractive alternatives to toxins and microalgae characterization. In this chapter, different approaches to monitor and specific detection of shellfish toxins and dinoflagellates are reviewed taking into account the availability of resources. The potential use of several techniques for marine biotoxins or phytoplankton characterization, such as biosensors, applied immunology, PCR, sequencing, radioisotopic, flow cytometry, metabolomics, and proteomics will be explained along the chapter. Marine phytoplankton comprised both prokaryotic and eukaryotic species that share a common ability to photosynthesize and thus thrive in the upper, euphotic zone of the worlds' oceans. Together, the prokaryotic and eukaryotic phytoplankton support marine food webs, including valuable fisheries and play a key role in the regulation of global biogeochemical cycles. Identification and quantification of individual species remains a difficult task. The recent development and application of advanced technologies from the generically defined *-omics* sciences coupled with bioinformatics platforms has been included in this chapter in order to understand the ecology and evolution of phytoplankton species and bloom dynamics. Dinoflagellate toxins are structurally and functionally diverse, and many present unique biological activities. At present, marine biotoxins include an extraordinary potential source of new bioactive compounds for pharmaceutical or medical uses constituting an emergent field of interest. Sometimes, the toxin itself is directly used as a drug, although more often the toxin lead compound provides a design idea for the development of a drug molecule. The biological activities of these phycotoxins are harmful to the target organisms, since the function of the toxin is either to protect the toxic species from attack by a predator or to immobilize the potential preys. Despite the damage in the target organism, toxins have a great potential for not target organisms, in particular for therapeutic purposes in humans. For instance, the target of many marine neurotoxins is the Na^+ channel, though the sites of interaction and, thus, the pharmacological effects differ among compounds. In this context, phycotoxins can be invaluable tools to recognize and identify ionic channels and the specific role of each channel subtype in controlling cell function, exocytosis, or the Ca^{2+}-dependent release of a given neurotransmitter. The literature and information regarding the biological activities and the potential application of these phycotoxins has been gathered in this book section.

37.1 Marine Toxins

Marine biotoxins are produced by certain species of toxic phytoplankton and can accumulate in various marine species: fish, crustacean, or molluscs such as mussels, clams, oysters, and scallops. In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. However, when contaminated shellfish is consumed by humans this may lead to a severe intoxication or even death, causing public health and economic problems.

Currently, five groups of marine toxins are regulated in Europe: amnesic shellfish poisoning toxins (ASP), paralytic shellfish poisoning toxins (PSP), okadaic acid (OA) and pectenotoxin group (DSP), azaspiracid group, and yessotoxin group (EC/853/2004) [37.1]. Another group, neurotoxic shellfish poisoning (NSP) toxins (e.g., brevetoxin) is regulated in United States, New Zealand, and Australia. Many cases of tetradotoxin food poisoning are reported in Southeastern Asia as well as ciguatera fish poisoning (CFP) that was limited to tropical and subtropical areas. Other emerging toxins (Palytoxin, cyclic imines,) are not on the radar of the European Legislation or in other regions of the world. Scientists and legislative authorities are aware of these toxins but do not have sufficient amount of information related to their activity, estimation of the toxicity and risk assessment in humans [37.2].

37.2 Lipophilic Toxins

37.2.1 Okadaic Acid-Group (OA) Toxins and Pectenotoxin-Group Toxin (PTXs)

OA-group toxins are a class of marine biotoxins that include OA and dinophysis toxins (DTX1, DTX2, and DTX3). These toxins cause Diarrhetic Shellfish Poisoning (DSP), which is characterized by symptoms such as abdominal pain, diarrhea, nausea, and vomiting. These toxins are produced by planktonic dinoflagellates, *Dinophysis* genus, and the benthic *Prorocentrum* genus and can be found mainly in shellfish.

They were first reported in Netherlands in 1960s [37.3, 4] and in Japan in 1978 [37.5]. Nowadays, their distribution is considered worldwide, Europe, Japan, North and South America Australia, Indonesia, and New Zealand [37.6].

OA-group toxins are heat-stable polyether compounds [37.7]. While OA and DTX2 only differ by the position of one methyl group in the molecule, DTX1 has one additional methyl group. DTX3 represents a wide range of derivatives of OA, DTX1, and DTX2 that form *acylated* analogues when they are esterified with saturated and unsaturated fatty acids. Acylated analogs were reported to contribute considerably to the concentration of total OA-group toxins [37.7, 8].

OA and DTXs act by inhibiting serine/threonine phosphoprotein phosphatases PP1 and PP2A.These enzymes perform the dephosphorylation of numerous proteins; this function is closely related to many essential metabolic processes in eukaryotic cells [37.9, 10].

Pectenotoxins (PTXs) frequently co-occur with the OA-group. The presence of PTXs in shellfish was discovered due to high acute toxicity in mouse bioassay (MBA) after intraperitoneal (*i.p.*) injections of lipophilic extracts. Animal studies indicate that they are much less potent via the oral route and they do not induce diarrhea. PTXs have been detected in microalgae and/or bivalve mollusks in Australia, Italy, Japan, New Zealand, Norway, Portugal, and Spain and they are produced by *Dinophysis* spp.

PTXs are cyclic polyether macrolides that include over 15 analogs. PTX1 to PTX 5 were originally isolated from Japanese scallops, *Patinopecten yessoensis* [37.11]. More recently, other PTXs were found but only four analogs (PTX2, PTX12, PTX11, and PTX13) have been identified as actual biosynthetic products of the algae. Other PTXs seem to be either product of the shellfish metabolism or artifacts [37.12].

37.2.2 Yessotoxin-Group Toxins (YTXs)

Yessotoxins (YTXs) are a group of structurally related polyether toxins produced by the dinoflagellates *Protoceratium reticulatum*, and *Lingulodinium polyedrum*, and *Gonyaulax spinifera* [37.13]. YTXs had traditionally been included within the DSP group but the EU has excluded the YTXs from the DSP group in 2004, and nowadays, YTXs are considered as a separate group in the current regulation [37.1].

YTXs was first isolated in Japan, in 1986 [37.14] from the digestive gland of *P. yessoensis*, a scallop that gave its name to the toxin. More recently, YTXs have been reported worldwide, in Korea [37.15], Chile [37.16], and New Zealand [37.17]. In Europe, it has been described in mollusks in Norway, Italy, Spain, and Russia [37.13].

YTXs are disulfated polyethers, with a characteristic ladder-shape formed by 11 adjacent ether rings of different sizes and a terminal acyclic unsaturated side chain consisting of nine carbons and two sulfate ethers [37.13, 14]. The presence of the sulfo-ether group makes these molecules the most polar of the lipophylic toxins. More than 90 YTXs congeners have been reported but structures for most of them have not been determined and only 30 have been isolated [37.13, 18].

There is discrepancy between the toxicity via oral administration and *i.p.* injection and the precise mechanism of action is not yet known [37.13]. Several studies have been carried out to clarify this, it seems clear that YTXs do not inhibit phosphatases PP1 and PP2A [37.19]. They modulate Ca^{2+} homeostasis in human lymphocytes [37.20] and produce a cytotoxic effect during in vitro experiments in various cellular models, F-actin decrease, alter cell adhesion and cause apoptotic events [37.6].

37.2.3 Azaspiracid-Group Toxins (AZAs)

Azaspiracids (AZAs) are a group of marine biotoxins that accumulate in shellfish and represent an emerging human health risk [37.21]. They were originally isolated from blue mussels from Killary Harbour (Ireland) and produced an outbreak of shellfish poisoning in the Netherlands, in 1995. Afterward, seafood contamination by AZAs have been reported in different coastal localizations, including Europe, North and South America, Africa, and Japan [37.22–26].

There have been several attempts to identify the AZA-producing organism(s). *James* et al. [37.27] have detected the presence of AZA-1, 2, and 3 in extracts from the heterotrophic dinoflagellate *Protoperidium crassipes*. However, this organism is supposed to act as a vector and nowadays, *Azadium spinosum* is considered the organism that produces these compounds [37.28].

AZAs are nitrogen-containing polyether toxins involving a unique spiral ring assembly containing a heterocyclic amine and an aliphatic carboxylic acid moiety [37.6, 27, 29]. They have been identified as AZA1 to AZA5 and numerous natural analogs and artifacts of methanolic solution (methyl ester analogs). AZA4 and AZA5 seem to be a biotransformation product of primary compounds into shellfish.

AZA1 is highly potent and capable of producing an important degradation of the intestinal tract and finally the death of rodent. In vitro assays, AZAs elevate caspase activity, increase cellular concentrations of Ca^{2+} and cAMP, induce irreversible cytoskeletal rearrangements, deplete cellular ATP, inhibit neuronal ion flux and bioelectrical activity, inhibit cell–cell adhesion and stimulate cholesterol biosynthesis [37.21, 25]. Human symptoms of intoxication include nausea, vomiting, and stomach cramps, but thus far, no deaths have been attributed to AZAs [37.25].

37.3 Hydrophilic Toxins

37.3.1 Saxitoxin-Group Toxins (STXs)

Saxitoxin (STXs)-group toxins include various naturally occurring neurotoxic alkaloids that induce the so-called paralytic shellfish poisoning (PSP) in humans. PSP is characterized by symptoms varying from a slight tingling sensation or numbness around the lips to fatal respiratory paralysis [37.30]. The intake of toxins necessary to induce PSP symptoms varies greatly. This may be due to differences in susceptibility as well as a lack of precision in exposure assessments due to problems with sampling and analysis of contaminated shellfish at the time of intoxication.

They have been detected in bivalve mollusks from various areas of the world. Several decades ago, few localizations appeared to be affected by PSP; but nowadays they are found worldwide [37.31–33].

STXs are mainly produced by toxic dinoflagellates belonging to the genus Alexandrium: e.g., *A. tamarensis*, *A. minutum* (*syn. A. excavata*), *A. catenella*, *A. fraterculus*, *A. fundyense*, and *A. cohorticula*, *Gymnodimium, Pyrodinium* and they have also been identified in some cyanobacteria which may occur in fresh and brackish waters.

Most of STX-group toxins are water-soluble and heat-stable nonproteinaceous compounds whose basic structure is composed of a 3,4-propinoperhydropurine tricyclic system. Up to 57 different STX analogs have been described and they can be divided into subgroups based on substituent side chain such as carbamate, sulfate, hydroxyl, hydroxybenzoate, or acetate and also decarbamoyl variants [37.6]. STX, NeoSTX, GTX1, and dc-STX seem to be the most toxic analogs. These neurotoxins produce a blockade of ion conductance through the voltage-gated Na^+ channel (VGSC) in nerves and muscles fibers [37.30].

37.3.2 Domoic Acid

Domoic acid (DA) is a potent neurotoxic amino acid that accumulates in high concentrations in shellfish, anchovies, and sardines that feed on the toxic phytoplankton. DA can affect marine animals, seabirds, and humans via consumption of this contaminated shellfish causing amnesic shellfish poisoning (ASP). DA was first reported in Canada in 1987 when it caused various human deaths after consuming DA-contaminated mussels [37.34]. Effects on both gastrointestinal tract and nervous system were observed including vomiting, nausea, diarrhea, abdominal cramps, and hemorrhagic gastritis; neurological symptoms are headache, dizziness, vision disturbances, disorientation, loss of short-term memory, motor weakness, seizures, profuse respiratory secretions, hiccoughs, unstable blood pressure, cardiac arrhythmia, and coma. Neurological symptoms may occur after a delay of a few hours or up to 3 days [37.35]. DA especially harms the hippocampus and amygdaloid nucleus in the brain and damages the neurons by activating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, causing an influx of Ca^{2+} and cell degeneration [37.36, 37].

DA is produced by the red alga *Chondria armata* and also from the diatoms *Pseudo-nitzschia* spp. and

Nitzschia [37.38]. It is a water-soluble and heat-stable cyclic amino acid and it is structurally very similar to another known neurotoxin, the kainic acid [37.39]. Several isomers of DA (epi-domoic acid (epi-DA), (domoic acid C5′-diastereomer) and isodomoic acids A, B, C, D, E, F, G, and H (iso-DA A–H)) have been reported as well [37.7]. Iso-DA A, B, and C have not been detected in shellfish tissue. DA transforms into epi-DA through long-term storage [37.40] and degrades and transforms to epi-DA and iso-DAs through exposure to ultraviolet light [37.41–43]. In addition, the epimerization is also accelerated by heating [37.44].

37.4 Other Toxins

37.4.1 Ciguatoxins-Group Toxins

Ciguatoxins (CTXs) are a class of marine biotoxins that cause CFP. CTXs occur in fish as a result of biotransformation of precursor gambiertoxins produced by the benthic dinoflagellate *Gambierdiscus* spp. They are mainly associated to tropical and subtropical areas, but recently CTX-group toxins were identified for the first time in fish in Europe [37.45]. Various congeners of CTXs have been identified according to differences in their molecular structure: Pacific (P-CTX), Caribbean (C-CTX), and Indian (I-CTXs) [37.46].

Symptoms of the intoxication of ciguatera in humans include gastrointestinal and neurological effects [37.47]. Although, *Gambierdiscus toxicus* has been traditionally considered the main species responsible for CTXs production, nowadays it is known that *Gambierdiscus* spp. shows a high degree of complexity and it is composed of genetically and morphologically toxin producer species. Thus, distantly related groups co-occur across geography. Ten *Gambierdiscus* species were reported in the Atlantic and in the Pacific region [37.48, 49], and other new species were found in European Atlantic waters and in the Mediterranean Sea [37.50].

The congeners of CTXs are lipophilic polyethers compounds consisting of 13−14 rings fused by ether linkages into a rigid ladder-like structure. CTXs are colorless, tasteless, odorless, and relative heat stable, devoid of heteroatoms other than oxygen and bear few conjugated bonds. Nowadays, over 50 chemical congeners of CTXs have been identified whose toxicity can vary significantly: more than 100-fold among species of *Gambierdiscus* spp. compared with a 2 to 9-fold within species variation due to changing growth conditions [37.49]. The chemical structures of more than 20 P-CTXs analogs, main CTX in Pacific areas, have been identified, and structural modifications are seen in both termini of the toxin molecules, mostly by oxidation. Two CTXs from Caribbean sea (C-CTXs) were first isolated by *Vernoux* and *Lewis* in 1997 [37.51], and then were identified structurally. Other additional congeners were identified in 2002 [37.52]. More recently, four Indian Ocean CTXs (I-CTXs) have been reported [37.53], but their structural determination remains to be established.

The VGSC (Na_V) is the primary molecular target of CTXs, and their binding to the neurotoxin receptor site 5 of Na_V causes the opening of the ion pore, activation of the channels, and Na^+ entrance into the cells. Membrane depolarization and functional impairment of excitable cells are produced due to Na^+ entry into the cell. Secondary responses observed in cells exposed to CTXs include Ca^{2+} into the cell by reverse action of Na^+/Ca^{2+} exchangers eventually leading to muscular contraction and neurotransmitter release. Na^+ influx also affects the entrance of water into the cell, leading to cell swelling, blebbing, and cytotoxicity [37.45].

37.4.2 Brevetoxins (BTX)

Brevetoxin-(BTX) group toxins are neurotoxic polyether biotoxins which can accumulate in shellfish and fish. They are primarily produced by a dinoflagellate *Karenia brevis* (formerly called *Gymnodinium breve* and *Ptychodiscus brevis*) first identified in the Gulf of Mexico in 1947 [37.54–56]. However, other algae species (*Chattonella antiqua*, *Chattonella marina*, *Fibrocapsa japonica*, *Heterosigma akashiwo*) have also been reported to produce BTX-like toxins [37.57]. BTXs have been described in several localizations such as North America, New Zealand, Australia, Japan and Scotland [37.57–59]. *K. brevis* or *K. brevis*-like species have also been reported from Japan, New Zealand, West-Atlantic, Spain, Portugal, and Greece [37.57]. Nevertheless, no intoxication outbreaks in humans or occurrence of BTX-group toxins in shellfish or fish have been reported in Europe [37.55].

They are grouped into types A and B based on their molecular backbone structures. BTX-1 (or PbTx-1)13 (type A) and BTX-2 are considered to be the parent toxins from which other BTX-group toxins derive [37.60]. BTX A has a backbone of 10 fused cyclic ether rings; BTX B (most abundant in *K. brevis*) has a backbone of 11 fused cyclic ether rings [37.60–62]. It is important to highlight that BTX-group toxins are metabolized in shellfish and fish, yielding several metabolites of BTX-group toxins that finally affect to consumers [37.55, 63].

Ion channels are the principal target of BTXs by binding to receptor site 5 of the VGSC in cell walls [37.60] leading to an uncontrolled Na^+ influx into cells and depolarization of neuronal and muscle cell membranes. The response to BTXs in different cells, organs or in vivo models is dependent on various factors, such as the existence of different VGSC subtypes expressed.

37.4.3 Tetrodotoxin (TTXs)

TTX (11-[^{3}H]-tetrodotoxin) is a neurotoxin that has been identified from taxonomically diverse marine organism. Its name derives from *Tetraodontiformes*, an order that includes pufferfish, ocean sunfish or mola, triggerfish, among others. However, TTX has been described in a diverse range of phylogenetically unrelated organism, such as newts, frogs, nematodes, starfish, crabs, mollusk, and others. It has also been suggested that TTX is produced by symbiotic bacteria. Actually, there has been a debate between source of production of TTX from animals or bacteria, and nowadays it has not been firmly established a conclusion. TTX may serve as an antipredator defense, offensive weapon, or for within- and between-species communication [37.64].

Although TTX is associated to tropical waters, mainly South Asia and more specifically Japan, it has been also identified in Mexico and United States [37.65]. In Europe, TTX has been reported in migrant puffer fish in Greek waters [37.66] and in a gastropod *Charonia lampas lampas* in Portugal [37.67].

TTX is a heat-stable and water-soluble heterocyclic guanidine with a highly unusual structure containing a single guanidinium moiety attached to a highly oxygenated carbon backbone that consists of a 2,4-dioxaadamantane structure with five hydroxyl groups [37.68].

Tetrodotoxin binds to site 1 of the fast VGSC [37.33]. Site 1 is located at the extracellular pore opening of the ion channel. The binding of any molecules to this site temporarily disables the function of the channel. Saxitoxin, neosaxitoxin also bind the same site although TTX has different affinities for the variant Na^+ channel isoforms that confer resistance to various species [37.69]. TTX causes paralysis of voluntary muscles (including the diaphragm and intercostal muscles, stopping breathing), and reduced blood pressure, predominantly by vasodilatation. Early symptoms of this effect might include among others weakness, tingling of the lips, and dizziness [37.70]. Gastrointestinal symptoms are often severe and include nausea, vomiting, diarrhea, and abdominal pain. Cardiac arrhythmias may precede complete respiratory failure and cardiovascular collapse.

37.4.4 Palytoxin (PITXs) and Analogs

Palytoxin (PlTX) is one of the most poisonous nonprotein substances known to date. PlTX was first isolated and purified from *Palythoa toxica* [37.71] and currently present a worldwide distribution [37.71, 72]. PlTXs have also been detected in other marine zoanthids (soft corals) of the genus *Palythoa* (e.g., *P. tuberculosa*, *P. vestitus*, *P. mammilosa*, *P. carobaeorum*, *P. aff. Margaritae*) and benthic dinoflagellates of the genus *Ostreopsis* (e.g., *Ostreopsis siamensis*, *O. mascarenensis*, *O. ovata*) [37.73]. Blooms of *Ostreopsis* spp. have also been reported in European countries such as Spain, France, Italy, and Greece [37.74].

The PlTX-group toxins are complex polyhydroxylated compounds with both lipophilic and hydrophilic areas. They are white, amorphous, hygroscopic solids. At least eight different PlTX analogs are known: PlTX, ostreocin-D, ovatoxin-A, homopalytoxin, bishomopalytoxin, neopalytoxin, deoxypalytoxin, and 42-hydroxypalytoxin [37.75].

The main accepted molecular action of palytoxin is blockage of the Na^+/K^+-ATPase pump [37.76–78]. This binding inhibits the active transport of Na^+ and K^+ across the cell membrane that is essential for cell homeostasis, by transforming the pump into a nonspecific permanently open ion channel [37.79]. Symptoms of PlTX-group toxins intoxication are not well defined, but include weakness and myalgia, fever, nausea, and vomiting.

37.4.5 Cyclic Imines (CIs)

Cyclic imines (CIs) are a recently discovered group of marine biotoxins formed by: spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs), pte-

riatoxins (PtTXs), prorocentrolides, and spiroprorocentrimine. These toxins are macrocyclic compounds with imine- (carbon–nitrogen double bond) and spiro-linked ether moieties. They are grouped together due to that imino group acts as functioning pharmacophore [37.80] and the similarities in their *i.p.*, toxicity in mice [37.6, 81].

SPXs are the largest group of cyclic imines and together with GYMs are the best characterized. SPXs were described for the first time in contaminated shellfish, in 1991, in Canada [37.82]. These toxins are metabolites of dinoflagellates *Alexandrium ostenfeldii* and *Alexandrium peruvianum* and are sometimes found in the presence of other toxins such as PSP toxins [37.83]. Up to 14 SPXs analogs have been described of which 13-desmethyl SPX C is the most commonly found in shellfish [37.81].

GYMs are produced by the dinoflagellates *Karenia selliformes* and first isolated in oysters from New Zealand [37.84]. PnTXs and PtTXs are the CI structurally and synthetically more related to SPXs. PTXs were first discovered in extracts from the digestive glands of pen shell, *Pinna attenuata* in China and Japan [37.85]. Seven PnTXs analogs (PnTXs A-G) have been characterized [37.81]. Pteriatoxins A, B, and C, were isolated from the Okinawan bivalve *Pteria penguin* in 2001 [37.86]. *Torigoe* et al. [37.87] isolated the Prorocentrolide A for the first time from *Prorocentrum lima* and then the Prorocentrolide B was first isolated from *P. maculosum* in 1996 [37.88]. Finally, Spiro-prorocentrimine was isolated from a laboratory-cultured benthic *Prorocentrum* species of Taiwan by *Lu* et al. [37.89]. At present, it is accepted that nicotinic and muscarinic acetylcholine receptors are their principal target.

37.4.6 Maitotoxins (MTXs) and Gambierol

Maitotoxins (MTXs) and gambierol are a group of water-soluble toxins produced by species of dinoflagellates of the genus *Gambierdiscus*, which grow on algae in tropical waters around the world. *Gambierdiscus* is known to produce both MTX and CTXs, which accumulate in the body of herbivorous fish and are transmitted through the tropical food chain to carnivorous species.

MTX is a polyketide-derived polycyclic ether integrated by four rigid polyether ladders connected by mobile hydrocarbon chains. Maitotoxin includes 32 ether rings, 22 methyls, 28 hydroxyls, and 2 sulfuric acid esters and has an amphipathic structure [37.90–92].

Maitotoxin activates Ca^{2+} permeable, nonselective cation channels, leading to an increase in levels of cytosolic Ca^{2+} ions. It is thought that maitotoxin leads to the formation of pores on these ion channels. Finally, a cell death cascade is activated, resulting in membrane blebbing and ultimately cell lysis [37.93]. Maitotoxin is known to activate cytosolic Ca^{2+}-activated proteases calpain-1 and calpain-2, contributing to necrosis [37.94].

Gambierol was described as a potent potassium voltage-gated channel blocker that exhibits potent acute lethal toxicity against mice (minimal lethal dose: 50 µg/kg, *i.p.*) and it is suspected to participate in the symptoms of CFP.

37.5 Biotechnological Techniques Used to Study Toxic Microalgae and Marine Biotoxins

The potential use of several techniques for marine biotoxins or phytoplankton characterization are explained along the chapter. In general, the sensitivity of the analytical methods and bioassays depends on the toxins studied. Combination of several approaches, including chromatography-based techniques, to identify individual toxins and the overall toxicity of the mixture, should provide an ideal alternative. Thus, a synergistic complement can be obtained by coupling biological and or functional assays with analytical techniques based on the physicochemical properties of the toxins.

37.5.1 Immunological Methods

The main advantage of the immunological techniques is the variety of immunoglobulins for the specific detection of different targets. These are characterized by sensitivity, specificity, fast performing, and automation. However, the corner stone for these methods seems to be the quality of the antibody presenting cross-reactivity limitations.

An unequivocal identification of phytoplancton and detection of phycotoxins can be obtained by using a species-specific antibody against the target organism

or group of toxins. Specific polyclonal or monoclonal antibodies can be reactive to one epitope and therefore more specific. Combination of some of them, allows the identification, counting, and examination of marine phytoplankton. Use of antibodies with fluorescent markers has made possible to apply different technologies, such as microscopy, flow cytometry, or flow cam. The light emitted by a labeled antibody can be detected by a fluorescent microscope, equipped with a UV light, a flow cytometer a confocal microscope or a flow cam. For instance, several monoclonal antibodies were obtained against different species of *Alexandrium* allowing the unequivocal identification of the dinoflagellate *A. minutum* [37.95]. Then, using immunofluorescence could help to identify different species in natural samples during coastal monitoring.

To discriminate and count small cells or a large number of samples, flow cytometry can be used. Designed to automate the rapid analysis and identification of cells, flow cytometry is a reliable method for the routine monitoring of the abundance of phytoplankton species, leading to an early detection of HABs. This is an optimal instrument for analyzing cells ranging from 0.5 to 20 μm in diameter (concentrations between 10^6 to 10^9 cells/L [37.96].

In this context, a modified flow cytometer, known as FlowCam, was developed to handle larger cells. It is an integrated system combining the capabilities of flow cytometry, microscopy, imaging, and fluorescence that counts, takes images and analyses the particles that range in size from 20 to 200. In addition, to monitor harmful species of microalgae, other potential applications of immunodetection include studies of plankton community structure and ocean optics. Other authors used Imaging FlowCytobot that combines video and flow cytometric technology to capture images of nano- and microplankton and to measure the chlorophyll fluorescence associated with each image. The images are of sufficient resolution to identify many organisms to genus or even species level [37.97].

The use of immunological assays has become a promising alternative for detecting small molecules such as marine biotoxins and many kits are commercially available.

Enzyme-Linked Immunosorbent Assays (ELISAs)

The enzyme-labeled immunosorbent assay ELISA is a powerful analytical tool for natural toxins detection. Specific antibodies recognize toxins and this bound complex is quantified by labeling the free component with a reporter enzyme, usually horseradish peroxidase (HRP) acting as an amplifier to produce many signals. The use of the 96-well microtitre polystyrene plate, the automation and the competitive principle are the most commercial immunoassays for natural toxins.

The ASP ELISA for the determination of DA was approved by the EU as an alternative to the official chemical method HPLC-UV detection [37.98]. This kit is a direct competitive immunoassay where free DA in the sample competes with DA-conjugated protein coated on plastic wells for binding to antidomoic acid antibodies in the solution. Other ELISA tests were developed as the Ridascreen Fast PSP, a competitive ELISA for the quantitative analysis of STX and related toxins. A monoclonal antibody with high affinity against brevetoxin B was used to develop a competitive ELISA for detection of brevetoxins in molluscs [37.99]. Production of monoclonal antibodies for immunoassays by immunizing mice with a synthetic hapten conjugate, instead of the natural toxins, allowed the detection of Pacific ciguatoxins using a direct sandwich ELISA [37.100, 101]. Recently, an improvement of the efficiency and simplification of a colorimetric competitive indirect immunoassay ELISA was carried out based on direct labeling via a covalent bond to the anti-OA antibody [37.102, 103] and a chemiluminiscent ELISA method for this toxin was also developed [37.104].

The challenge of designing antibodies with the optimal specificity and matching toxicity with toxic levels is further complicated by the structural and toxicological diversity within a toxin group together with a potential chemical conversion and biotransformation. However, since recombinant antibodies have been successfully constructed against PITX [37.105], similar strategies could help the challenge for complex toxins groups. Lack of standardization and transferability of in house ELISA tests between laboratories should be overcome by increasing the focus on validation, based on new strategies for production of high-quality antibodies. Nevertheless, a comparative evaluation of ELISAs and EU reference methods for the detection of the hydrophilic toxins ASP and PSP was carried out in many seafood products [37.106]. The high correlation coefficient between official and immunoassays proves that these tests can be used as screening systems in a variety of species without matrix interference. However, the best sensitive bioassay will depend on the toxins evaluated. In this context, the advantages and disadvantages of ELISA and cytotoxicity assays to detect BTXs were recently reviewed [37.107].

Phage Display Technology

The phage display technology refers to the expression of peptides, proteins, or antibodies on the surface of filamentous bacteriophage viruses. This in vitro strategy provides a valuable system for easy and rapid generation of specific antibodies fragments directed against different antigens, including difficult targets as small molecules. Semisynthetic phage display libraries were used to select recombinant antibodies against PlTX. Some antibodies proved high affinity for immobilized and free PlTX in a competitive ELISA with a low detection limit, a very reproducible standard curve and a wide working range [37.105].

Biosensors

Biosensors are measuring systems based on a biological recognition component with three functioning parts: a biological element, cells, microorganisms, antibodies, the transducer, capable of measuring a biomolecular interaction and converting it into a signal, and the electronics, responsible for the display of the results easily interpreted.

Most of the biosensors developed for shellfish toxins are immunosensors and the optical biosensor with surface plasmon resonance (SPR) is the most widely used in the large majority of reported platforms [37.108]. It has been incorporated into a wide range of devices to measure the analytes present in a sample. For most biomolecules, the change in response is proportional to the mass of the material bound to the surface of the sensor chip. The measure of the binding response against time is called *sensorgram* [37.109]. Small molecular weight compounds, as many drugs and toxins, are very difficult to measure. Then, an inhibition assay approach that relies upon the immobilization of analytes to the chip surface and the injection of the binding partner over the chip surface was developed. Using this assay format, the level of binding to the surface is inversely proportional to the concentration of the target analyte present in the sample. There are three key elements that determine the way in which the methods perform.

- The binding protein, often an antibody, is essential in terms of delivering a specific molecular interaction with the target analyte.
- The surface chemistry is a very important parameter in an SPR assay. Small compounds can be immobilized via the production of an analyte–protein conjugate and this, in turn, is immobilized onto the surface on a sensor chip via an amine or carboxyl coupling strategy. While this approach can be relatively straightforward, it has two main disadvantages. Firstly, during the regeneration of the chip surface, the immobilized conjugate can alter its three-dimensional structure and, thereby, bring about a different level of interaction with antibodies during subsequent interaction measurements. Secondly, the lifespan of an immobilized conjugate on a chip surface tends to be very short. The sensor chips tend to be the most expensive part of the assay, resulting in a high costper test. A much better way to produce chip surfaces is through the direct immobilization of the analyte onto the sensor surface, being much more robust and stable than the conjugate approach.
- The type of method used for sample preparation has to be compatible with this analytical approach. In general, due to the advantages of SPR over conventional immunoassays, i. e., as they are nonequilibrium based and flow through based, in many cases the requirement for a highly purified sample extract can be avoided [37.96].

Some examples of biosensors for DA, OA, PSP, or TTX have been developed, tested and/or validated [37.96, 110–113]. However, one challenge for these assays is the ability to simultaneously measure multiple biotoxins in a single bioanalytical system as was performed in a Framework 7 Project entitled CONFFIDENCE (www.conffidence.eu). Within this project, the SPR methods developed for DSP, ASP, and PSP are being combined onto a single sensor chip using a multichannel biosensor developed as part of another European project Biocop (www.biocop.org).

Comparison of ELISA and SPR biosensor technology for detection of PSP toxins shows that the method of choice will depend on the end-user needs. Some authors point out the reduced manual labor and simplicitiy of SPR biosensor compared to ELISA, ease of sample extraction, and superior real time semiquantitave analysis [37.114], although lower costs and high sensitivity are some advantages of the ELISA.

DNA-biosensors have specific probes that target only DNA-sequences present in the organism of interest and can be used on-site and therefore circumvent the need to return samples into the laboratory. A DNA-biosensor was adapted by *Metfies* et al. to the electrochemical detection of the toxic dinoflagellate *A. ostenfeldii* [37.115]. The technical background of this device is explained in detail in the German patent application DE 10032 042 A1 (Elektrochemischer Ein-

wegbiosensor für die quantitative Bestimmung von Analytkonzentrationen in Flüssigkeiten). The device could facilitate the work that must be undertaken in the course of monitoring toxic algae by eliminating the need to count algae and reduce the toxins tests.

An immunosensor based on magnetic beads as support to immobilize OA on the surface of SPE and then to perform indirect competitive immunoassay using differential pulse voltammetry as a method of electrochemical detection [37.116]. Modifications of this automated flow-through amperometric immunosensor were performed for highly sensitive detection of OA [37.117, 118]. Other sensors include the molecularly imprinted polymer (MIP)-based sensors that use templates to provide polymers with specific recognition properties and chemosensors based on the photoinduced electron transfer (PET) sensing of the toxins by synthetic fluorophores.

Lateral Flow Immunochromatography

Lateral flow immunochromatographic assays are based on a competitive immunoassay format, similar to the home pregnancy test and provide a qualitative indication to the presence of OA and analogs, and DA. These methods also faced the same antibody cross-reactivity challenge.

37.5.2 Enzymatic, Colorimetric, and Other Tests

Several studies based on the mechanism of action of OA-group toxins, inhibition of the enzyme PP2A, were designed and adapted. Some systems as fluorimetry or colorimetry were the most common employed [37.119, 120], although other as electrochemical biosensors were also reported [37.102, 121].

Numerous colorimetric protein phosphatase inhibition assays have been recently reviewed [37.102, 103]. An enzymatic colorimetric test, OkaTest, based on the OA-group of toxins mechanism of action, was recently internationally validated to be used as an alternative or complementary to the reference method for monitoring the OA toxins group in molluscs [37.122] according to Commission Regulations (EC) No. 2074/2005 [37.123] and No. 15/2011 [37.124].

Recently a ligand binding assay was developed to detect cyclic imine in the frame of the Atlantox project, within the Atlantic Area Operational Programme (Atlantox 2008-1/003): www.atlantox.com. This assay, under patent, is based on the mechanism of action of these toxins that act on the nicotinic acetylcholine receptor.

A fast, simple, and sensitive capillary electrophoresis-based immunoassay with electrochemical detection was developed for STX and analogs in shellfish. The method was based on competitive reactions between HRP-labeled antigen and free antigen with a limited amount of antibody [37.125].

The feasibility of OA detection in real-time by using an acoustic wave platform with a microfluidic feature and an ELISA-like protocol was recently reported [37.126].

37.5.3 Receptor Binding Assay (RBA)

The receptor binding assay (RBA) is a high-throughput method for detection of toxins in extracts of seawater and shellfish. This method is based upon the competition between radiolabeled and unlabeled toxins for available receptor sites. The radioactive toxin is displaced from its receptor by toxin present in an unknown sample, thereby, reducing the total radioactivity. Thus, the amount of radioactively labeled toxin that is displaced is proportional to the amount of toxin in the unknown sample. Then, the toxin present in an unknown sample can be quantified by comparison to a standard curve obtained using pure toxin.

RBA was developed from the isolation of VGSC obtained from animal tissues, and was basically implemented by competitive measures of radioactivity using tritiated saxitoxin ([^{3}H]-STX) or brevetoxin (PbTx). Vieytes et al. published a rapid and sensitive method that used Na^+ channels obtained from rat brain membranes to detect PSP toxins. Na^+ channels were coated onto microtiter plates and were used to develop a direct solid-phase binding assay [37.127]. Years later, more developed method was described by *Doucette* et al. based on microplate scintillation technology. Good quantitative agreement of the assay with MBA and HPLC analysis of crude extracts of contaminated shellfish, as well as PSP toxin-producing algae, was observed [37.128]. Usup reported about binding properties of six saxitoxin congeners using [^{3}H]-STX and a preparation rich in Na^+ channels. In this study, EC50 values ranged from 4.38 nM for STX to 142 nM for GTX5 [37.129].

RBA has been refined in the last decade for the detection of PSP toxins. These methods suffer from limitations such as the availability of radio-labeled materials. Restrictions on the world-wide distribution of [^{3}H]-STX imposed by the international Chemical

Weapons Convention in 1997 served to other radiolabeled toxin development such as 11-[^{3}H]-tetrodotoxin (TTX) [37.130, 131]. In 2004, thanks to collaborative efforts among the US Food and Drug Administration (FDA), International Atomic Energy Agency (IAEA), and the National Oceanographic and Atmospheric Administration (NOAA), the worldwide unavailability of ^{3}H saxitoxin was solved temporarily. A relative stable [^{3}H] STX is now distributed worldwide by IAEA and was used in collaborative trials of the PSP toxins using receptor binding assay [37.132]. The assay used a competition among PSP toxins, in shellfish extracts, and a labeled ^{3}H STX diHCl for binding to VGSC in a rat brain membrane preparation. Quantification of binding was carried out using a microplate or traditional scintillation counter. The study focused on the ability to measure the PSP toxicity of samples below, near, or slightly above the regulatory limit of 800 µg STX diHCl equiv/kg [37.1]. The correlation with the MBA (OMA 959.08) yielded a correlation coefficient (r2) of 0.84, while correlation with the precolumn oxidation HPLC method (OMA 2005.06) yielded a (r2) of 0.92. This method has been accepted as official method of analysis AOAC (OMA 2011.27) [37.133].

Similar to PSP toxins method; BTX RBA is a functional bioassay in which an unknown quantity of nonradiolabeled BTX competes with radiolabeled BTX [^{3}H]PbTx-3, for the site 5 receptor of VGSC. Although, the assay using direct detection of a radiolabeled probe, bound to rat brain membranes, is a simple and robust format of pharmacological assays; other variations have been also tested using phospholipid vesicles [37.134] and membranes from other animals than rats [37.135, 136]. More recently other study has compared various extracts of BTX using four independent methods: RBA, radioimmunoassay (RIA), neuroblastoma (N2A) cytotoxicity assay, and liquid chromatography/mass spectrometry (LC-MS). As determined by LC/MS, the RBA, RIA, and N2A cytotoxicity assay detected 73, 83, and 51% of the total BTX concentration [37.137].

RBA designed for CTXs traditionally measures the binding competition between CTX in the sample and a [^{3}H]PbTx standard for the Na^+ channel receptor. The use of [^{3}H]PbTx is a necessity, as nowadays no tritiated CTXs is available. It should to be noted that RBA was successfully applied for monitoring programs on ciguatera risk in three islands of French Polynesia [37.138, 139].

In the case of ASP toxins, the RBA quantifies DA activity by the competitive displacement of radiolabeled kainic acid from a cloned glutamate receptor by DA in a sample. In 1997, *Van Dolah* et al. [37.140] reported the further development of the receptor assay using a cloned rat GLUR6 glutamate receptor. The limit of detection and selectivity of the assay were optimized through inclusion of the glutamate decarboxylase (GLDC) pretreatment step to eliminate potential interference due to high concentrations of endogenous glutamate in shellfish. However, as GLDC was difficult to obtain from suppliers, other variations were introduced [37.141].

Due to limitations of the standard AOAC MBA for estimation of toxins levels there was a need for alternative testing protocols. The relevance of RBA tools is high, but the need for specific equipment and licensing requirement for the use of radiolabeled compounds may lead to obvious limitations in the application of this assay. Some main disadvantages include the high dependency on the receptor source, it does not provide any information on the toxin profile, it cannot be easily automated, and it has been validated only for a few toxins. However, RBA is adequately sensitive and more specific than MBA [37.45]. Attempts to modify this assay to use label other than tritiated forms has led to the development of a competitive RBA based upon the use of BTX-B2 labeled with a chemiluminescent acridinium moiety. The acridinium brevetoxin-B2 seems to be a promising alternative to the conventional radioactive ligand in order to avoid constraints associated with RBA [37.46, 142].

37.6 Biotechnology Application for Phytoplankton Detection, Monitoring, and Toxins Production

Recent development and application of advanced technologies from the generically defined *-omics* sciences (genomics, transcriptomics, proteomics, and metabolomics) coupled with bioinformatics platforms has already provided deep and often revolutionary shifts in understanding the ecology and evolution of phytoplankton species and bloom dynamics [37.143].

Harmful algal blooms (HABs) are natural events that have become common in coastal waters worldwide during the last years, being recurrent in Euro-

pean coastal waters. The toxic microalgae responsible for these blooms are mainly dinoflagellates, which display heteromorphic life cycles, including motile planktonic stages, as well as immotile benthic resting cysts [37.144, 145].

Monitoring of harmful species is important to understand population dynamic activities related to the exploitation of coastal areas. To distinguish species by their morphological characteristics, cells must be examined by light, epifluorescence, or electron microscopy. This can be time consuming, and requires taxonomic expertise. Moreover, in some cases, morphology-based diagnostic tests lead to misidentification due to the existence of morphologically closely related species. Molecular methods, generally based on the DNA probe hybridization and PCR techniques, have been developed for the rapid and sensitive identification of HAB species [37.145–147]. The target DNA genes are generally within ribosomal RNA operons, which are repeated in high copy number in the microalgae genome and can be genus or species specific, as 18S, 28S, 5.8S, ITS1, and ITS2 among others.

37.6.1 Genomics

Conventional genomics is often based upon the sequencing and annotation of whole genomes, with subsequent bioinformatics focus on the structure and function of key groups of genes. However, the ability to perform genetics experiments with microalgae has been accelerated in recent years by the availability of genome sequences. The application of emerging genomics techniques to phytoplankton research is likely to become widespread in the future.

Sequencing

Currently, there are more marine cyanobacterial genome sequences available than eukaryotic phytoplankton genome sequences, primarily due to differences in genome size and complexity. The average cyanobacterial genome size is 2–3 million base pairs (Mbp), although they range up to 9 Mbp. In contrast, eukaryotic genomes tend to be orders of magnitude larger [37.148]. Completed genome projects have focused on species with relatively small genomes, ranging from 12 [37.149] up to 57 Mbp [37.150]. The recent availability of marine phytoplankton genome sequences has prompted a wealth of research in diverse fields including ecology, evolution, biochemistry, and biotechnology.

Concerning eukaryotic marine phytoplankton, it must be considered that it includes phylogenetic lineages resulting from primary, secondary, and tertiary endosymbiosis events. This phylogenetic and physiological diversity is translated into a wide range of potential genome sizes, structures, metabolic pathways, life histories, and evolutionary relationships. Of the four major lineages of eukaryotic phytoplankton, whole genome sequences are currently available for two; the heterokonts [37.150, 151] and the prasinophytes [37.149, 152]. Additional members of the heterokont and haptophyte lineages are in the pipeline for sequencing or draft genomes are being analyzed (e.g., the haptophyte *Emiliania huxleyi*; http://genome.jgi-psf.org/Emihu1/Emihu1.home.html) [37.148].

Genomics are more than simply genome sequencing including transcriptional analyses of both sequenced [37.153, 154] and unsequenced organisms [37.155], and also targeted metagenomics of ecologically important but uncultured phytoplankton [37.156].

One of the challenges in examining structural diversity in eukaryotic phytoplankton is the large number of novel genes identified in each newly sequenced genome that have no homology to genes with known or suspected functions. Often, they are similar only to genes of unknown function in closely related organisms. Depending on the genome, the number of novel genes ranges from 20 to nearly 40% highlighting the limitations of homology-based approaches. Differences in gene structure (e.g., novel splice sites, signal peptides, and untranslated regions) constitute a challenge to gene-calling algorithms designed for higher plants and metazoans. One solution has been the development of tiling arrays for sequenced genomes [37.148] that differs from traditional microarrays in the nature of the probes. Instead of probing for sequences of known or predicted genes that may be dispersed throughout the genome, tiling arrays probe intensively for sequences which are known to exist in a contiguous region. This is useful for characterizing regions that are sequenced, but whose local functions are largely unknown. Using tiling arrays *Mock* et al. [37.154] predicted 3470 new genes in the *Thalassiosira pseudonana* genome that were not previously identified using standard gene-calling algorithms. This 33% of increase in the total number of genes predicted in the *T. pseudonana* genome suggests that gene density and number are not fully captured using standard gene-finding algorithms. Sequenced genomes also highlight the phyloge-

netic and metabolic diversity that characterizes marine phytoplankton. Description of new lineages, such as the uncultured picohaptophytes suggests that probably many ecologically important lineages have yet to be discovered [37.156].

Investment in genetic techniques both available and robust in a diverse array of marine phytoplankton will greatly help in understanding gene function, especially for the large number of hypothetical and conserved hypothetical genes [37.148]. New approaches have emerged and will continue to emerge that rely on genome sequence data. For example, whole genome microarrays include a range of targets such as all annotated genes or even the whole genome. New directions in this area will use genome information from diverse keystone species to create microarrays for use in characterizing environmental samples; mRNA sequencing approaches such as Illumina sequencing generate only short reads and can take advantage of available genomes to examine gene expression. For example, mRNA from two conditions is used to generate cDNA libraries that are sequenced to obtain quantitative gene expression profiles that can be compared.

The application of these emerging genomics techniques to phytoplankton research is likely to become widespread in the future. One challenge beyond the genomics revolution is to apply these techniques in the marine environment to examine phytoplankton physiology in situ. This will mean synthesizing many different types of data simultaneously such as real-time physical and chemical environmental variables, community species composition, gene expression profiles, and protein signatures. This system-level approach has the potential to greatly expand our understanding of how phytoplankton acts in their environment [37.148].

PCR and Real-Time PCR (qPCR)

qPCR is a very promising technique in terms of sensitivity, specificity, enumeration capability, and costs reduction [37.145, 157]. Molecular approaches, when used together with other morphological characters, may help to solve taxonomical problems. The nuclear rDNA internal transcribed spacer regions and the 5.8S rRNA gene are widely used to characterize the phylogenetic aspects of a great variety of these organisms [37.147, 158–160].

Even though these applications are very valuable regarding human health, the use of PCR and qPCR for the direct detection and identification as well as monitoring of toxic phytoplankton is of special interest. Several studies have been developed for simplex and multiplex detection of different groups, genus and/or species, as *Alexandrium*, *Ostreopsis*, or different cyanobacteria [37.161–164]. It is important to highlight the fact that environmental samples often contain complex mixtures of organic matter, which may inhibit PCR/qPCR, thus an internal amplification control (IAC) should be included to avoid misinterpretation of false-negative results. Additionally, different strategies have been applied to overcome the problem of PCR/qPCR inhibition caused by contamination substances: the use of thermostable DNA polymerase, commercial nucleic acid extraction kits that remove inhibitors and facilitate purification, dilution of template DNA prior to PCR/qPCR assays and the use of bovine serum albumin in the PCR/qPCR assay [37.144, 163]. Several studies have been published applying PCR/qPCR for monitoring HAB worldwide, from the Mediterranean Sea to Australia [37.157, 164–166].

Regarding toxin quantification/detection, it is more often performed by other techniques, but different studies have also been performed applying PCR/qPCR. In 2011, *Murray* et al. developed a method targeting the unique core gene *sxtA* to identify saxitoxin-producing HABs in Australian marine water [37.165]. Their method was tested against different species and strains of *Alexandrium*, *Ostreopsis*, *Gymnodinium* among others. *Al-Tebrineh* et al. also developed a qPCR method capable of detecting SXT producing species, but they took advantage of the higher throughput of multiplex formats of qPCR and also targeted the genes coding for microcystin (*mcy*), nodularin (*nda*), cylindrospermopsin (*cyr*) in a multiplex qPCR experiment to specifically detect toxin producing species [37.163].

Microarrays

In a microarray study, gene-specific probes are spotted onto precise positions on a solid surface and targeted with an unknown DNA or RNA sample. Microarrays represent the scaling up, miniaturization, and automation of well-known hybridization techniques. Recently, a FP7 European research project called MIDTAL (Microarrays for the detection of toxic algae) aimed to construct a universal microarrayfor the detection of harmful algae and their toxins (htpp://www.midtal.com/). Results obtained showed the third generation of the MIDTAL microarray that includes between 140 and 163 probes for various toxic algal species including ASP, DSP, and PSP producers. The designed microarray has great potential to be used as a monitoring tool for toxic algae detecting multiple species simultaneously although improvements in RNA extraction

and tests are still needed [37.167]. The most obvious advantage of the microarray is its ability to identify species that cannot be identified with traditional methods, such as *Pseudo-nitzschia*, *Alexandrium*, and *Heterosigma* [37.168, 169].

Other Genomic Techniques

Other techniques different from sequencing and PCR have been also applied in phytoplankton studies. These include restriction fragment length polymorphism (RFLP), the first DNA-based technique used in phytoplankton studies; denaturing gradient gel electrophoresis (DGGE); single stranded conformation polymorphism (SSCP); random amplified polymorphic DNA (RAPD); amplified fragment length polymorphism (AFLP); Microsatellites or short sequence repeats (SSRs); molecular probes and single nucleotide polymorphisms (SNPs). Most of these techniques were quite useful to study phytoplankton especially when no sequences were available because they can be used without prior knowledge of the genome [37.170].

37.6.2 Transcriptomics

Transcriptomic studies gather efforts to isolate all the RNA transcripts, i. e., the transcriptome, from a sample of cells, and determine the presence and abundance of each particular species of gene-encoding RNA or mRNA. These studies help to elucidate genes involved in nutrient acquisition and metabolism (including those involved in synthesizing or regulating the synthesis of toxic molecules), aid in annotating genomes, and also have the potential to find novel transcripts or transcripts with unusual structures that may hint at the mechanism of gene regulation [37.143, 171, 172].

For many organisms with either large and/or complex genomes, transcriptome sequencing may be a more immediate and cost-effective route than whole genome sequencing to obtain ecological and evolutionary information.

An expressed sequence tag or EST is a short subsequence of a cDNA. They may be used to identify gene transcripts in gene discovery and sequence determination. The identification of ESTs has proceeded rapidly, with approximately 74.2 million ESTs now available in public databases (e.g., GenBank 1 January 2013, all species). The cDNAs used for EST generation are typically individual clones from a cDNA library. The resulting sequence is a relatively low quality fragment whose length is limited by current technology to approximately 500–800 nucleotides. Because these clones consist of DNA that is complementary to mRNA, the ESTs represent portions of expressed genes. They may be represented in databases as either cDNA/mRNA sequence or as the reverse complement of the mRNA, the template strand. ESTs can be mapped to specific chromosome locations using physical mapping techniques, such as radiation hybrid mapping, Happy mapping, or FISH. Alternatively, if the genome of the organism that originated the EST has been sequenced, one can align the EST sequence to that genome using a computer.

EST analysis of the dinoflagellate *A. minutum* revealed 192 genes that were differentially expressed between isolates that produced PSP toxins and those that did not [37.173]. In terms of the functional ecology of *A. minutum*, the 192 genes are putative candidates for genes involved in toxin synthesis and regulation or acclimation to intracellular PSP toxins. The sequences in the EST library also suggest that PSP toxins generated by this alveolate did not arise from a recent gene transfer from cyanobacteria, as was previously hypothesized [37.173]. Other dinoflagellate EST sequencing projects have yielded further insights into toxin production, predator–prey interactions, genome architecture, and trophic status. As sequencing costs drop and capabilities increase, transcriptome analyses of marine phytoplankton species and strains within species will soon become not only feasible but also common. This approach has also been recently applied to *A. ostenfeldii* [37.143, 174].

37.6.3 Proteomics

The field of proteomics is complementary to genomics and transcriptomics since it provides additional information on gene expression and regulation. Proteomics include the determination of protein expression levels and protein–protein interaction studies. Moreover, proteomics aim to identify posttranslational modifications of proteins, as well as the organization of proteins in multiprotein complexes and their localization in tissues [37.172].

In the past decade, genomics began to explore the diversity, cellular evolution and adaptive abilities of marine phytoplankton. Although this has provided the community with a huge amount of information, it is not necessarily being translated into biochemical expression or phenotype. Genomics demonstrates which genes are shared, but proteomics can show clearer relationships by illustrating functional similarities and phe-

notypic variances. With a well-designed experiment, researchers can examine the conditions under which a protein is expressed; its cellular location, the relative quantities, and what protein–protein interactions take place [37.175].

Chan et al. [37.176, 177] studied protein profiles under different environmental conditions found in *Prorocentrum triestinum*. He showed both constancy of protein profiles and variability of the relative abundance of some proteins under different and culture conditions. Differentially expressed protein patterns of *P. triestinum* indicated that *P. triestinum* growing under nitrogen-limited conditions possessed higher growth potential since its protein expression profiles are more similar to that of the blooming stage. These results are consistent with the observations that it is possible to rapidly trigger a bloom by the addition of a supply of nitrates. A more detailed analysis of the differential synthesis of proteins in these various stages and studies to elucidate the functions of these proteins will be critical for understanding the molecular mechanisms involved in blooming. Phytoplankton proteomic approaches that use whole genome sequences to examine protein expression by matching protein MS/MS spectra to those predicted from whole genome sequences are already available [37.150]. Cell surface proteins (CSPs) of *Alexandrium catenella* have been also studied in detail. Protein profiles at different toxin biosynthesis stages, were analyzed and 53 differentially expressed proteins were identified. These were involved in various biological processes, nine of which might be involved in the PST biosynthesis of *A. catenella* using the quantitative proteomic approach to compare protein profiles at different toxin biosynthesis stages.

Proteomics studies should help to reveal the toxin biosynthesis mechanisms and pathways in dinoflagellates [37.178]. Proteomics has provided valuable information on the effects and also the mode action of the dinoflagellate metabolite yessotoxin on a human liver cell line. Also, analysis of the plastid proteome of the Florida red tide dinoflagellate *K. brevis* detected an electron transfer protein (plastocyanin) inherited from green algae that may contribute to ecological success in iron use. Nevertheless, much promise remains to be fulfilled in the application of proteomics to studies of HAB ecophysiology and bloom dynamics [37.179].

37.6.4 Metabolomics

The term metabolomics is usually defined as the comprehensive and quantitative analysis of all small molecules in a biological system [37.180]. Metabolomics allows monitoring and mapping the dynamic range of bioactive secondary metabolites under different conditions. The most commonly used gas chromatography-time-of-flight mass spectrometry (GC-TOF-MS)-based method facilitates the identification and robust quantification of a few hundred metabolites. The merging of metabolomics and metagenomics has the potential to be a particularly powerful combination in shedding light on planktonic and microbial interactions in marine ecosystems. Statistical evaluation of data provides insight into the metabolic changes within the cells as well as into the released metabolites. The knowledge of the complex patterns of chemicals released by microalgae will reveal new mechanisms for processes such as: community function, food location, or complex defensive and allelopathic interactions [37.181].

A great number of metabolic pathways and regulatory mechanisms could be predicted based on the genomic sequences. Nevertheless, some of the genes might not be functional in vivo. Recent technical developments make metabolomics a suitable tool to define their genes function and regulation in microalgae. Analyses of diatom genomes have suggested tight connection between a number of novel pathways and regulatory steps in diatom metabolism. However, there are still several important open questions to be addressed. Given the massive recent acceleration in genome sequencing capacities we will shortly have an enormous resource of genetic information for marine phytoplankton. Combining this information with details on their metabolic complement will greatly enhance our understanding of the metabolic processes by and how they interact with their environment, as well as the evolution of their underlying metabolic pathways [37.182].

Metabolomics approach has already been successfully applied to the dinoflagellate endosymbiont *Symbiodinium* [37.179]. As an emerging technique, metabolomics have not been widely applied in toxic phytoplankton studies, but some research groups are developing databases for identification of marine biotoxins using metabolomics approaches [37.183].

37.7 Potential Pharmacological Uses of Phycotoxins

In the past few decades, extensive studies have been devoted to investigate the toxicology and pharmacology of biotoxins [37.184, 185]. A renewed interest is seen in marine alkaloids and their analogues, including the STX-group toxins (STXs), with regards to their use as therapeutic agents or as a drug lead [37.186]. STXs bioactivity studies and molecular modeling could lead to the design of unnatural analogs with improved pharmaceutical characteristics. Recently, a group of toxins isolated from marine cone snails (genus *Conus*), known as conotoxins, have been shown to contain over 2000 peptide analogs. Conotoxins are able to specifically target a broad range of ion channels and membrane receptors and are currently under investigation for possible clinical trials. In 2004, a synthetic version of a conotoxin analogue, ω-conotoxin MVIIA, also known as ziconotide (trade name Prialt) was the first marine natural product to be approved for use by the US FDA [37.186]. Ziconotide acts by targeting N-type voltage sensitive Ca^{2+} channels and is used for the treatment of chronic pain in spinal cord injury.

Ion channels triggered by voltage changes along the cell membrane are termed voltage-gated ion channels (VGICs), include channels that conduct Na^{+}, Ca^{2+} and K^{+}. Activation of Na^{+} or Ca^{2+} VGICs produces cell stimulation by membrane depolarization, while K^{+} channel activation results in inhibitory hyperpolarization. Toxins that bind VGICs can serve as powerful molecular probes to study the channel structure and function, as well as serve as models for studying evolutionary targets and drug discovery [37.187]. Some marine biotoxins are classified as neurotoxins due to their ability to interact with these channels [37.188]. As we will discuss later in this section many marine biotoxins have affinity by one or more VGICs and therefore could have an important pharmaceutical potential.

37.7.1 Lipophilic Toxins (LTs)

Okadaic Acid-Group (OA) Toxins and Pectenotoxin-Group Toxins

OA is a marine biotoxin that presents a great value in medical research and has facilitated the understanding of several cellular processes [37.189]. OA-group toxins have interest due to their high cytotoxic activity and the extraordinary ability to promote tumors [37.190, 191]. It is considered that their effect varies depending on the cell type and concentration used [37.192].

It has been a model for analyzing therapeutic effects of some drugs in treating neurodegenerative diseases [37.193]. OA acts as a potent neurotoxin that induces apoptotic events in various cell lines and promotes tumors in several organs. Mechanisms are dependent on cell type, the antioxidant status of each cell and the ability to metabolize OA [37.194–199].

OA-group toxins are specific inhibitors of PP1 and PP2A that are involved in the regulation of many cellular processes and modulate the extent of phosphorylation or dephosphorylation of proteins. Other cellular targets for OA have been suggested [37.189, 200–203]. OA has been used to analyze the mechanisms by which the conjugated linoleic acids can act as antitumor agents in breast cancer cells due to its activity as an inhibitor of PP2A [37.189]. In addition it has been reported that the extent of OA-induced injuries and the toxin organotrophicity are dose-related and may be determined by the administration route. After intravenous administration, OA acts as a hepatotoxin with undetectable effects on the intestine but also has an impact on cytoskeletal elements at sublethal doses [37.204].

PTXs are potent cytotoxic compounds against several human cancer cell lines. They are hepatotoxic whose mechanism of action has not yet been fully elucidated [37.205–207]. Among other effects, the induction of apoptosis in rat hepatocytes, in salmon and p53-deficient cell lines have been reported [37.208].

PTX2 is an inhibitor of actin and cytokinesis and it is known to be used as a chemotherapeutic agent against tumors type p53 [37.209]. It was demonstrated that a loss of p53 sensitizes tumor cells to actin damage. PTX2 was first identified as a cytotoxic entity in marine sponges, which depolymerizes actin filaments and it was found to be highly effective and more potent to activate an intrinsic pathway of apoptosis in p53-deficient tumor cells compared to those with functional p53 both in vitro and in vivo. Other agents that depolymerize or knot actin filaments were also found to be toxic to p53-deficient tumors. In p53-deficient cells, PTX2 triggers apoptosis through mitochondrial dysfunction, and this is followed by the release of proapoptotic factors and caspase activation. Furthermore, Bax activation and Bim induction was observed only in p53-deficient cells after PTX2 treatment. Therefore, these results suggest that Bim triggers apoptosis by activating Bax in p53-deficient tumors upon actin damage, and that actin inhibitors may be potent chemotherapeutic agents against p53-deficient tumors.

Recent studies demonstrated that this congener do not induce diarrhea or any other signs of disease by oral administration. Body distribution of PTX2 and PTX2 seco acid (PTX2SA) was analyzed in mice after oral and intraperitoneally administration. Cytotoxicity assays in vitro have been the first step to the discovery of many drugs used in antitumor therapy. However, more information is needed about its toxicity to humans and their pharmacokinetics [37.210].

Yessotoxins-Group Toxins

YTXs are polyether compounds that were discovered due to their high acute toxicity during MBA by *i.p.*, injection for lipophilic toxin analysis of mollusks [37.211, 212]. There are no published reports of human poisonings caused by YTXs and no available data on chronic exposure, carcinogenicity, or genotoxicity [37.213]. Various authors have suggested that the target organ following *i.p.* administration is the heart [37.214]; however, other authors consider that YTX is involved in neurological disorders and its main target is the thymus [37.215]. *Tereo* et al. have reported severe microscopic alterations in cardiac muscle [37.214]. Difference between these studies is not clear and has been proposed that discrepancies may be due to different purities of the toxins and the use of different strains of mice [37.213]. YTX administration to human lymphocytes leads to an increase in cytosolic Ca^{2+} concentration [37.20] and in specific activity of cyclic nucleotide phosphodiesterase 3′, 5′ [37.216]. Other studies have reported that cellular Ca^{2+} homeostasis is not related to its cytotoxic effect [37.217]. Furthermore, YTX causes dose-dependent decrease in the levels of GMPc and AMPc after incubation due to the activity of phosphodiesterases. These effects depend on Ca^{2+} and can be changed by phosphodiesterases [37.216, 218].

YTX induced a decrease in F-actin filaments in several cell models as cerebellar granule cells (CGCs), mouse fibroblast, rat L3, mouse myoblast cell lines, and MCF-7 human breast adenocarcinoma cells [37.217, 219]. However, this seems not to be a universal effect, since no effects over F-actin levels were detected in M17 neuroblastoma cell line, in rabbit enterocytes, or in human Caco-2 cells [37.220, 221]. YTXs cause selective disruption of the E-cadherin-catenin in epithelial cells and could potentially suppress tumor functions of E-cadherin [37.222]. Pathway degradation of E-cadherin is affected by YTX at long incubation times and low doses. Since E-cadherin has been linked to tumor spreading and metastasis, it has been a concern if the disruption of E-cadherin by YTX could affect tumor expansion in YTX-contaminated mollusk consumers. However, in vivo experiments demonstrated that although the resulting molecule of E-cadherin disruption had increased, YTXs did not induce any effect on E-cadherin system in in vivo experiments [37.223].

YTX-induced apoptotic events in different cellular lines, including cancer cells, primary cell cultures, and cell lines. There are reports related to YTX-induced apoptosis in rat hepatocytes, rat glioma cells, HeLa S3 cells, rat cerebellar neurons, BE(2) neuroblastoma cell line, L6 myoblast cell line, BC3H1 myoblast cell line, mouse fibroblast NIH3T3 cell line, CaCo-2 cells, MCF-7 cancer cells, and HepG2 cell cultures. In this sense, YTX could be used in therapeutic applications, for instance, as an antitumor drug [37.224]. Thus, the European patent application EP1875906 [37.225] considers to use YTX as an antitumor drug, and protoceratin I, the major compound with cytotoxic activity against human tumor cell lines isolated from *P. cf. reticulatum*, was proved to be identical to homo YTX [37.226].

It has also been investigated the activity of various YTXs analogs on MCF-7 tumor cell lines; structural modification in homoYTX do not change the activity of the toxin, while the side chain structure of carbon 9 seems essential. Thus, carboxiYTX and hidroxihomoYTX present less activity than YTX [37.227]. These data have been confirmed by kinetic studies between phosphodiesterases and YTX, hidroxiYTX and carboxiYTX, proving the lower affinity between these proteins and the last two analogs [37.228].

Azaspiracid-Group Toxins

Molecular targets of these toxins are not well known, but some studies were focused on the study of second messengers: increased intracellular Ca^{2+} and AMPc [37.229–232], effects of reorganization of the actin cytoskeleton [37.233–236] and effects on adhesion by activation of adhesion protein fractionation (claudins and cadherins) [37.234, 237, 238].

Although, symptoms observed during episodes of AZAs poisoning were similar to those typically observed with DSP, administration of toxic AZAs materials to mice led to great differences with the DSP. Mice injected with low doses of AZAs did not present diarrhea [37.239], and in a few hours develop slowly progressive paralysis, difficulty in breathing, and finally death [37.240]. A 200 mg/kg *i.p.* dose appears to be the minimal lethal dose in mice to AZA1 while AZA3 and AZA2 showed a higher toxicity (110 and 140 mg/kg, respectively) [37.240, 241]. Damages in liver, pancreas,

spleen, thymus and stomach were observed. Moreover, after administration of AZA sublethal doses by gavage, alterations were observed in the small intestine, affecting epithelial cells with a degree of damage progressively increasing from 4 to 24 h after administration [37.242, 243]. AZA cytotoxicity was first observed by *Flanagan* et al. [37.244] in hepatoblastoma and bladder carcinoma cells exposed to contaminated crude mussel extract. *Twiner* et al. [37.235] observed that AZA1 causes cytotoxicity in kidney, lung and immune, neuronal, and pituitary cells. However, neurons appear to be particularly sensitive to AZAs, causing irreversible morphological changes with relatively short exposure times and cytotoxicity with longer exposures to these toxins [37.231, 236]. It also requires longer exposure times to achieve complete cytotoxicity in primary cultures of cerebellar granule cells [37.200]. Also, they induce apoptosis through caspase activation [37.210, 245].

AZAs are implicated in upregulation on gene expression and protein levels of low-density lipoprotein receptor (LDLR). This effect seems to be a response to the decreased level in intracellular cholesterol caused by AZA1 [37.25]. It was shown that T lymphocytes exposed to AZA1 up regulated gene expression and protein levels of LDLR due to decreased levels of intracellular cholesterol. Although premature to determine decisively, yet consistent with currently available in vitro data, AZA1 appears to be targeting a membrane protein such as a claudin, cadherin, or LDLR, in turn eliciting an effect on intracellular signaling molecules and the cytoskeleton, ultimately resulting in cytotoxicity.

37.7.2 Hydrophilic Toxins

Saxitoxin-Group Toxins (PSP)

STX-group toxins are closely related water-soluble tetrahydropurine compounds acting by interfering with VGSC functioning. The toxin acts from the exterior of the cells by getting access to the extracellular cavity of the channel and binding to the so-called site 1 of the α-subunit in the Na^+ channel [37.246]. Site 1 is shaped by a short portion (SS2) of the amino acid stretches connecting the S5 and S6 trans-membrane helices in the four domains of the α subunit, giving rise to a cavity that accommodates the toxin. STX then forms hydrogen bonds and electrostatic interactions with the side chains of several amino acids that participate to the ion selectivity filter of the channel. The interaction of one STX molecule with the site 1 of the α-subunit in the Na^+ channel essentially plugs the channel and blocks its ion flux. The loss of Na^+ conductance in excitable cells prevents membrane depolarization and the transmission of the action potential, representing the molecular basis of the toxic effects of STX. As a consequence of VGSC blockade, a progressive loss of neuromuscular function ensues, leading to the reported neurotoxic symptoms that can result in death by asphyxia. Skeletal analogs of STX with a fused-type tricyclic ring system, designated FDSTX, were synthesized as candidate Na^+ ion channel modulators [37.247]. Their inhibitory activity on Na^+ ion channels was examined by means of cell-based assay. Two of the analogs showing moderate inhibitory activity were further evaluated by the use of the patch-clamp method in cells that expressed $Na_V1.4$ (a TTX-sensitive Na^+ channel subtype) and $Na_V1.5$ (a TTX-resistant Na^+ channel subtype). These compounds showed moderate inhibitory activity toward $Na_V1.4$, and weaker inhibitory activity toward $Na_V1.5$. Uniquely, however, the inhibition of $Na_V1.5$ by one of the analogs was *irreversible*.

Some STXs have demonstrated pharmaceutical potential as a long-term anesthetic in the treatment of anal fissures and for chronic tension-type headache. The recent elucidation of the STX biosynthetic gene cluster in cyanobacteria and the identification of new STX analogs will present opportunities to further explore the pharmaceutical potential of these intriguing alkaloids [37.186]. A specific suite of analogs can be isolated from a single STXs-producing organism, which is directly a result of the evolution of genes present within the organism's genome. Naturally occurring STXs can also be precursors for extracellular metabolic or chemical transformations into new analogs. Knowledge of these transformations may have important implications for the detection, toxicity, and removal of STXs from a contaminated source. Other medicinal uses for STXs may become more established by screening the bioactivity of less toxic analogs, since their use as a potential local anesthetic has long been known. The characterization of STX biosynthesis genes [37.165, 248] and their potential use in combinatorial biosynthesis, together with the constant discovery of novel analogs (either natural or transformed), is likely to expand the possibilities for their pharmaceutical use.

STX also has a huge pharmaceutical potential for its ability to induce anesthesia through interaction with the VGSC [37.186]. It has been suggested that site 1 blockers prolong the duration of anaesthesia in a synergistic manner when combined with other local anaesthetics. In spite of this, the push for STX to enter clinical tri-

als has been hindered by its systematic toxicity. The use of STX as a slow release, prolonged anesthetic was recently demonstrated using a novel controlled release system in male rats. Liposomal formulations of STX, either alone or in conjunction with dexamethasone and/or bupivacaine, were able to block the sciatic nerve in rats for long periods with no damaging myotoxic, cytotoxic, or neurotoxic effects and little associated inflammation. Liposome formulations of STX for slow and site-directed release for prolonged anaesthesia have been postulated as a putative treatment of localized and severe joint pain.

GTX2 and GTX3 also have clinical potential and have been utilized for the treatment of anal fissures by their direct injection into both sides of the fissure. A success rate of 98% with remission after 15 and 28 days for acute and chronic conditions respectively was observed. Both toxins have also been used in the treatment of chronic tension type headache, with 70% of patients responding to treatment. These studies recognize that several STXs have potential as future pharmaceutical leads; therefore, more investigation is needed in this field [37.6].

NEO-STX has being developed as a local anesthetic. Doses of 100 μg have been used by local infiltration in anesthetized adult humans without adverse effect. *Wylie* et al. hypothesized that similar doses could cause significant respiratory, neuromuscular, and cardiovascular impairment [37.249].

Domoic Acid (ASP)

DA belongs to the kainoid class of compounds, which is a class of excitatory neurotransmitters. Pharmacological studies reported that DA isomers are not as toxic as DA because they bind less strongly to the kainate receptor proteins than DA itself; however, 5′-epi-domoic acid has a similar toxicity [37.35]. *Mayer* and *Hamann* [37.250] reviewed the available literature on pharmacological and toxicological studies with marine natural products including articles reporting on the bioactivity and/or pharmacology of 106 marine chemicals. They included DA in the group of compounds that affect the immune system. Moreover they reported that DA at in vitro concentrations that were toxic to neuronal cells (1 mM) was shown to trigger a limited activation of rat brain microglia, an immune cell type that contributes to circa 10% of the total glial population in the central nervous system, and the concomitant release of two potentially neurotoxic mediators, namely TNF-α and matrix metalloproteinase-9.

DA acts as a potent glutamate receptor agonist. Although mechanisms of domoic acid neurotoxicity are poorly defined, an excitotoxic mechanism is believed to be involved. DA inhibits astrocytes glutamate uptake in a dose-dependent manner, leading to accumulation of glutamate in the extracellular compartment and thus producing neurotoxicity [37.251].

In vitro studies have shown that, depending on its concentration, DA causes necrotic or apoptotic cell death in mouse cerebellar granule neurons (CGN). Necrosis is observed at higher concentrations of DA (10 μM) and involves activation of both AMPA/kainate and *N*-metyl-D-aspartic acid (NMDA) receptors, the latter activated by DA-induced glutamate release. In contrast, apoptotic damage predominates at lower concentrations of DA (e.g., 100 nM) and is solely due to activation of AMPA/kainate receptors [37.252]. Both, necrosis and apoptosis have been shown to be mediated by oxidative stress. Upon oral administration, DA is poorly absorbed, has a short half-life in blood, and poorly permeates the blood–brain barrier, limiting the access of low levels of DA in the central nervous system, but this might not be the case during development, a period of suggested particular sensitivity to DA neurotoxicity. The authors evaluated if a prolonged exposure to low DA levels would result in neurotoxicity and/or in altered susceptibility to a subsequent exposure in cultured mouse CGNs. Neurons from wild-type mice and from mice lacking the glutamate cysteine ligase (GCL) modifier subunit (*Gclm*$^{-/-}$) (already known to be highly susceptible to DA neurotoxicity) were compared. They found that prolonged exposure to low-level DA in vitro provides protection against toxicity of a higher DA acute exposure, apparently due to the ability of chronic low dose of DA to up-regulate glutathione (GSH) synthesis. Genetic conditions leading to low GSH levels, which already increase susceptibility to DA neurotoxicity, could potentially negate such protective mechanisms [37.252].

37.7.3 Other Toxins

Ciguatoxins (CTXs)

CFP is the most frequently reported seafood-toxin illness in the world. It produces gastrointestinal, cardiovascular, neurologic, and neuropsychiatric symptoms which can last from days to even months. CTXs derive from benthic dinoflagellates and are transferred through the food web from the algae to herbivorous and carnivorous fish, and finally to humans.

CTXs are among the most potent natural substances known. One of the Pacific Ocean ciguatoxins, P-CTX-1, poses a health risk at concentrations as low as 0.08–0.1 μg/kg although CTXs rarely accumulates in fish at levels that are lethal to humans [37.253]. CTXs activate the VGSCs in cell membranes, which increases Na^+ ion permeability and depolarizes the nerve cell. This depolarization is believed to cause the array of neurological signs associated with CFP. Voltage-clamp studies suggested that CTXs cause spontaneous, and enhance evoked, action potentials by lowering activation thresholds and delayed repolarization of VGSCs. Several studies further elucidated cellular effects and molecular mechanisms of CTXs actions including selective binding and competition with BTX for site 5 on VGSCs, elevation of intracellular Ca^{2+} levels, stimulation of spontaneous and evoked neurotransmitter release from synaptosomes and motor nerve terminals, axonal and Schwann cell edema, induction of TTX-sensitive leakage current in dorsal root ganglion neurons and blockade of voltage-gated potassium channels [37.254]. Neurological symptoms are consistent with CTXs' interactions with VGSCs; however, all the symptoms produced during CFP may not be exclusively due to VGSCs [37.46]. The chronic fatigue syndrome that may last for weeks or months has been related to high nitric oxide (NO) production. Penil and muscular relaxation, as well as L-type Ca^{2+} channel activation, were shown to be NO-mediated via the nitric oxide synthetase pathway.

CTX also caused rapid hypothermia in mice, which lose their ability to regulate their own body temperature and became poikilothermic. This reduction in body core temperature was found to be due to neuroexcitatory actions of ciguatera on regions of the brain stem receiving vagal afferents and ascending pathways associated with visceral and thermoregulatory responses [37.255]. These authors reported a complete review on the pharmacological effects of CTXs, including cardiovascular and gastrointestinal effects, effects on other smooth muscle, actions on ganglionic and neuromuscular transmission and on VGIC, and other Na^+-dependent actions. Despite the multiple pharmacological effects no medical applications of these toxins have been reported so far.

Brevetoxin-Group Toxins

BTXs are lipid soluble cyclic polyethers (M.W. ≈ 900) and potent neurotoxins that open VGSCs leading to Na^+ influx into the cell. BTXs exert their toxicity by interacting with neurotoxin receptor site 5 associated with domain IV of the α subunit of the VGSC. BTXs binding to tissues that contain VGSCs on excitable cells results in membrane depolarization, repetitive firing, and increase in Na^+ currents [37.256]. At the Na^+ channel, at least four actions of BTXs have been identified leading to the depolarization of excitable cell membranes: shift of the voltage dependence, inhibition of inactivation, increase of mean open times and multiple subconductance levels. A description of these actions and the mammalian BTXs toxicity was recently review [37.63].

BTXs exert a variety of actions in vivo affecting both the peripheral as well as the central nervous systems, probably due to their high lipid solubility and ability to penetrate the blood–brain barrier. Humans exposed to the toxin usually exhibit neurotoxic shellfish poisoning symptoms, which include nausea, difficulties in movement, and seizures [37.257]. Cellular effects associated with exposure to BTXs have been observed in the immune system of many species, although the mechanisms of action of BTXs on immune cells and immune competence are not well understood. Several potential mechanisms for BTX immunotoxicity have been suggested, including the inhibition of cathepsin, apoptosis, release of inflammatory mediators, and oxidative stress [37.58]. In vitro experiments have demonstrated possible DNA damage, chromosomal aberrations and effects on cellular growth [37.256, 258]. Other immune system effects include mast cell degranulation [37.259] and histamine release which may contribute to observed airway responses following the inhalation of aerosolized BTXs.

There are over 10 different BTXs isolated in seawater blooms and *K. brevis* cultures, as well as multiple analogs and derivatives from the metabolism of shellfish and other organisms [37.58]. *Cao* et al. [37.260], quantified the potencies and efficacies of an array of lipophilic VGSCs gating modifiers by measuring Na^+ influx in murine neocortical neurons. An assay using a Na^+-sensitive fluorescent dye to primary cultures of neurons afforded a highly quantitative assessment of the increments in neuronal Na^+ level produced by gating modifier toxins. The relative efficacies differ substantially; the rank order of efficacy of several naturally occurring BTXs and semisynthetic analogs as Na^+ channel gating modifiers was PbTx-1 > PbTx-desoxydioxolane > PbTx-2 = PbTx-3 < PbTx-3α-naphthoate. *K. brevis* cultures also produce shorter ring structures, hemi-BTXs and brevenals, likely to be incomplete products of the BTX biosynthetic pathway. These smaller cyclic ether ring

structures exhibit reduced biological activity, yet they are still consistent with a site of action common to the BTXs [37.63]. An interesting discovery was the finding that brevenal (produced both in *K. brevis* laboratory cultures and in the environment during *K. brevis* blooms), is a brevetoxin antagonist. This is a case of a toxin producing organism that also secretes its own antagonist. Brevenal is synthesized by *K. brevis* in significant amounts and it acts at a different receptor site on nerve cells than the brevetoxins. It is a new and important natural product which could treat chronic obstructive pulmonary disease and cystic fibrosis [37.58].

Taupin [37.261] studied the activity of four generic formulae of BTX derivatives – formulae I–IV – and their compounds on the growth of neurites. He reported that BTX derivatives stimulate neurite growth, particularly the growth of minor processes from which the axons form, on neurons in primary cultures. The activity is mediated by VGNC and the *N*-methyl-D-aspartate-mediated intracellular Ca^{2+} pathway. They proposed the use of BTX derivatives for enhancing neuronal growth and for the treatment of neurodegenerative and neurological disorders, such as Alzheimer's disease, amyotrophic lateral sclerosis, cerebral strokes, traumatic brain, and spinal cord injuries.

Tetrodotoxin

TTX is a very well-known marine neurotoxin due to its frequent involvement in fatal food poisoning, unique chemical structure, and specific action of blocking Na^+ channels of excitable membranes [37.262]. The toxicity of TTX relates to two factors: paralysis of skeletal muscle, including the diaphragm and intercostal muscles, leading to respiratory failure, and reduced blood pressure, predominantly due to vasodilatation. Early signs of this effect might include weakness, tingling of the lips and dizziness [37.70]. Pharmacological activity of TTX on various animals, their organs and tissues has been extensively studied, and it was found that TTX strongly inhibits nerve conduction and has inhibitory effects on Na^+-K^+-ATPase activity. Recently, TTX has been shown to block L-type Ca^{2+} current (ICa) in canine cardiac cells [37.263]. These authors studied the TTX-sensitivity of Ca in isolated canine ventricular myocytes as a function of channel phosphorylation, extracellular pH, and the redox potential of the bathing medium using the whole cell voltage clamp technique. They demonstrated that TTX inhibits L-type Ca channels in the heart by binding to its selectivity filter.

TTX has been extensively used to elucidate the role of specific VGSCs subtypes in a wide range of physiological and pathophysiological processes in the nervous system. VGSCs play a key role in pain and TTX-sensitive subtypes have received much attention over the past few years because these channels have been strongly implicated in normal and pathological pain. Since TTX blocks this subset of VGSCs in a highly selective manner, this toxin may have a potential role in relieving pain [37.264, 265]. *Nieto* et al. [37.266] evaluated the effect of the acute systemic administration of low, nontoxic, doses of TTX on the expression of different signs (heat hyperalgesia, mechanical- and cold allodynia) of paclitaxel-induced neuropathic pain in mice. They reported that low doses of TTX can be useful to prevent and treat this pain since inhibited hyperresponsiveness to thermal and mechanical stimuli. Their findings suggest that TTX-sensitive subtypes of Na^+ channels play a role in the pathogenesis of chemotherapy-induced neuropathic pain.

Hagen et al. [37.267] evaluated the analgesic activity of TTX administered subcutaneously in a randomized, placebo-controlled, parallel design study, in patients with moderate or severe unrelieved cancer pain. This trial suggested that TTX could potentially relieve moderate to severe, treatment-resistant cancer pain in a large proportion of patients, and often for prolonged periods following treatment. In a later study [37.268] it was assessed the long-term safety and efficacy of subcutaneous TTX treatment in reducing the intensity of chronic cancer-related pain. They conducted a multicenter open-label longitudinal trial, in which 30 µg TTX was administered subcutaneously for 4 days in a heterogeneous cohort of patients with persistent pain. Onset of pain relief in no more than half the patients was typically cumulative over days, and after administration ended, the analgesic effect subsided over the course of a few weeks. Toxicity was usually mild or moderate and remained so through subsequent treatment cycles, with no evidence of cumulative toxicity or tolerance. They concluded that long-term treatment with TTX is associated with acceptable toxicity and, in a substantial minority of patients, resulted in a sustained analgesic effect. *Berde* et al. [37.70] reported that prolonged duration percutaneous sciatic nerve blockade could be achieved in rats using combinations of site 1 Na^+ channel toxins with either bupivacaine or epinephrine. Addition of either bupivacaine or epinephrine increases the LD_{50} of TTX in rats, i. e., reduces systemic toxicity from TTX, and also increases the potency of TTX by producing sensory blockade, thereby substantially improving the therapeutic index of TTX.

Thus, combination of formulations seems desirable from the standpoint of both efficacy and safety. TTX, formulated as Tectin, underwent Phase III trials as an injectable systemic analgesic for chronic cancer pain. *Berde* et al. [37.70] examined dose–duration relationships and sciatic nerve histology following local nerve blocks with combinations of Tectin with bupivacaine 0.25% solutions, with or without epinephrine 5 μg/mL in rats. Three-way combinations of TTX, bupivacaine and epinephrine produced significant prolonged sciatic nerve blockade in rats, compared to bupivacaine plain or bupivacaine + epinephrine. Their studies indicate that clinically relevant combinations of Tectin with commercially available bupivacaine 0.25% and epinephrine 5 μg/mL warrant further study for prolonged-duration local anesthesia. Moreover they suggest that a single-injection approach to prolonged local anesthesia would provide good pain relief as a component of multimodal analgesic regimens, reducing postoperative opioid requirements and improving the course of postoperative recovery and acute rehabilitation.

A review by *Nieto* et al. [37.269] focuses on the preclinical and clinical evidence supporting a potential analgesic role for TTX. In addition they compiled data on the contribution of specific TTX-sensitive VGSCs to pain. They conclude that the therapeutic use of TTX as an analgesic agent seems hopeful although further preclinical and clinical research is needed to clarify its potential use during painful conditions. *Wang* et al. [37.270] examined the effect of TTX, applied topically to the rat cornea, on the duration of corneal anesthesia, administered with either proparacaine (PPC) or the chemical permeation enhancer octyl-trimethyl ammonium bromide (OTAB). The effect of test solutions on corneal healing was also studied. Combination of TTX and PPC resulted in corneal anesthesia that was 8 to 10 times longer in duration than that from either drug administered alone, whereas OTAB did not prolong anesthesia. The rate of corneal healing was moderately delayed after coadministration of TTX and PPC. They concluded that coadministration of TTX and PPC significantly prolonged corneal anesthesia, but in view of delayed corneal reepithelialization, caution is suggested in the use of the drug combination.

Bragadeeswaran et al. [37.262] isolated three TTX-producing bacteria (*Kytococcus sedentarius*, *Cellulomonas fimi* and *Bacillus* species) from the pufferfish *Arothron hispidus*. Their partially purified filtrates exhibited hemolytic activity on chicken and human erythrocytes and also presented ATPase, Mg^{2+}-ATPase, Na^+K^+-ATPase, and AchE enzymatic activities. The authors also evaluated if the viability of leukemia cell line (P388) was adversely affected upon adding the crude microbial extracts and found a dose-dependent growth inhibition. These TTX-producing bacteria hold promise for the development of effective antitumor compounds.

Palytoxin and Analogs

PlTXs are a group of complex, extremely potent, and marine natural products. PlTX is a large (molecular weight: 2681), water-soluble polyalcohol first isolated from zoanthids (genus *Palythoa*) even though dinoflagellates of the genus *Ostreopsis* are postulated as the PlTX origin [37.77]. PlTX has been found in a variety of marine organisms ranging from dinoflagellates to fish and it is implicated in seafood poisoning with potential danger to public health. The most commonly reported complications of PlTX poisoning appears to be rhabdomyolysis, a syndrome injuring skeletal muscle, causing muscle breakdown, and leakage of large quantities of intracellular (myocyte) contents into blood plasma. Other symptoms associated with PlTX poisoning in humans are characterized by abdominal cramps, nausea, vomiting, diarrhea, paresthesia, bradycardia, renal failure, cyanosis, and respiratory distress [37.79]. The latter two precede death in fatal cases.

Toxicological studies with these compounds show repeatedly low LD_{50} values in different mammals, revealing an acute toxic effect on several organs, as demonstrated by different routes of exposure. Acute oral administration in mice induced histological and ultrastructural changes in several organs, such as liver, pancreas, cardiac, and skeletal muscle cells. The recognition of its wide distribution coupled with the poisoning effects that these toxins can have on animals, and especially on humans, have concerned the scientific community [37.79]. The toxin is known to affect cellular functions by selective binding to the Na^+-K^+ ATPase and converting the pump into a nonselective channel [37.195, 271]. The induced ion flux alterations are reported to target pathways involved in cytoskeletal dynamics within different cellular models [37.77]. Na^+-K^+ ATPase also functions as a signal transducer to relay messages from the plasma membrane to the intracellular organelles. The toxicological effects of acute oral administration of 42-OH-PlTX were evaluated in mice, as well as its in vitro delayed hemolytic effects [37.272]. The compound showed a toxicity profile comparable with that of the parent compound palytoxin (LD_{50} = 651 and 767 mg/kg, respectively), both

as lethality and acute pathological effects. Also the in vitro hemolytic activity of 42-OH-PlTX was similar to that of PlTX in agreement with the common mechanism of action on the Na^+/K^+ pump, to which the two compounds bind with the same affinity.

Franchini et al. [37.204] used the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) to verify the effects of PlTX in mortality, delayed growth, and embryo malformation. The toxicological effects of PlTX evaluated by FETAX assay revealed evident impacts on embryo mortality, teratogenesis and growth. The histological analysis of the surviving young larvae revealed structural changes compared with controls in the nervous and muscle tissue, even if some specimens did not show any significant histopathological modifications. A general reduction in the size of the main inner visceral organs (i. e., intestine, pancreas, and liver) was reported, but no morphological changes. Severe injury to the heart structure was observed in some specimens.

PlTX is a novel skin tumor promoter, which has been used to probe the role of different types of signaling mechanisms in carcinogenesis. The multistage mouse skin model indicates that tumor promotion is an early, prolonged, and reversible phase of carcinogenesis. Understanding the molecular mechanisms underlying tumor promotion is therefore important for developing strategies to prevent and treat cancer [37.273]. PlTX stimulates a wide range of cellular responses that are likely to play a role in carcinogenesis. Stimulation of arachidonic acid metabolism and the production of prostaglandins modulation of the epidermal growth factor receptor, and modulation of mitogen-activated protein (MAP) kinase cascades are the best studied [37.273]. MAP kinases are a family of serine/threonine kinases that relay a variety of signals to the cellular machinery that regulates cell fate and function. This author [37.274] investigated how palytoxin stimulates MAP kinase activity and how MAP kinases mediate the response of cells to PlTX concluding that MAP kinases appear to be important mediators of PlTX-stimulated signals. The central role of MAP kinases in regulating a variety of critical cellular functions, ranging from enzyme activity to gene expression and ultimately to proliferation and apoptosis, may help explain how palytoxin can stimulate the variety of effects that are characteristic of tumor promoters. Other author suggests that PlTX conjugates could be used as prodrugs in cancer chemotheraphy due to its high cytotoxicity in vitro indicating that targeting the prodrug to a tumor, and there releasing the toxin, selective destruction of neoplastic cells could be achieved [37.275].

Many findings on the involvement of PlTX and analogs acting on different cellular targets and modulating the actin cytoskeleton within different cellular models were published [37.77, 276, 277]. *Louzao* et al. suggest that also cytoskeleton is necessary for PlTX activity and that this may find important applications in biomedical research, with particular interest toward the discovery and development of cytotoxic compounds providing novel antitumor therapy [37.77].

Cyclic Imines

CIs toxins are a very diverse group of marine biotoxins containing an imino functional group as part of its cyclic structure. In many of them, it was found that the toxicity is greatly diminished if imino group is reduced or destroyed [37.278]. Nowadays, there is no evidence of human poisoning caused by cyclic imines but the toxicity observed in experimental animals requires a deeper study of their activity and distribution [37.279]. Only the toxicity of spirolides (SPXs) and gymnodimines (GYMs) has been studied in depth. Spiroimine moiety has been established as the common pharmacophore unit of CIs group, although structural change in other parts of the molecules can also affect the toxicity [37.280]. Primary studies on mechanism of action of CIs suggested that it could be related to the interference on transmission of nervous impulses at neuromuscular junction level. It has been demonstrated that SPXs and GYMs act as potent antagonists of nicotinic (nAChRs) and muscarinic (mAChRs) acetylcholine receptors [37.281–283]. *Kharrat* et al. [37.281] demonstrated that GYM A broadly interferes in neuromuscular transmission by directly blocking muscle-type nAChRs. GYM A interacts with a wide range of muscular and neuronal types of nAChRs, larger than other well characterized toxins that also act on nicotinic receptors [37.281]. *Bourne* et al. [37.282] confirmed the interaction of SPX and GYM with several muscle and neuronal types of nAChRs by competition binding experiments against labeled α-bungarotoxin and epibatidine. No agonist effect was observed with SPX or GYM, but both toxins showed a dose-dependent antagonism in muscle- and neuronal-type receptors. The study concluded that SPX and GYM toxins display high affinity and potent antagonism but limited selectivity for the muscle type versus neuronal subtypes of nAChR [37.282].

SPXs effects on mAChRs were studied by *Wandscheer* et al. in a human neuronal model. They observed that 13-desmethyl SPX C caused a dose- and time-dependent inhibition on neuroblastoma to acetylcholine

extracellular stimulation. They demonstrated by competitive binding assays that the 13-desmethyl SPX is a competitive antagonist of mAChRs and also causes an internalization of the M3-type muscarinic receptor that might contribute to the inhibitory effect of the toxin on the Ca^{2+} response to acetylcholine stimulation [37.283].

Maitotoxins and Gambierol

Many bioactive compounds have been isolated from *Gambierdiscus* spp., i. e., maitotoxins (MTXs), gambierols, and gambieric acid. MTX increases intracellular Ca^{2+} and is considered one of the most potent marine biotoxins when injected intraperitoneally (ip) in mice, with an LD_{50} (24 h) of 50 ng/kg. However, MTX has no proven role in human intoxication due to its low capacity for accumulation in fish flesh and low oral potency [37.46]. At cellular level, MTX causes an increase in Ca^{2+} and Na^{+} by activating Ca^{2+}-permeable nonselective cation channels, resulting in potent membrane depolarization. This causes, in excitable cells, the opening of voltage-gated Ca^{2+} and Na^{+} channels. The massive influx of ions could exceed cell buffering mechanisms affecting cell viability [37.284]. Due to its ability to activate Ca^{2+}-permeable nonselective cation channels, MTX is considered a powerful tool in the study of Ca^{2+}-dependent mechanisms. Another MTX effect is the formation of cytolytic/oncotic pores that finally conduce to cell membrane blebbing and oncotic cell death. For this reason, MTX could be a unique tool to explore oncotic/necrotic cell death [37.284].

Gambierol has moderate inhibitory activity against VGSCs and modified Ca^{2+} homeostasis. High concentrations of this toxin activate Na^{+} channels in human neuroblastoma cells, and, as a consequence, gambierol induces a cytosolic Ca^{2+} increment [37.285]. *Cagide* et al. tested the effect of gambierol on the shape and F-actin cytoskeleton of neuroblastoma. Cells remained with spread morphology and a well-defined actin cytoskeleton, without showing evidence of alterations. Moreover, gambierol was able to produce depolarization, as well as a Ca^{2+} influx, in neuroblastoma cells and scarcely reduced their metabolism [37.286]. *Alonso* et al. [37.287] designed and synthesized truncated skeletal analogs of gambierol with inhibitory activity against voltage-gated potassium (Kv) channels. They examined the effect of these compounds in an in vitro model of Alzheimer's disease obtained from triple transgenic ($3 \times$ Tg-AD) mice, which expresses amyloid beta ($A\beta$) accumulation and tau hyperphosphorylation. In vitro preincubation of the cells with the compounds resulted in significant inhibition of K^{+} currents, a reduction in the extra- and intracellular levels of $A\beta$, and a decrease in the levels of hyperphosphorylated tau, reducing these two cellular pathologies. Thus, these compounds could be useful chemical probes for understanding the function of Kv channels and for elucidating the molecular mechanism of $A\beta$ metabolism modulated by NMDA receptors. Moreover, since both $A\beta$ peptides and phosphorylated tau proteins are supposed to interact with NMDA receptors and to be implicated in the glutamate enhancement of AD pathology, they could have a potential pharmacological use.

Gambieric acids isolated from the culture medium of *G. toxicus* were described as potent antifungal polyether compounds [37.46].

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38. Marine Microbial Enzymes: Current Status and Future Prospects

Barindra Sana

Due to unique metabolic capabilities the marine microorganisms are excellent natural resource for screening of novel molecules. Marine microbial enzymes are of special interest for their distinct habitat-related features. Published literatures agree with potential biotechnological, biomedical and industrial applications of several marine microbial enzymes. These enzymes attracted substantial attention for their novel chemical and stereochemical characteristics that may be exploited in chemical or pharmaceutical synthesis. A couple of marine microbial enzymes are already being used for industrial and pharmaceutical purposes. Marine biotechnology research is boosted by recent development of several molecular biology and bioinformatics tools. Study of marine microbial enzymes is now focused on multiple directions including screening, recombinant production, characterization and structure analysis of enzymes, and optimization of process parameters for their systematic and inexpensive production. Published literatures suggest potential applications of marine microbial enzymes in wide range of industries but more focused application-oriented research is essential for exploitation of their full biotechnological potential.

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38.1 Overview

In recent years, enzymes attracted huge attention for their potential industrial, pharmaceutical, cosmetics, and daily-life applications. Enzyme-based industrial processes are ecofriendly and less energy consuming. High specificity of enzymatic reactions minimizes bi-product formation in industrial processes and thus prevents wastage of expensive raw materials. High substrate specificity also suggests minimum side-effects of enzyme-based therapeutic systems. Extraordinary enzymes can be used for unique bioprocesses development or treatment of life-threatening diseases. However, in most cases industrial processes could not be operated in mild conditions or sometime substrates for industrial processes have complex chemical structure and no known enzyme is suitable to catalyze the reaction for such processes. Hence, there is a constant urge for discovering new enzymes that are active on novel substrates or can catalyze reactions at extreme environment. Screening of large number of microorganisms is the most promising method for searching novel enzymes and marine microorganisms are revealed as the source of numerous novel biocatalysts. This chapter focuses on distinct features of marine microbial enzymes, their current uses, current research

status and future prospects with reference to major challenges for commercialization of marine microbial enzymes and some potential way to overcome them.

For the enzyme-based bioprocess development, the enzymes should be stable and active at the industrial reaction conditions – many marine microbial enzymes showed that potential. They are found to be suitable for various industrial process development, biomedical applications, organic synthesis, and biotechnological research [38.1–3]. Activity and stability at extreme conditions are the most useful features of marine microbial enzymes. They are reported to be active at extreme pH or temperature, in the presence of organic solvents or high salt concentration – each of these characteristics could be potentially exploited for some industrial applications [38.3–5]. Several marine extremozymes are key tools of current biotechnological research while others are being used in textile industry, biofuel industry, and pharmaceutical industry [38.4, 6]. Several other enzymes are reported to have potential industrial applications, and currently being studied for feasible large-scale production and industrial process development. Solvent-tolerant marine microbial enzymes are suitable for synthesis of pharmaceutically important stereoselective compounds.

Isolation of a novel enzyme with unique application is a challenging job and search for marine microbial enzymes got extreme importance for their vast potential application. Instead of huge potential of engineered enzymes fewer researches are being conducted for protein engineering and directed modification of the isolated marine enzymes. Bulk production at reasonable cost, development of large-scale downstream processing, and feasible industrial processes development are also important considerations for industrial exploitation of any enzyme. Unfortunately, such important industrial aspects are grossly neglected in the field of marine biotechnology. Recently, a few research groups are focusing on scale-up and downstream processing of marine microbial products but still the efforts cannot match with the huge number of potential industrial enzymes that are being reported from marine microbial environments. Future prospects of marine microbial enzymes are crucially dependent on the success of specific researches, which are focused on large-scale production, engineering, downstream processing, and industrial exploitation of these enzymes.

38.2 Marine Extremozymes and Their Significance

Marine microbial enzymes attract special interest due to their habitat related properties that enable them to be active in extreme environments. In the course of evolution, marine microorganisms faced extreme environmental challenges and adapted their metabolic pathways to survive in diverse environments. As a result, they produce unique metabolites, which are seldom reported from terrestrial sources. Several marine enzymes were reported to be stable and active at extreme temperature or pH. Some are tolerant to extreme saline condition, active in the presence of different chemicals or even in organic solvent system. A number of marine enzymes have novel stereochemical properties that can catalyze (stereoselective) chemical reactions, which are impossible otherwise. Importance of these habitat-related characteristics is vastly recognized from general biotechnological perspective and blending of two such features in a single enzyme is extremely useful for the development of unique bioprocesses. For example, thermostable alkaliphilic proteases are used as additive in laundry detergents and these enzymes can be useful in dry-cleaning if they can work in the presence of organic solvents. Another example is the use of thermostable solvent-tolerant enzymes in the biofuel industries and these enzymes have potential application for biofuel production from seaweed biomass if they are active in saline environment. The following sections highlight on the importance of various extremozymes with special reference to the marine isolates.

38.2.1 Thermostability

Thermostable extremozymes can be used in industrial reactions that are not feasible at ambient temperature. Higher temperature is preferable in many chemical reactions due to higher solubility of substrates, lower viscosity, better mixing, faster reaction rate, and decreased risk of microbial contamination. Moreover, thermostable enzymes are easy to purify by heat treatment. Thermostable proteases, lipases, amylases, cellulases, chitinases, carragenases, xylanases, polymerases, and oxidases have several applications in food processing industry, detergent industry, paper industry, textile industry, biofuel industry, and biotechnological research. Thermostable marine microbial enzymes are reported mostly from ther-

mophilic members of *Thermotoga*, *Thermus*, *Thermococcus*, *Pyrococcus*, *Bacillus*, and *Sulfolobus* family. Thermostable archaeal enzymes were reported to have higher stability towards high pressure, detergents, organic solvents, and proteolytic degradation [38.7]. Several thermostable enzymes were screened from hyperthermophilic archaea of deep-sea hydrothermal vents [38.8–10]. A crude esterase from *Pyrococcus abyssi* exhibited 22 h half-life at 99 °C that can retain entire initial activity after 8.5 h incubation at 90 °C [38.8]. A thermostable amylase from *Rhodothermus marinus* was reported to produce branched glucan from amylase and showed maximum activity at 80 °C [38.11]. *Taq* polymerase and *Pfu* polymerase are two mostly used thermostable DNA (deoxyribonucleic acid) polymerases isolated from two thermophilic archaea *Thermus aquaticus*, and *Pyrococcus furiosus* respectively [38.4, 12]. The latter species was isolated from a geothermally heated marine sediments having temperature in between 90−100 °C.

38.2.2 Cold Adaptivity

Cold adaptive enzymes are essential to catalyze the bioprocesses that need to be performed at low temperature to avoid degradation or evaporation of reaction components or products. Low-temperature reactions are preferred in industrial processes because it minimizes corrosion of the metallic reactors. Many cold active enzymes can be easily inactivated by mild heat treatment that can prevent undesired prolonged activity; this property is also useful in multistep process if the enzyme has undesired effect in subsequent reaction step. For example, stonewashing with cellulases in the textile industry where long-term enzyme activity could lead to the loss of mechanical resistance of the cotton fibers. Cold adaptive enzymes are abundantly isolated from a broad range of psychrophilic marine microorganisms including the members of the genus *Psychrobacter*, *Methanococcus*, *Alteromonus*, *Aquifex*, and *Bacillus*. Arctic and Antarctic microorganisms are well-known source of cold adaptive enzymes. A cold adaptive alkaline phosphatase isolated from an Antarctic bacterium shown interesting property for application in molecular biology research [38.13]. It is highly active at low temperature and after use can be easily inactivated by mild heat treatment (at 55 °C) that eliminate the need of separating the DNA after alkaline phosphatase treatment. Several others cold adaptive enzymes were reported to have potential applications in baking industry, bioremediation and as cold-wash additive [38.14–16]. Cold adaptive polysaccharolytic enzymes are being studied for applications in textile and paper industries. Several cold active lipases were reported from Antarctic deep-sea sediments metagenome and microbial isolates including *Pseudomonas* and *Psychrobacter* species [38.17–19]. Some of these enzymes retained their activity at very low temperature but inactivated at ambient temperature. A cold-active lipase was reported from a psychrophilic isolate of the Southern Ocean deep sea that retains 37% of its maximum activity at 0 °C [38.20]. Cold active lipolytic enzymes could be exploited in specific industrial processes such as bioremediation of fat-contaminated aqueous systems [38.16]. A cold active esterase was reported from *Pseudoalteromonas arctica* that showed optimum activity at 25 °C and retained 50% of maximum activity at 0 °C [38.21]. It can hydrolyze several members of nonsteroidal anti-inflammatory drugs including ibuprofen.

38.2.3 Extreme pH Tolerance

In general, proteins are unstable at extreme acidic or basic environments and average enzymes have optimum activity near neutral pH. Several bioprocesses are favored in acidic or alkaline conditions where most typical enzymes are inactivated – such reactions can be catalyzed only by enzymes that can tolerate extremely low or high pH, respectively. Many marine microorganisms can survive at extreme acidic or alkaline conditions and, extracellular enzymes from these microorganisms are often active at extremely low and extremely high pH, respectively. Detergent industry consumes more than 30% of industrial enzymes as additive in laundry detergents that includes alkaliphilic protease, lipase, amylase, and cellulase. These enzymes also have potential application in contact lens cleaning. Several thermostable alkaliphilic proteases from marine microorganisms have potential application as cleansing enzyme [38.22–24]. Alkaline phosphatase is another useful alkaliphilic enzyme frequently reported from marine microorganisms [38.5, 25]. *Kobori* et al. (1984) reported an alkaline phosphatase from marine microorganisms that can be inactivated by mild heat treatment (at 55 °C) and has potential application in biotechnological research [38.13]. Acidophilic digestive enzymes are useful in food and pharmaceutical industries. Numerous alkaliphilic enzymes were reported from marine *Bacillus* whereas marine fungi produce a range of acidophilic enzymes. Several thermostable alkaline proteases from marine *Bacillus* are reported to

be active in the presence of surfactants and bleaching agents; potentially they can be used as additive in commercial detergent [38.22, 23, 26]. An alkaline protease from marine shipworm bacterium was reported to have strong cleansing power and it can degrade lysozyme more efficiently than subtilisin [38.24]. This enzyme is active in the presence of hydrogen peroxide that is usually used to sterilize contact lenses. Two thermostable alkaliphilic amylases from marine microorganisms are active in the presence of surfactants and commercial detergents that suggests their potential applications in detergent industry [38.27, 28].

38.2.4 Halotolerance and Organic Solvent Stability

Most characteristic feature of the marine environment is high salinity and extracellular enzymes from marine microorganisms are reasonably active in the presence of high salt concentration. Salts present in reaction systems usually as contaminant, reactant, or product; sometimes salts are used to stabilize proteins essential in the bioprocess. Salt-tolerant enzymes can be exploited to catalyze the bioprocesses where reaction mixture contains high salt concentrations that inhibit most common industrial enzymes. Halotolerant proteolytic enzymes are used in peptide synthesis and fish/meat processing industries. Salt-tolerant polysaccharolytic enzymes are being studied for application in biofuel production from marine microalgae and seaweed biomasses. A halotolerant amylase-producing marine bacterium was reported to be used in saccharification of marine microalgae in the presence of high NaCl concentration [38.29]. Halotolerant enzymes are uniquely adapted to function in conditions with low water availability and some of them are inactive without high salt concentration. An amylase from marine *Streptomyces* sp. D1 was reported to retain almost 100% activity in the presence of 7% (w/v) NaCl after 48 h incubation but its activity drastically decreases after 48 h incubation without NaCl [38.27].

Several halotolerant enzymes are reported to be active in the presence of organic solvents, which is likely to be related with their adaptability in low water containing reaction system [38.30, 31]. An organic solvent tolerant lipase was reported from the marine *Aeromonus hydrophila* where the enzyme production was highly dependent on NaCl concentration within the production media. However, tolerance to both high salt and organic solvents gives a new blend of extremophilic properties in these enzymes that can be exploited for certain applications. In organic systems, enzymes may catalyze reactions even impossible in pure aqueous media. Organic solvent-tolerant enzymes can catalyze reactions in biphasic mixtures and some of them are useful for synthesis of chirally pure drug molecules. An organic solvent-tolerant enantioselective alcohol dehydrogenase from *P. furiosus* showed potential applications in reduction of ketones with low solubility in aqueous buffers [38.3]. This enzyme is highly stable and active in organic solvents like *iso*-propanol, DMSO, methyl *tert*-butyl ether, and hexane. Catalytic properties, substrate specificity, and enantioselectivity of this enzyme are vastly studied. It catalyzes reduction of aryl ketones for production of enantiomerically pure chiral alcohols and enzyme activity increases at enhanced temperature without affecting enantioselectivity. Several organic solvent tolerant esterase/lipase were isolated from marine microorganisms that have potential application in biodiesel production and synthesis of chirally pure compounds [38.32, 33].

38.2.5 Barophilicity

The effect of pressure on a reaction is dependent on sign and magnitude of the activation volume. Reactions accompanied by negative volume change are promoted by increased pressure and biocatalysis of these reactions may require barophilic enzymes. Deep-sea piezophilic microorganisms are natural source of barophilic enzymes. Pressure-tolerant enzymes could be useful in food processing industries, in particular where high pressure is applied for processing and the sterilization of food materials [38.34]. Biotechnological aspects of a few high-pressure-tolerant marine microbial enzymes are being studied but this group of extremozymes is yet to be understood in terms of their structure, activity, flexibility, and potential applications [38.35]. A protease produced by a deep-sea fungus that was isolated from 5000 m depth of Central Indian Basin reasonably retains its activity at elevated pressure as high as 300 bar [38.36]. The barophilic and extremely thermophilic protease isolated from another deep-sea microorganism *Methanococcus jannaschii* is an excellent example of extremozyme from marine microorganism [38.37]. Enzyme activity and thermostability of this protease increased with pressure – raising the pressure to 500 atm at 125 °C enzyme activity and thermostability enhanced by 3.4-fold and 2.7-fold, respectively. Similar behavior was observed in

a partially purified hydrogenase from the same organism [38.38]. The hydrogenase activity was increased more than three times when the pressure was raised from 7.5 to 260 bars at 86 °C. Thermostability of this hydrogenase was also stabilized by enhanced barophilic pressure.

38.3 Current Use of Marine Microbial Enzymes

Detail discussions on the research and applications of marine enzymes are presented in some recent reviews [38.16, 39–41]. From these discussions, it is clear that biochemical characterization of several marine microbial enzymes showed their potential biotechnological or biomedical applications but most of them are yet to be exploited for industrial purposes. Only a few marine microbial enzymes are currently used in industrial and research purposes.

Until now, thermostable DNA polymerases are most widely used marine microbial enzymes. PCR (Polymerase Chain Reaction) is a very strong tool of molecular biology research but wide use of PCR was practically impossible without a thermostable DNA polymerase. Two marine microbial enzymes, the Vent polymerase from *Thermococcus litoralis* (New England Biolab) and *Pfu* polymerase from *Pyrococcus furiosus* (Stratagene) solved this problem. *Pfu* polymerase is one of the most widely used DNA polymerase in molecular biology research. These enzymes showed optimum activity at ≈72 °C and are stable at >95 °C. They are almost solely used enzymes for this purpose. *Tth* DNA polymerase from *Thermus thermophiles* is another marine microbial enzyme used for biotechnological research. It is a 5′→3′ polymerase enzyme and showed reverse transcriptase activity in the presence of Mn^{2+} ion. *Tth* polymerase has optimal activity at 70–80 °C and is used for some special PCR applications including one-step reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR [38.42]. ArcticZymes AS, a subsidiary of Biotec Pharmacon ASA announced several marine enzymes for molecular biology research though exact source of the enzymes were not revealed. They are marketing uracil DNA glycosylase and several nucleases including thermolabile DNAses and salt-active nucleases [38.43].

Vernium Corporation revealed the discovery of a thermostable α-amylase from deep-sea microorganism [38.6]. The enzyme with brand name Fuelzyme is highlighted as a next generation α-amylase for starch liquefaction. By a unique mechanism of action it randomly hydrolyzes α-1,4 linkage of starch and produces lower molecular weight soluble dextrin and oligosaccharides of uniform size. It is active over a broad range of temperature and is used to increase fuel ethanol yields due to improved starch hydrolysis at much lower concentration in comparison to other competitive enzymes. Currently this enzyme is being marketed by Verenium Corporation and is expected to significantly improve the efficiency and economics of fuel ethanol production.

Two recombinant lipases *Candida antarctica* Lipase A and Lipase B are being marketed by Sigmaaldrich. These enzymes were originally reported from the marine fungie *C. antarctica* isolated from Lake Vanda in Antarctica. They are used industrially for the resolution of chiral compounds and the transesterification production of biodiesel. Lipase B is an enantioselective enzyme that can catalyze the synthesis of esters of ethyl D-glucopyranoside from fatty acids larger than octanoic acid. It can catalyze a wide variety of organic reactions including enantioselective syntheses. It is used in enzymatic acylation of nelarabin for production of a highly soluble antileukemic agent, nelarabin 5′-monoacetate [38.44]. The end-product has better bioavailability than nelarabin. This enzyme is tolerant to organic solvents and the best substrate conversion is achieved when the reaction takes place in anhydrous dioxane. It can convert almost 99% substrate to the desired stereoisomer, which is almost impossible in conventional chemical acetylation processes.

38.4 Current Research Status of Marine Microbial Enzymes

Extracellular enzymes from marine microorganisms need to be exposed in extreme environments and logically most extremozymes belong to this category. Several preliminary researches were concluded with enzymatic activity of intact microorganisms or extracellular culture supernatants. For many years, research articles reported mainly physicochemical characterization of crude, partially purified, or purified enzymes.

Recent development of biological and engineering tools is slowly leveraging the marine biotechnology research. Cloning and overexpression of marine-derived enzymes is a common practice now a day. Structures of a few marine enzymes were resolved and a marine microbial enzyme is reported to be engineered to achieve the desired characteristics [38.45–47]. Bioinformatics is playing a key role for the development and analysis of huge databases, which are useful for sequence analysis, analysis of gene expression, identification of microorganisms, prediction of structure and function of unknown proteins and, in primer design and mutant construction for protein engineering. Metagenome analysis becomes a routine technique for searching proteins from uncultivable marine microorganisms. On the other hand, several mathematical models are used for optimization of media composition and fermentation parameters. Marine-derived enzymes are also being studied for potential nontraditional applications including waste management and biofuel production [38.48–50].

38.4.1 Genetic Engineering and Related Research

Molecular biology and recombinant DNA technology are widely used in current research on marine microbial enzymes. Large-scale enzyme production from a native host is less economic due to low productivity and wastage of nutrients for cellular maintenance. Marine microorganisms often need expensive media components and sometime special fermentation conditions for production of desired enzymes. Cloning of marine-derived genes is essential for enzyme production in normal expression hosts – it can bypass the challenges of large-scale cultivation of marine microorganisms and extracellular enzyme purification from the complex marine media.

Alkaline protease genes were amplified from complementary DNA (cDNA) and genomic DNA of the marine yeast *Aureobasidium pullulans* 10 and *A. pullulans* HN2-3 [38.51, 52]. The gene from *A. pullulans* 10 was cloned and overexpressed in *Yarrowia lipolytica* as an extracellular protein that was purified from the culture medium. The gene from *A. pullulans* HN2-3 was cloned into a surface display vector pINA1317-YlCWP110 and expressed in *Y. lipolytica* cell for production of the surface-displaying alkaline protease [38.52]. The overexpressed protease on *Y. lipolytica* surface can produce bioactive peptides that showed angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant activity. Other marine enzymes may be useful for production of bioactive peptides from various raw materials using different surface displaying proteases. *Tsujibo* et al. cloned two alkaline serine protease encoding genes from a marine *Alteromonas* sp. strain O-7 and overexpressed into *E. coli* [38.53, 54]. The amino acid sequence revealed that both the proteins are composed of four domains including a signal peptide and a mature protease region with high sequence homology to other class-I or class-II subtilases. Another alkaline protease gene was isolated from the marine fungus *Engyodontium album* and cloned in *E. coli* system [38.55]. Homology modeling of translated amino acid sequence suggests the gene encode an extracellular protease that belongs to the subtilase family of serine protease. Comparative homology modeling revealed that the protease has broad substrate specificity with preference for bulky hydrophobic residues at P1 and P4. Also the model suggests that the protease structure is stabilized by two disulfide linkage and more than two Ca^{2+} binding sites. Partial sequence of a lipase-encoding gene from an Antarctic *Psychrobacter* sp. was identified by PCR-based screening using primers obtained from multiple sequence alignments of a group of prokaryotic lipases [38.56]. By application of a fast and efficient genome-walking technique, a complete 1293 bp gene sequence was obtained and, the promoter and downstream sequences of the gene were also determined. The gene was cloned into pMAL-c2E expression vector and a recombinant MBP fusion protein was over expressed in *E. coli*. The fusion protein showed high lipolytic activity and retained the psychrophilic property with optimum activity at 20 °C. Oxidative stability of an α-amylase was studied by site-directed mutagenesis [38.46]. The enzyme was purified from a deep-sea marine *Bacillus* isolate and reported to retain almost complete activity even after 1 h incubation with 1.0 M H_2O_2. Mutagenesis study showed that the presence of a nonoxidizable Leu198 in place of a conservative methionine residue results exceptional oxidative stability of this enzyme.

Several marine enzymes were identified by metagenomic approach. *Hardeman* et al. (2007) reported a marine lipase from a metagenomic library of Baltic Sea sediment bacteria [38.57]. A library of 47 000 clones was generated by extracting the prokaryotic DNA and cloning it into a pCC1FOS plasmid. The inserts within the clones are ranging from 24 to 39kb. The lipolytic gene was detected by activity-based screening followed by subcloning and sequencing of positive clones. The isolated gene with 978 bp ORF encodes a 35.4 kDa lipase that shows 54% amino acid similarity and several

conserved region with a *Pseudomomas putida* esterase. In another study, two novel esterase genes were isolated by functional screening of a marine microbial metagenomic library [38.58]. Two esterase containing 277 and 328 amino acids were purified and biochemically characterized. Site-directed mutagenesis showed one of the esterase contained classical catalytic triad containing S146–D222–H255 and the second esterase contained an unusual catalytic triad made up with S–E–H. The latter esterase was highly stable in the presence of organic solvents. *Xu* et al. (2010) reported cloning, expression, and characterization of another novel esterase from marine sediment microbial metagenomic library [38.59]. The expressed protein was a psychrophilic esterase that showed 71% sequence identity to another esterase (ADA70030) identified from marine sediment metagenome. Another esterase was reported from metagenomic library constructed from bacteria associated with the marine sponge *Hyrtios erecta* [38.60]. The esterase gene was PCR-amplified, cloned in pET22b plasmid and overexpressed in *E. coli*. The purified protein is moderately thermostable and active in the presence of high NaCl concentration. One α-amylase encoding gene was isolated from a marine metagenomic library, cloned in pUC19 vector and overexpressed in *E. coli* [38.61]. The full-length amino acid sequence showed very low similarity with known α-amylases but revealed the presence of a conserved catalytic domain of α-amylase superfamily. It exhibited the highest sequence identity (50−72%) to four putative glycosidases from four different marine bacteria.

38.4.2 Structural Study

Structure elucidation of marine microbial enzymes is rarely reported instead of more than two decades extensive research in the field of marine biotechnology and characterization of numerous marine microbial enzymes. Understanding of 3-D structure of enzymes is extremely useful to study structure activity relationship and improvement of the enzyme activity by protein engineering. Structure of the catalytic domains gives an idea regarding substrate specificity of enzymes while other domains determine several important features need to be considered for enzyme activity such as stability and ligand binding of the enzyme.

Crystallization and preliminary X-ray analysis were done to study catalytic domain of a β-1,3-xylanase from the marine bacterium *Vibrio* sp. AX-4 [38.47]. The protein contains an N-terminal catalytic module and two *C*-terminal xylan-binding modules. Successful crystallization was possible with a deletion mutant composed of a catalytic module without a xylan-binding module. The crystal diffracted to 1.44 Å resolution and the crystal data analysis suggests that the crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 51.6$, $b = 75.8$, $c = 82.0$ Å. A similar study was done with a methanol dehydrogenase from the marine bacterium *Methylophaga aminisulfidivorans* MP^T [38.45]. The mechanism of activity of this enzyme was predicted from preliminary X-ray crystallographic analysis of the native heterotetrameric $\alpha_2\beta_2$ methanol dehydrogenase complex. X-ray diffraction data were collected to 1.7 Å resolution and it was revealed that the crystal belongs to the monoclinic space group $P2_1$ (unit-cell parameters $a = 63.9$, $b = 109.5$, $c = 95.6$ Å, $\beta = 100.5°$). Matthews coefficient and solvent content of the asymmetric heterotetrameric complex were calculated as 2.24 $Å^3\,Da^{-1}$ and 45.0%, respectively. In two other studies, psychrophilic alkaline proteases from two different marine microorganisms (Antarctic *Pseudomonas* sp. and Chinese Yellow Sea *Flavobacterium* YS-80 sp.) showed a homologous structure [38.62, 63]. Both the proteases have two domain structures where N-terminal domains have proteolytic activity and the C-terminal domains are responsible for stability of protein structure. Several Zn^{2+} and Ca^{2+} ions are associated with crystal structure of both the proteins. The tertiary structures of these psychrophilic proteases are comparable to their mesophilic counterparts. The Yellow Sea *Flavobacterium* protease is more thermostable and is more rigid in structure than Antarctic *Pseudomonas* protease but is more flexible than its mesophilic counterpart. These properties are consistent with the respective habitat of the enzyme producing microorganisms and would be helpful for elucidating the structure-environment adaptation of these enzymes. Crystal structure of the Yellow Sea *Flavobacterium* protease was solved at 2.0 Å resolution and was refined to a crystallographic R_{work} and R_{free} of 0.16 and 0.21, respectively. Structure of the psychrophilic Antarctic *Pseudomonas* protease was studied in two crystal forms with 2.1 Å and 1.96 Å resolutions. Comparative studies showed that the overall structures of this psychrophilic enzyme is similar to its mesophilic counterparts but conformation of its substrate-free active sites are similar to substrate-bound form of the mesophilic proteases.

38.4.3 Fermentation and Related Research

Continuous supply in ample quantity and cost of large-scale production are major challenges for commercial-

ization of most marine natural products. Optimization of media composition and fermentation parameters is key consideration for the scale-up of microbial enzyme productions. Cultivation of most marine microorganisms needs special media composition and in many cases physical conditions of their native habitat have influence in optimum fermentation conditions. Cost-effective production media are reported for solid-state and submerged fermentation of several marine microbial enzymes. High NaCl concentration, addition of trace metals or sea water was reported to enhance productivity of several enzymes.

Media and fermentation parameters for protease production by the marine yeast, *A. pullulans* was optimized in a 2 L fermenter [38.64]. Maximum protease activity was achieved after 30 h fermentation at 24.5 °C in a medium containing 2.5 g soluble starch, 2.0 g $NaNO_3$ and 100 mL seawater (per liter) with initial pH 6.0. Thermostable alkaline protease production by a marine *Bacillus* sp. was optimized and scaled up in a stirred tank bioreactor using cheap and readily available substrates [38.23]. Maximum protease production was achieved after 40 h fermentation at 42 °C with an aeration rate of 1.5 vvm and constant agitation at 400 rpm in a soybean-casein medium (pH 9.6). Protease production by the marine bacterium *Teredinobacter turnirae* was optimized for solid-state fermentation as well by immobilization the microbial cells on solid matrixes [38.65, 66]. Media composition, initial pH, inoculums concentration, and effect of different salts and carbon sources were studied to optimize protease production by solid state fermentation in a soybean-based media. The maximum protease production was obtained using 1% (w/v) of soybean concentration, initial pH 7.34, and 2.5% (v/v) inoculum level. In the other study, material and particle size of the immobilizing media and, method and conditions for cell immobilization was optimized for repeated batch fermentation of the alkaline protease by *T. turnirae*. Ceramic support, different size of broken pumice stone and silicone foam enhanced enzyme production by more than 200%, when compared to free cells. Electron micrograph evident *T. turnirae* colonization in porous support matrices and five repeated batch fermentation was achieved without significant decrease of the enzyme production. When the same strain was immobilized in calcium alginate beads maximum protease activity was achieved at 3% (w/v) sodium alginate and 3% $CaCl_2$ concentrations with a 1 : 2 ratio of cell and alginate. Protease production was independent on bead size but decreases significantly when the beads were treated with glutaraldehyde. The immobilized cells were used for eight cycles each lasting 72 h. Protease production increased with increased number of fermentation cycles and maximized at fourth cycle when the enzyme activity reached about 3.5 times than that of the first cycle. *Estrada-Badillo* et al. (2003) studied the effects of agitation and aeration rate on biomass and extracellular protease production by a marine *Vibrio harveyi* sp. using a seawater/Zobell-based medium [38.67]. Maximum protease activity was obtained at 700 rpm agitation and 0.5 vvm air flow rate. Addition of skim milk in Zobell medium enhanced the extracellular protease production by fivefold. Growth of the culture was highly dependent on agitation rate and most protease activity was achieved at stationary phase after 3 h culture. *Sarkar* et al. (2011) developed a special conico-cylindrical flask with an inner arrangement to enhance protease production by an intertidal marine isolate [38.68]. The conico-cylindrical flask was attached with eight equidistant rectangular strips mounted radially on a circular disk to provide additional surface area for microbial attachment. 35% higher protease production was achieved in this system in compare to Erlenmeyer flask. The influence of surface material (hydrophobic versus hydrophilic) was also studied for growth and protease production by this marine isolate. Esterase fermentation of the marine *Bacillus licheniformis* MP-2 strain was optimized by response surface methodology [38.69]. Eleven factors related to esterase fermentation were studied with Plackett–Burman design. Amount of peanut cake, soybean cake, and inoculum volume was observed to have major influence on the MP-2 esterase fermentation. The critical level of these three parameters was further studied by response surface methodology. The optimal process conditions were determined by solving the quadratic regression model equation and analyzing the response surface contour plots. Practical enzyme production was increased from 258.8 to 318.2 U mL^{-1} using peanut cake, soybean cake and inoculum ratio of 0.9%, 2.29%, and 5.98%, respectively, while theoretical optimum esterase activity of MP-2 was predicted as 334.46 U mL^{-1} under these conditions. A similar approach was reported for optimization of fermentation parameters and media composition for a cold adapted amylase production by marine bacterium *Wangia* sp. 52 [38.70]. At optimized condition experimental amylase production reached very close to the yield predicted by statistical model. Tenfold increase of the amylase production was achieved in compared to the control shake flask culture. An efficient, simple and reliable

production process was developed for a thermostable esterase production by a thermophilic marine *Pyrococcus* sp. [38.71]. The gene was cloned and overexpressed in *E. coli* and, fermentation and downstream processing of the recombinant protein was scaled-up to industrial scale. This method is potentially competitive for industrial application due to high enzyme concentration (2 g/L) and product recovery yield (>70%).

38.5 Future Prospects

Several marine microbial enzymes showed immense potential for industrial applications but there is lack of effort for their commercialization. Significant numbers of enzymes are not further reported after physicochemical characterization though some interesting enzymes went through cloning and gene over-expression, lab-scale product optimization, and structure elucidation. However, scale-up of marine enzyme production or large scale process development with marine enzyme is rarely reported [38.72]. Only a few marine-derived enzymes are already reported to be used as industrial enzymes and in biotechnological research. Few more enzymes are in trial for industrial or biomedical applications but a countable numbers are facing challenges in early stage development on their way to practical application. A major challenge for commercializing marine enzymes comes from the lack of cost-effective large scale production methods. Extremophiles are source of most potential commercial enzymes but their optimum growth and enzyme production often need extreme environments. This problem can be overcome by cloning and expression of the respective gene in a suitable host microorganism that can grow in ambient environmental condition. However, to ensure continuous supply of enzymes in large quantity, more intensive research is essential in the area of scale-up and downstream processing. Industrial process development with existing potential enzymes is another big challenge. More research efforts can be diverted for industrialization of the existing potential enzymes than searching for an unknown one but matching an enzyme with a process is not always an easy job. Though many industrial plants can be modified to accommodate the limitations of an existing enzyme, in most cases it is not cost effective. Finding a more suitable enzyme for the existing industrial process may be easier but an extremely uncertain approach. Marine ecosystem has a lot room for searching novel enzymes while protein engineering may be complementary research strategy. Enzymes can be engineered to increase their activity, enhance tolerance to organic solvents or other chemicals, to manipulate substrate specificity and to enhance tolerance to extreme physical conditions. Manipulating activity or physical characteristics of an enzyme is tough but systematic approach. Protein engineering became more practicable with current tools of biotechnology and it is advancing every days – it may be the future of industrial enzyme development. Understanding of 3-D structure and their analysis by bioinformatics tools would be helpful to design engineered enzymes with improved properties. Unfortunately, structural biology and protein engineering is grossly neglected in marine biotechnology. Marine biotechnology is no more a nascent research field – sufficient work is done with marine microbial enzymes to step forward for exploring the chance of using multidisciplinary knowledge and techniques of biotechnology for their commercialization.

After detail characterization several marine microbial enzymes reported to have potential applications in food processing industry, chemical and pharmaceutical synthesis, and bioremediation. Potential applications of psychrophilic xylanases were reported from Antarctic microorganisms in baking industry and the enzymes are already patented [38.14, 73, 74]. Organic solvent tolerance is a common feature of many marine microbial enzymes. Numerous marine microbial esterases and lipases showed high tolerance to organic solvents [38.33]. These enzymes can catalyze some reactions ever impossible in aqueous systems and have potential applications in chemical synthesis. They are also well known for catalyzing chemical reactions for enantioselective product formations; these enzymes can be exploited for synthesis of enantiospecific drug molecules [38.75]. An organic solvent-tolerant enantioselective alcohol dehydrogenase from the hyperthermophilic marine archaeon *P. furiosus* is being considered for its potential applications in industrial biocatalysis [38.3]. Catalytic properties, substrate specificity, and enantioselectivity are widely studied for this enzyme. It catalyzes reduction of aryl ketones for production of enantiomerically pure chiral alcohols. The activity of this enzyme increases at enhanced temperature without affecting enantioselectivity. Marine microbial enzymes can also be exploited for biomedical application. L-asparaginase is a drug of choice in combination therapy for treatment of children lym-

phoblastic leukemia [38.76]. L-glutaminase also has a similar antineoplastic activity. Both of these enzymes were reported from marine sources. The L-glutaminase from the marine bacteria *Pseudomonas fluorescens* was studied for antineoplastic activity [38.77, 78]. However, potential immunogenic issues need to be addressed before use of these microbial enzymes in human system. Some marine microorganisms showed potential application for bioremediation of industrial and agricultural wastages [38.48, 79–82]. Use of marine enzymes is proved to be advantageous for degradation of organic wastes generated in seafood farming [38.83]. A strain of the marine fungus *Geotrichum marinum* was reported to degrade crude petroleum and petroleum products [38.84]. It can be useful in pollution control for treatment of effluents from petrochemical industries. Oil spillage on sea surface is frequently reported from damaged ships or broken pipelines, which cause severe damage to marine ecology. A salt-tolerant petroleum-degrading enzyme would be extremely useful to degrade the residual petroleum products – may be the solution is hidden under the sea.

38.6 Conclusion

Scientific publications suggest that the enormous biodiversity of the marine ecosystem is an excellent source of novel enzymes. The extremophilic nature and versatile biocatalytic capabilities confirm potential industrial application of marine microbial enzymes. Marine microbial enzymes already provided some extremely important tools for biotechnological research. A few marine enzymes are also being used in biofuel and pharmaceutical industries. Several marine microbial enzymes showed potential applications in chemical and pharmaceutical synthesis, food processing industries, and environmental managements. With the development of high-throughput screening techniques there are lot room for discovering novel enzymes from vast marine ecosystem. The advancement of genetic engineering, better understanding of protein structure, and availability of several biotechnology tools is leading to the development of engineered enzymes with improved properties such as higher catalytic activity, better tolerance to organic solvents and more stability to adverse environments. Published research works suggest extensive industrial scope of marine microbial enzymes but further application-oriented researches are essential for exploitation of their full biotechnological potential.

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39. Marine-Derived Exopolysaccharides

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Marine biotechnology still remains a new and emergent science, which is closely linked to marine biodiversity and to the technological capacities of investigating more atypical ecosystems. Marine microorganisms show a unique biodiversity since they have to adapt to various marine environmental conditions such as low or high temperatures, alkaline or acidic water, high pressure, and limited nutrients. Marine natural products, especially marine polysaccharides, are attracting more and more attention. Microbial polysaccharides are of growing interest for many sectors of industry, resulting in isolation of new exopolysaccharide (EPS)-producing bacteria. The diversity of these polysaccharides arises from the structural variations (glycosidic bonds, side branching chains, monosaccharidic content) controlled through a genetic basis. A lower molecular weight and functionalized derivatives together with the native form of the polysaccharide have been shown to possess a variety of biotechnological activities. Therefore, the biophysical and biological properties have made them useful in many pharmaceutical, food, and industrial applications. This chapter gives information on EPS-producing bacteria from the marine environment, as well as on the carbohydrate molecule they produce, including the chemical composition or structure when available, the putative pathways of biosynthesis, and the potential applications in industry with a focus on healthcare and glycosaminoglycan-like compounds.

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39.1 In Search of New Polysaccharides

Polysaccharides constitute the most abundant and the most diverse materials found on earth and in the oceans. Polysaccharides (PSs) form a class of biotechnological molecules that find applications in many industrial fields. They consist of monosaccharides (sugar units) that are linked to each other, generally in a repeated sequence order. By composition, PSs may be divided into homopolysaccharides, composed of single monosaccharide type and heteropolysaccharides with several different monosaccharides. Interest in carbohydrates was first very low due to their extreme structural complexity and the lack of understanding of their key role in different biological systems or processes. The type of glycosidic bond, the chain length, the degree and

type of branching, as well as the presence of organic or chemical groups are important features of the molecular structure, which are also of great importance for the biophysical or biological properties. Except for the PS used traditionally in the food industry, the structure of most PS, as well as the structure–function relationships are mostly unknown.

For biotechnological purposes, marine PSs have been studied in animals (chitin from crustaceans, chondroitin sulfate from fish, sulfated PSs from sponge and glycosaminoglycans from scallops and abalone), plants (ulvans, agarans, carrageenans, alginates, fucans, laminarin from seaweeds), and microorganisms such as fungi, microalgae, and bacteria. In bacteria, several kinds of PS exist, depending on their cellular localization and structure. Exopolysaccharides (EPSs) represent an important component of the biofilm matrix and serve as functional elements for adhesion, protection, and recognition. They also have chelating properties especially of heavy metals. The main advantages of microbial EPSs over plants or macroalgal and animal PSs lie on a stable supply independently of climatic or physiologic variations. Moreover, new structures have been described in bacteria, in particular in marine bacteria, giving the opportunity for the development of new applications, especially in the pharmaceutical field.

Marine-derived PSs and their oligosaccharidic derivatives have been studied for a variety of biological activities such as antithrombotic, antitumor, antiviral, antioxidant, and immunomodulatory effects. A high specificity linked with highly beneficial properties compared with the reference molecules already existing on the market are the major conditions of the development of new molecules, especially if these molecules present a cheaper production cost.

There is in particular a big diversity in the abundant microbial life in the oceans. The marine environments include coastal ecosystems, the deep ocean with hydrothermal vents, sediments, and the cold water of the poles. The extremes of temperature, pressure, and acidity met in the ocean, and the availability of the sources of energy and nutrients results in different strategies of microorganisms to survive. The combination of the diversity, the quantity, and the rate of metabolism makes the oceanic world a vital for the earth. Indeed, marine microbes are responsible for the global cycle of nutriments and other elements and are involved in the chemistry of the ocean, the composition of the atmosphere, and climate. As microorganisms quickly reproduce when conditions are favorable, the huge number of generations allows, by selection and natural evolution, the appearance of new species or other groups of the classification. The microbial genetic diversity also results in a biochemical diversity offering opportunities of biotechnological and pharmaceutical applications.

This chapter will focus on PS from bacterial microorganisms, presenting an overview of microbial marine biodiversity, structures of the produced polymers, some molecular aspects of their biosynthesis, as well as some of their applications driven by their features and biological properties with a focus on the human health domain.

39.2 Marine Biodiversity

For a long time, oceans have been considered as unlimited to human beings, who have always wanted to exploit the attractive resources. However, the industrial exploitation of the oceans only began 100 years ago. Studies to understand oceans and their ecosystems with the aim to exploit molecules produced by marine organisms or to develop new drugs inspired by marine molecules have started to reveal secrets, new biodiversity, and innovative molecules of biotechnological interest.

39.2.1 Ecosystems in the Oceans

The oceans cover 71% of the earth's surface, with an average depth of 3800 m. Depths reach 6000 m for the abyssal plains; the deepest point is the Mariana Trench in the Pacific Ocean (estimated to be 11 000 m). Smaller seas of salt water also exist; they are partly or fully enclosed by land.

Except on the surface and in temperate or tropical areas, most of seawater is characterized by a temperature lower than 5 °C, low nutrients, and no sunlight to allow photosynthesis. The open ocean is a huge ecosystem but particular habitats sometimes qualified as extreme have also been discovered: underwater salt lakes, volcanos, mountains, hot smokers, cold seeps, ...

In the deep sea, pressure is very high, the temperature is between 2 and 5 °C, and there is total darkness. A remarkable variety of deep-sea habitats exist: cold seeping waters, sinking particles, sediments, animal

guts and surfaces, deep sediments, and hydrothermal vents [39.1]. These latter areas are unstable due to high volcanic activity; they are characterized by a large gradient of temperatures [39.2], high amounts of dissolved compounds, gas, as well as very low pH values. The first deep-sea hydrothermal vent was discovered in 1977, near the Galapagos islands, on the East Pacific Rise, and the first *black smoker* in 1979 on the East Pacific Rise, 21° North. Deep-sea hydrothermal vents are mainly located on the active Mid-Atlantic Ridge, the East Pacific Rise, and back-arc basins (Fiji, Okinawa, Lau Basin). They come from the seeping of seawater down into the crust through rock cracks in the seafloor. During course of the water, several chemical processes enrich it in minerals, such as calcium, sulfates, magnesium, sodium, hydrogen sulphide, and metals, and it also becomes hotter. Jetting upwards by chimneys called black or white smokers formed by compound deposits or diffusing out of cracks, the hydrothermal fluid gives rise to the development of a luxuriant life contrasting with the apparently deserted surrounding water. Various vent fauna, bivalves, crustaceans, and worms take their energy and resources from endosymbiotic and episymbiotic chemoautotrophic microorganisms capable of oxidation of H_2 and sulfur compounds [39.3–5]. To survive under extreme conditions, such organisms must be adapted by appropriate metabolic pathways and protective mechanisms.

Some other volcanic areas also exist near the coast [39.1]. Shallow hot springs have been studied [39.6, 7]. Cold seeps are also characterized by hydrocarbon-rich fluids emanating from the ocean floor and are also the center of ecosystems with a large biodiversity [39.8, 9].

Near-shore sediments and deep-sea sediments have also been explored (the Ocean Drilling Project) and have revealed microbial life down to 1600 m in the sediment under the sea floor [39.10].

In polar areas, the environment is perennially cold, and in some cases permanently covered with ice; therefore, microbes need to adapt to cold temperatures and fight against freezing. However, even in these extreme conditions, some bacteria have been collected at around 0 °C in the Antarctic [39.11–13].

High saline water in inland water (the Dead Sea) together with high pH, high saltwater lakes situated underwater of seas such as the Black Sea, the Red Sea, the Mediterranean sea, or Gulf of Mexico have also been explored [39.14].

French Polynesia microbial mats are characterized by variable parameters such as pH values between 6 and 10.5, salinity ranging from 5–42 $g\,L^{-1}$, and temperatures between 20 and 42 °C. Microbial mats are laminated abundant communities of phototrophic and chemotrophic microorganisms. *Pseudomonas*, *Alteromonas*, *Paracoccus*, and *Vibrio* bacteria from these environments have been shown to produce EPSs [39.15, 16].

Marine surfaces and especially marine eukaryotes are covered with microorganisms embedded in a matrix forming a biofilm. The microbial epibiotic consortia differ significantly from microorganisms living in the surrounding environment and highlight the close relationships between microbial epibionts and hosts [39.17].

From deep-sea vents to surface-sea ice and open ocean, nutrient sources and physic-chemical conditions may be highly fluctuant, making quick adaptive responses necessary; the adaptation is possible on the basis of genetic diversity and has led to huge biochemical diversity.

39.2.2 Microbial Diversity and the Limitation of Collections

A high diversity of ecosystems exists within the world's oceans; they are different from terrestrial and freshwater environments; some endemic species have, therefore, been identified resulting in specific diversity.

Most of the biodiversity is microbial. The genetic diversity of microorganisms is far larger than that of plants and animals all together. Due to their large population size and since they grow rapidly, prokaryotes have an enormous potential to accumulate mutations, allowing them to adapt, and thus acquire genetic diversity. By weight, more than 95% of all living organisms found in the oceans are microbial. Microorganisms are the most abundant life form in the ocean, they are the basis of the marine food web, drive energy and nutrient cycling, and are at the origin of life on the earth [39.18]. This genetic resource is mainly unknown and underexploited and will probably be the major source for biochemical diversity resulting in novel molecules in the future.

Isolating new species requires the ability to cultivate them. Cultivation efficiency only allows the isolation of 1–5% of microbial species present in marine ecosystems [39.19]. Therefore, microbial diversity is largely undiscovered. From oceanographic cruises or sampling campaigns, several academic or industrial teams all over the world have developed marine microbial collections. These they are a good source of screening for innovative biomolecules. These collections, except

private ones, offer marine microorganisms and other services such as various screening. However, these collections shelter only cultivable microorganisms and, therefore, are limited. Nevertheless cultivation is a prerequisite to isolate compounds of biotechnological value such as PSs [39.20].

Culture-independent molecular techniques such as metagenomics, also named environmental genomics, have been adopted to explore the actual diversity of natural assemblages of microbes. Their sensitivity allows the detection of taxa at very low abundance. They have also revealed the incredible richness of marine life.

For several years, J. Craig Venter's Sorcerer II (Venter's Global Ocean Sampling Expeditions) sailed all around the sea world to collect samples of near-surface sea water every 200 miles. After filtration and freezing to collect and store microorganisms in the samples, DNA was extracted in the J. Craig Venter Institute laboratory and sequenced. Results revealed the power of metagenomic approaches to obtain a good estimation of microbial diversity and biochemical diversity; they also revealed a high proportion of new bacterial and viral sequences, which suggested that until now marine microorganisms have not been well isolated, especially viruses [39.21]. Many of these sequences must have potential as sources of new drugs and they are also studied for protein evolution, discovery of new enzymes, and microbial ecology concerns.

Tara Oceans expeditions [39.22] started on 2004 to study the impact of climate change on marine ecosystems, especially marine plankton including microorganisms such as viruses and bacteria, and other organisms such as medusas. It sailed all over the seas to collect samples and data and provide them to the world's scientific community. In 9 years, 8 expeditions have been organized with the aim to establish a time zero of marine ecosystems, to discover new organisms in each size class, to obtain a good estimation of oceanic biodiversity and its distribution, and to develop new models to estimate this biodiversity.

Deep-sea microbial diversity has been explored by metagenomic [39.23] or cultivation approaches [39.24, 25]. Sea floor sediments as well as hydrothermal vents with black or white smokers have been sampled. Studies on the genomic diversity of water columns [39.26] or sea floor sediments [39.27] have also been carried out as well as on microbial communities as associations with marine invertebrates, sponges [39.28] or with corals [39.29, 30].

In all cases, sequences related to fungi, bacteria, archaea, viruses and phages have been detected in variable ratios in a site-specific manner or isolated on culture media. The orders Thermococcales, Archaeoglobales, and Methanococcales are widely encountered in extremophilic archaea. On the other hand, the vast majority of bacterial sequences are mainly assigned to Thermotogales: *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, and *Actinobacteria*, and 80–95% of marine bacteria are Gram-negative rather than Gram-positive.

Global Ocean Sampling Expeditions revealed that the archaea are nearly absent from the list of dominant organisms in the near-surface samples. Based on 16S ribonucleic acid (RNA) data, the most abundant phyla or classes are *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Actinobacteria*, and *Planctomycetes* [39.21]. The sequences mainly cluster within *Alphaproteobacteria* and *Gammaproteobacteria*. However distribution of these ribotypes is not homogeneous all over the ocean and different microbial communities can be distinguished.

The diversity of seawater habitats results in diversity of the organisms, and subsequently of the metabolites they produce. Thus marine genomics provide sequence data for microbial ecology but also biodiscovery [39.31]. This renders marine ecosystems really interesting to discover innovative molecules for biotechnological purposes. Extremophiles will provide new molecules or pathways for novel biotechnological processes but are also models to investigate how biomolecules are stabilized under extreme conditions. Several studies on marine biodiversity for biotechnological applications have been carried out on particular habitats such as deep-sea hydrothermal vents for extremophiles and thermostable enzymes, polar areas for psychrophilic ones, for exopolysaccharides [39.1, 32–36], microbial mats from French Polynesia [39.16].

These bacteria may produce new enzymes and bioactive compounds, including exopolysaccharides (EPSs) with innovative structures (Table 39.1). Many known marine bacteria can produce molecules of biotechnological interest, some of which have unique properties, and the search for new microorganisms of biotechnological value is still promising.

Table 39.1 Some marine bacteria, cyanobacteria, and marine archaea, and the EPS they produce

Microorganism	Origin	EPS composition or repeating unit structure	References
Marine bacteria			
Alteromonas hispanica	Hypersaline lake, Southern Spain	Glc/Man/Rha/Xyl 18.1/62.7/6.9/12.3	[39.37]
Alteromonas infernus	Animal population, Guaymas Basin, Gulf of California	Sulfated PS [SO_3Na] ↓ 2 [→4)–β–Glc*p*–(1→4)–α–Gal*p*A–(1→4)–α–Gal*p*–(1→] 3 ↑ *1* β–Glc*p*–(1→6)–α–Gal*p*–(1→4)–β–Glc*p*A–(1→4)–β–Glc*p*A 2 3 ↑ ↑ *1* *1* α–Glc*p* α–Glc*p*	[39.38, 39]
Alteromonas macleodii subsp. *fijiensis*	Deep-sea hydrothermal vents, North Fidji Basin	→4)–β–D–Glc*p*–(1→4)–α–D–Gal*p*A–(1→4)–α–Gal*p*–(1→ 3 ↑ *1* α–D–Glc*p*A 3 ↑ *1* β–D–Glc*p*A 4 ↑ *1* 4,6–Pyr–β–D–Man*p*	[39.40, 41]
Alteromonas macleodii subsp. *fijiensis biovar deepsane*	Deep-sea hydrothermal vent, East Pacific Rise	16 to 18 Monosaccharides, including seven types of monosaccharide. Two fragments have been identified: β–D–Glc*p*A–(1→3)–α–D–Gal*p*–(1→4)–D–Glc*p*A *A* 3 *B* *C* ↑ *Lac* SO_3 ↓ *2* β–D–Glc*p*–(1→4)–α–D–Gal*p*A–(1→4)–α–D–Gal*p* *G* *D* 3 ? *E or E'F* ↓ *R*	[39.42, 43]
Alteromonas sp. Strain 1644	Polychaete tissue, East Pacific Rise	Glc/Gal/GlcA/GalA and 3-*O*-[(R)-1-carboxyethyl]-D-glucuronic acid	[39.44]
Bacillus licheniformis	Shallow hydrothermal vent, Vulcano Island, Italy	Tetrasaccharide repeating unit, Man/Glc 1/0.2	[39.6, 45, 46]
Bacillus thermoantarcticus	Sea sand in Ischia Island, Italy	2 Sulfated EPS: EPS1 contains α-Man and β-Glc (1.0/0.7) EPS2 contains α-Man and pyruvic acid	[39.47]
Bacillus thermodenitrificans	Shallow hydrothermal vent, Vulcano Island, Italy	2 EPS with uronic acid and sulfate	[39.46]
Geobacillus tepidamans V264	Bulgarian hot spring	Galacto-glucan, α linkage Glc/Gal/Fuc/Fru (1/0.07/0.04/0.02)	[39.36, 48]
Geobacillus thermodenitrificans		Man/Glc	[39.49]
Geobacillus sp.	Ischia Island	EPS 1 Man/Glc/Gal (0.5/1/0.3) EPS 2 Man/Glc/Gal (1 : 0.3 :trace) EPS 3 Gal/Man/GlcN/Ara (1 : 0.8/0.4/0.2) with pentasaccharide unit	[39.36]

Table 39.1 (continued)

Microorganism	Origin	EPS composition or repeating unit structure	References
Marine bacteria			
Geobacillus sp. 4001	Shallow marine hydrothermal vent, Italy	Mannan Man/Glc/Gal/NMan	[39.7]
Geobacillus sp. 4004	Shallow marine hot spring (Ischia, Italy)	Gal/Man/GlcN/Ara (1.0/0.8/0.4/0.2)	[39.46, 50]
Hahella chejuensis	Sediment of Marado, Cheju Island, Korea	EPS-R Glc/Gal/Rib/Xyl (0.68/1.0/trace/trace)	[39.46, 51]
Halomonas sp. AAD6		Levan Fru, β-2,6 linkage	[39.52]
Halomonas alkaliantarctica	Sediments in salt lake in Cape Russell, Antarctica	Glc/Fru/GlcN/GalN (1/0.7/0.3/0.2) xylan-mannan fructo-glucan	[39.46]
Halomonas almeriensis		Man/Glc and trace of Rha	[39.53]
Halomonas anticariensis	Saline wetland, Malaga, Spain	Glc/Man/AcGal	[39.46, 54]
Halomonas maura		Mauran Man/Gal/Glc (1/0.6/0.2)	[39.55]
Halomonas ventosae	Saline wetland, Malaga, Spain	Glc/Man/Gal	[39.46, 54]
Iodomarina fontislapidosi	Spanish hypersaline water	Glc/Man/Gal Anionic PS	[39.37]
Iodomarina ramblicola	Spanish hypersaline water	Glc/Man/Gal Anionic PS	[39.37]
Olleya marilimosa CAM030	Southern Ocean	Man/GlcA/GalNAc/Glc/GlcNAc/Ara/Gal/GalA/Xyl/Rha 48/10/10/9/8/6/4/2/2/1	[39.56, 57]
Paracoccus zeaxantifi-ciens subsp. *payriae*	Microbial mats, French Polynesia	Sulfated PS	[39.16, 58]
Pseudoalteromonas marinoglutinosa KMM232 Mucoid colonies		Sulfated PS $\rightarrow$3)–β–D–Man*p*–(1$\rightarrow$4)–α–L–Rha*p*–(1$\rightarrow$ 2 | SO_3H	[39.59]
Pseudoalteromonas sp. HYD721	East Pacific Rise	$\rightarrow$4)–β–D–Man*p*–(1$\rightarrow$4)–β–D–Glc*p*–(1$\rightarrow$4)α–D–Gal*p*–(1$\rightarrow$4)–β–D–Glc*p*–(1$\rightarrow$ 2 3 $\uparrow$ $\uparrow$ 1 1 α–L–Rha*p* β–D–Gal*p* 3 $\uparrow$ 1 β–D–Glc*p*A 4 $\uparrow$ 1 [SO_3H]$\rightarrow$3–β–D–Man*p*	[39.60]
Pseudoalteromonas ruthenica SBT033	Sea water, coast of India	Man/Glc/Gal/Xyl, uronic acids	[39.61]
Pseudoalteromonas sp. strain CAM025	Sea water in Southern Ocean	Neutral sugar, uronic acid, acetyl, sulfate Glc/GalA/Rha/Gal (1/0.5/0.1/0.08)	[39.11, 56]
Pseudoalteromonas sp. strain CAM036	Sea water in Southern Ocean	Neutral sugar, uronic acid, acetyl, succinyl, sulfate GalA/Glc/Man/GalNAc/Ara (1/0.8/0.84/0.36/0.13)	[39.11, 56]
Pseudoalteromonas sp. strain SM9913	Deep-sea sediment, Yellow Sea, China	Glc with α-1,6 linkage and high degree of acetylation Glc/t-Ara/t-Glc/t-Gal/Xyl/Glc/Glc (0.62/0.11/0.11/0.03/0.04/0.05/0.05)	[39.46, 62]
Shewanella colwelliana	Eastern oyster		[39.63]
Thermotoga maritima		Glc/Rib/Man (1/0.05/0.02)	[39.64]

Table 39.1 (continued)

Microorganism	Origin	EPS composition or repeating unit structure	References
Marine bacteria			
Vibrio alginolyticus	Marine fouling material, Bengal	Glc/AraNRibN/Xyl	[39.65]
Vibrio diabolicus	Deep-sea hydrothermal vent, East Pacific Rise	→3)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*A-(1→4)-α-D-Gal*p*NAc-(1→	[39.66, 67]
Vibrio furnissii	Coastal regions, India	Neutral sugars (Glc/Gal), uronic acids	[39.68]
Vibrio harveyi		Neutral sugars, uronic acids, sulfate	[39.69]
Vibrio parahaemolyticus	Marine natural biofilm	Neutral sugars (Glc/Gal/Ara/Man), uronic acids	[39.70]
Marine cyanobacteria			
Aphanothece halophytica		Xanthan-like behavior	[39.71, 72]
Cyanothece sp. 113	Salt lakes in China	α-D-1,6-homoglucan	[39.73]
Cyanothece sp. Strain ATCC51142		Sulfated PS with uronic acids and methyl sugars	[39.74]
Arthrospira platensis	Blue-green cyanobacterium	Spirulan aldobiuronic acid, acofriose, sulfate	[39.75]
Marine archaea			
Haloarcula spp. T5		AcGlc/Man/Gal (1/0.6/0.3) pentasaccharide repeating unit	[39.76]
Haloferax denitrificans	San Francisco Bay	→ 4)-β-D-Glc*p*A2,3NAc-(1→4)-β-D-Glc*p*A2,3NAc-(1→4)-α-D-Glc*p*A2,3NAc-(1→3)-α-D-Gal*p*-(1→, where D-Glc*p*A2,3NAc is 2,3-diacetamido-2,3-dideoxy-D-glucopyranosiduronic acid	[39.77]
Haloferax gibbonsii		Man/Glc/Gal/Rha (0.6/0.3/1/0.3) heptasaccharide with side chains	[39.78]
Haloferax mediterranei	Mediterranean Sea	Man/Glc/Gal/amino sugars/ uronic acids, sulfate, 4-D-GlcNAcA-β-1,6-D-Man-α-1,4-D-GlcNAcA(3S)-1,	[39.79]
Sulfolobus solfataricus MT4 and MT3		MT4 Glc/Man/NGlc/Gal (1.2/1.0/0.18/0.13) and MT3 Glc/Man/NGlc/Gal (1.2/1.0/0.77/0.73) Sulfated PS	[39.80]
Thermococcus litoralis		Man	[39.64]

EPS were chosen when some structural data were available, CPS and O-antigens were excluded.
(3S): 3-*O*-sulfo, AcGal: acetylgalactose, AcGlc: acetylglucose, Ara: arabinose, Fru: Fructose, Fuc: L-Fucose, Gal: galactose, GalA: galacturonic acid, GalNac: *N*-acetylgalactosamine, GalNac: *N*-acetylgalactosamine, Glc: glucose, GlcA: glucuronic acid, GlcN:glucosamine, GlcNAc: *N*-acetylglucosamine, GlcNAcA: *N*-acetyl-glucuronic acid, Gro: Glycerophosphate, Man: mannose, ManN: mannosamine, p: pyranose, Rha: rhamnose, Rib: ribose, SO_3: sulfate, t- :terminal, Xyl: xylose)

39.3 Bacterial Polysaccharides

Different types of PSs exist within or around the bacterial cell depending on their location:

i) Polysaccharides found in the cytoplasm that serve as carbon and energy reserve,
ii) Cell wall PSs including teichoic acids and peptidoglycans,
iii) Extracellular PSs which are released outside the cell: exopolysaccharides (EPSs) or capsular polysaccharides (CPS).

CPSs are extracellular but remain attached to the cells through covalent bonds to other outer surface polymers thus forming the capsule.

Intracellular PSs are produced when bacteria are grown in excess of sugar and may serve as reserve, since the cell also produces enzymes to degrade them. They usually appear as granules of glycogen or amylopectin type PSs and may be related to sporulation of *Clostridium* sp., for example. However, bacteria synthesize only very few intracellu-

lar PSs but they produce many diverse extracellular PSs.

The cell wall is complex and rich in carbohydrate compounds. Among these, peptidoglycans are linear macromolecules consisting of PSs cross-linked by peptide moieties. The PS part is composed of alternating *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) linked by β-1,4-glycosidic linkages. Carboxylic groups from *N*-acetylmuramic acids are involved in the linkage to peptidic chains. Teichoic acids, which are only found in Gram-positive bacteria, may be glycerol phosphate units, or ribitol phosphate units linked by phosphodiester bonds. Glycerol and ribitol may also be associated with glucose, galactose, or *N*-acetylglucosamine. Teichoic acids are closely connected to the peptidoglycan network and make it more cohesive. Gram-negative bacteria also produce lipopolysaccharides (LPS) whose lipidic part (lipid A) is set inside the external membrane. They are composed of two carbohydrate parts, the O-specific chain, and the core [39.81]. The O-specific chain is highly variable, especially in pathogens such as *Vibrio cholerae* [39.81]. Lipid A is composed of aliphatic chains linked to glucosamine residue. LPS is also called endotoxin.

The following paragraph deals specifically with extracellular PSs and EPS.

39.3.1 Bacterial Exopolysaccharides

From different carbon sources, bacterial cells synthesize extracellular PSs; sugar units are synthesized within the cytoplasm before being excreted outside and polymerized. Extracellular PSs may remain attached to the cell membrane, forming a capsule or a slime, while the other part is released into the surrounding environment.

Depending on the enzymatic machinery, EPS are usually constituted of a regular repeating unit. They may be linear or branched. Homopolysaccharides are composed of a unique type of sugar residue type, while heteropolysaccharides contain different ones [39.82].

In heteropolysaccharides, glucose, galactose, and glucuronic or glacturonic acids are common. However, other neutral or acid sugars, hexosamines or *N*-acetylhexosamines, and some rarer sugars are also found. The presence or absence of a repeating unit containing up to 16 or 18 monomers [39.42], linear or branched, the presence of organic (lactate, pyruvate, *N* and *O*-acetates...) or chemical (sulfate, phosphate...) groups give a very large structural diversity of the molecules with consequently very diverse physicochemical or biological properties [39.83].

Depending on both sugar chain and osidic linkages, the behavior of the homopolysaccharides in solution may be different [39.84, 85]. Scleroglucan or pullulan are soluble and highly viscous, while curdlan lacking side-chains is poorly soluble and forms a gel; bacterial cellulose is insoluble; however, these three PSs are only composed of glucose. The presence of side-chains renders the polymer insoluble; acyl and non-sugar substituents may affect the physical features as well as uronic acids. β-1,4-linkages are rigid, while α-1,4-bonds found in dextrans confer flexibility of the structure [39.85].

EPS chain length may vary from one species to another, but also within a species; the molecular weight may range from 50 000 to over several million g mol^{-1}.

39.3.2 EPS from Marine Bacteria

A number of species of marine microorganisms can produce EPSs: cyanobacteria, bacteria, and archaea [39.86]. These strains are screened for mucoid phenotypes on a solidified medium supplemented with a carbohydrate source, for a high viscosity in liquid medium, by staining specifically EPSs.

Chitin, the main marine polymer composed of β-1,4-linked *N*-acetylglucosamine residues, is one of the most abundant PSs in nature after cellulose and is usually prepared from the shells of crabs and shrimps [39.87, 88]. Until now, unlike for cellulose [39.89, 90], no bacterium has been shown to produce chitin. Nevertheless, many marine bacteria species exhibit chitinolytic activity and also produce chitosan from chitin, the *N*-deacetylated product [39.91]; bacteria are, therefore, involved in the turnover of the PS in the marine environment [39.87]. Some non marine bacteria species still produce polyglucosamine exopolymers with a structural homology with chitosan [39.92].

Alginates, another kind of the most common marine PSs, are currently mainly obtained through brown seaweed harvesting; the non-marine bacteria *(Pseudomonas aeruginosa* and *Azotobacter vinelandii)* are also capable of producing acetylated alginates that bind some divalent ions but fail to form gels in the manner of their algal analogs.

Polysaccharide-producing bacteria have been isolated from the Gulf of Naples [39.7], from microbial mats [39.16, 58, 93], the Antarctic [39.11], and deep-sea hydrothermal vents [39.43, 94–98]. Psychrotolerant *Pseudoalteromonas*, *Shewanella*, *Polaribacter*, or

Flavobacterium sp. have been isolated from Antarctic water [39.56] and are able to produce high molecular weight EPS.

Some EPS-producing marine bacteria are listed in Table 39.1. The vast majority of marine bacteria producing exopolysaccharides are *Alteromonas* or the *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, and *Vibrio* species. In contrast only a few extremophilic bacteria or archaea have been studied for EPSs [39.36, 55, 64].

EPS production by microorganisms has the advantages of a rapid production (a few days compared to the 3–6 months in the case of plants), of a production in bioreactors on different substrates of possibly hydrocarbon residues and of easier extraction since they are exuded in the extracellular environment.

39.3.3 Benefits for the Bacterial Cell

Many marine bacteria produce EPS as a strategy for growth and survival, adhesion to surfaces, and resistance to adverse conditions. EPSs have also been involved in virulence and host contamination. The physiologic roles of EPS for bacteria have not been completely identified, but a protective or adhesive role has frequently been proposed. Most of the time, EPSs are not degraded by the bacteria producing them and thus do not represent a carbon source [39.99]. In a variable nutrient environment such as the ocean, bacteria need greater adaptability in the pathways involved in the detection of nutrients and in metabolism to utilize them. This could explain the diversity of already found polysaccharidic structures.

Therefore, EPSs have an effect on the interactions between the cells and the surrounding environment [39.86]. CPS or slime may help bacterial cells to overcome the various stresses encountered in the environment or to adhere to the surfaces, providing survival advantage. In aquatic environments, the majority of bacteria adhere in a selective manner or not to inert surfaces or living organisms. In biofilms, microbial cells aggregate within a matrix of extracellular polymeric substances composed of PSs, proteins, nucleic acids, peptidoglycan and lipids. Extracellular PSs are generally considered to be the main part of the biofilm matrix [39.100] and are involved in the flexibility of the biofilm shape under shear force as well as in some of the functional properties of the matrix [39.101].

Moreover, anionic EPSs slimes or from capsules allow the aggregation and storage of the soluble nutriments necessary for the growth of the cell or other particles and may chelate metals and ions [39.11]. Bacterial symbionts have been involved in the resistance of polychaetes to the high metal levels around deep-sea hydrothermal vents [39.3] and in environments anthropogenically polluted with metals [39.102]. In other respects, several anionic EPSs from marine bacteria have been shown to bind toxic compounds [39.13, 37, 62, 94]. The presence of bacteria on marine worms may protect them against toxic metals.

39.3.4 Putative Pathways of Biosynthesis

Oceans contain huge bioresources. To exploit them in a manner respectful to the environment, researchers are trying to decipher their production mechanisms. The first particular mechanism of biosynthesis was encountered for homopolysaccharides such as dextran, glucan, levan, inulin, and fructan; it involves an extracellular enzyme (glycan-sucrase) secreted at the cell surface of Gram-positive bacteria such as *Leuconostoc* and other lactic acid bacteria; it has not been yet described in Gram-negative bacteria [39.84, 103]. A specific glycosyltransferase (GT) transfers the sugar residue to the nascent polymer from a disaccharide, usually saccharose.

The other kinds of biosynthesis concern homo and heteropolysaccharides with repeating units. This biosynthesis usually involves three steps: sugar precursor production, repeating unit synthesis, export and polymerization (Fig. 39.1). The whole process is not fully understood, in particular for polymer exportation and synthesis regulations.

The synthesis of heteropolysaccharides has been widely studied in Gram-negative bacteria (*Escherichia coli* [39.104]) as well as lactic acid bacteria and appears to involve a common mechanism. Several enzymes are involved in the assemblage of the repeating unit; they are encoded by genes located within large EPS gene clusters ranging up to 15–20 kb. They are sometimes located on plasmids, especially when considering lactic acid bacteria [39.105]. The biosynthetic machinery involves activated sugar precursors such as nucleoside sugar diphosphate (UDP-sugar), which are produced in the central metabolic pathways. EPS biosynthesis from cytoplasmic activated UDP-sugar precursors is common. However, alternative activated sugar donors are encountered: nucleoside sugar monophosphates, lipid sugar phosphates or pyrophosphates, or unsubstituted sugar phosphate [39.106]. EPSs are usually synthesized inside the cytoplasm in the form of repeating units by glycosyltransferases before being ex-

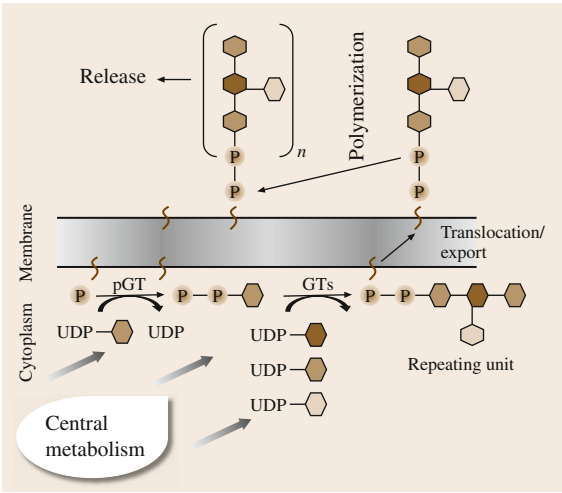

Fig. 39.1 Common biosynthetic mechanism of PSs with repeating units (the biosynthesis begins within the cytoplasm with the synthesis of activated precursors. The repeating unit is then assembled by GT on a carrier located on the membrane. The repeating unit is then translocated across the membrane, polymerized on the nascent polymer within the periplasm or outside the cell, and then released in the surrounding environment or slime)

creted [39.104]. The repeated unit is assembled on a carrier molecule, anchored in the cytoplasmic membrane. Different molecular acceptors on the membrane for the carbohydrate in formation have been proposed; in particular, a lipidic transporter such as undecaprenyl phosphate or isoprenoid pyrophosphate is used in EPS biosynthesis [39.95]. The first monomer is linked to the lipid-carrier by the priming-glycosyltransferase or phosphoglycosyltransferase. The following sugar monomers are linked by appropriate GTs to the growing repeating unit. After completion, the repeating unit is exported outside the cell and subsequently polymerized by addition to the reducing end of the growing EPS chain on the outer face of the cell membrane (Fig. 39.1). Regulations are also involved but not well understood. In particular, a chain length determination factor regulates the extension of the PS molecule; the efficiency of this regulation may depend on whether or not the chain length feature is important for the cell. Various exportation mechanisms have been described; some of them involve ATP (adenosine triphosphate)-binding-cassette (ABC)-transporters and others an externalization of the lipid carrier by a Wzx flippase [39.104, 107].

A specific biosynthesis mechanism involves a bifunctional glycosyltransferase called synthase. Cellulose [39.108] and hyaluronan (HA) [39.109, 110] are examples of the molecular pathway of that biosynthesis. The EPS chain is built by repetitive, non-processive addition of monosaccharides to the nascent polymer. Therefore, it seems that this mechanism can only exist for simple heteropolysaccharides (up to two sugar residues in the repeating unit). The EPS is subsequently exported out of the cell by an ABC transporter; synthase has also been described to be able to form a pore across the cell membrane, allowing the polymer to be exported while synthesized [39.107, 110].

Alginate is a linear, anionic heteropolysaccharide consisting of β-D-mannuronic acid (ManA) and α-D-guluronic acid (GulA). The primary structure of alginate relies on ManA-blocks containing homogeneous $(ManA)_n$, GulA-blocks or $(GulA)_m$, and alternating ManA–GulA-blocks. The relative amount of ManA and GulA as well as the length and distribution of the blocks are dependent on the producing species but are fundamental to the swelling and gelling features, as well as the solubility [39.111]. The bacterial alginate biosynthesis mechanism is particular since the polymer is not constituted of a repeating unit [39.112].

Except for xanthan production (up to 23 g L^{-1} in the culture broth) or pullulan production, production yields upon fermentation seem low (up to 4 g L^{-1} usually). However, an improved activity or a new one resulting in new applications may have a good chance of success for industrial applications.

Engineering of microbial production as well as of genetic material of bacteria, together with the exploration of biodiversity, are different promising approaches to find new PSs with innovative properties.

39.4 Applications of EPSs

Several PSs are well established in different industrial fields. The large range of these existing applications together with the potential ones reflects the many proposed functions in nature as well as their biochemical biodiversity.

Polysaccharides as texture agents of thickening or stabilizing additives are already widely used, such as xanthan, gellan, or some algal PSs (agars, alginates, carrageenans). Therefore, a large number of applications exploit the rheological properties of EPSs. Marine

EPSs having particular functional properties render them more resistant to extreme temperatures, pH, or salinity are of great value for industry [39.113].

Alginate PSs form hydrogels and have found applications in explosives as gelling agents, in the paper industry and textile printing as water-holding agents, in antifoams and lattices because of their emulsifying properties, and in cleaners and ceramics as stabilizers. Alginates are also used in the controlled release of active ingredients that are entrapped in calcium alginate beads, and as new biomaterials for cell immobilization and tissue engineering [39.111]. Research on chitin and chitosan have resulted in applications in various industrial and biomedical fields [39.88].

In plant and animal cells, PSs participate in many central biological processes through the interaction with key proteins such as chemokines, cytokines, growth factors, enzymes, and adhesion molecules involved in cell development, cell signaling, and cell integrity. Therefore, glycobiology, which studies these interactions, and impact on human health have resulted in an increased demand for PSs for therapeutic purposes in cancer, inflammatory diseases, pathogen infections, and thromboembolic disease [39.114, 115]. The potential in this domain of bacterial PSs is now well recognized [39.116, 117].

EPS from the bacteria *Xanthomonas campestris* (xanthan), *Sphingomonas paucimobilis*, or *Sphingomonas* (formerly *Pseudomonas*) *elodea* (gellan), *Acetobacter xylinum* now called *Gluconacetobacter xylinus* (cellulose), *Rhizobium* sp. (succinoglycan), *Agrobacterium* sp. (previously *Alcaligenes faecalis*) (curdlan), and from the fungi *Sclerotium rolfsii* (scleroglucan) and *Aureobasidium pullulans* (pullulan), as well as dextrans from various bacteria have been commercialized and are the most utilized PSs [39.84, 118]. Microbial hyaluronic acid (*Streptococcus* sp.) is also at commercial level and is mainly used in cosmetics, in ophthalmology, and in wound healing [39.119].

39.4.1 Food Products

Polysaccharides are used as ingredients in food products, as carbohydrate and dietary fibre source but also as hydrocolloid to influence rheology and texture. Therefore, in addition to the sensory benefits of EPS in food products, they also impact human health and nutrition [39.120]. EPSs are also produced in situ by bacteria involved in food processing, such as probiotic lactic acid bacteria, and act as functional or prebiotic ingredients. Alginates and various vegetal gums are thickeners, gelling agents, and emulsifiers in food products [39.121].

39.4.2 Environment

The biotechnologies applied to the domain of the environment are being developed and concern essentially the depollution and the rehabilitation of ground, water, and effluents by means of microbial techniques (bioremediation, biodetoxification). Thus, research projects relative to the applications of the bacterial biopolymers in the problems of environmental protection have been conducted for a few years. These concerns are mainly due to the biosorbent properties of the PSs to remove toxic metal pollutants [39.113].

39.4.3 Cosmetics

Cosmetics is a fast growing branch of industry that responds to a societal demand. The lifetime of new molecules ranges between 3–5 years, leading to a constant search for new molecules. Polysaccharides act in feeding, regenerating, and maintaining the skin. Among the most popular products are molecules with moisturizing, smoothing, and/or antiwrinkle activity, as well as those active in the cellular repair or the regeneration of dermic cells and UV protection. Polysaccharides represent more than 20% of the molecules used in the cosmetic field (hyaluronic acid, chitosan, and β-glucane).

The PS deepsane is produced by *Alteromonas macleodii* subsp. *fijiensis biovar deepsane* [39.43] and is used in cosmetics. This EPS shows a protective action of keratinocytes, which are the main cells of the skin, from a proinflammatory agent. Protective effects were also found for cells sensitive to ultraviolet attacks and involved in the cutaneous immune defense system. This EPS would, therefore, contribute to the repair of the skin [39.122].

39.4.4 Medical Applications

The most expected contribution from biotechnologies in the healthcare field concerns the development of new molecules with new biological activities or a better profit–risk ratio. This search inevitably passes by a phase of bioprospecting, and the marine environment constitutes a privileged field of investigations. Therefore, bacterial EPSs offer new and innovative approaches to handle a large number of diseases and to replace certain drugs. Moreover, bacterial EPSs are not

dependent on climatic or physiologic variation, since they are produced in a controlled environment in bioreactors.

The first kind of medical applications derives from the functional properties of the PS molecule (rheology, water-holding, or gelification) [39.123]. As an example, dextran, even widely used as chromatographic supports for purifications of molecules, is also developed in the therapeutic field as artificial plasma. Some bacterial EPSs may also serve as encapsulating agents for a controlled release of drugs near the target, in medical textiles for wound care, in artificial skin and tissue engineering scaffolds [39.121]. *Streptococcus zooepidermicus* produces a hyaluronate with a structure identical to the mammalian one. This EPS is used in ophthalmic surgery. Some other uses are under study, such as various biomaterials and prosthetic surgery to improve biocompatibility. Hydrogels based on natural polymers are close to living tissues and can help during the healing process. Most of these PSs are high molecular weight PSs.

Biological properties constitute the basis for the second type of applications. Some pathogenic bacteria exhibit PSs on their surface; this is the principal factor for immune response. Consequently, they are potential vaccines. Examples of PS vaccines are those against pneumonia (*Streptococcus pneumoniae*) and pneumococcal disease (*Neisseria meningitidis* serogroups A, C, Y, and W135).

Some EPSs have been studied for their antiviral effects [39.124–126], e.g., microbial β-1,3-D-glucans, including levan as well as sulfated dextran [39.127, 128]. Other bacterial PSs exhibit diverse bioactivities, which could also find interest in the biomedical field as therapeutics. Curdlan [39.127] shows antitumor activity. The marine *Geobacillus thermodenitrificans* produces an EPS having immunomodulatory and antiviral effects [39.49]. Some PSs may exhibit innovative structure similar to heparin, a mammalian PS with biological activities such as anticoagulant, antitumor or antiviral. Indeed within the structural diversity, some homologies of new bacterial EPSs have been identified with molecules from animal and bacterial molecules might advantageously replace animal ones. CPSs from *Escherichia coli* are usually antigenic but the *Escherichia coli* K5 strain produces an extracellular PS, called K5, whose structure, [4-D-GlcA β-1,4-D-GlcNAc α-1,]$_n$, is similar to a heparin precursor, *N*-acetylheparosan [39.129]. Because of this structural relationship, chemically and/or enzymatically modified K5 PS is of considerable interest for biomedical applications. Such a project is currently being undertaken by several research teams. This concern is developed in the following paragraph with a focus on marine EPSs.

39.5 Marine EPSs as Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAGs) constitute a class of glycans found ubiquitously in mammalian tissues as components of proteoglycans that have various cellular and intercellular matrix functions. Vertebrates utilize GAGs in structural, recognition, adhesion, and signaling roles. GAG chains are negatively charged by the presence of uronic acids and sulfate groups. They are made up of hexuronic acid and *N*-acetylhexosamine in alternating linear sequence. These PSs exhibit polydisperse high molecular weights in the range of several thousands to

Table 39.2 Structural diversity of GAGs

Name	Hexuronic acid/hexose	Hexosamine	Major repeating unit
Chondroitin sulfate	GlcA or GlcA(2S)	GalNAc or GalNAc(4S) or GalNAc(6S) or GalNAc(4S,6S)	GlcA β-1,3 GalNAc(4S) β-1,4
Dermatan sulfate	GlcA or IdoA or IdoA(2S)	GalNAc or GalNAc(4S) or GalNAc(6S) or GalNAc(4S,6S)	IdoA α-1,3 GalNAc(4S) β-1,4
Keratan sulfate	Gal or Gal(6S)	GlcNAc or GlcNAc(6S)	Gal β-1,4 GlcNAc(6S) β-1,3
Heparin	GlcA or IdoA(2S)	GlcNAc or GlcNS or GlcNAc(6S) or GlcNS(6S)	IdoA(2S) α-1,4 GlcNS(6S) α-1,4
Hyaluronan	GlcA	GlcNAc	GlcA β-1,3 GlcNAc β-1,4

(2S): 2-*O*-sulfo, (4S): 4-*O*-sulfo, (6S): 6-*O*-sulfo, Gal:D-galactose, GalNac: D-*N*-acetylgalactosamine, GlcA: D-glucuronic acid, Glc: D-glucose, GlcNAc: D-*N*-acetylglucosamine, GlcNS: D-*N*-sulfoglucosamine, IdoA: L-iduronic acid

millions g mol^{-1}. GAG structural diversity comes from isomers differing in sulfate presence and position and uronic acid epimerization (Table 39.2). Among them, heparin and heparan sulfate (HS) represent the most heterogeneous structural group because of different sulfation degrees and position as well as various extents of uronic acid epimerization. Postpolymerization modifications of the PS backbone varies depending on the tissue and developmental stage [39.130]. HA is the only non-sulfated GAG.

Heparin is widely used in the clinical treatment of thrombosis as an intravenous anticoagulant with 100 000 kg produced annually through extraction from porcine intestinal mucous membranes.

39.5.1 Biological Activity and Structure–Activity Relationship

The high negative charge of GAG molecules allows binding to many cellular compounds (receptors, growth factors, ...), which results in various bioactivities [39.131]. Medical applications for GAGs are manifold. They are used as surgical aids, moisturizers, for drug delivery, in tissue engineering, as anticoagulants, and for their anticancer activities [39.123, 130, 132, 133]. These biological activities are dependent on the key structural features. Advances in the understanding of the relationships between biological activity and structure are important for the development of safer bioactive analogs.

Sulfation of carbohydrate compounds enhances their biological activity such as their antiviral [39.125, 126] or anticoagulant activity [39.115]. Although the mechanisms are not clear, it has been proposed that sulfates contribute to a molecular conformation that is essential for the activity which, in the case of spirulan, is dependent on calcium ion chelation by sulfates [39.125].

HA, heparin, and chondroitin sulfate (CS) are currently used in various medical applications. Linear HA is mainly used in cosmetics to reduce wrinkles, in drug delivery, in ophthalmology, and in wound healing for its hydratation capability and viscoelasticity [39.119]. Derivatized HA and cross-linked HA are also used in healthcare as non-surgical dermal tissue fillers and in the treatment of osteoarthritis and in tissue engineering [39.119].

The key pentasaccharide of heparin has been well studied and shown to be involved in the interaction with antithrombin, inhibiting blood coagulation [39.134]. In addition, some trisaccharides have been identified to be responsible for binding with fibroblast growth factors having an important role in cell proliferation, differentiation, and migration, as well as angiogenesis and, therefore, can inhibit cancer tumor development. The production of low molecular weight heparinoid would allow us to obtain more efficient preparations with lower side effects by targeting the structural features involved and consequently to obtain more specific preparations [39.131]. In addition, low molecular weight compounds are easier to inject as therapeutics drugs. The use of EPSs as active ingredients does, indeed, concern mainly low molecular weight polymers with bioactivity in cardiovascular and cancer domains.

39.5.2 Modifications to Create GAG-Like Molecules

When an active drug is extracted from animals (heparin from porcine intestinal mucosa, HA from rooster comb, CS from bovine trachea or shark cartilage) or marine invertebrates (scallop, whelk, crustacean, sea squirt), a risk of allergic response to contaminating compounds, or of contamination by non-conventional agents, e.g., prions or viruses [39.130], exists and some new molecules are searched for. In actual fact, GAGs also exhibit undesirable side effects such as hemorrhagic risk; therefore, new analog drugs are being developed [39.132].

For future applications, GAGs derived from bacteria seem to be safer materials with higher purity. Molecules similar to GAGs have been found in microbial sources [39.130]. As has already been presented, *Streptococci* as well as *Pasteurella* produce an EPS identical to HA acid [39.119]. Recently, the recombinant production of HA has been achieved in *Bacillus subtilis* [39.135] and *Lactococcus lactis* [39.136]. Both HA produced by extraction from rooster combs or by microbial fermentation are structurally identical and it can, therefore, be used for its physical properties such as viscosification in ophthalmology, orthopaedic surgery, rheumatology, dermatology, plastic surgery, and wound healing without any modifications of the structure [39.137].

The marine bacterium *Vibrio diabolicus* isolated on the Pompei worm from deep-sea hydrothermal vents has been shown to produce the HE800 polymer constituted of a tetrasaccharidic repeating unit [3-D-GlcNAc-β-(1,4)-D-GlcA-β-(1,4)-D-GlcA-β-(1,4)-D-GalNAc-α-(1] [39.66, 67]. This EPS shows structural similarities to HA. Bioactivity in bone re-

pair has also been described [39.138]. The HE800 EPS in its native form has found application in skin and cartilage therapy [39.133]. Microbial-derived HA has now been commercialized and even if research on marine bacteria may produce analogs, research is also directed at producing heparin via microbial fermentation [39.139].

Beside natural the PSs available, some PS derivatives exhibiting enhanced bioactivity or features can be generated by chemical, biochemical, and genetic methods, or a combination thereof to modify the PS structure through depolymerization and regioselective functionalization. The current challenge, in addition to improving the productivity of EPSs from bacteria, is to produce EPSs of a structure and size that allow the desired activity. In particular, the sulfation degree and pattern must be controlled since the biological activity of heparinoid relies greatly on the specific modification pattern (*N*-deacetylation, *N* and *O*-sulfation, and epimerization of glucuronic acid to iduronic acid). Tailoring the structure is currently performed in chemical ways, but enzymatic ways are also under study.

Pullulan was modified by chemical sulfation to produce new anticoagulant drugs as a potential heparin substituent [39.140]. Some analogs of GAG sugar backbones have also been found in bacteria such as *Escherichia coli* K4 and K5, as well as in marine bacteria. *E. coli* K4 and K5 have been shown to produce PSs with a basic structural similarity to chondroitin sulfate and heparin, respectively [39.139, 141]. The *E. coli* K5 polysaccharidic antigen is similar to a precursor of heparin (desulfoheparin). Much in vitro chemoenzymatic production of heparin, in which the EPS backbone of *E. coli* K5 is subjected to chemical and/or enzymatic modification, have been described [39.110, 139, 142–145].

Alteromonas infernus, a deep-sea marine bacterium has been isolated from seawater samples collected around a dense population of giant worms *Riftia pachyptila* [39.38]. It produces an anionic complex heteropolysaccharide with a high molecular weight and around 10% sulfate content [39.39]. Some other sulfated EPSs from marine bacteria have been described in *Alteromonas macleodii* strains and *Pseudoalteromonas* sp. [39.42, 60]. There are very few bacterial sulfated PSs whose structure has been determined (Table 39.1), but they provide the basis for future applications and basic research on GAG-like molecules from marine environments. On the other hand, L-iduronic acid resulting from 5-epimerization of glucuronic acid has been described in one bacterial PS [39.146]. Since sulfated PSs and L-iduronic acid exist in bacteria, one can expect the isolation of enzymes involved in their biosynthesis in the near future. These enzymes would then be very useful tools to produce engineered GAG-like PSs.

The *A. infernus* EPS repeating unit is a nonsaccharide composed of glucuronic acid, galacturonic acid, and neutral sugars (Gal and Glc), as well as one sulfate group on a galacturonic acid residue (Table 39.1). This PS prepared in a hydrogel exhibited interesting activity in cartilage tissue engineering applications [39.147]. Chemically oversulfated low molecular weight derivatives have been synthesized by two processes that differ in the order of the same steps (radical depolymerization and sulfation). Surprisingly, the two kinds of derivatives did not exhibit the same biological features: one was able to stimulate the proliferation of mature endothelial cells, whereas the other was not [39.148], which suggested that the modification pattern catalyzed by both processes is not the same; therefore, the sulfation pattern appears to be critical for bioactivity. However, *lteromonas infernus* EPS derivatives presented anticoagulant activity similar to that of heparin with a lower hemorrhagic risk [39.149]. These derivatives have also shown interesting properties for cell therapy and tissue engineering [39.133], and also in cartilage regeneration [39.150].

These studies have highlighted the crucial roles of molecular weight and the the sulfation pattern [39.151]. Chemical reactions may be selective to some extent but are not specific enough for such molecules. In particular, it is very difficult to obtain compounds with specific sulfated positions [39.151, 152]. Therefore, the use of enzymatic methods in the modification process leading to bioactive oligosaccharidic derivatives may have clear advantages, such as a better control of reaction selectivity and an easier reaction in a single step (without functional group protection and de-protection steps) under mild conditions. Most enzymes involved in GAG modification are very specific, especially sulfotransferases [39.114, 153, 154]. Moreover, the PAPS (3′-phosphoadenosine 5′-phosphosulfate) molecule required for the sulfation reaction as the sulfate donor is expensive. Therefore, approaches to regenerate this cofactor have been developed [39.153, 155]. However, finding active enzymes on each PS backbone is likely to be the main bottleneck of such a strategy; therefore, enzyme design and optimization by molecular techniques will surely need to be envisaged.

In the future, one can also expect that genetic engineering will be performed to tailor carbohydrate

biomolecules in vivo. This will depend on a deep understanding of biosynthetic mechanisms of EPSs to be able to engineer them and of how the structure of EPSs determines the final activity. The development of improved characterization methods is also required, especially to determine the distribution of functional groups as well as new methods to modify the PS backbone in a regiospecific manner.

39.6 Conclusion

Life emerged from the ocean; nowadays, oceans shelter large biomasses that are mainly microscopic. Among these, marine bacterial diversity, associated with the original ecosystem and biochemical metabolim, is a huge reservoir for the identification of new species and the extraction of new molecules of biotechnological interest, resulting in both fundamental and applied research. It is pointed out that the ocean provides renewable resources for different industrial fields.

The putative or demonstrated benefits of some new PSs and their derivatives are still being studied. Crucial knowledge has been gained on how the structural composition influences the functional properties and on how these features can be applied in biomedical research. In vitro and in vivo structural design of PSs to tailor targeted PS derivatives might produce important bioactive materials in the future. The growing demand will probably lead to the establishment of an adequate and efficient microbial production process.

Only a few marine products have reached commercial production. Indeed, the search for new molecules for healthcare rarely encounters success, and it is always a long time before they are released on the market. The number of marine molecules used as drugs is very low; anticancer, antiviral, and pain-fighting molecules are available on the market, but none are carbohydrates. However, oceans are not only a source of molecules but also a source of inspiration for the synthesis of new drugs [39.156].

In biotechnology, the development of new marine molecules is linked to the progress in the sustainable production process. Moreover, one will have to take into consideration the evolution of legislation regarding the use of natural products, intellectual property rights, the availability of the resource, the ease of implementation, and naturally the production costs.

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40. Sulfated Polysaccharides from Green Seaweeds

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With the growing interest in the development of novel bioactive compounds from marine resources, underutilized marine green algae have been considered as valuable sources of structurally diverse bioactive compounds. Among their various constituents, water-soluble sulfated polysaccharides possess innovative and unique structures and numerous beneficial biological activities, such as anticoagulation, antioxidation, anticancer, and antivirus activities. The sulfated polysaccharides derived from green seaweeds, therefore, have great potential as ingredients in nutraceutical, pharmaceutical, and functional food products. An overview of the diverse structural features and biological activities of sulfated polysaccharides from green seaweeds along with a brief mention of the structure–bioactivity relationship are presented in this chapter.

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40.1 Overview

Green algae are part of a large group called Chlorophyta and are very common algae distributed worldwide. Most of them live in freshwater and some (about 10%) can be found in seawater or brackish water, particularly in estuaries. The chlorophyll a, b in green algae absorb red light, which is available in shallow water but absent in deep water. Therefore, green seaweeds are commonly found on the surfaces of rocks in middle to low intertidal zones [40.1]. Green seaweeds are known as an indicator for water recycling in aquatic environments such as with integrated invertebrates or aquacultured fish systems because of their harmful effects which could cause hypoxia and death of aquatic organisms [40.2]. Most of the collected green seaweed biomass has been considered to be of little value and has thus been used as a compost or animal feeds, or otherwise simply wasted. In some countries, however, green seaweeds, particularly the *Ulva* and *Enteromorpha* species, are used as food ingredients and nutritional supplements [40.3, 4]. Recently, commercial exploitation has made green seaweeds attractive to produce functional or health promoting foods due to their nutritional bioactive constituents, such as dietary fiber, proteins, polysaccharides, essential minerals, antioxidants, vitamins, and bioactive peptides [40.2, 5–11].

Among the various components in green seaweeds, the cell wall polysaccharides representing 38–54% of the dry algae matter display physicochemical and biological features that are of potential interests for different applications. Four types of cell wall polysaccharides are reported to be included in the biomass of *Ulva* sp.: water soluble sulfated polysaccharides, water insoluble cellulose, alkali soluble xyloglucan, and glucuronan [40.12]. Most work on the polysaccharides of green seaweeds have focused on sulfated polysaccharides because of their various biological activities,

such as anticoagulation, anticancer, antihyperlipidemia, and immunomodulation activities [40.13–16]. Sulfated polysaccharides from marine seaweeds are considered to be a unique compound with distinct structures and novel bioactivities. So far, studies on sulfated polysaccharides from marine seaweeds have mainly focused on those of brown seaweeds, known as fucoidans, sargassan, and glucuronoxylofucan, and those of red seaweeds including agar and carrageenans, due to their potent bioactivities [40.17–19]. Green seaweeds such as *Ulva* sp., *Enteromorpha* sp., *Capsosiphone* sp., and *Codium* sp. have also been found to be rich sources of sulfated polysaccharides [40.20–23]. Among sulfated polysaccharides, ulvan consisting of sulfated-rhamnose and uronic acids such as glucuronic acid and iduronic acid has been isolated from *Ulva* and *Enteromorpha* sp., and sulfated rhamnan and sulfated galactans were obtained from *Monostroma* sp. and *Codium* sp., respectively [40.12, 24, 25]. Ulvans are also known to form gels in the presence of borate and calcium ions and to resist digestion and fermentation by human digestive enzymes and colonic flora [40.26, 27]. Their resistance to digestion and fermentation is due to their structural features rather than their chemical compositions [40.27]. Numerous research efforts have shown that the structural characteristics of sulfated polysaccharides from green seaweeds vary mostly depending on their origins [40.12]. It has been reported that the bioactivities of sulfated polysaccharides are dependent, among other factors, on their glycosidic linkages, molecular weight, sulfate content, and conformation [40.13, 28]. Therefore, a basic understanding of the primary and secondary structures of the sulfated polysaccharide may lead to the successful interpretation of their biological activities. The objective of this chapter is to review the fine structures, bioactivities, and structure-function relationship of sulfated polysaccharides originating from various green seaweeds.

40.2 Extraction and Chemical Composition of Sulfated Polysaccharides

The extraction of alcohol from a raw alga before extraction of the sulfated polysaccharides has been reported to enrich the cell walls of the polysaccharides, thus increasing the total sugar content in the material and decreasing the content of minor sugars and glucose [40.29]. Therefore, this preliminary step appears to be desirable to obtain the less contaminated sulfated polysaccharides from green seaweeds. The extraction of the sulfated polysaccharides from green seaweeds has generally been conducted by H_2O in the presence of chelating agents [40.26]. The enzyme treatment was often included in the extraction procedures using amyloglucosidase, cellulase, and protease in order to improve the extraction efficiency of the sulfated polysaccharides entrapped in the cell walls [40.26, 29]. The extraction of the sulfated polysaccharides was also carried out in an acidic condition (acidic pH below the pK_a of glucuronic acid) to prevent their aggregation [40.26, 30]. The acidic condition seemed to be also efficient to improve their extraction yield. However, the decrease in the molecular weight of polysaccharides is a drawback of this treatment if texturing ability is required [40.29]. After the extraction, the crude extract of sulfated polysaccharides is often purified using ion-exchange chromatography. The yield of extracted polysaccharide usually ranges from 8 to 29% of the dry weight of the algae, depending on the extraction and purification procedures applied (Fig. 40.1) [40.26].

Sulfated polysaccharides from green seaweeds mainly consist of rhamnose, xylose, and uronic acids such as glucuronic and iduronic acids in the major repeating units, and include minor amounts of other sugars, such as glucose, galactose, mannose, and arabinose [40.31, 32]. In addition, the presence of sulfate groups has been identified as the main constituent of polysaccharides [40.29, 33].

40.3 Structural Characteristics of Sulfated Polysaccharides

Despite the structural variation originating from methodology, taxonomy, and ecophysiology, the structural features of sulfated polysaccharides of green seaweeds varies mostly depending on their origins [40.12]. According to *Zhang* et al. [40.21], the proximate composition and the methylation analysis demonstrated that the polysaccharide from *Monostroma latissimum* was a high rhamnose-containing sulfated polysaccharide and included 1,3-; 1,2-; 1,2,3-; 1,2,3,4-; and 1,2,4-linked rhamnose. This indicates that the sulfated polysaccharides from *M. latissimum* are branched polysaccharides with major monosaccharide unit of

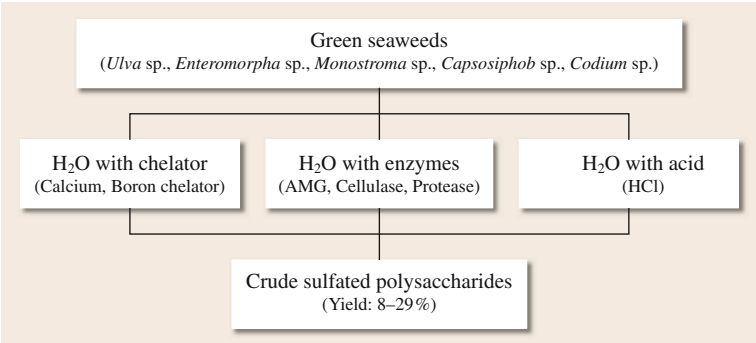

Fig. 40.1 Extraction methods of sulfated polysaccharides from green seaweeds. The extraction of the sulfated polysaccharides was generally treated with H_2O, with chelating agents, enzymes, and acid conditions

rhamnose. It was also found that the sulfate substitution mainly occurred at C-3 or C-4 of the 1,2-linked rhamnose residues [40.21]. The sulfated polysaccharide from *Monostroma nitidum* exhibited a high proportion of 1,2 and 1,3-linked L-rhamnose. The sulfate substitutions were deduced to be at C-2 and C-4 of 1,3-linked L-rhamnose or to be at C-3 and C-4 of 1,2-linked L-rhamnose residues [40.13]. On the other hand, a heteroglycan obtained from *Enteromorpha compressa* contained the major units of 1,4 and 1,2,4-linked rhamnose and 1,4-linked xylose. Sulfates were present either at C-3 of 1,4-linked rhamnose unit or C-2 of 1,4-linked xylose unit [40.32].

Determining the sugar sequence in the sulfated polysaccharides from marine seaweeds is a major challenge. Accurate determination of the glycosidic linkages of the sulfated polysaccharides requires desulfation without cleavage of the chain linkages. The solvolytic desulfation procedure using a pyridinium salt has been preferably used to desulfate the sulfated polysaccharides because of their mild reaction, minimizing the chain degradation [40.34]. However, the desulfation has also been carried out by methyl sulfoxide-pyridine because the method was rapid and complete, resulting in a better recovery yield (55–57.5%) [40.20].

The chemical structures of sulfated polysaccharides from various green seaweeds are shown in Fig. 40.2. The major repeating sugar sequence of the sulfated polysaccharides from *Ulva rigida* was determined as β-D-GlcpA-(1 → 4)-α-L-Rhap 3-sulfate (A_{3S}). It was found that on the sugar sequence GluA could occur as a branch on C-2 of rhamnose. In addition, GluA could flank on the sugar sequence having an extra 4-linked β-D-GlcpA residue, which might be a cleavage site by glucuronan lyase [40.31, 40]. The sulfated polysaccharides from *Ulva armoricana* includes a large amount of α-L-IdopA-(1 → 4)-α-L-Rhap 3-sulfate (B_{3S}), which is resistant to the glucuronan lyase, indicating the major structural differences among the sulfated polysaccharides from the *Ulva* species [40.35]. Also, those of *Ulva clathrata* are deviants of the A_{3S} and B_{3S} ulvanobiuronic acids [40.4] and the mixed structures of the ulvanobiuronic acid 3-sulfate type A and B were observed in those from *Ulva rotundata* [40.41].

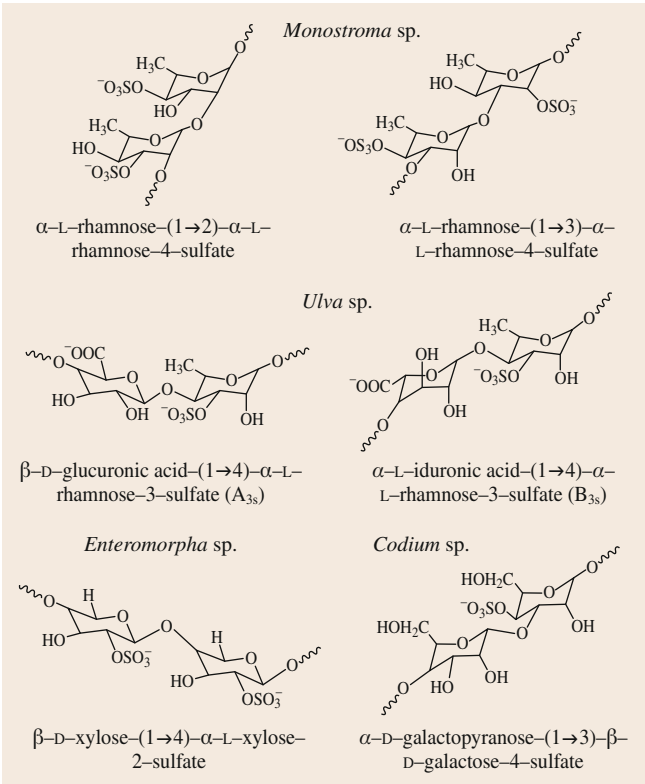

Fig. 40.2 The chemical structure of sulfated polysaccharides from green seaweeds

Table 40.1 Representative chemical structures from different green seaweeds

Species	Oligosaccharide/repeating structure	References
Monostroma nitidum	α-L-Rhap-(1 → 2)-α-L-Rhap-3-sulfate	[40.13]
	α-L-Rhap-(1 → 2)-α-L-Rhap-4-sulfate	
	α-L-Rhap-(1 → 3)-α-L-Rhap-2-sulfate	
	α-L-Rhap-(1 → 3)-α-L-Rhap-4-sulfate	
Monostroma latissimum	α-L-Rhap-(1 → 2)-α-L-Rhap-4-sulfate	[40.21]
	α-L-Rhap-(1 → 2)-α-L-Rhap-3-sulfate	
	α-L-Rhap-(1 → 3)-α-L-Rhap-(1 → 2)-α-L-Rhap-4-sulfate	
	α-L-Rhap-(1 → 4)-α-L-Rhap-(1 → 2)-α-L-Rhap-4-sulfate	
Enteromorpha compressa	α-L-Rhap-(1 → 4)-β-D-Xylp-2-sulfate	[40.32]
	β-D-Xylp-(1 → 4)-α-L-Rhap-3-sulfate	
	α-L-Rhap-(1 → 2)-α-L-Rhap-(1 → 4)-β-D-Xylp-2-sulfate	
Ulva rigida	β-D-GlcpA-(1 → 4)-α-L-Rhap-3-sulfate	[40.31]
Ulva armoricana	α-L-IdopA-(1 → 4)-α-L-Rhap-3-sulfate	[40.35]
Codium isthmocladum	β-D-Galp-(1 → 3)-β-D-Galp-4-sulfate	[40.36]
Codium fragile	β-D-Galp-(1 → 3)-β-D-Galp-*O*-3-sulfate	[40.37]
	β-D-Galp-(1 → 3)-β-D-Galp-(1 → 6)-β-D-Galp-*O*-3-sulfate	
Capsosiphon fulvescens	(1 → 4)-β-D-Manp	[40.38]
	(1 → 4)-β-D-Manp-(1 → 2)-β-D-Manp	
	(1 → 4)-β-D-Manp-(1 → 2)-β-D-Xylp	
	(1 → 2)-β-D-Manp-(1 → 4)-β-D-Manp-(1 → 2,3S)-D-Rhap	
	(1 → 2)-β-D-Manp-(1 → 4)-β-D-Manp-(1 → 2)-D-Rhap-(1 → 4)-β-D-Manp	
Ulva pertusa	(1 → 4)-α-L-Rhap-(1 → 4)-β-D-Glc-(1 → 2)-β-L-Rhap-(1 → 4)-β-D-Xylp	[40.39]

A large amount of galactose is included in the sulfated polysaccharides from *Codium* sp., which reveals their significant heterogeneity. Sulfated galactan from *Codium isthmocladum* is composed preponderantly of 3-linked β-D-galactopyranose residue mainly sulfated at C-4 position and, in lower amounts, it is sulfated and glycosylated at C-6. In addition, pyruvate groups have also been found, forming five-membered cyclic ketals as 3,4-*O*-(1′-carboxy)-ethylidene-β-D-galactose residues [40.36]. Such a highly complex, pyruvylated and sulfated galactan has also been found in *Codium yezoense* in which the sulfated galactan has essentially linear backbone segments of 3-linked β-D-galactopyranosyl units with short oligosaccharides attached at C-6. Sulfate groups are substituted mainly at C-4 and in lower amounts at C-6. The sulfated galactan from *Codium fragile* also consist of 3-linked β-D-galactopyranose residue but are highly ramified at C-6 through β-D-Galp units; sulfate esters have mainly been found at 4-*O*-positions of nonreducing terminal galactose residues [40.37]. Therefore, the most dominant component in the backbone of sulfated galactan from *Codium* sp. appears to be the 1,3-linked β-D-Galp 4-sulfate units [40.42]. Structural analysis of the sulfated polysaccharides from *Capsosiphon fulvescens* has revealed that their main glycosidic linkages were β-(1 → 4)-D-Manp, (1 → 2)-D-Rhap, (1 → 2,3-sulfate)-D-Rhap and (1 →)-β-D-Xylp with minor (1 → 2), (1 → 3) and (1 → 2,4)-D-Manp residues [40.38]. In addition, *Tabarsa* et al. [40.39] reported a unique structural feature of sulfate position on glucose and glucuronic acid at O-3 position from the sulfated polysaccharides in *Ulva pertusa*, which are mainly composed of α-(1 → 4)-L-Rhap, β-(1 → 4)-D-Glc, β-(1 → 2)-L-Rhap, and β-(1 → 4)-D-Xylp residues with branches at O-2 position of rhamnose. The sulfate groups are mostly found on glucuronic acid at O-3 position (Table 40.1).

The variations on chemical compositions and repeating sequences of the sulfated polysaccharides from green seaweeds clearly demonstrate the differences in glycosidic linkages, monosaccharide distribution, branching, and sulfation patterns. It appears that such variations mainly derive from the differences in their species. However, whether their varied nature and proportion of chemical features are species-specific or methodological differences remains to be clearly established.

40.4 Biological Activities of Sulfated Polysaccharide from Green Seaweeds

Recently, various sulfated polysaccharides isolated from marine seaweeds have attracted much attention in the areas of food, cosmetics, and pharmacology due to their physicochemical and biological properties. Among the sulfated polysaccharides from various marine algae, carrageenans from red algae and fucoidans from brown algae are commercially available from various cheap sources and are widely used as food additives and ingredients in novel drugs and functional foods [40.43–45]. Sulfated polysaccharides from green seaweeds also display physicochemical and biological features that are of potential interest for functional food, pharmaceutical, and chemical applications [40.12]. In addition, most of the positive health effects are generated by the presence of sulfate groups in the polysaccharides [40.46]. In vitro and in vivo beneficial biological effects of sulfated polysaccharides have been reported in broad ranges of biological activities including antioxidant, anticoagulant, anticancer, antihyperlipidemic, and immunomodulating activities (Table 40.2) [40.18, 21, 47–49].

40.4.1 Antioxidant Activity

Excessive amounts of reactive oxygen species (ROS) generated in living organisms during metabolism may be a primary cause of bio-molecular oxidation, which may lead to various diseases, such as cancer, stroke, and diabetes, as well as the degenerative processes associated with aging (Fig. 40.3) [40.50–53]. Antioxidants may have a positive effect on human health by protecting the human body against damage by ROS.

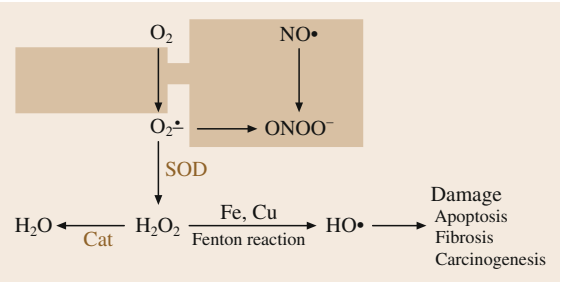

Fig. 40.3 Cellular generation of reactive oxygen species (ROS) and antioxidant system. O_2 = oxide, H_2O_2 = hydrogen peroxide, OH^- = hydroxyl radical, NO = nitric oxide, $ONOO^-$ = peroxynitrite

In addition, deterioration of some foods has resulted from the oxidation and/or rancidity of lipids due to the formation of lipid peroxidation products, which causes a decrease in the nutritional value of lipid containing foods. The negative effects of oxidative stress in foods have been mitigated by many synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG). However, the use of these synthetic antioxidants is under strict regulation due to their potential health hazards [40.54, 55]. Therefore, concerns related to synthetic antioxidants have led to a considerable interest in the development of safe and inexpensive alternatives from natural origins.

Antioxidant activity of sulfated polysaccharides has been determined by various methods such as ferric reducing antioxidant power (FRAP), nitric oxide (NO) scavenging, lipid peroxide inhibition, 1,1-diphenyl-2-picryl hydrazil (DPPH) radical, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

Table 40.2 Biological activities of water soluble sulfated polysaccharide from various green seaweeds

Biological activities	Species of green seaweed	References
Antioxidant activity	*Ulva pertusa*	[40.47, 56, 57]
Immunomodulatory activity	*Enteromorpha prolifera*	[40.9, 15]
	Monostroma nitidum	[40.14]
	Ulva rigida	[40.18]
	Capsosiphon fulvescens	[40.22]
Anticoagulant activity	*M. nitidum*	[40.13, 24, 58]
	Monostroma latissimum	[40.21]
Antihyperlipidemic activity	*U. pertusa*	[40.16, 49]
Anticancer activity	*M. nitidum*	[40.14]
	Enteromorpha intestinalis	[40.48]
	C. fulvescens	[40.59]
Antiviral activity	*Gayralia oxysperma*	[40.19]
	M. nitidum	[40.23]
	Caulerpa racemosa	[40.60]

radical, superoxide, and hydroxyl radicals scavenging assays. Various types of sulfated polysaccharides from brown and red algae, including fucoidans, laminarans, and carrageenans, have shown potential antioxidant activity [40.61–63]. *Kim* et al. [40.64] reported that the sulfated polysaccharides of a brown alga, *Sargassum fulvellum*, exhibited stronger NO scavenging activity than commercial antioxidants such as BHA and α-tocopherol.

The sulfated polysaccharides from a green seaweed, *Ulva pertusa*, are also potent free radical scavengers. The antioxidant activity of the sulfated polysaccharides depends on their structural features, such as molecular weight (M_w), degrees of sulfate group, and functional groups. It was observed that the lower M_w sulfated polysaccharides showed stronger antioxidant activity [40.56]. It seemed that the lower M_w polysaccharides might incorporate into the system more efficiently and donate protons to scavenge free radicals more effectively compared to the high M_w polysaccharides. It was also found that the antioxidant activity was deeply affected by the amount and distribution of functional groups inside the polysaccharide. The enhancement of their antioxidant activity may be desirable by the envisaged application and has been successfully investigated both by increasing the degree of sulfate group and by introducing suitable functional groups (acetyl and benzoyl) that can boost the activity of the native polysaccharides [40.47, 57].

40.4.2 Immunomodulatory Activity

Macrophage cells are known to be important components of host defense against bacterial infections and various types of cells, including cancer cells [40.48]. Activated macrophages release cytokines such as tumor necrosis factor-α (TNF-α), PGE_2, and various interleukins (IL-12, IL-6, and IL-1β) to kill external pathogens and inhibit cancer cell growth (Fig. 40.4). Sulfated fucans from *Fucus vesiculosus* has been reported to activate macrophages, producing nitric oxide (NO) through the expression of inducible nitric oxide synthase (iNOS) induced by IFN-γ [40.65]. According to *Lee* et al. [40.23], the sulfated polysaccharides from a green seaweed, *Codium fragile*, also enhanced the production of NO and various cytokines by activating macrophages. The authors observed an increased release of proinflammatory (IL-1, IL-6, IL-12, and TNF-α) and anti-inflammatory (IL-10) cytokines, which suggests that the sulfated polysaccharides might possess the potent immunomodulating activity by stimulating macrophages while preventing potential detrimental inflammatory effects from excessive macrophage activation. Similar trends of pro and anti-inflammatory cytokine releases were observed by the sulfated polysaccharides from *Capsosiphon fulvescens* and *U. pertusa* [40.38, 39]. It appears that the induction of various cytokines by sulfated polysaccharides can be attributed to the up-regulation of their messenger ribonucleic acid (mRNA) [40.9, 14, 15]. Sulfated polysaccharides from *Enteromorpha prolifera* also stimulated a macrophage cell line Raw 264.7, inducing considerable NO and various types of cytokine production via their up-regulated mRNA expression. In addition, an in vivo experiment exhibited an increase in IFN-γ and IL-2 secretions, implying that sulfated polysaccharides can activate T cells by up-regulating the Th-1 response (Fig. 40.4). These results, therefore, suggest that sulfated polysaccharides might be an immunostimulator with an immunomodulatory effect through cell-mediated immune response [40.15].

40.4.3 Anticoagulant Activity

Blood coagulation proceeds by coagulation factors both through intrinsic and extrinsic pathways in order to stop the flow of blood inside the injured vessel walls when an abnormal vascular condition and exposure to nonendothelial surfaces at sites of vascular injury occur (Fig. 40.5). Blood coagulation can be prolonged or stopped with endogenous or exogenous anticoagulants by inactivating and restricting the coagulation factors [40.66]. Heparin, a glycosaminoglycan of animal origin with carboxylic acid and sulfates, has been used for more than 50 years as a commercial anticoagulant for the prevention of venous thromboembolic disorders [40.67]. However, several side effects and the high cost of the extraction of heparin have led to demands for alternative sources of anticoagulants with safe and economic values.

Since the investigation of the potential anticoagulant activity of fucoidan from marine brown algae [40.68], it has been reported that sulfated polysaccharides may be alternative sources for developing novel anticoagulant drugs. A few studies have reported that sulfated polysaccharides may not interfere with and/or inhibit the extrinsic coagulation factors directly related with the prolongation of prothrombin time (PT) [40.69, 70]. Therefore, the anticoagulant activity of sulfated polysaccharides is mostly determined by prolongation of activated partial thrombinplastin time (APTT)

and thrombin time (TT), which suggests inhibitions of the intrinsic factors and thrombin activity of the sulfated polysaccharides. Various anticoagulant sulfated polysaccharides have been isolated and characterized from marine algae. Among them, sulfated galactans known as carrageenans from red algae and fucoidans from brown algae are identified with high anticoagulant activity [40.71–75].

The sulfated polysaccharide isolated from the green seaweed *Monostroma nitidum* also has potent anticoagulant activity, showing a sixfold higher heparinoid activity than that of standard heparin [40.58]. Due to the heparinoid-like structure, the sulfated polysaccharides from *Monostroma latissimum* partially affect both the activated thromboplastin time (APTT) and thrombin time (TT), displaying the inhibition of both the intrinsic pathways of coagulation or thrombin activity and the conversion of fibrinogen to fibrin [40.21]. High anticoagulant activity is also observed by the sulfated polysaccharides from *M. nitidum*, which mediated thrombin inhibitors by heparin cofactor II [40.13].

The anticoagulant activity of sulfated polysaccharides depends on their structural features such as M_w, and the degree and position of the sulfate groups. According to *Zhang* et al. [40.21], the decrease in the molecular size of sulfated polysaccharides dramatically reduces their anticoagulant activities, which indicates that M_w of sulfated polysaccharides has an important effect on the anticoagulant activity. The considerable anticoagulant activity of sulfated polysaccharides is also closely correlated to the content and position of the sulfate groups inside the polysaccharide chains [40.76].

40.4.4 Antihyperlipidemic Activity

Sulfated polysaccharides are known to resist degradation of human endogenous enzymes and be able to reduce cholesterol levels like dietary fibers [40.78]. According to *Guillon* and *Champ* [40.79], dietary fibers with ion-exchange capacity are more potent to lower cholesterol levels, therefore, sulfated polysaccharides appear to have the potential to be used as dietary fibers. During the last decade, many new classes of hypolipidemic agents have been widely used for the improvement of hyperlipidemia associated with atherosclerosis [40.80–82]. Currently, the most commonly used lipid regulators are statin and fibrates. However, recent reports of undesirable side effects of some *super statins* required the development and utilization of effective

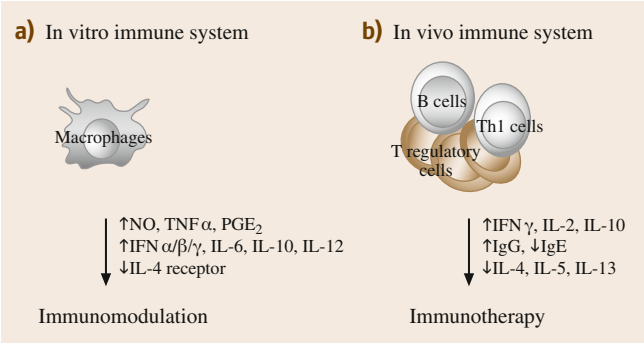

Fig. 40.4 (a) In vitro and (b) in vivo immune systems of sulfated polysaccharides from green seaweeds. NO = nitric oxide, TNF = tumor necrosis factor, PGE_2 = prostaglandin E_2, IFN = interferon, IL = interleukin, Ig = immunoglobulin

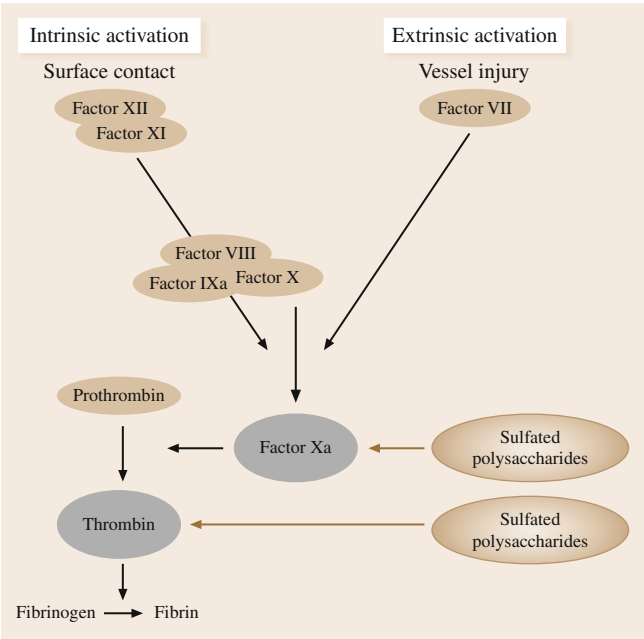

Fig. 40.5 Intrinsic and extrinsic pathways of blood coagulation systems [40.77]

and natural lipid regulators, protecting the human body from hyperlipidemia [40.16, 83]. It has been observed that porphyran from the red algae *Porphyra yezoensis* reduced the secretion of an essential component of very low density lipoprotein (VLDL) synthesis, apolipoprotein B100, mainly through suppression of lipid synthesis in human liver derived cells, revealing its use as a potent antihyperlipidemic agent [40.84, 85]. The

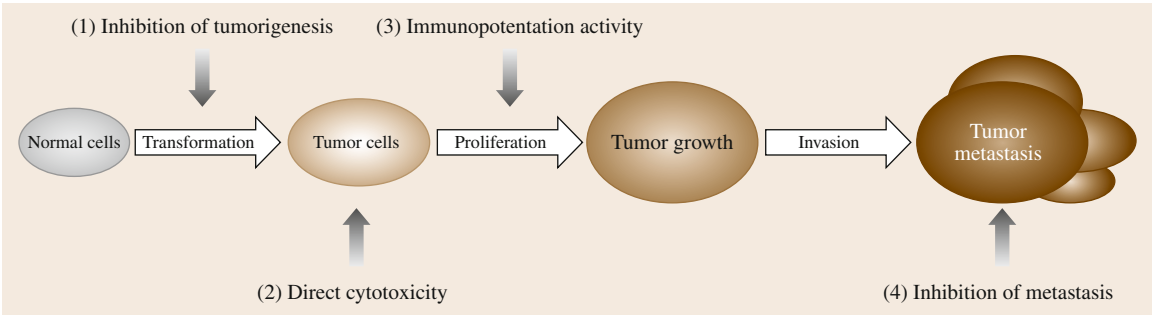

Fig. 40.6 The different inhibition pathways of sulfated polysaccharides from green seaweeds in cancer development. (1) the prevention of tumorigenesis, (2) direct cytotoxicity, such as the induction of tumor cell apoptosis, (3) immunopotentiation activity in combination with chemotherapy, and (4) the inhibition of tumor metastasis

high M_w sulfated polysaccharides from *Ulva pertusa* also demonstrated the effective reduction of serum total and LDL-cholesterol (LDL: low density lipoprotein) levels. On the other hand, the low M_w ulvan derivatives increased serum HDL-cholesterol (HDL: high density lipoprotein) but decreased serum TG level [40.49]. *Qi* et al. [40.16] reported that highly sulfated polysaccharides isolated from *U. pertusa* considerably decreased triglycerides (TG) and LDL-cholesterol levels. These results revealed that M_w and the sulfate contents of sulfated polysaccharides may be determinant factors on lipid metabolism.

40.4.5 Anticancer Activity

The anticancer activity of polysaccharides was first reported by *Nauts* et al. [40.86]. Since then, numerous studies have suggested that polysaccharides have anticancer effects, such as:

1. The prevention of tumorigenesis by oral consumption of active preparations.
2. Direct cytotoxicity, such as the induction of tumor cell apoptosis.
3. Immunopotentiation activity in combination with chemotherapy.
4. The inhibition of tumor metastasis (Fig. 40.6). [40.87, 88].

Most natural anticancer compounds are able to manipulate the growth of cancer cells with no or minor side effects. Identification of novel effective natural cancer therapeutic agents has become an important worldwide strategy in cancer prevention. Several studies have revealed that sulfated polysaccharides from brown and red seaweeds has direct cytotoxic effects on cancer cell lines, as well as suppressive activity of tumor growth in mice [40.61, 89]. In addition, antimetastatic activity of fucans has also been reported, which appears to block the interaction between cancer cells and the basement membrane [40.90].

Sulfated polysaccharides from green seaweeds also exhibit anticancer activities. According to *Karnjanapratum* and *You* [40.14], the sulfated polysaccharides from *Monostroma nitidum* have direct cytotoxic effects on human gastric cancer cells. *Kwon* and *Nam* [40.59] reported that the sulfated polysaccharide from *Capsosiphon fulvescens* inhibits the proliferation of human cancer cells, and they observed a marked increase in caspase-3 activation but a considerable decrease in Bcl-2 expression, and the phosphorylation of the insulin-like growth factor-I receptor (IGF-IR). In addition, sulfated polysaccharides suppress IGF-I-stimulated recruitment of p85 to IGF-IR and IRS-1. These results suggest that the sulfated polysaccharide from *C. fulvescens* might inhibit cell proliferation through the induction of apoptosis by inhibiting IGF-IR signaling and the PI3K/Akt pathway. On the other hand, the sulfate polysaccharide isolated from *Enteromorpha intestinalis* has in vivo anticancer activity with no direct cell cytotoxicity by increasing the relative spleen and thymus weight, and facilitating the release of tumor necrosis factor α (TNF-α) [40.48].

40.4.6 Antiviral Activity

Sulfated polysaccharides from various marine species have been shown to inhibit the replication of enveloped viruses such as flavivirus, togavirus, arenavirus, rhabdovirus, orthopoxvirus, and herpesvirus families [40.91]. According to *Harden* et al. [40.92], the evaluation of sulfated polysaccharides containing

seaweed extracts from *Undaria pinnatifida, Splachnidium rugosum, Gigartina atropurpurea*, and *Plocamium cartilagineum* against HSV-1 and HSV-II revealed that their antiviral activities are potent when they are added during the first hour of viral infection; however, they were not effective if added later. It has been reported that the chemical structures including the degree of sulfate content, molecular weight, constituent sugars, conformation, and dynamic stereochemistry are important factors to determine their potency of antiviral activity [40.93, 94]. Sulfated polysaccharides such as fucoidans and carrageenans have been extensively investigated for their antiviral activities. They appear to inhibit the entry of enveloped viruses, including herpes and human immunodeficiency virus (HIV), into cells [40.91]. In addition, *Hidari* et al. [40.95] found inhibitory potency of fucoidans on dengue virus type 2 infection, suggesting that fucoidans are able to directly bind to virus particles.

Compared to research on sulfated polysaccharides from brown and red seaweeds, little research has been performed on sulfated polysaccharides from green seaweeds. According to *Lee* et al. [40.96], the sulfated polysaccharides from various green seaweeds have antiherpes simplex virus type 1 (HSV-1) activities. The water soluble sulfated polysaccharides not only show the inhibition of the early stages of HSV-1 replication, but also interfere with late steps of virus replication. The homogeneous sulfated heterorhamnan obtained from *Gayralia oxysperma* also has high and specific activity against HSV-1 and HSV-2. The early step of HSV-2 replication is significantly interfered with through the presence of the sulfated polysaccharide from *Monostroma nitidum*. However, it has no effect on the replication of influenza A virus [40.23]. A sulfated heteropolysaccharide isolated from *Caulerpa racemosa* has been shown to be a selective inhibitor of TK-acyclovir-resistant strains of HSV-1 and HSV-2 [40.60]. It was reported by *Cassolato* et al. [40.19] that high M_w and sulfation (75% and 80% of the rhamnosyl and uronic acids, respectively, are substituted by sulfates) of the polysaccharide are desirable with potent antiherpetic activity, implying a correlation between M_w and the degree of sulfation, and the antiviral activity.

40.5 Conclusion

In order to gain a deeper insight into the potentiality of using sulfated polysaccharides from green seaweeds in various bioapplications, the structural characteristics and the biological activities have been discussed in this chapter. Most of the positive health effects induced by these polysaccharides appear to be generated by the presence of sulfate groups inside the structure. However, due to the heterogeneities and difficulties in identifying the structural features, the structure–bioactivity correlation is not clearly understood. Therefore, further systematic work is required to explore their structural diversity in relation with their bioactivities. In addition, the elucidation of the interaction mechanism between the polysaccharides and the receptors triggering the bioactivity may provide a better understanding of the relationship between structure and bioactivity. This will provide the key to improving the biological activity of the sulfated polysaccharides from green seaweeds and broaden their utilization as a material in various bioapplications.

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Application

Part G

Part G Application of Marine Biotechnology

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41. Marine-Derived Pharmaceuticals and Future Prospects

Kalpa W. Samarakoon, Don A. S. Elvitigala, You-Jin Jeon

Over the last decades, the ocean has been identified as a sustained source for the requirements of human beings. The marine environment has an enormous biodiversity and is a source with huge potential for scientific applications. Among the potentials, the pharmaceutical perspective has been identified as having an important and substantial role for future therapeutic uses. Marine-derived secondary metabolites have become a promising source for the design and development of drugs. Therefore, in this chapter, we describe the recent findings of marine derived-secondary metabolites, including halogenated terpenes, steroids and sterols, and polyphenols, along with their therapeutic potentials. Marine proteins and lipids are also targeted to speculate on their role in human health. Moreover, molecular biology approaches and future trends in pharmaceuticals with a view of current drug developments will also be discussed.

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41.1 Marine Bioresources

Nature is an attractive prolific source with a tremendous chemical diversity of the organisms living on earth. More than 70% of the earth's surface is covered by oceans and it is conceived that life originated from the ocean. Despite the richness of plant and animal diversity in terrestrial life, the demand to explore natural products in the marine environment has been increased. Interestingly, many marine organisms and microorganisms have a greater chemical diversity and are richer sources of secondary metabolites than terrestrial organisms [41.1]. This fact can be reflected through evolutionary process of natural selection and progression of self defense mechanisms against predators in the life cycle. Intriguingly, new therapeutic candidates are discovered as novel marine metabolites from this exceptional reservoir of bioactive secondary metabolites, as frequently reported in literature [41.2]. Therefore, promising biological activities associated with these chemical entities are useful for finding new drugs with greater efficacy for the treatment of human diseases [41.3]. Hence, marine organisms, such as marine bacteria, fungi, sponges, algae (macro or micro), crustaceans, mollusks, fish, and small vertebrates in the ocean are indispensable sources of functional metabolites.

The emerging diversity of the marine environment and its untapped resources are becoming of great interest and have attracted the curiosity of scientific community. In fact an impressive amount of exploration of biochemicals has been carried out by many research pioneers from different countries in the last few decades. Especially, scientists from the USA, Europe, Korea, Japan, and China have contributed to a great deal of research effort to bring these amazing marine biofunctional ingredients to pharmacological applications. Thus, this chapter focuses on the pharmaceutical

value of some marine secondary metabolites, proteins, and lipids, as well as molecular biology approaches to sustain these bioresources for future prospects. Furthermore, this chapter presents recent developments of drug design and discoveries of pharmaceutical innovations for future therapeutic use.

41.2 Marine Secondary Metabolites

Marine natural products or secondary metabolites are quite species specific and have been employed for the defense mechanisms against predation, infection, parasitism, or interspecies communication and maintenance of homeostasis in marine organisms [41.4]. With the huge diversity of marine organisms, there is an extraordinary potential for the discovery of various natural products. Indeed, evolutionary selection has pronounced that exposure to extreme environmental conditions such as a broad range of variable pHs, high hydrostatic pressure, and very low and very high temperatures may change the morphology of marine organisms. In fact, our understanding of the adaptation strategies can gain a better insight into discovering novel secondary metabolites from marine systems. Most marine secondary metabolites play a physiological role in marine ecological systems and are known to be involved in specific binding mechanisms with desired target receptors. These physiological functions may have been conceived to comply with biochemical and physiological phenomena of humans. Furthermore, these biomolecules are found in their terrestrial analogs and furnished the focus of formulating new classes of drugs [41.1, 5]. Thus, identification and isolation of many of these compounds can play a substantial role in the designing and development of pharmaceutical products.

Over the last three decades, more than 35 000 novel marine natural products have been isolated and structurally identified from marine sources [41.6]. Reported bioactivities of marine secondary metabolites have commonly shown anticancer activity [41.7], matrix metalloproteinase inhibitory activity [41.8], antioxidant activity [41.9], antiviral activity [41.10], anti-inflammatory activity [41.11], hepatoprotective activity [41.12], antibiotic [41.13], and antidiabetic activity [41.14]. However, with higher demands on global pharmaceutical market value, the design and development of new drugs need to be increased massively. In fact, major research areas with respect to the burden of human health diseases such as cardiovascular diseases, cancers, gastrointestinal disorders, and infective and central nervous system diseases need to be met with innovative drugs and medicines with selective and specific mechanisms of actions. In order to achieve these targets, mining of bioactive molecules should be carried out using high-throughput screening. Thus, recently reported marine secondary metabolites or drug-like molecules and their pharmacological effects are open for further discussion.

41.2.1 Halogenated Terpenes

Terpenes are derived from five carbon isoprene molecules and described as a unique type of secondary metabolites. The wide range of chemical and structural diversities of terpenoid compounds are associated with significant biological properties. Due to structural variations, certain classes of terpenes have been reported, from 5 carbon units (C5; hemiterpenoid) to 40 carbon units (C40; tetraterpenoid) [41.15]. Figure 41.1 depicts an isolated diterpenoid, flexibilide **(1)** and dihydroflexibilide **(2)** from soft coral *Sinularia flexibilis* showing tissue necrosis effects due to toxicity against near sea organisms [41.16]. In addition, in the marine sponge *Dysidea* sp. a major sesquiterpene, 7-deacetoxyolepupuane **(3)** was isolated, showing an allelopathic effect [41.17].

The biomedical potential of terpenes isolated from marine seaweeds has been studied recently. The marine red algae, genus *Laurencia* (family Rhodomelaceae, order Ceramiales) was reported to have around 135 species worldwide [41.18]. It could be a major source of isolated halogenated terpenes and was found to possess biofunctional effects, which could be easily used as drug-like molecules for the pharmaceutical industry [41.11]. Hence, *Laurencia* spp. was the first recognized source of halogenated natural products [41.6]. In this case, isolated laurinterol **(4)** was the first reported brominated terpene. Despite that, recently isolated terpenes, 5β-hydroxypalisadin B **(5)**, palisadin B **(6)**, palisol **(7)**, and pacifigorgiol **(8)** from *Laurencia snackeyi* were reported to show potential in vitro anti-inflammatory effects. In addition, a novel brominated diterpene, **(9)** with antibacterial activity was isolated from Malaysian *Laurencia* spp. [41.13].

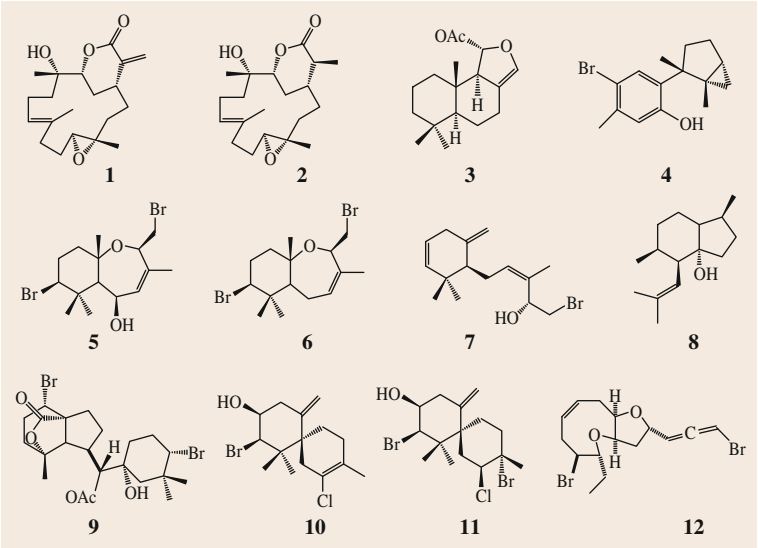

Fig. 41.1 Chemical structures **(1–12)** of halogenated terpenes as possible analogs for future pharmaceuticals from marine bioresources

Furthermore, interesting halogenated metabolites, elatol **(10)** and isoobtusol **(11)** with an antibacterial effect have reported from *Laurencia majuscule* [41.19]. *Ji* et al. reported on the isolation of halogenated terpenes including seven diterpenes, two polyether triterpenes, and one C_{15}-Acetogenin, neolaurallene **(12)** from *Laurencia saitoi* [41.20]. These molecules are described as halogenated and cyclic terpene structures with a unique substrate selectivity that confers the stereo-specific and regiospecific reactivity for promising bioactivities.

41.2.2 Steroids and Sterols

A steroid comprises a characteristic arrangement of four cyclic rings joined together. Sterols are a form of steroids and contain a hydroxyl attachment at the 3-carbon position. Steroids are derived biochemically from the squalene precursor, and a few important sterols are identified such as, acylated, alkylated, sulfated, free sterols, and sterol glycosides [41.21]. The exploration of marine steroids and sterols started when a significantly larger amount of structural variations was found in the marine ecosystem than in terrestrial life. Thus, these steroids and sterols are gaining attention in the newest drug design and developments [41.22]. The number of different biological activities of these compounds were attributed to the unusual side chains that attached to the steroid skeleton. In particular, because they have unusual carbon atoms at C-24 as quaternary alkyl, cyclopropane, and cyclopropene rings, allenes and acetylenes have offered a wide range of biosynthesis applications for drug designers [41.23].

Among the marine organisms, sponges are the richest source with a high content of sterols (Fig. 41.2). The marine sponge *Petrosia weinbergi* was found to contain major sterols, isofucosterol **(13)**, and clionasterol **(14)** with antiviral activities [41.21]. Microalgae sterols usually represent C-27 ≈ C-29 skeletons with different alkylations at C-24, along with double bonds in the rings (Δ5, Δ8, or Δ8, Δ14), and in the side chains (Δ22, Δ24). In fact, dinoflagellates possess a typical steroid composition and are commonly used as biomarkers (e.g., dinosterols **(15)**) [41.24, 25]. The unique structure of oxysterols has shown a protective effect against β-amyloid peptides and is found in plaques in Alzheimer's diseased brains. However, only the isolated oxysterols, 5α-cholestane-hexanol **(16)** are found in starfish, *Hippasteria kurilensis* [41.26]. A low amount of sterols is found in brown algae, and the only dominant sterol is reported as fucosterol **(17)**. Fucosterol isolated form *Pelvitia siliquosa* shows antidiabetic activity [41.27] and antioxidant activity [41.28]. Moreover, saringosterol **(18)**, a derivative of fucosterol, was found to inhibit the growth of *Mycobacterium tuberculosis*, and is isolated from the brown algae *Lessonia nigrescens* [41.29]. According to further studies, this compound has not shown cytotoxicity for the mammalian cell, thus representing a good candidate for tuberculosis drugs. The starfish *Linckia laevigata* was reported to bear the most different type of sterols, for example, linckoside

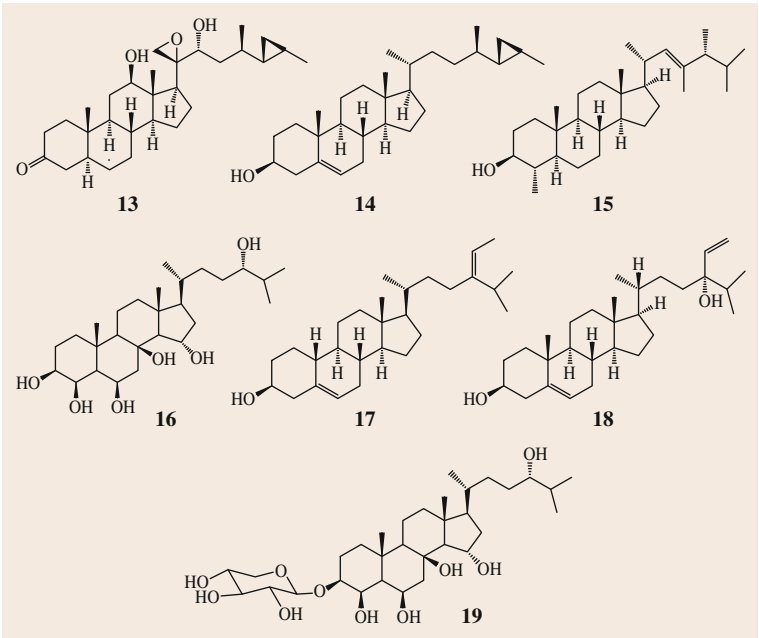

Fig. 41.2 Isolated different steroid and sterol skeletons **(13–19)** from marine bioresources

L1 **(19)** identified as polyhydroxylated steroid glycosides [41.30]. This compound was reported to be neuritogenic and thus might help to treat damaged neuronal cells and can potentially be used as a drug for neurodegenerative disease. Each of these steroid molecules shows significant variations throughout the marine ecosystem and shows a broad range of bioactivities. Therefore, these biomolecules may contribute as templates or drug analogs for future therapeutic uses.

41.2.3 Polyphenols

Polyphenols are a class of large secondary metabolites associated with multiple phenol structural units. Polyphenols serve as a reactive species, tend to be oxidized and are attributed as antioxidants, which can commonly be found in plant materials. However, marine seaweeds have had a wide acceptance with the latest studies reporting that they contain phenolic compounds or polyphenols [41.31]. In fact, polyphenols can interact with tannins and may be derived as a special form of secondary metabolites in brown algae, known as phlorotannins [41.32]. These molecular components make the integral part of the cell walls of brown algae. Basically they play an important role as secondary metabolites to as protection from ultraviolet (UV) radiations and oxidative stress.

Among the high biodiversity of marine seaweeds, brown algal species such as *Ecklonia cava*, *Eisenia arbora, Ecklonia stolinifera* and *Eisenia bicyclis* have been reported that comprise of high content phlorotannins [41.33–35]. Figure 41.3 shows that the isolated phlorotannin structures from marine brown algae and phloroglucinol **(20)** is the basic unit which is polymerized through the acetate-malonate biosynthesized pathway to form phlorotannins. Functional benefits from different phlorotannins which accumulated in marine brown algae are widely studied recently. There are four subclasses of phlorotannins reported in marine system such as phlorethols (with ether linkages), fucols (with a phenyl linkage), fucophloroethols (with an ether and phenyl linkage) and eckols (with a dibenzodioxin linkage) [41.36].

Eckol **(21)** isolated from *E. bicyclis* was reported to show anti-diabetic properties as inhibitory activity against glycation and α-amylase [41.33]. Moreover, a brown alga *E. cava* has been provisioned to extract eckol and dieckol **(22)** and showing the inhibitory effect of melanogenesis and protective effects against photo-oxidative stress induced by UV-B radiation [41.34]. Inducing apoptosis by dioxinodehydroeckol **(23)** and 7-phloroeckol **(24)** were isolated from *E. cava* and having a promising potential to use as anticancer drugs for human breast cancers [41.7]. In addition, *Ecklonia stolonifera* extract phlorotannins were identified

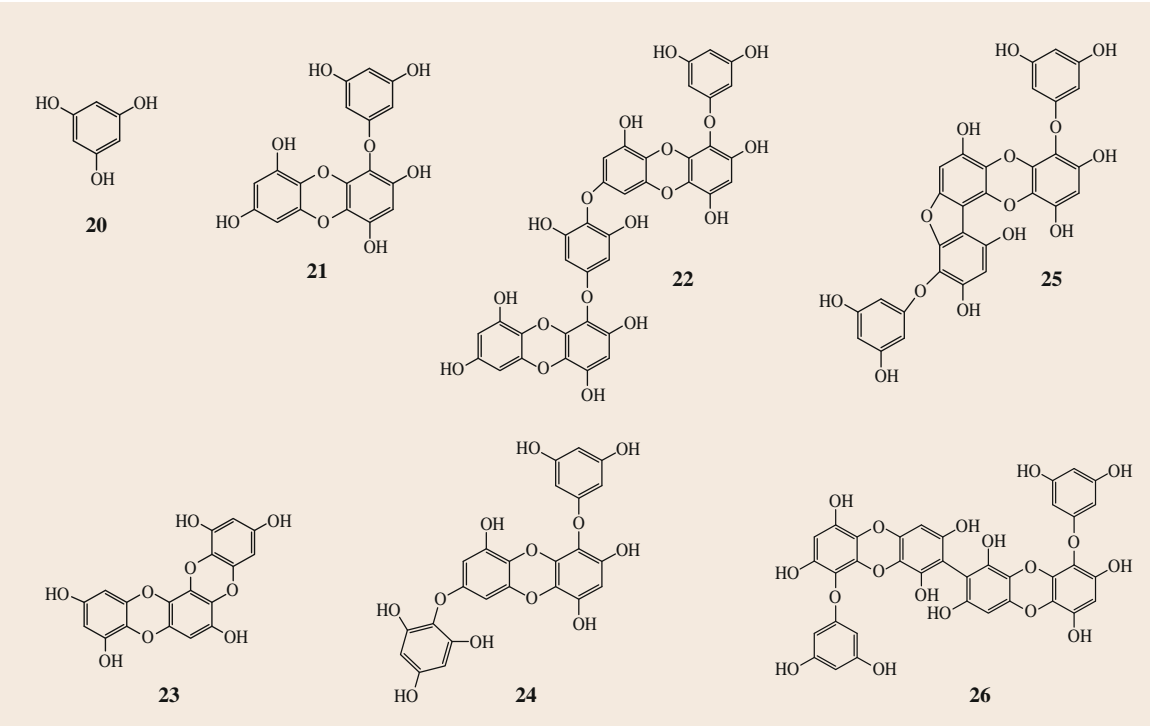

Fig. 41.3 Phloroglucinol derivatives (phlorotannins) **(20–26)** isolated from marine brown algae

as phlorofucofuroeckol A **(25)** with the lowering effect of Cu^{2+} induced low-density lipoproteins (LDL) oxidation [41.37]. Moreover, angiotensin converting enzyme (ACE) inhibitory activity was also proven by this molecule [41.38] and confirmed that the future opportunistic for drug development against cardiovascular diseases. *Ahn* et al. (2004) reported on the promising effect of inhibitory activity on human immunodeficiency virus type 1 (HIV-1), reverse transcriptase (RT), and protease from the *E. cava* phlorotannins, including eckol, phlorofucofuroeckol A and 8,8′-bieckol **(26)** [41.39]. There is an abundant chemical distribution of phlorotannins among brown seaweed, which is described as a multifunctional approach for biological and pharmacological applications. Most of the burden of human health issues can be managed using phlorotannins, which will be a good candidate for future drug design strategies.

41.3 Marine Proteins

Importantly, most marine bioresources are composed of a higher content of proteins than other components. Marine microalgae, muscle tissues of fish, shellfish, and other invertebrates show a range of 15–65 w/w% proteins [41.75]. On the other hand, marine fish and shellfish waste as processing by products are also considered to contain large quantities of high-quality proteins (10–23 w/w%). These sources are considered as being a potential source forming active proteins and bioactive peptides [41.76]. The significance of marine-derived proteins and peptides are due to their advantage over conventional drugs with a high bioavailability and biospecificity to the targets. In addition, the properties of low toxicity, structural diversity, and little or no accumulations in the body tissues of these molecules make them promising candidates for therapeutic purposes [41.77]. Marine enzymes are described as a promising biotechnological tool that can be synthesized from marine bioresources [41.78]. Interestingly, their high speci-

Table 41.1 Pharmaceutical value of marine proteins and lipids isolated from marine bioresources

Functional ingredients	Source of origin	Marine source	Pharmaceutical value	Reference
Marine peptides				
LEQ/GMNNLTP	*Nannochoropsis oculata*	Microalgae	ACE inhibitory	[41.40]
VECYGPNRPEF	*Chlorella vulgaris*	Microalgae	ACE inhibitory	[41.41]
VEGY	*Chlorella ellipsoidea*	Microalgae	ACE inhibitory	[41.42]
LNGDVW	*Chlorella ellipsoidea*	Microalgae	Antioxidant	[41.43]
AKYSY	*Porphyra yezoensis*	Macroalgae	ACE inhibitory	[41.44]
YNKL	*Undaria pinnatifida*	Macroalgae	ACE inhibitory	[41.45]
VLSGGYYMAMYTLV	*Theragra chalcogramma*	Fish – Alaska pollack	Ca-binding	[41.46]
CAAP/VCSV	*Paralichthys olivaceus*	Fish – olive flounder	Antioxidant	[41.47]
LGLNGDDVN	*Conger myriaster*	Fish – conger eel	Antioxidant	[41.48]
VKAGFAWTANQQLS	*Katsuwonus pelamis*	Fish – tuna	Antioxidant	[41.49]
TFPHGP/HWTTQR	*Syngnathus schlegeli*	Fish – pipefish	Antihypertensive	[41.50]
LLEYSI/LLEYSL	*Crassostrea gigas*	Shellfish – oyster	Anti HIV-1 protease	[41.51]
EVMAGNLYPG	*Mytilus edulis*	Shellfish – blue mussel	ACE inhibitory	[41.52]
MEGAQEAQGD	*Acaudina molpadioidea*	Sea cucumber	ACE inhibitory	[41.53]
AHIII	*Styela clava*	Sea squirt	Antihypertensive	[41.54]
MLLCS	*Styela plicata*	Sea squirt	ACE inhibitory	[41.55]
DDTGHDFED/TGEAM	*Brachionus rotundiformis*	Rotifer	ACE inhibitory	[41.56]
Marine enzymes				
Chymotrypsin	*Gadus morhua*	Fish – Atlantic cod	Protein hydrolysation	[41.57]
Transglutaminase	*Cyprinus carpio*	Fish – carp	Modification of viscoelastic properties-transferase	[41.58]
α-D-glucosidase	*Aplysia fasciata*	Shellfish – mollusc	Enzymatic synthesis of oligosaccharides	[41.59]
Citrate synthase/pyruvate kinase	*Meganyctiphanes norvegica*	Shellfish – northern krill	Metabolic key enzymes	[41.60]
Cholinesterase	*Mytilus edulis*	Shellfish – blue mussel	Aquatic biomarker	[41.61]
(2′-5′) oligoadenylate synthetase	*Geodia cydonium*	Sponge	Immunologically related proteins	[41.62]
ATP *N*-glycosidase	*Axinella polypoides*	Sponge	Nucleosidase enzymatic activity-transferase	[41.63]
Fibrinolytic	*Basillus clausii* ICTF-1	Marine bacteria	Treatment of cardiovascular diseases	[41.64]
Hydrolases	*Oscillatoria willei* BDU 130511	Cyanobacteria	Bioremediation	[41.65]
Marine lipids				
Galactolipids	*Phaeodactylum tricornutum*	Microalgae	Apoptosis inducing	[41.66]
EPA (eicosapentaenoic acid)	*Phaeodactylum tricornutum*	Microalgae	Reduce the risk of heart disease	[41.67]
GLA (γ-linolenic acid)	*Spirulina platensis*	Microalgae	Reduce the risk of heart disease	[41.68]
Oleic acid	*Haematococcus pluviales*	Microalgae	Antioxidant	[41.69]
Palmitic, α-linolenic & oleic acids	*Dunaliella salina*	Microalgae	Antimicrobial	[41.70]
Sterols and fatty alcohols	*Euphasia Superba*	Shellfish – krill oil	Reduce the risk of heart disease	[41.71]

Table 41.1 (continued)

Functional ingredients	Source of origin	Marine source	Pharmaceutical value	Reference
Marine lipids				
EPA and DHA	*Perna canaliculus*	Shellfish – Green-lipped mussels	Reduce the risk of heart disease	[41.72]
EPA and DHA	*Oncorhynchus gorbuscha*	Fish – salmon	Reduce the risk of heart disease	[41.73]
Diacylglycerol (DAG)	*Katsuwonus pelamis*	Fish – tuna	Anti-obesity	[41.74]

ficity and high reactivity at low concentrations may encompasses the efficacy of marine enzymes in different applications, including molecular biology research, food processing, diagnostics and pharmacology [41.79]. The interesting proteins, enzymes, and bioactive peptides isolated from different marine sources with their therapeutic uses are presented in Table 41.1.

41.4 Marine Lipids

The consumption of seafood has been proven to prevent lifestyle diseases and give long-term health benefits [41.80]. One of the characteristic components in marine resources are lipids. The lipid factions of marine bioresources consist of polyunsaturated fatty acids (PUFA), including omega-3 and omega-6 fatty acids [41.81]. Over many years, there impressive work on PUFA has been carried out, and they have been proven to reduce or prevent the burden of diseases. Especially, the consumption of seafood once or twice a week may have a protective effect against coronary heart disease, may reduce the death risk by 36% and 17% from total mortality due to a high content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the diet [41.82]. These health benefits may bring about relievable effect against physiological conditions [41.83] such as diabetes [41.84], cancers [41.85], and inflammatory diseases [41.86].

As presented in Table 41.1, many food components serve the essential fatty acid molecules for the food industry. These PUFA sources can be used directly in pharmaceutical applications and, on the other hand, may be utilized as food supplements or in neutraceutical applications.

41.5 Molecular Biology Approaches

Molecular biology approaches in biotechnology and pharmacology are interesting. In this regard, the structures and functions of macromolecules like nucleic acids and proteins, with respect to their roles in life processes are investigated. However, applications of this promising discipline of marine bioresources such as microorganisms, algae, invertebrates, and vertebrates including economically important fish species have become valuable recently. Marine ecosystems consist of a huge diversity of organisms and are a prominent source of a wide array of therapeutic agents that can be developed as pharmaceuticals. Novel developments in molecular biology, including genomics, metagenomics, transcriptomics, proteomics, and bioinformatics, along with the refinement of technologies, such as genetic engineering and bioactivity screening offer unique opportunities to establish marine natural products as interesting drug candidates.

Several explorations have been made based on genomic and metagenomic approaches, including the identification of the putative bryostatin polyketide synthase gene cluster from the marine bacterium *Candidatus Endobugulase ertula*. In fact, this gene cluster encodes crucial constituents in the biosynthesis of pharmaceutically potent bioactive compounds, known as bryostatins [41.87]. Moreover, polyketide bio-synthesis was already characterized from an noncultivable bacterial symbiont of the marine sponge; *Theonellaswinhoe*, with respect to its corresponding genes, such as polyketide synthase (PK) and non-ribosomal peptide synthase (NRPS) which are known to produce antitumor molecules [41.88–90]. In addition, polyke-

tide coding genes were identified from *Streptomyces* species, proving the secretory properties of antitumor and antibacterial agents from marine bacteria as their secondary metabolites [41.91]. Particularly, PKs and NRPS can be used in screening approaches to pre-select new isolates with promising bioactivities for natural product analysis. Moreover, the discovery of novel antimicrobial peptides (AMP) originating from marine invertebrates; such as penaedin, crustin, and defensin, etc., is facilitated by the development of new drug candidates by molecular biological approaches. Especially, the isolation of complementary DNA (cDNA) transcripts or express sequence tag (EST) clones which show homology with already characterized AMP sequences furnishes ease and success of such investigations of AMPs from marine species as desirable [41.92]. Marine organisms, especially marine microorganisms, algae, and sponge-like invertebrates can also be considered prospective candidates to be subjected to genetic improvements, as described above, to improve them as sustainable resources of bioactive compounds. Finally, the overall insights on the impact of molecular biology on the development of marine bioresources points us to a flourishing future, especially with respect to drug discovery programs.

41.6 Future Trends in Marine Pharmaceuticals

Over the last few decades, the number of marine natural products isolated from marine bioresources has increased significantly [41.93]. However, a limited number of natural products gained market value throughout this period. It is believed that there is a lowering of the ecological niche of sample sources or a lack of sufficient quantities of the compounds and difficulties with isolation and purification. In addition, to face the challenges in vivo, pre-clinical and clinical phase studies, drug-like activities need to be proven prior to the commercial chemical synthesis. Nevertheless, these obstacles in the chemical synthesis of the desired drug-like molecules may be described as the major issue while determining bioactivities with the conformational changes of the molecules. Moreover, to obtain regulatory approval of a drug from the FDA for sale and marketing, from the discovery of natural products until the success of preclinical, clinical, and phase studies may cost about US$ 802 million and takes about an average of 10 ≈ 15 years [41.94]. Despite this, some marine drugs with prominent pharmaceutical value are available in

Table 41.2 Marine-derived pharmaceutical agents

Compound name	Trade name	Marine organism	Approval or clinical status	Medicinal value
Ziconotide	Prialt	Cone snail – *Conus magus*	Approved by FDA in 2004	Analgesic (Chronic pain)
Cytarabine	Cytosar-U	Sponges – *Cryptotheca crypta*	Approved by FDA in 1969	Cancer and leukaemia
ET-743	Yondelis	Tunicate – *Ecteinascidia turbinate*	Approved by EMEA in 2007	Ovarian cancer
Pseudopterosin A	Resilience	Sea fan – *Pseudopterogorgia elisabethae*	Approved for skin care products	Anti-inflammatory
Diazonamide A	NA	Sea squirt – *Diazona angulata*	NA	Cytotoxic and antimicrobial
Vidarabine	Vira-A	Sponge – *Tethya crypta*	Approved by FDA	Antiviral
Eribulin mesylate	E7389	Sponge – *Halichondria okadai*	Phase III	Cancer treatment
Soblidotin	TZT-1027	Bacterium – *Symploca sp.*	Phase III	Cancer treatment
Plitidepsin	Aplidin	Tunicate – *Aplidin albicans*	Phase II/III	Cancer treatment
Kahaladide F	NA	Mollusc – *Elysia rubefescens*	Phase II	Antitumor
Aplyronine A	NA	Sea hare – *Aplysia kurodai*	Phase I	Antitumor
Dideminin B	NA	Tunicate – *Tridemnum soldium*	Phase II	Antitumor
Granulatimide	NA	Tunicate – *Ascidian didemnum*	Phase II	Cancer treatment

FDA: Food and Drug Administration, EMEA: European Medicines Agency, NA: Not applicable

the market and some of which are presented in Table 41.2.

The drug development process can be provided with recent molecular biological strategies, including genomics, metagenomics, transcriptomics, proteomics, and bioinformatics. The current contributions from the diverse areas of secondary metabolites are promising for future pharmacological developments. In fact, the knowledge of the advances of biotechnology and pharmacology may lead to a better understanding of the infrastructures of scientific communities and strengthen the development of innovative drugs in the future.

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42. Marine Functional Foods

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In line with consumer awareness that a relationship between diet, health, and disease prevention exists, the research and development of new functional foods have been the target of many studies over the last years. The increasing ageing of populations, the decrease in quality of life due to stress, the high incidence of so-called modern diseases (cardiovascular disease, obesity, cancer, diabetes, and allergies) represent the driving forces in the quest for different foods and diets to promote healthy active ageing, improve well-being, and counteract the incidence of many diseases. The marine environment provides a huge source of many healthy foods, including seaweeds, which is an example of a marine product that has been part of the diet in several countries around the world. Additionally, marine specimens are also sources of a plethora of chemicals, many of them with biological properties and, therefore, called bioactive compounds. These chemicals can be extracted and incorporated in several food matrices leading to new potential functional foods. This chapter is a review summarizing those marine organisms with healthy properties that are consumed all over the world, as well as the potential of using marine organisms as sources of food ingredients towards applications in new functional foods. Recent findings on functional foods based on marine-derived ingredients are described and discussed.

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42.1 General Overview

Since ancient times, man has always had the perception that a direct relationship between diet and human health exists. As *El-Sohaimy* [42.1] described in his review on functional food and nutraceuticals, the phrase postulated by Hippocrates over 2500 years ago *Let food be thy medicine and medicine be thy food* is a clear indication of this perception. Despite the fluctuations in the economy, wars, and social politics that occurred in

the last century, the human diet has evolved over the last 100 years and nowadays evidence shows that consumers are able to make informed choices based on their improved knowledge on the existing relationship between diet, health, and disease prevention. Consumption of healthy foods such as cereals, vegetables, and fruit, as well as marine foods rich in polyunsaturated fatty acids (PUFAs) goes beyond meeting basic nutritional needs; they are also crucial for health promotion and/or maintenance, as well as for disease risk reduction [42.2]. Additionally, research progress over the past decade has correlated diet and some chronic diseases that still have high mortal rates and are extremely expensive for public or private healthcare systems. Nutritional science has been evolving from a focus on identifying nutrients and the respective amounts to prevent deficiency diseases to a focus on improving quality of life and health [42.3]. According to several authors several foods can play an important role by preventing or hindering the progression of chronic diseases such as atherosclerosis, obesity, diabetes, hypertension, osteoporosis, cancer, and cardiovascular disease [42.4–7], as well as relieving symptoms in osteoarthritic patients, for example [42.8]. However, independent of a higher level of nutritional knowledge, today's consumer and populations as a whole still need to pinpoint strategies that may enable us to overcome modern age diseases such as those previously mentioned. For example, the positive role of functional foods and nutraceuticals in the primary prevention of cardiovascular diseases was recently reviewed by *Allisa* and *Ferns* [42.6].

From a different standpoint, the increasingly ageing population as a consequence of medicinal advances in the last years, also demands an active response from food formulators and offers an excellent opportunity for the development of adapted foods and diets in order to contribute to healthy, active ageing and improved well-being [42.9].

Based on the above rationale, research, development, and commercialization of functional foods has been in the limelight for the last years and also offers an alternative way for some producers and industries to diversify their products, especially those based on marine-derived products [42.1, 2]. However, much has yet to be done regarding the acceptance of functional foods, namely in terms of health claims as stated in current legislation. Claims should be based on solid scientific evidence, which to date is often lacking [42.1] but is becoming increasingly important. A regulatory framework protecting consumers, promoting fair trade, and encouraging product innovation in the food industry is urgently needed [42.3]. *Hayes* [42.10] reviewed the claims and current legislation on marine-derived functional foods that have been applied in China, Japan, United States of America (USA) and in the European Union (EU). These authors also describes the recently approved health claims in the EU related with omega-3 fatty acids and with chitin-glucan, which is a known fiber-boosting weigh management food ingredient.

As discussed by *Freitas* et al. [42.11], there is still no recognized standard definition for functional foods – in general, the term refers to a food, and according to Health Canada, a functional food is similar to a conventional food, which is consumed as part of a usual diet that either provides physiological benefits or reduces the risk of chronic disease beyond basic nutritional functions [42.12]. According to the Food Agriculture Organization (FAO) [42.13], functional foods are those foods similar to conventional food in appearance, intended to be consumed as part of a normal diet containing biologically active compounds that offer potential for enhanced health or reduced risk of disease. *Ramesh* et al. [42.3] presented six proposed definitions of functional foods established by six different agencies; in five of them the concept that they should provide health benefits beyond basic nutritional functions is clearly present. For nutraceutical and/or dietary supplements, there is also no consensual definition and there is still ambiguity about the regulatory requirements concerning these compounds [42.14]. In certain countries, nutraceuticals and functional foods are still used interchangeably [42.2] since some nutraceuticals are used as conventional foods or as sole items of a meal or diet. According to *El-Sohamy* [42.1], nutraceuticals and functional foods are two recent designations used to describe health-promoting foods or their extracted compounds.

From the point of view of the authors, nutraceuticals or dietary supplements are those defined as functional ingredients but sold as powders, pills, and other medicinal forms not associated with food. Nevertheless, in functional foods, nutraceuticals, or dietary supplements, the main focus is to improve health and diminish as much as possible the risk of disease through prevention toward improvement of quality of life and well-being contributing to an increased healthy longevity [42.2].

Taking in consideration the previous discussion, functional foods belong to foods that besides their nutritional effects, have demonstrated that they improve the state of health or well-being, reduce the risk of disease, as well as benefit one or more functions of the human

organism. According to *Freitas* et al. [42.11], functionality could be intrinsic to a feature introduced in the food matrix, improving health or reducing any adverse health effect, accomplished, for example, by:

i) Elimination or promotion of a chemical change of a harmful ingredient.
ii) Addition of new health-promoting food ingredients or probiotic microorganisms in an effective concentration.
iii) Addition of an existing health-promoting food ingredient, increasing its concentration.
iv) Increasing the bioavailability or stability of the health-promoting food ingredient.

The development of foods containing a group of compounds with desired properties is, in general, particularly challenging for biotechnology and the food industry.

Functional ingredients exist in many different reservoirs from both terrestrial and marine environments [42.11], it being known that the terrestrial environment (for example, fruit, vegetables, cereals, and mushrooms) is far more explored than the marine environment (for example, fish, sponges, macro and microalgae) for bioactive compounds. There are many functional marine ingredients that are presently known, yet it is of general consensus that other marine ingredients remain to be evaluated and new sources to be discovered. According to *Zhang* et al. [42.15] bioprocessed marine products are alternative sources for synthetic ingredients that can contribute to the consumer's well-being as a part of nutraceuticals and functional foods. By citing *Gates* [42.16] with his statement *The ocean is considered Mother Nature's medicine cabinet*, the importance of the marine environment as a major reservoir of bioactive compounds that have potential to be applied in several fields such as medicine or food becomes quite clear. For example, the marine environment represents a huge source of many healthy foods, including seaweeds, a marine product that has been part of the diet in some countries around the world. According to *Zhang* et al. [42.15], to date more than 20 000 new compounds have been isolated from marine biomaterials.

The characteristics of the marine environment such as various degrees of temperature, salinity, light, pressure, and nutrients are of special importance, since due to their broad range of values marine organisms had to evolve some protective mechanisms and metabolites [42.17, 18]. Therefore, research on the identification of biologically active marine-derived compounds can be seen as an almost unlimited field. In fact, in the last years much research has been pursued to discover and produce marine-derived bioactive compounds [42.19, 20]. Recommendations detail that although functional ingredients do not need to be nutrients they should be dietary, biologically active components added to a food vehicle or present in unmodified whole food [42.13]. Recent advances in genetics, namely in nutritional genomics, have also spiked interest in functional ingredients [42.14], since it has been shown that food may play a role in the individual needs and predispositions [42.11]. Marine specimens are sources of a plethora of chemicals, many of them with biological properties that can be extracted and incorporated in several food matrices, leading to new potential functional foods.

42.2 Marine Sources as Healthy Foods or Reservoirs of Functional Ingredients

Marine sources are well known for their phenomenal biodiversity, which offers a strong basis for their use as a natural source of healthy foods as well as of many novel functional food ingredients with biological properties. Crustaceans, macro or microalgae, fish, and fish by-products, as well as bacteria and fungi are the most representative groups of organisms of potential interest as healthy food or as a source of functional ingredients, which include polysaccharides, chitin, proteins and peptides, lipids, pigments, vitamins, minerals, and phenolic compounds (Fig. 42.1).

The interest in functional food marine-derived ingredients with biological properties derived from lipids, proteins, polysaccharides, and pigments, has increased tremendously in the last years due to their beneficial effects in terms of disease prevention or maintenance of health and well-being. Considering this perspective the next sections describe, in a summarized way, the properties of the above-mentioned groups of marine organisms: seaweeds, microalgae, fish and fish-by-products, crustaceans, as well as marine fungi and bacteria. The target of much scientific debate, there have been many

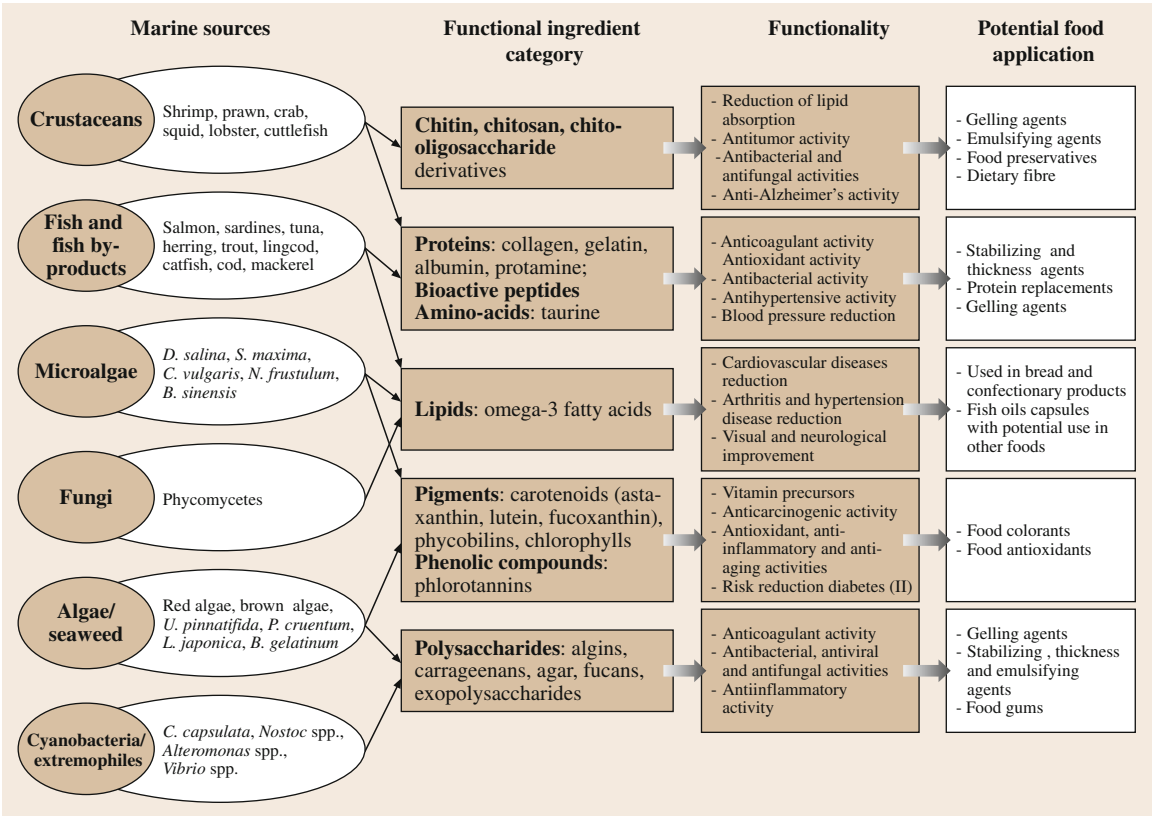

Fig. 42.1 Main marine functional ingredients with potential food applications, sources, and inherent functionality (after [42.11, 21, 22], courtesy of Elsevier)

recent reviews concerning these organisms, some of which will be mentioned in this chapter.

42.2.1 Seaweeds

Considering their great taxonomic diversity, algae, in particular, edible marine macroalgae or seaweeds, are a very interesting source of healthy foods as well as a natural source of compounds with biological activity that could be used as functional ingredients constituting a research area with much to explore for food purposes. According to *Chojnacka* et al. [42.23], there are about 10,000 identified species of algae and about 5% of them are used as food for either humans or animals. More than one hundred macroalgae species are used worldwide, especially in Asian countries as sea vegetables [42.24]. Seaweeds are of potential interest for incorporation in diet as foods because they are claimed to have a low cholesterol content, fight obesity, reduce blood pressure, tackle free radicals, and promote healthy digestion [42.25]. Seaweeds are known to be of low calorie content, rich in polysaccharides, minerals, vitamins proteins, steroids, and dietary fibers [42.26]; furthermore, some of them have demonstrated biological properties such as antibacterial, antioxidant, anti-inflammatory, anticoagulant, antiviral and/or apoptotic activities [42.27]. According to *Lordan* et al. [42.26], the ingestion of such compounds has the advantage of presenting high bioavailability, of natural origin and, in general, their isolation does not require an effectively high cost and, more importantly, affects some pathologies positively. In *Holdt* and *Kraan*'s [42.28] review one can find an exhaustive description of bioactive compounds that are present in seaweeds, their application in functional foods, as well as emerging legislation issues. The perspective of seaweeds as a sustainable functional food for complementary and alternative therapy has been reviewed by *Mohamed* et al. [42.29].

In fact, research advances have shown that seaweed can be used as a rich source of polysaccharides,

pigments such as carotenoids, phycobilins and chlorophylls, as well as of phenolic compounds (Fig. 42.1) that can be incorporated in several food products. Furthermore, seaweeds extracts are practically fat and calorie-free, making them increasingly pursued for commercial purposes. Macroalgae, i. e., the *Sargassum* species (Phaeophyceae, brown algae), have been found to be good sources of dietary fiber and carotenoids with antioxidant activity and play important roles in the prevention of neurodegenerative diseases [42.30–32]. Some algae live in complex habitats where they are subject to extreme conditions, being forced to quickly adapt to new environmental conditions, and in order to survive they produce a wide variety of biologically active secondary metabolites [42.25] like acetogenins, terpenes, derivatives of amino acids, phenols, and polyphenols, which are compounds that, in general, differ from plant products because they are often halogenated.

It is known that not all algae use and need the same light intensity to perform photosynthesis and, therefore, are generally classified in three divisions by the presence of specific pigments: brown macroalgae (phylum Ochrophyta, class Phaeophyceae), red macroalgae (phylum Rhodophyta), or green macroalgae (phylum Chlorophyta) [42.26, 33]. According to *Bocanegra* et al. [42.34], green macroalgae abound more in coastal waters because they absorb large amounts of light energy, whereas red and brown algae are found at greater depths where penetration of sunlight is more limited. Brown algae owe their color to the presence of the carotenoid fucoxanthin. Food reserves of brown algae are characteristically complex polysaccharides including laminarins, fucans, and cellulose, as well as higher alcohols; many bioactive metabolites with different pharmacological activities such as antioxidant, anti-inflammatory, antitumor, cytotoxic antifungal, and nematocidal activities, have been isolated from these algae [42.32, 35]. Green algae owe their color to the dominant presence of chlorophylls *a* and *b*, and the main polysaccharides present are normally ulvans. In turn, red algae, which are also considered an important source of many biologically active metabolites [42.35] possess phycoerythrin and phycocyanin as the main pigments, and the primary polysaccharides are agars and carrageenans [42.27].

42.2.2 Microalgae

Microalgae are microscopic marine organisms ($< 20\,\mu m$) that can be found in benthic and littoral habitats but also in the ocean identified as phytoplankton comprising blue–green algae (phylum Cyanobacteria, class Cyanophyceae), diatoms (phylum Ochrophyta class Bacillariophyceae), dinoflagellates (phylum Myzozoa, class Dinophyceae), as well as green and yellow–brown flagellates (chlorophyta, prasinophyta, prymnesiophyta, cryptophyta, and others) [42.26, 36, 37]. Microalgae play a key role in the productivity of oceans, constituting the basis of the marine food chain [42.35] but are also considered important producers of some highly bioactive compounds. For example, they can be used to improve the food nutritional profile due to their abundance in PUFAs and pigments such as carotenoids and chlorophylls [42.35]. Chlorophyll a, phycocyanins, and phycoerythrin (phycobilins) are the pigments of interest found in blue–green algae, which possess a secondary metabolism responsible for the production of distinct compounds in terms of health. In fact, not all of these compounds are desirable in terms of public health, for example, some are strong hepatotoxins or neurotoxins, however, other compounds are of potential interest because they present biological properties such as anticancer, antifungal, antibacterial, and immunosuppressive properties; this subject was reviewed by *Gamal* [42.35]. Hence, due to this large diversity and depending on the species, microalgae can be used as a nutritional supplement or represent a source of natural food colorants as soon as their safety is assured [42.37].

Diatoms are also specific organisms that dominate the phytoplankton of cold and nutrient rich waters [42.38]. Diatoms are photosynthetically organisms with a high degree of flexibility to adapt to different environments, which is of interest for biotechnological applications. In addition, they are able to produce compounds for functional foods [42.39]; some of the most important functional ingredients are PUFAs such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and other omega-3 FAs, as well as the antioxidants fucoxanthin and chlorophyll. Dinoflagellates are also recognized as eukaryotic primary producers in marine coastal waters containing chlorophyll and carotenoids as well as PUFAs [42.38].

42.2.3 Fish and Fish By-Products

All over the world several million tons of fish and fish by-products are discarded as waste representing a large environmental problem. However fish and fish

by-products are known sources of potential bioactive ingredients such as fish oils rich in PUFAs from fish livers, calcium from fish bones, protein hydrolysates of high biological value, peptides with biological properties such as antihypertensive activity, amino acids such as taurine, which have antioxidant activity and positive effects on cardiovascular system, as well as vitamins and minerals [42.30, 40–50]. According to *Ferraro* et al. [42.42] fish heads, viscera, skin tails, blood, and seafood shells possess a plethora of compounds with the potential to be used as functional food ingredients with biological properties. In a review by *Kim* and *Mendis* [42.43] the bioactive compounds resulting from marine processing by-products are described; these can be obtained by extraction and purification procedures enabling the preparation and isolation of bioactive peptides, oligosaccharides, as well as fatty acids (FAs) suitable for biotechnological applications.

42.2.4 Crustaceans

Chitin is extractable from crustaceans, being the second most abundant natural polymer [42.44]. Chitosan is a biodegradable and biocompatible polymer chitin derivative, which results from crab, shrimp, and cuttlefish, and from processing shells and bones; it has been associated with antibacterial activity that can be used as a food preservative. Its ability to absorb fat justifies its potential use as an anticholesterol agent [42.45]. The biological activities of chitosan and chitooligasaccharides (antioxidant antitumor, anticancer, hypocholesterolemic, immunity-enhancing, antimicrobial) and the potential application in food, among other applications, are reviewed by *Xia* et al. [42.46].

42.2.5 Marine Fungi and Bacteria

Marine bacteria and fungi have drawn increasing attention from researchers from all over the world since they are considered as sources for new marine natural compounds [42.20, 47]. Marine extremophilic bacteria, for example, are of particular interest since they have metabolic pathways adapted to various extreme marine environments. According to *Laurienzo* [42.48], extremophiles are still a huge source of unknown and uncultivated bacteria with much to be explored in terms of bioactive compounds; many microbial enzymes and exopolysaccharides from extremophiles have potential and unique properties. Bacteria derived from intestinal tracts of marine organisms such as fish have also been researched with interest, since these strains may be new probiotics or have additional functions such as antibacterial activity [42.49]. For example, it was observed that *Lactococcus lactis* subsp. *lactis* isolates from the intestinal tract of freshwater fish possess different phenotypic properties, suggesting additional functions in comparison to those derived from a cheese starter [42.49].

Much interest has also been focused on marine fungi, which have been studied for their metabolites [42.50, 51]. *Masuda* et al. [42.52] reported a unicellular marine fungi with high concentration of γ-amino-butyric acid (GABA), which is a promising functional and healthy food ingredient. In addition, marine fungi are a promising source of novel bioactive compounds with anticancer, antibacterial, antiplasmodial, anti-inflammatory, and antiviral properties [42.20]. Compounds isolated from marine fungi have been the target of reviews [42.53], as well as the analytical techniques to search for bioactive compounds [42.20].

42.3 Food Marine-Derived Ingredients with Biological Properties

As discussed before, polysaccharides, proteins, peptides, amino acids, FAs, pigments, and phenolic compounds are some of the major groups of compounds that have been studied for their biological properties as well as extracted and purified for food and medicinal applications The literature on marine-derive biological compounds is extremely dense and in the last years the number of publications that have been written on this subject is outstanding. Therefore, in the following sections, the authors intend to give an overview of the main marine-derived compounds with the potential to be used as functional food ingredients to be incorporated in new food products; although the last topic to be covered it may be considered the main one of this chapter.

42.3.1 Polysaccharides

Polysaccharides, polymers of monosaccharides linked by glicosidic bonds, have been used in numerous commercial applications such as stabilizers, thickeners, and emulsifiers in foods and beverages [42.28, 54]. Several polysaccharides have been associated with biological properties such as antitumor, antiviral, antimutagenic, or hematopoietic activities [42.23]. The most known

and studied polysaccharides with potential interest as marine-derived functional ingredients are of macro and microalgae origins and the focus will be mostly, but not exclusively, on these.

The total concentration of polysaccharide in seaweeds can range from 4–76% of dry weight [42.28] depending on the macroalgae species, geographical origin, season, water temperature, location, method of cultivation, harvesting time, and other factors [42.26, 55]. Agar, algins, and carrageenans are examples of polysaccharides derived from algae that are widely used as stabilizers and thickeners in gels, as well as in foods [42.38]. Sulfated fucans, such as ulvans from green algae, carrageenans from red algae, and fucoidans from brown algae, have been known to act as modulators of coagulation as well as to reveal anti-inflammatory, antithrombotic, antioxidant, antidiabetic, and anticancer activities, among others [42.56, 57].

Soluble polysaccharides from algae have tremendous potential as dietary fiber for human nutrition and are being evaluated as possible new prebiotic compounds; the use of algal fiber promotes control and maintenance of intestinal flora [42.17]. For example, sulfated polysaccharides can also act as prebiotics exerting growth-promotion of beneficial bacteria in the gastrointestinal tract, imparting health-improving benefits to the host [42.28].

Seaweeds are recognized as a major source of sulfated polysaccharides with various biological activities, the structure of these compounds varies according to the species of algae. Sulfated polysaccharides found in red algae are manly galactans consisting entirely of galactose or modified galactose units, whereas in brown algae are fucans, which comprise families of polydisperse molecules based on sulfated L-fucose; heterofucans are also called fucoidans. In turn, the major water-soluble polysaccharides found in green seaweed are ulvans [42.58]; some of them are a significant source of sulfated galactans particularly in *Codium* species (Chlorophyta, green algae) [42.59, 60]. Sulfated polysaccharides from algae have been the target of much research in the last years because of their potential in terms of biological properties; anticoagulant, antioxidant, antitumor, anti-inflammatory, antiviral, antiproliferative, antipeptic, and antiadhesive activities have been associated to these compounds [42.61] and were recently reviewed by *Chojnacka* et al. [42.23].

Sulfated galactans are widely used in the food industry due to their jellifying and thickening properties [42.62]. Carrageenan and xylomannan sulfate are other sulfated polysaccharides that are found in some red seaweeds and that possess antiviral properties.

The carrageenan hydrocolloid is a linear sulfated polysaccharide of D-galactose and 3,6-anhydro-D-galactose extracted from some red algae species. There are three general forms (kappa, lambda, and iota) but, in general, seaweeds do not produce pure carrageenans, but rather a range of hybrid structures [42.63, 64]. Each of the copolymers kappa and iota carrageenan jellify with different properties and they are used in food production due to their excellent physical and functional properties (thickener, jelling, and stabilizer) in products such as yogurt, puddings, ice cream, sausages, hams, jams, among others. They also possess several applications in non-food industries, as well as pharmaceutical products with anticoagulant and anti-inflammatory properties [42.65].

Fucoidan is a type of complex sulfated polysaccharide that contains α-1,3-linked sulfated L-fucose as its main sugar unit and sulfate ester groups [42.55]. Antitumor and antiangiogenic activities are affected, increasing the number of sulfate groups in the fucoidan molecule [42.66], but fucoidan has also been reported to possess diverse biological activities such as anticoagulant, antioxidant[42.67], antitumor, anti-inflammatory, and antiviral activities [42.61, 68]. A review focusing fucoidan as a natural bioactive ingredient for functional foods was recently published by *Thanh-Sang* and *Kim* [42.69].

Agar is a water soluble long-chain polysaccharide which dissolves or disperses in water. This polysaccharide is widely used in the manufacture of gelatin. Agar consists of two fractions: agarose and agaropectin, considered a complex mixture of polysaccharides; agaropectin has low gelling power [42.28]. Agar-agar has been reported to decrease the concentration of blood glucose, to exert an antiaggregation effect on red blood cells, and to have antioxidant, antitumor as well as antiviral activities [42.28].

Laminarin is one of the major polysaccharides found in brown algae, which has been identified as a modulator of intestinal metabolism through its effects on intestinal pH, mucus composition, and short-chain fatty acid production [42.27, 70] with antiviral and antibacterial properties. The antioxidant activity of laminarin depends on its molecular weight and chemical structure. Its chemical structure consists of β-(1 $\rightarrow$ 3)-linked glucose in the main chain and random β-(1 $\rightarrow$ 6)-linked side chains [42.71], and their content in seaweeds is about 10% of the dry weight, but could seasonally reach up to 32% [42.28].

Alginates can be extracted from brown seaweeds, in which they can constitute up to 47% of dry biomass. Alginates are important soluble fibers due to their biodegradability, immunogenicity, and ability to form gel with a variety of cross-linking agents being used in several fields like the food industry, medicine, and biotechnology [42.72]. The acid form (alginic acid) is a polymer consisting of two types of hexuronic acid monomers linked by $1 \rightarrow 4$ bonds: β-D-mannuronic acid and α-L-guluronic acid, respectively [42.73]. This polysaccharide has shown antitumor, anticoagulant, and antiviral activities; furthermore, evidence shows its capacity to prevent obesity as well as large intestine cancer and diabetes and to reduce low-density lipid (LDL)-cholesterols in rats [42.74–77]. Alginic acid can also apparently reduce hypertension and porphyran from red seaweeds has been related with immune-regulatory, antioxidant, and antitumor activities [42.29].

Besides the previously mentioned anticoagulant [42.78, 79], anti-inflammatory [42.80], antiviral [42.81, 82], and antitumor activities [42.55] of fucoidans and alginic acid derivatives produced by brown seaweeds (*Ecklonia cava*, *Ascophylum nodusum* and *Undaria pinnatifida*, as well as other species), these have also been demonstrated to play an important role as free-radical scavengers and antioxidants preventing oxidative damage [42.83]. Evidence from different studies suggests that the antioxidant and anticoagulant activities of polysaccharides are strongly dependent on the content of sulfate groups, the molar ratio of sulfate/fucose and sulfate/total sugar, and the molecular weight [42.67].

Starch, glucose, sugars, and other polysaccharides can be found in microalgae [42.26]. Exopolysaccharides produced by cyanobacteria and by extremophilic deep-sea bacteria have been regarded with potential to be used as food functional ingredients. For example, polysaccharides produced by *Cyanospira capsulata* strains were reported to have interesting viscosity properties similar to xantham gums whereas exopolysaccharides produced by *Pseudoalteromona*s, *Alteromonas*, and *Vibrio* (bacteria) have the potential to be used as thickening agents [42.38].

Complex polysaccharides from *Chlorella pyrenoidosa* (Chlorophyta, green algae) produces complex polysaccharide such as β-1,3-glucan with potential immunostimulating properties and possess antioxidant properties [42.26, 37].

Another marine polysaccharide of interest is chitin, which is one of the major structural components of crustaceous shells and shellfish wastes. Chitin possesses a similar structure to cellulose built from n-acetyl-glucosamine monomers. Shrimp, crab, lobster, prawns, and crayfish have been reported to contain between 14 and 35% chitin [42.26]. Chitin, chitosan, and derivatives such as chito-oligosaccharides have potential to be used as functional ingredients since they may increase dietary fiber and act as a lipid absorption reducer. Chitin-glucan has been recently approved with a food claim by the European Union (EU) as a fiber-boosting weigh management product [42.10]. Several biological properties been attributed to chito-oligosaccharides, such as antimicrobial, angiotensin-I-converting enzyme inhibition, anticancer, antioxidant, hypoglycemic, hypocholesterolemic, and anticoagulant activities [42.84]. According to *Chen* et al. [42.85], chitosan could be used as a novel dietary fiber supplement for the prevention of Blackfoot disease since it is an effective adsorbent of humic acid in the gastrointestinal tract. Recently, *Alishhi* and *Aïder* [42.86] reviewed the various potential applications for chitosan including as a functional food.

42.3.2 Proteins, Peptides, and Amino Acids

The role of protein in the diet has been acknowledged worldwide, and currently dietary proteins are one of the major sources of bioactive compounds with a beneficial physiological impact on the health of humans and animals [42.87]. According to *Kim* et al. [42.88] marine-derived products seem to be one of the major sources of food-derived bioactive proteins and peptides. Proteins and peptides with biological properties have been isolated from macroalgae, microalgae, fish and fish by-products [42.26, 89]. In general, a higher content of protein is found in green and red seaweeds in comparison to brown seaweeds [42.28]. According to some authors, microalgae can be used as an alternative protein source because of the high protein content in the various species; 60–70% of protein with all essential amino acids and good bioavailability has been reported for some strains of *Spirulina* (Cyanobacteria, blue–green algae) [42.90, 91]. Collagen, gelatin, albumin, and protamine are some proteins that can be extracted from fish and fish by-products with several applications such as foaming capacity, gel forming, stabilizing agents, and antimicrobial activity; fish muscle proteins can be hydrolyzed enzymatically as a protein biomass of great interest [42.26, 38].

Extraction of protein from most seaweed is difficult due to the presence of large amounts of polysaccharides in the cell wall, such as alginates in brown algae

or carrageenans in red algae. Enzymatic hydrolysis by digestive enzymes or by proteolytic microorganisms has been a successful strategy to enhance extraction of peptides from seaweeds [42.92]. Some of the considered important bioactive proteins that can be extracted from macroalgae are lectins and phycobiliprotein [42.93]. Macroalgal lectins have been detected and isolated from red algae; lectins are generally bound to polysaccharides and participate in many biological processes like intercellular communication and have antibacterial, antiviral, or anti-inflammatory activities [42.23]. Lectins interact with specific glycan structures linked to soluble and membrane-bound glycoconjugates. It seems that it is the protein carbohydrate interactions that are responsible for lectin involvement in numerous biological processes such as cell–cell communication, host–pathogen interactions, cancer metastasis, induction of apoptosis and differentiation, as well as recognizing and binding carbohydrates [42.28]. Bioactive lectins are found in macroalgal species such as *Ulva* sp. (Chlorophyta), *Eucheuma* sp., and *Gracilaria* sp. (Rhodophyta), and have biotechnological applications in several scientific and medicinal fields of research [42.28]. Bioactive properties like mitogenic, cytotoxic, antibiotic, antinociceptive, anti-HIV, anti-inflammatory and antiadhesion activities have been reported for lectins [42.94, 95]. In turn, phycobiliproteins are oligomeric proteins, built up from two chromophore-bearing polypeptides found in macroalgae which have antioxidant, antidiabetic, and anticancer activities [42.89, 93].

Bioactive peptides usually contain 3–20 amino acid residues, and their activities are based on their amino acid composition and sequence and they are related to some biological functions such as antihypertensive, immune modulator, antioxidant, anticancer, antithrombotic, and antimicrobial activities [42.26, 96]. Bioactive peptides isolated from fish, molluscs, crustaceans, macroalgae, and microalgae proteins have been shown to possess anticancer, hypocholesterolemic, anticoagulant, antimicrobial, and antioxidant activities, among others [42.26, 96, 97]. *Suetsuna* et al. [42.98] isolated ten dipeptides from seaweed *Undaria pinnatifida* (Phaeophyceae) with four of these dipeptides containing the greatest ACE-inhibitory potential; their hypotensive effects were investigated in vivo and their results demonstrated a decrease of blood pressure in hypertensive rats. Numerous studies have been carried out to date that report on the antioxidant potential of macroalgae protein hydrolysates. *Harnedy* and *Fitzgerald* [42.99] reported a description of antioxidant potential of diverse macroalgae protein hydrolysates. In fact, several reviews have been published in the last 3 years on proteins and bioactive peptides from marine sources: on algae [42.99, 100], on marine organisms [42.96, 101], on processing marine waste and shellfish [42.102], and on fish-derived antioxidant peptides [42.103].

From microalgae such as *Navicula incerta* (Bacillariophyceae) and *Chlorella ellipsoidea* (Chlorophyta), antioxidative peptides have also been identified with demonstrated in vitro antioxidant properties [42.104, 105]. The high protein content found in some species of microalgae makes them of potential interest for their exploration as a source of bioactive peptides [42.87].

Bioactive peptides isolated from various fish protein hydrolysates have been reported as possessing several biological properties such as antithrombotic, antihypertensive, antioxidant, and anticoagulant activities. Several studies have reported on peptides with antioxidant activities from the skin of Pacific cod (*Gadus macrocephalus*), backbones (*Nemipterus japonicus*, *Exocoetus volitans*), muscles (*Nemipterus japonicas)*, and viscera *(Magalaspis cordyla)* [42.106–109].

In terms of amino acids, most seaweed species contain all the essential amino acids and are a rich source of the acidic amino acids. Brown seaweeds are a rich source of threonine, valine, leucine, lysine, glycine, cysteine, methionine, and histidine, among others [42.28]. In the majority of peptide sequences in antioxidant marine-derived peptides, the presence of hydrophobic amino acids such as leucine, valine, and proline is common, which contributes greatly to their potential antioxidant activity [42.110] and has a major role in the inhibition of lipid peroxidation [42.107]. Taurine (2-aminoethanesulfonic acid) is also an amino acid with potential interest for humans since it is mainly obtained through diet. The highest amount of this amino acid is found in seafood, namely in shellfish, especially scallops, mussels, and clams [42.111]. Taurine has the ability to conjugate bile acids, regulate blood pressure, act as an antioxidant and an anti-inflammatory agent [42.111] and has been associated with several positive effects on the cardiovascular system. According to *Elvevoll* et al. [42.112], supplementation with omega-3 FAs and taurine improved the blood lipid profiles to a greater extent than those supplemented with omega-3 FAs alone (one group ($n = 39$) received fish pâté ($36\,g\,d^{-1}$) enriched in omega-3 FAs (1.1 g EPA + DHA/day) and another group ($n = 41$) received an identical pâté enriched both in omega-3 FAs and taurine ($425\,mg\,d^{-1}$) for 7 weeks).

42.3.3 Fatty Acids

Phospholipids and glycolipids are the major classes of lipids found in algae, accounting for only 1–5% of cell composition [42.23]. Since at lower temperatures algae are able to accumulate PUFAs, species found in cold regions contain more PUFAs than those in moderate climates. PUFAs are known to regulate a variety of body functions such as blood pressure, blood clotting, and the proper development and function of brain and nervous system [42.113], and also play an important role in regulating inflammatory response through the production of inflammatory mediators termed eicosanoids [42.114]. PUFAs account for almost half of the lipid fraction, with much of it occurring in the form of omega-3 and omega-6 fatty acids such as EPA and arachidonic acid (ARA) [42.115]. The predominant FA in various seaweeds is EPA (C20:5, n-3), which is at concentrations as high as 50% of the total fatty acid content [42.28]. Red and brown algae are rich in omega-3 FAs (EPA and α-linolenic acid), omega-6 FAs (ARA and α-linoleic acid), along with relatively high levels of oleic and palmitic acids [42.116], whereas green seaweeds are more characterized by the presence of hexadecatetraenoic, oleic, and palmitic acids. EPA and DHA are found at high levels in various species of macroalgae but also in microalgae with relatively high oxidative stability compared to fish oils [42.117]. Under optimal culture conditions *Chlorella minutissima* can produce up to 45% of its total content of FAs and several species of diatoms, and dinoflagellates such as *Nitzschia frustulum*, *Navicula incerta*, and *Gymnodinium simplex* also have a high content of EPA and DHA [42.38]. High levels (2–30%) of long-chain omega-3 PUFAs can be found in tissues in many marine fishes mainly due to a transfer through the aquatic food chain with a good content of EPA and DHA. The liver of lean white fish such as cod species, and muscle from herring and mackerel are some examples of good sources of marine functional lipids [42.43]. Marine lipids and the long-chain omega-3 PUFAs are extensively reviewed by *Larsen* et al. [42.118].

It is important from a nutritional standpoint that an appropriate balance between omega-3 and omega-6 is maintained, since these FAs work together to promote health; most omega-6 FAs (precursors of arachidonic acid and prostaglandin E2) tend to promote inflammation and tumor growth [42.119], hence the intake of macroalgae and microalgae rich in omega-3 fatty acids would help to achieve this balance. *Van Ginneken* et al. [42.120] studied the efficiency of using marine macroalgae as a source of PUFAs and found a very low content of omega-6 fatty acids (only 3%) and, therefore, a very low omega-6 : omega-3 ratio compared to that indicated by experimental evidence as being optimum from a human body homeostasis point of view (ratios of 4 : 1–5 : 1 and no higher than 10 : 1 are suggested); this achievement is important and evidence that seaweeds lipids contents may help the prevention of inflammatory, cardiovascular, and neural disorders.

42.3.4 Pigments

Carotenoids, phycobiliproteins, and chlorophylls comprise the major categories of photosynthetic pigments that can be found in marine organisms [42.38]. The biological importance of macroalgae and their pigments can be found in *Gamal* [42.35] and *Pangestuti* and *Kim*'s [42.121] reviews.

Carotenoids are a group of pigmented compounds that act as a light energy harvester in photosynthetic marine organisms and are potent antioxidants that inactivate reactive oxygen species. Carotenoids include, for example, fucoxanthin and β-carotene in brown seaweeds; green seaweeds possess β-carotene, lutein, and violaxanthin, red seaweeds contain, for example, β and α-carotene and lutein, whereas astaxanthin is found in salmon [42.26, 118, 122, 123]. Some of the most important carotenoids are β-carotene, fucoxanthin, and tocopherol [42.23]. Fucoxanthin, present in species such as *Undaria pinnatifida* (Wakame), *Hijikia fusiformis* (Hijiki), *Laminaria japonica* (Ma-Kombu), and *Sargassum fulvellum* (Phaeophyceae), has been indicated to reduce obesity by inhibiting intestinal lipase activity [42.124] as well as to possess antiangiogenic and antiviral properties [42.123]. Fucoxanthin has also been shown to have an important antioxidant activity, as well as antidiabetic, antiphotoaging, and anticancer properties; these and other health properties of fucoxanthin were recently reviewed by *D'Orazio* et al. [42.122]. Based on the plethora of properties D'Orazio inclusively named fucoxanthin the treasure of the sea. Xantophylls, another type of carotenoids are the main pigment found in brown seaweed, namely in *Undaria pinnatifida*, and have been related with some activity against cerebrovascular diseases [42.125]. The biological functions and commercial applications of marine carotenoids can be found in the review by *Vílchez* et al. [42.126].

Phycobiliproteins are classified as protein–pigment complexes (red, phycoerythrobilin; purple–deep blue,

phycocyanobilin) found in algae, which are used as natural color pigments in food products [42.38].

Chlorophylls are green pigments that are used as natural colorants in foods and beverages in the food industry [42.38] and have been shown to possess some biological activity whereby they exhibit anticancer properties. In an era where the trend to natural products prevails in food product development, the use of these compounds as natural colorants instead of their synthetic counterparts is an effective alternative.

42.3.5 Phenolic Compounds

Polyphenols or phenolic compounds are found at high levels in algae that have very strong antioxidant properties and also other biological activities like anti-inflammatory or antimutagenic ones. In algae, these compounds are associated with chemical defenses to external conditions such as stress and herbivores [42.127]. Seaweed species such as *Undaria* sp., *Laminaria* sp., and *Fucus* sp. (Phaeophyceae, brown algae), *Porphyra* sp. (Rhodophyta, red algae) are rich sources of polyphenolic compounds [42.26]; cathechins, flavonols, and phlorotannins have been explored as functional food ingredients [42.118, 128].

The phlorotannins that have been identified in several brown algae are highly hydrophilic compounds with antioxidant, anti-inflammatory, antidiabetic, antitumor, antihypertensive, and antiallergic activities, and are inhibitors of hyaluronidase and of metalloproteinase enzymes, two virulence factors that may be expressed by some microorganisms [42.22, 129].

42.3.6 Minerals

It is well known that seaweeds are recognized as being rich in minerals (Ca, Mg, Na, P, and K) and trace elements (Zn, I, and Mn), which is related to their capacity to retain inorganic marine substances due to polysaccharides in the cell surface, accounting for up to 36% of dry matter in some species [42.26]. They are one of the most important sources of calcium and potassium [42.115], and some seaweeds are indicated to be used as food supplements to meet the recommended daily intake of some minerals and trace elements.

Selenium, iodine, and also zinc, magnesium, and calcium are other minerals that are more abundant in some seafood than, for example, meat [42.118]. Increased dietary intake of selenium has been related to protection against several cancers. In some countries or populations with low iodine intake, the seaweeds are an alternative way to deliver iodine trough diet instead of fortified salt with iodine. Seaweeds are one of the best natural sources of iodine [42.16].

42.4 Functional Foods Incorporating Marine-Derived Ingredients

As discussed throughout this chapter, marine resources are indeed a source of high value-added compounds with biological properties to be used as functional food ingredients. Several types of polysaccharides, such as sulfated polysaccharides, chitin or chitosan, proteins and protein hydrolysates, peptides, amino acids such as taurine, omega-3 oils, carotenoids, and other bioactive compounds [42.30] are examples of compounds that can be added at different stages, from processing to storage, of the food production process [42.11]. Marine-derived functional ingredients with biological properties constitute a research area with much to explore for food purposes. On the other hand, many foods already widely consumed all over the world are a potential vector for incorporating new marine-derived functional ingredients, which will enhance their value at both the nutritional and economic levels (Fig. 42.2). Since dairy products are widely accepted by consumers, the use of this type of product to deliver bioactive compounds has received attention from the food industry in the last years [42.130, 131]. Functional foods and natural health products are an emerging field in food science due to their increasing popularity with health-conscious consumers and are a source of new opportunities for the agri-food sector. In several countries of the EU, these types of products are already part of an industry that is already generating millions of euros per year. Food products containing marine-derived chitin, chitosan, as well as oils rich in omega-3 fatty acids, are some food products that are being commercialized in several markets around the world including Japan, the USA, and some European countries [42.30].

The consumption of functional foods can provide various nutritional/health benefits, since diet controls and modulates many functions of the body, maintaining its good health or homeostasis. Enhancement of immunity stimulation and antioxidant activity are the most studied health benefits and have driven con-

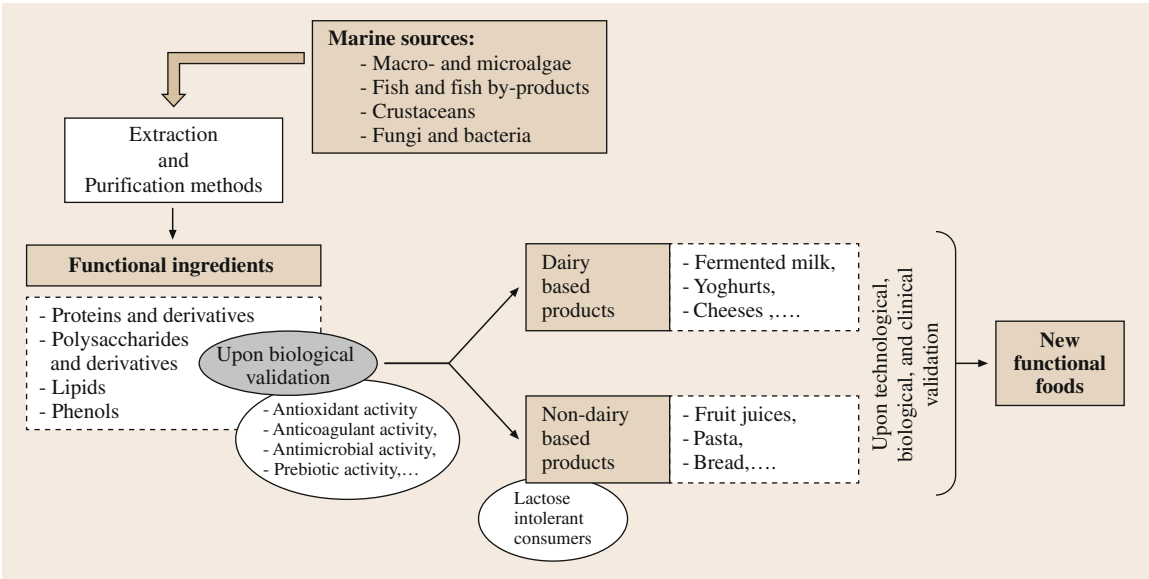

Fig. 42.2 New potential functional foods based on dairy or non-dairy products via incorporation of functional marine-derived ingredients

sumers to be more aware that diet can serve both nutrition and health promotion goals [42.11]. Nowadays, marine-derived functional ingredients such as fish oils, fish proteins, and seaweeds themselves have found application in bakery, dairy, confectionary, and pasta products. They are added as fortificants and nutritional enrichments in food, leading to the so-called functional foods. According to *Kadam* and *Prabhasankar* [42.30], more and constant efforts in research and design of novel functional foods based on marine functional ingredients is needed to contribute to the reduction of health problems through diet. Despite the scientific interest in the use of marine-derived food ingredients, there still are various challenges ahead that have to be overcome to design new functional foods, namely:

i) Efficient extraction methods and purification steps, to obtain food grade validated extracts or purified compounds with biological properties (antioxidant, antibacterial, prebiotic, and others). Isolated functional ingredients should rely upon food methods compatible with economically viable yields. Hence, different extraction methods must be applied in order to maximize the extraction efficiency of functional ingredients with biological properties.
ii) To design functional foods based on the incorporation of marine-derived functional ingredients upon biological validation. According to *Siegrist* et al. [42.132], consumers are more and more inclined to buy functional foods with physiological health claims.
iii) Foods should have good sensorial characteristics in order to be accepted by the consumer. In general consumers are not able to compromise taste for health [42.133]. This is, in fact, one of the most important challenges to overcome in the case of some of the marine compounds to be used, for example, fish oil.

42.4.1 Foods Incorporating Marine Organisms: Seaweeds

Dietary minerals (sodium, potassium, and iodine), fibers, as well as polyphenolic compounds are some compounds in which seaweeds are rich. Thus making use of seaweeds or their extracts to supplement foods of particular interest [42.17] enables the development of new functional foods from a nutritional standpoint. The possibility of adding seaweeds to food as natural antioxidants, antimicrobial and texturing agents, are other aspects of significant importance from an industrial point of view [42.17].

Meat and meat products, which are a good source of protein and vitamins but deficient in fibers and, in general, contain excess sodium are good candidates to

be supplemented with seaweeds to overcome the technological problem associated with low-salt meat products [42.17, 134]. According to *Cofrades* et al. [42.134] due to consumer awareness of the negative perceptions of meat in terms of overall fat content, in particular saturated fatty acids, cholesterol, and sodium, the meat industry should adapt to new meat functional foods to address consumer concerns without making radical changes in their eating habits. Several studies based on the formulation of meat-based functional foods incorporating marine compounds have been reported, some of which are described in Table 42.1. In general, all studies presented as main objective the development of low-salt meat products with improved nutritional profile by addition of different species of seaweeds [42.135–137]; some of these studies go one step further in terms of also reducing fat content by replacement with olive oil [42.138] or with konjac gel [42.139]. The major results reported in these studies are encouraging, and according to *Confrades* et al. [42.136], incorporation of seaweeds allows reformulation strategies to design and develop functional meat products with lower sodium, fat, and cholesterol content, and simultaneously with relevant contribution to the intake of dietary fiber, polyphenols, minerals, and unsaturated FAs acids. Technologically, the incorporation of seaweeds enables overcoming some of the negative sensorial impacts caused by low levels of sodium related to water-binding and fat-binding properties. In terms of organoleptical properties, some of the functional meat products developed have attained sensorial acceptability, but this is a subject that should be evaluated in a well-based sensorial analysis.

Pasta products incorporating seaweeds have also been developed and two studies thereof are described in Table 42.1. In fact, the design of functional foods based on incorporation of marine ingredients such as seaweeds, has been more successful in bakery and pasta products and was recently reviewed by *Kadam* and *Prabhasankar* [42.30]. For example the incorporation of 10–20% edible seaweed wakame (*Undaria pinnatifida*) in pasta has not only received sensorial acceptance but has also resulted in a product with improved amino acid and fatty acid profiles, an increase of antioxidant activity, and a higher content of fucoxanthin (concentration and activity were not affected by processing) and fucosterol [42.141]. Dietary ingestion of wakame has also been reported to reduce blood pressure [42.31].

According to *Gupta* and *Abu-Gannam* [42.17], seaweeds are currently receiving considerable consideration for the nutritional enrichment of meat, bakery, and pasta products because, as was previously demonstrated, the addition of seaweeds or seaweed extracts can not only improve the quality of the product but may even enhance its safety at high doses with respect to natural antioxidants and antimicrobials.

42.4.2 Foods Incorporating Marine-Derived Ingredients: Polysaccharides

Depending on their chemical composition and structure, complex marine-derived polysaccharides can possess several biological properties, as was described above. Therefore, their incorporation in food matrices is of potential interest to develop new functional foods. In Table 42.2 lists some studies based on the incorporation of marine-derived polysaccharides as functional ingredients such as alginates and carrageenan in bread, meat, or liquid foods. New types of healthier breads, especially gluten-free bread, a rising market due to the increasing diagnoses of celiac disease among adult individuals [42.142], has been a challenge to bakers and scientists. Agar-agar and carrageenan are two hydrocolloids that can be used as thickening, swelling, stabilizing, or humectant agents which can be used in gluten-free baking [42.142]. Different types of bread with the incorporation of sodium alginate and carrageenan has been attempted and investigated in terms of baking properties [42.143–145]. In general, marine derived polysaccharides are able to impart acceptable textural and sensorial characteristics. However, none of these studies carried out any nutritional evaluation that could enhance some healthy aspects due to the incorporation of marine-derived polysaccharides. Meat sausages as well as milk and apple juice were other food matrices tested with supplementation of carrageenan, chitosans, and chito-oligosaccharides (Table 42.2). Satisfactory technological and sensorial results were achieved by *Ayadi* et al. [42.146], but again no nutritional evaluation was performed. *Fernandes* et al. [42.147] studied the incorporation of commercial crab shells chitosans and chito-oligosaccharides in apple juice and milk to ascertain the influence of food components on their antimicrobial activity, evaluating in parallel the acceptance by a sensory panel. Once again, no nutritional evaluation was considered. In Japan, several foods (potato chips, soybean paste, and noodles) with added chitosan are available as cholesterol-lowering functional foods [42.148].

Results reported by *Fernandes* et al. [42.147] are an example of the difficulties that need to be overcome in

Table 42.1 Functional foods based on incorporation of seaweeds

	Product	Seaweed	Description	Major achievements	Sensory Characteristics	Reference
Meat	Frankfurter sausages	*Himanthalia elongata*	Low-fat frankfurters added with 5.5% seaweed	Addition of seaweed provided Ca-rich, low-Na sausages, better Na/K ratios, and a higher fiber content	No sensory analysis presented	[42.135]
			Low-fat, low-salt frankfurters added with konjac glucomannan gel (0–19.3%) and seaweed (0–3.3%)	• Incorporation of combination of konjac gel and seaweed increased cooking loss and reduced emulsion stability • Replacement of pork fat, reducing fat content in 15%, did not impart noticeable changes in the sensory quality of the frankfurters	• No significant differences among samples in terms of texture • No noticeable changes in sensory quality as a consequence of fat reduction and fat replacement • Samples with added seaweed with low score in terms of overall acceptability	[42.139]
	Beef patties	*Undaria pinnatifida*	Low-salt and low fat beef patties added with 3.3% of seaweed and partial fat replacement with olive oil	• Patties with seaweed presented lower thawing and cooking losses and were softer • Incorporation of seaweeds increased mineral contents but imparts similar Na/K ratio • Good technological, sensorial and nutritional properties in beef patties with seaweed and olive oil emulsion	• Freezing did not affect sensorial parameters studied • Total replacement of fat by olive oil emulsion improved appearance • Incorporation of seaweed affected the sensory properties	[42.138]
	Restructured poultry steaks	*Himanthalia elongata*	Low-salt restructured poultry with microbial transglutaminase and seaweed (3%)	• Addition of a cold binding agent (MTGase/caseinate) did not affect water binding properties • Incorporation of seaweed caused a slight increase in purge loss but reduced cooking loss • Addition of both seaweed and binding agent increased the Kramer shear force	• Addition of seaweed and MTGase/caseinate significantly affected most sensorial parameters • Flavor acceptability, juiciness, and general acceptability scored with values higher than 5 • Low-salt restructured products with seaweed and MTGase/caseinate were considered sensorial acceptable	[42.136]
	Pork patties	*Laminaria japonica*	Reduced-fat pork patties with added seaweed (0–5%)	Reduced-fat pork patties with seaweed had significantly higher moisture, ash, carbohydrate content, and lower protein and fat content, cooking loss, hardness than regular-fat control samples	Pork patties with fat content reduced to 10% and supplemented with 1–3% seaweed has improved quality characteristics, similar to control patties with 20% fat content.	[42.137]
Pasta	Fresh noodles	*Monostroma nitidum*	Noodles added with 4–8% seaweed powder with or without eggs	Addition of seaweeds increased crude fiber content, higher cooking yields and decreased springiness and extensibility	Sensory scores varied with the parameters evaluated, percentage of seaweed and addition of eggs; Higher scores for color and wetness were obtained in egg noodles with 8% of seaweed	[42.140]
	Pasta	*Undaria pinnatifida*	Pasta prepared with semolina and seaweed blends: 100 : 0, 95 : 5, 90 : 10, 80 : 20, and 70 : 30	• Fucosterol and fucoxanthin content higher in pasta with seaweeds, which were not affected by cooking step • Improved nutritional, amino acid, and FA profiles by incorporation of seaweed.	Pasta with 10% seaweed was considered acceptable sensory-wise with mild seaweed flavor tasting similar to control pasta	[42.141]

Table 42.2 Functional foods based on incorporation of marine-derived polysaccharides

	Product	Marine-derived polysaccharide	Description	Major achievements	Sensory characteristics	Reference
Bread	Bread	Sodium alginate κ-carrageenan	Bread produced with different hydrocolloids of 0.1–0.5% (flour basis)	• All hydrocolloids were able to reduce loss of moisture content during bread storage, reducing dehydration rate of crumb • Higher moisture content and specific volume index in bread with k-carragenaan than with sodium alginate • Alginate showed antistaling effect retarding the crumb hardening • No nutritional evaluation	• Bread with 0.5% alginate were scored higher in terms of visual appearance, crunchiness and overall acceptability than control bread or bread with 0.1–0.5% κ-carrageenan	[42.140]
	Wheat dough, Chinese steamed bread	Sodium alginate	Wheat dough Chinese steamed bread added with sodium alginate (0.2 w/w %) and konjac glucomannan (0.8 w/w %)	• Addition of alginate sodium and konjac glucomannan produced dough with rigid and weak network • Lower spread ratio and specific volume Chinese steamed bread with addition of alginate sodium and konjac glucomannan but softer and more resistant to staling through storage than control samples • No nutritional evaluation	• No sensory analysis presented	[42.141]
	Wheat Bread	Carrageenan	Wheat bread added with enzymes and hydrocolloids	• Lower volume and specific volume was observed in bread with carrageenan (0.075% flour basis) but higher when conjugated with xanthan gum (0.15%, flour basis) than in standard bread • Lower water losses in bread with carrageenan alone or mixed with xanthan gum than in standard bread • No nutritional evaluation	• Higher scores for sensorial properties (taste, color, shape, aroma, elasticity, hardness) in bread with carrageenan, especially when mixed with xanthan gum in comparison to standard bread	[42.143]
Meat	Sausages	Carrageenan	Turkey meat sausages with addition of carrageenan (0–1.5 w/w%)	• Carrageenan causes a decrease in emulsion stability but an increase in water holding capacity, hardness, and cohesiveness • Addition of 0.2–0.5% of carrageenan increases gel elasticity; higher percentage of carrageenan causes opposite effect • Higher percentage of carrageenan leads to a progressive appearance of an additional carrageenan gel network • No nutritional evaluation	• Addition of carrageenan did not cause significant effect in taste but improves sausage appearance and texture	[42.144]

the development of new functional foods. The antibacterial effect against pathogenic *Staphylococcus aureus* and *Escherichia coli* was dependent on the Gram nature of the bacteria the chitosans' molecular weight, yet their addition to apple juice led to some unpleasant off-flavors which steadily increased in intensity with the chitosan molecular weight (Table 42.2). In fact, the incorporation of marine ingredients in foods of different nature could lead the consumer to expect a negative influence on food [42.133]. The importance of the food vector has been shown in many studies and, indeed, a high correlation between the food vector and the origin of the functional ingredient is required in order to promote the acceptance of functional food [42.132].

Table 42.2 (continued)

	Product	Marine-derived polysaccharide	Description	Major achievements	Sensory characteristics	Reference
Beverage	Milk, fruit juice	Chitosans chito-oligosaccharides (COS)	Cow milk and apple juice added with 0.5 w/v % chitosan with low to high molecular weight (MW) and COS lower than 3 and 5 kDa	• Antimicrobial effectiveness against to *Escherichia coli* and *Staphylococcus aureus* by medium and high Mw chitosans when incorporated in milk and apple juice, COS lost their antimicrobial activity upon both bacteria after 4–8 h in milk • Chitosans and oligomers are more effective as food preservatives in low pH foods and with low protein content • No nutritional evaluation	• Addition of chitosans to apple juice causes unpleasant off-flavors: astringency and after taste especially for higher MW • No sensorial analysis was performed on milk added with chitosans and COS because milk become unsuitable for consumption	[42.147]

42.4.3 Foods Incorporating Marine-Derived Ingredients: Fish Oils and Fatty Acids

Due to the richness of marine-derived lipids in FAs such as long-chain omega-3 PUFAs, many attempts have been made worldwide over the past years to enrich foods such as bread and other bakery products, dairy products such as cheese and yoghurts, as well as nutrition bars, with fish oils [42.149–151]. Most nutritional guidelines nowadays include a recommendation for higher intakes of long-chain omega-3 PUFAs for preventing cardiovascular diseases; however, their dietary intake remains low, particularly in Western countries [42.152]. According to some dietary guidelines, two servings of fish of approximately 227 g should be consumed per week to reduce the risk of mortality from coronary heart disease [42.152]. Due to the higher content in EPA, α-linolenic acid (ALA), and DHA in foods enriched with fish oils, these products are considered functional foods that lead to the reduction of cardiovascular diseases, cancer, and arthritis [42.30, 152] and are, in fact, an alternative way of increasing their intake. However due to the high instability of long-chain omega-3 FAs to oxidative deterioration, the enrichment of foods with long-chain omega-3 PUFAs is still technologically challenging.

In Table 42.3 several examples of foods enriched with fish oil or FAs are given. Cereals and dairy-based products, as well as beverages and salad dressings were enriched with neat oil, microencapsulated, or even as an oil-emulsion [42.149–151, 153–157]. The oxidation and appearance of off-flavors such as fishy off-flavor was variable and dependent on the food matrix. This was particularly evident in the study performed by *Let* et al. [42.157], who studied the enrichment with neat fish oil or with oil-emulsion of three food products: milk, yoghurt, and salad dressing. According to the authors, the application of neat fish oil was a good option for preserving the quality of yoghurt and salad dressing, whereas pre-emulsion may still be considered for the fish oil enrichment of products such as milk. *Let* and co-workers have published several research studies over the last years related to attempts to enrich milk with fish oil [42.158–164]. In these studies, several approaches were attempted and should be researched further to overcome the low oxidative stability of long-chain omega-3 PUFAs; according to the main achievements of previous studies, possible solutions may be based on the use of appropriate combinations of emulsifiers and stabilizers, homogenization of milk, or by pursuing encapsulation procedures [42.130, 152, 157, 162].

In the field of dairy products, cheeses have also been the target of enrichment with fish oil, unfortunately not all results have been acceptable for the consumer [42.154], nor were the cheeses analyzed in terms of sensorial characteristics [42.155]. However, results published by *Hughes* et al. [42.150] are promising for a soft goat cheese in which negligible oxidation occurred, and cheeses enriched with 60 g fish oil per batch were acceptable to the consumer (Table 42.3). Cereal based products like oat and soy bars and spaghetti seem to be the more suitable food products to deliver long-chain omega-3 PUFAs to the consumers [42.151, 153] with negligible levels of oxidation. According to *Hughes* et al. [42.151] consumer acceptable nutritional cereal bars have been successfully enriched with non-encapsulated, non-emulsified tocopherol-enriched fish oil, oxidatively stable over 10 weeks of storage, able to

Table 42.3 Functional foods based on incorporation of fish oils or FAs

	Product	Fish oil, FAs	Description	Major achievements	Sensory characteristics	Reference
Cereals	Nutrition bar	Purified, non-emulsified, tocopherol-enriched fish oil	Oat and soy-based nutrition bars (35 g serving) with added fish oil replacing canola oil in 0–60%	• No significant differences in terms of proximate composition, water activity, or ALA content • Higher content of EPA and DHA: bars with 20% fish oil with 178.1 mg/serving of EPA and DHA • Negligible oxidation in all bars over 10 weeks of storage	No significant differences in aroma, texture, flavor, and overall acceptability between cereal bars with 20% fish oil and control cereal bars	[42.151]
	Spaghetti	Microencapsulated refined marine oil containing a minimum of 30% EPA and DHA	Spaghetti enriched with 0.6, 1.2 and 1.8% (flour basis) of microencapsulated oil	• Higher fat, EPA, and DHA in spaghetti enriched with microencapsulated oil • Spaghetti with 1.2% microencapsulated oil had lower loss of EPA and DHA (<10%)	• Spaghetti with 1.2% of microencapsulated oil had higher score in terms of acceptability	[42.153]
			Spaghetti enriched with 1.2 (flour basis) of microencapsulated oil	• Functional spaghetti had a shelf-life comparable to control pasta • Light exposure cause a fast propagation of oxidation regardless the fat content and PUFA in functional spaghetti; packaging based on paperboard is recommendable • Microencapsulation provided effective protection against deterioration of EPA and DHA	• No sensorial analysis presented	[42.149]
Beverage	Fruit juice	DHA from microalgae	Water-fruit juice concentrate enriched with 0.08 g/100 g of DHA	• Oxidatively stable over 20 days of cold storage • Fatty acidity of fatty phase in acceptable range over 30 days of cold storage	• Appearance of extraneous fishy taste and flavor • Some color change in juices containing DHA supplemented	[42.154]
Dairy product	Soft goat cheese	Purified fish oil	Cheese fortified with 60, 80 and 100 g of fish oil per cheese batch	• Fat, EPA and DHA contents higher in enriched cheese samples with fish oil • EPA and DHA content did not differ significantly in enriched samples, independently of the fish oil addition • Average of 127 mg of EPA and DHA per 28 g serving • Negligible oxidation over 4 weeks cold storage	• No significant differences in appearance, color and aroma between enriched and control cheeses • Lower values were observed for taste and overall acceptability in enriched cheeses; best scored cheese with 60 g fish oil per batch	[42.150]

Table 42.3 (continued)

	Product	Fish oil, FAs	Description	Major achievements	Sensory characteristics	Reference
Dairy product	Soft fresh Hispanic cheese	Cod liver oil	*Queso fresco* enriched with 1 w/w % fish oil prepared from unprocessed milk (pressure = 0 MPa)	• Moisture and fat content did not differ significantly between cheeses fortified with fish oil and control cheese • Increase of peroxide value in cheese with fish oil through 30 days of cold storage • Hardness, cohesiveness, and gumminess values did not differ significantly between cheeses fortified with fish oil and control cheese	No sensorial analysis was presented	[42.155]
	Cream cheese	Cod liver oil	Cream cheese enriched with 1.3% fish oil as neat oil or pre-emulsified	Lipid oxidation increased over 20 weeks of cold storage in cream cheese enriched with fish oil added as neat oil or pre-emulsified	Unacceptable sensory perception of the fish oil enriched cream cheeses in the later part of storage period	[42.154]
	Milk	Refined cod liver oil	Pasteurized milk enriched with 1 v/v % net fish oil or 2% (by weight of final product) fish-oil-in water emulsion	Milk enriched with oil emulsion had higher values of peroxide values than milk enriched with net fish oil after 10 days of storage at 5 °C	Both milk enriched with oil emulsion or oil emulsion presented high levels fishy off-flavor after 29 days of storage	
	Yoghurt		Yoghurt enriched with 1 w/w % net fish oil or 2% (by weight of final product) fish-oil-in water emulsion	• Yoghurt enriched with oil emulsion had higher values of peroxide values than milk enriched with net fish oil after 20 days of storage at 5 °C • Yoghurt could be a suitable system for fish oil enrichment due to its high oxidative stability	Higher levels of levels fishy off-flavor in yoghurt enriched with oil emulsion than with net fish oil after 29 days of storage	[42.157]
Other	Salad dressing		Dressing enriched with 10 w/w % net fish oil or 20% (by weight of final product) fish-oil-in water emulsion	Dressing enriched with net fish oil had higher values of peroxide values than milk enriched with oil emulsion after 25 days of storage at room temperature	Higher levels of levels fishy off-flavor in dressing enriched with oil emulsion than with net fish oil after 29 days of storage	

deliver 178 mg of EPA and DHA per 35 g serving. According to *Kadam* and *Prabhasankar* [42.30], bakery and pasta products are the best food products to incorporate marine functional ingredients because they are widely consumed all over the world.

The enrichment of food products with long-chain omega-3 PUFAs can also be achieved indirectly by feeding animals, namely chickens and cattle, with marine sources such as macroalgae or microalgae, or fish oil. Omega-3-enriched eggs are an example of this approach and are already well commercialized in several countries around the world. *Carrillo* et al. [42.165] reported higher contents of long-chain omega-3 PUFAs in eggs laid by hens fed with marine algae and sardine oil. A higher DHA content was found in broilers fed on DHA-rich microalgae [42.166].

42.5 Current Understanding and Future Trends

Marine organisms and the hundreds of tons of marine by-products available annually are, indeed, tremendous sources for many new value-added healthy food ingredients and biologically active compounds constituting a research area with still much to explore for food purposes. On the other hand, many foods that are already widely consumed all over the world are a potential vector for incorporating new marine-derived functional ingredients that will enhance their value at both the nutritional and economic levels. Therefore, more research needs to be pursued to investigate marine resources as potential sources of functional food ingredients, as well as to develop new functional foods via supplementation of marine-derived ingredients. Functional foods as described are food products to provide physiological benefits or reduce the risk of chronic disease but to be consumed as part of a balanced diet. Seafood diets and marine-derived ingredients have been proven to prevent and protect against many diseases, namely the so called epidemic lifestyle diseases [42.40] such as cardiovascular diseases, obesity, and diabetes, among others. Therefore, it is of utmost importance that more efforts should be made to confirm health evidence regarding the health-promoting effects of marine functional foods accompanied by proper communication to the consumers. In fact, overall consumer interest in new healthy food ingredients is driving research and commercialization in the area of functional foods, and marine functional foods are no exception to this trend. Nevertheless, the introduction of marine-derived ingredients in the human diet will always be a complex subject due to several types of constraints that cover not only global issues including cultural and ethnic aspects such as diet type and habits or environmental aspects such as fears about sea pollution and the use of sources in a sustainable manner (leading to a more efficient utilization of the sea's natural resources), but also more specific aspects such as the organoleptic properties of food and legislation in itself [42.11]. Whereas edible seaweeds are a product with a very long tradition in human eating habits in China, Korea, Japan, and the USA, in Europe some countries still present legal obstacles, which will most certainly delay approval [42.28]. On the other hand, consumer acceptance of new functional foods with marine-derived functional ingredients will undoubtedly depend on the balance between cultural habits and diet, knowledge and perception about health benefits of functional food, and sensorial characteristics.

Despite the already commercially available marine-derived ingredients and functional foods, the search for new marine-derived products continues to be a challenge both for scientists and food engineers. The search and use of marine-derived functional ingredients demands appropriate and sustainable harvesting, and that extraction of compounds with bioactive potential be based on food grade compatible, efficient, and sustainable procedures followed by procedures to obtain concentrated extracts or purified compounds in a controlled manner. The validation of biological properties of extracts and/or purified functional marine-derived ingredients is determinant for the development of new functional foods based on their incorporation in food matrices, which in turn should be followed by characterization thereof and assessment of stability and in vitro bioavailability of functional ingredients; the proof of the specific health benefits through human clinical trials is also an important requirement.

The design of functional foods based on the incorporation of marine ingredients has been more successful in bakery and pasta products, for example, fish oils that are rich in omega-3 oils are already used in bread and other products, which shows a considerable improvement in EPA and DHA contents, which helps to prevent the risk of cardiovascular disease. However, this scenario goes beyond this type of product, including dairy products and others, but much can still be done to develop new and pleasant marine functional foods.

Legislation is another bottleneck for new functional foods with marine-derived functional ingredients, since it is continuously changing or being updated with the publication of new regulations, and in some aspects there are very complex measures and requirements to be applied in any particular country. Marketing food ingredients with health benefits now requires proper scientific substantiation; for example, in Europe it is now necessary to provide evidence of any benefit before any health claim can be made on a functional food product [42.10]. The European Food Safety Authority is the legal authority in Europe responsible for managing novel food and respective health claims, whereas China, Japan, Brazil, and the USA has their own individual regulatory authorities and laws regarding functional foods and ingredients and respective health claims [42.10]. In addition, commercialization of functional foods or bioactive compounds with health claims entails an extensive scientific dossier necessary to prove scientific evidence via human clinical trials,

as was previously mentioned, which is highly expensive [42.28], and the targeted market niche may not be enough to cover the economical investment.

Nonetheless, there is still a sea of opportunities to be explored, some of which have been duly covered in this book chapter. The coming decades will continue to see efforts toward a sustainable exploitation and management of marine resources and by-products for the production of value-added bioproducts to be used by the food industry (among others); an interesting and challenging time for scientists, engineers, and formulators in this field in anticipated.

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43. Marine Nutraceuticals

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Marine resources are gaining much attention as a gold mine of biologically active materials with healing power. Most of these metabolites have shown unique structural and functional features compared to their terrestrial counterparts. With the understanding of the requirement and the benefits of nutraceutical supplementation to prevent increasing incidences of life-style-related diseases, marine nutraceuticals has gained high demands worldwide. Among large number of marine nutraceuticals, omega-3 fatty acids, carotenoids, chitin oligosaccharides, glucosamine, collagen, and fucoidan are popularly consumed. This chapter presents a brief review of the beneficial effects of these marine nutraceuticals and their market positioning. Moreover, the need for research and development in this area to overcome the market challenges is also discussed.

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43.1 Marine Bioactives as Potential Nutraceuticals

Even though the popularity for health-promoting foods both in the common society and in the research field has raised recently, the concept drove back to thousands of years, which was well explained by the Hippocrates 2500 years ago as *let food be thy medicine and medicine be thy food*. Asian countries such as China, Japan, Korea, India, and Indonesia have used both terrestrial and marine resources as medicinal foods and traditional medicines. However, the awareness among the Western population has emerged much recently. With increasing incidences of life-style-related diseases such as cardiovascular disease, obesity, diabetes, and cancer have thrown a direct challenge to the food habits. While exploring the methods of disease prevention, use of nutraceuticals or functional foods was highly recommended. The term nutraceutical was coined by Stephen DeFelice, founder and chairman of the Foundation for Innovation in Medicine, USA in 1989 to define substances with *nutrition* and *pharmaceutical* values attached [43.1]. According to his definition a nutraceutical is any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and/or treatment of disease. Even though the term nutraceutical is being commonly used in the marketing, strict regulatory definition has not been adapted. The awareness and understanding of the health-promoting abilities of edible natural materials beyond their nutritional benefits, paved the way to a highly growing market for nutraceuticals, worldwide. Vast array of products such as dietary supplements, purified nutrients or food ingredients, herbal products, processed, and genetically

engineered products are marketed worldwide under the category of nutraceuticals. The admiration toward nutraceuticals is increasing as they are safe materials to be consumed with significantly less or no side effects compared to synthetic drugs. And also nutraceuticals provides cost-effectiveness over drugs as they are considerably cheaper materials [43.2]. The boundaries between functional foods and nutraceuticals are hard to identify except for their method of intake where functional foods are in a form of a food and nutraceuticals are in a form of a supplement such as capsules or pills [43.3].

Till recent past most of the marketed nutraceuticals are of terrestrial origin. However, currently nutraceuticals originated from marine sources are gaining higher attention worldwide. Marine environment represents great biological diversity compared to that of terrestrial counterpart. This has resulted in marine nutraceuticals with unique features [43.4]. Large number of research evidences show that the marine resources bear a higher potential to be developed as nutraceuticals. Marine nutraceutical could be simply defined as functional ingredients derived from marine sources which show a potent health-promoting activity other than its nutritional value. Omega-3-fatty acids from fish skin and microalgae, functional pigments such as carotenoids, functional polysaccharides such as fucoidan, chitin and glucosamine, collagen, bioactive peptides, and calcium are few popular marine nutraceuticals which have gained much popularity worldwide for their promising functionality. This chapter presents a brief overview of the established marine nutraceuticals in the market with special focus on production and their beneficial effects. Moreover, the potential for the future products, ongoing research and development in this area, and the challenges are also discussed.

43.2 Functional Carbohydrates

Carbohydrates are one of the most widely distributed macromolecules in marine environment and offer unlimited variation in their structure and function with the ability of their monomeric units to connect to one another by many different linkage types. However, the same complexity of structures and the lack of knowledge on the variations have limited the applications of carbohydrates as functional ingredients. Nevertheless, with extensive research on this area it was revealed that the marine carbohydrates bear diagnostic and therapeutic potentials [43.5]. Among the sources of marine polysaccharides seaweeds are widely used to produce bioactive sulfated polysaccharides [43.6]. Moreover, chitin and glucosamine extracted from sea food processing by-products have gained a considerable attention due to their therapeutic potential against adverse physiological conditions [43.7].

43.2.1 Chitosan and Chitosan Oligosaccharides (COS)

Chitin, chitosan, and chitin oligosaccharides (COS) have brought a revolution in marine nutraceutical industry with their wide array of application possibilities. Chitin is abundant natural polysaccharides in terrestrial sources, cell wall of fungi, and insects. It was found that the exoskeleton of crustaceans and other arthropods are a rich source of chitin. This finding led to the utilization of marine food processing by-products, such as shells of shrimp, crab, and lobsters as commercial sources of chitin. Furthermore, the utilization of these crustacean shell walls solves the serious problem of waste disposal in crustacean processing plants as shell waste of crustaceans accounts for more than 45% by weight [43.7, 8]. Chitin is the second most abundant natural polymer after cellulose which is made of *N*-acetylglucosamine arranged with β $(1 \rightarrow 4)$ glycosidic bonds between each monomer [43.9] (Fig. 43.1). Chitin could be deacetylated by the treatment of hot alkali and over 60% deacetylation results the polymer of D-glucosamine monomers which is named as chitosan (Fig. 43.1). Chitin itself has great industrial value owing to its nontoxicity, biodegradability, hydrophobicity, and physiological inertness. For instance, enzyme immobilized chitin is used for clarification of fruit juices and processing of milk in food industry [43.10]. However, applications of chitin and chitosan have limited due to its poor solubility; despite their promising functionality in promoting health. Even though, chitosan soluble in aqueous acid solutions such as formic acid and acetic acid when compared to chitin, both fail to be soluble in water [43.11]. To overcome this limitation low molecular weight COS were produced via chemical (acid hydrolysis) [43.12], physical (ultrasonic degradation, thermodynamic degradation) [43.13], or enzymatic hydrolysis [43.14] of chitosan. It has been reported that COS produced from chemical hydrolysis are not favorable as a bioactive material due to syn-

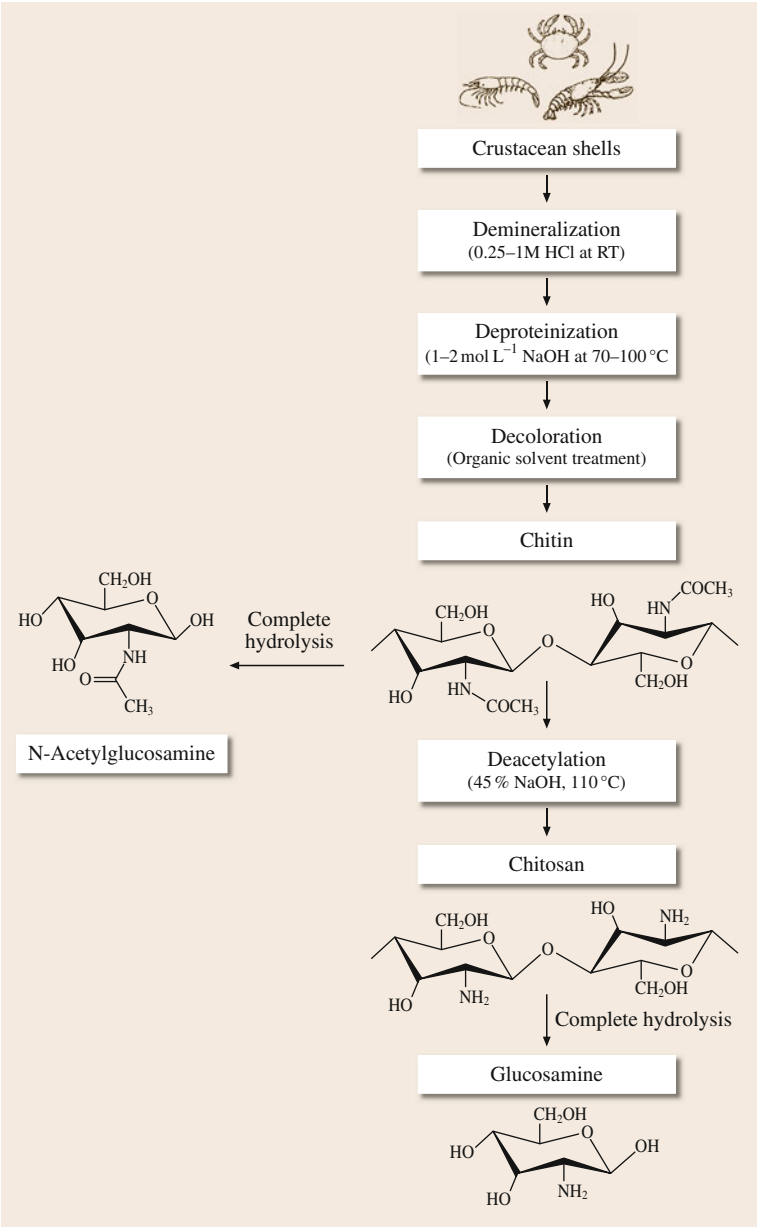

Fig. 43.1 Structures of chitin, chitosan, and glucosamine

thesis of other toxic compounds during the hydrolysis process [43.7]. Therefore, enzymatic hydrolysis for the production of bioactive COS is appreciated. COS have shown a wide array of biological activities. Evidently, COS is more potent and popular as a functional material due to its low molecular weight and water solubility. To enhance the functionality, COS derivatives have been synthesized by introducing structural modifications of COS by adding chemical groups (Fig. 43.2) [43.15]. A wide range of biological actives of chitin and chitosan have been reported. According to the review of *Aranaz* et al. the number of original articles and patents on chitin and chitosan after the year 2000, exceeds 50 000 [43.9]. However in this section, the potentials and applications of these molecules as nutraceuticals are discussed.

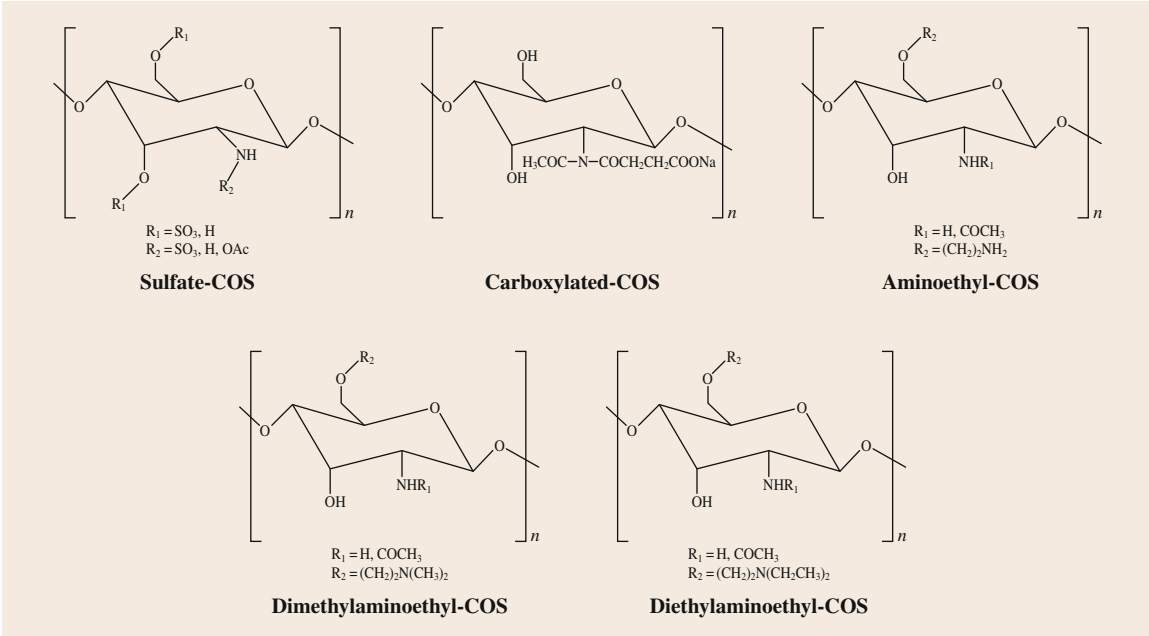

Fig. 43.2 Structures of bioactive chitosan oligosaccharide derivatives

43.2.2 Nutraceutical Potentials and Applications of COS and COS Derivatives

Biocompatibility, biodegradability, and nontoxicity of chitosan have gained growing interest in food and pharmaceutical industry. There have been a large number of studies carried out in the investigation of biological activities of chitosan and COS. They have shown promising activities against intracellular oxidative stress, aging, photo-aging, osteoarthritis, inflammation, asthma, allergy, diabetes, obesity, cancer, hypertension, and microbial infection. Table 43.1 gives an overview of biological activities of COS and its derivatives. The biological activities shown by both chitosan and COS could be attributed to its basic nature with overall positive charge and the molecular structure having reactive hydroxyl and amino groups [43.16]. One common attribute observed throughout these studies is that biological activities are greatly depend on molecular weight and degree of deacetylation of chitosan.

The anticancer and the wound-healing properties of chitosan and its derivatives are explained as their ability to stimulate the immune system. The chitosan-mediated immune-stimulation activates peritoneal macrophages and stimulate nonspecific host resistance followed by the removal of cancer cells [43.16]. In the same manner, the higher levels of macrophage production as a response to chitosan will release cytokines which are involved in the healing process [43.38]. The degree of deacetylation and the molecular weight are key factors for the biological activities of COS. Low molecular weight chitosans (below 5 kDa) with higher degree of deacetylation (90%) are found to be potent radical scavengers and enzyme inhibitors [43.39, 40]. This group of COS has effectively controlled the β-secretase enzyme and Angiotensin-1 converting enzyme activity which plays major roles in the progression of Alzheimer's disease and hypertension, respectively [43.27, 41].

Owing to their biological activities, both chitosan and its oligomers have been researched to develop as active ingredients in functional food and nutraceuticals [43.9, 42]. Chitosan have taken part of multiple formulations due to its promising hypocholesterolemic activity. The mechanism of hypocholesterolemic and antiobesity activity of chitosan and COS is explained as they are able to scavenge fat and cholesterol in the digestive tract and remove it through excretion. The positively charged amine groups on chitosan that could bond with the negative charges on fatty acids or bile acids by ionic interaction facilitate the excretion of fat and cholesterol from diets. The molecular weight (MW) and the degree of deacetylation (DD) are important determinants of hypocholesterolemic activity of chitosan.

Table 43.1 Biological activities of COS and COS derivatives

COS/COS derivative	Bioactivity	Reference
COS (< 1 kDa)	Antioxidant	[43.17]
N-acetyl-COS	Antioxidant	[43.18]
Gallate-COS	Antioxidant	[43.19]
Aminoethyl-COS (1−3 kDa)	Antioxidant	[43.20]
COS (< 1 kDa)	Photo-aging	[43.21]
Carboxylated-COS	ACE-inhiibtory	[43.22]
Aminoethyl-COS	ACE-inhibitory	[43.23]
COS (< 5 kDa)	Prevention of negative mineral balance	[43.24]
COS (3−5 kDa)	MMP-2 inhibitory activity	[43.25]
Carboxylated-COS	MMP-9 inhibitory activity	[43.26]
COS (3−5 kDa)	β-secratase inhibitory activity	[43.27]
Dimethylaminoethyl-COS Diethylaminoethyl-COS	Acetylcholinesterase inhibitory activity	[43.28]
Aminoethyl-COS	Anti-inflammatory	[43.29]
Aminoethyl-COS	Antineuroinflammatory	[43.30]
COS (< 1 kDa)	Antineuroinflammatory	[43.31]
Sulphated-COS	Antiarthritic	[43.32]
COS (1−3 kDa)	Antiallergy	[43.33]
Dimethylaminoethyl-COS Diethylaminoethyl-COS	Antitumor	[43.34]
COS (3−5 kDa)	Antidiabetic	[43.35]
COS (3−5 kDa)	Anti-HIV	[43.36]
COS (< 10 kDa)	Antimicrobial	[43.37]

It has been reported that chitosan showed higher fat-binding capacity than conventional cellulose and the capacity increased with both MW and DD. Further, the bile-salt-binding capacity of chitosan also greatly varying with the viscosity-average molecular weight, and the chitosan sample with the highest molecular weight showed the best binding capacity. It has been postulated that increased MW and DD increase the free amino groups which enhance the hypocolesterolaemic activity [43.43–45]. In relation to true commercial food application of chitosan, Japan food market has achieved considerable success. For instance, dietary cookies, potato chips, noodles, and pasta enriched with chitosan gained growing consumer attention. Incorporation of chitosan has also increased the shelf life of products via antioxidative and antimicrobial activity [43.46]. In dairy products such as yoghurts chitosan have ability to reduce the absorption of glucose and calcium than that of common dietary fibers [43.47]. Moreover, addition of chitosan into bakery products has been identified as one of the best options to utilize the physical properties as well as biological properties of chitosan. In addition, incorporation of chitosan into beverages such as orange juice and household products like vinegar to improve the functional properties and shelf life also became a viable approach to enjoy the biological activities of the chitosan.

Chitosan-based edible film is one of the promising approaches of chitosan in food industry. Both edible coatings and films have been developed using the film-forming properties of chitosan and it has been demonstrated that most mechanical properties are comparable to medium strength commercial polymers. Further, antimicrobial activity and antioxidant activity of chitosan enhance the quality and shelf life of products. It has been observed that other than the biological properties of chitosan and COS, they preserve and enhance the nutritional value of parent food [43.48–50]. All these evidences encourage the applications of chitosan and its derivatives as functional foods than supplementary pills which have lesser consumer demand, particularly in Asian population.

43.2.3 Glucosamine

Glucosamine (Fig. 43.1) is an amino sugar and one of the most abundant monosaccharaides. *N*-acetyl-glucosamine (2-(acetylamino)-2-deoxy-D-glucose) (Fig. 43.1), the monomer unit of chitin and chitosan, is an amide between glucosamine and acetic acid.

The extensive hydrolysis of chitin form crustacean exoskeleton produces glucosamine and *N*-acetyl-glucosamine which is the most common method of commercial production. However, glucosamine and *N*-acetylglucosamine are readily synthesized in the human body from glucose and higher glucosamine levels are found in bone tissues of humans. Glucosamine sulfate is a normal constituent of glycoaminoglycans in cartilage matrix and synovial fluid. It has been reported that the sulfate moiety strengthens the cartilages and facilitate glycosaminoglycan synthesis. Therefore in osteoarthritis conditions, supplementation of glucosamine is used to relieve from arthritic symptoms [43.51]. Clinical studies have also proved this hypothesis [43.52, 53] and currently, it is the most common nonvitamin, nonmineral, dietary supplements used by US adults. Moreover, glucosamine has been subjected to laboratory and clinical testing over all the other nutraceutical products in the world market. Other than the pain-relieving ability of glucosamine, it was found that it has cartilage building and lubricant properties for the joints [43.54]. In addition to the positive contribution for the joint pain relieving, it has shown anti-inflammatory activity by suppressing inflammatory activation of synovial cells, endothelial cells, and intestinal epithelial cells [43.55]. Due to these immense potentials, glucosamine has a high demand as a nutraceutical against inflammatory conditions in the market. Most of the commercially available glucosamine products; glucosamine hydrochloride, glucosamine sulfate, and *N*-acetyl-glucosamine are derived from hydrolysis of chitin even though several other sources have been identified. Even though a variety of glucosamine derivatives are available in the market as nutraceuticals, the sulfated form has been subjected to the majority of clinical trials. The recommended dosage of glucosamine nutraceuticals is 1500 mg per day and most of the time glucosamine-based nutraceuticals are well tolerated by the human body [43.56]. However, some mild gastrointestinal system related conditions among 12% of patients involved in random trials have been reported [43.57].

Other than the direct use as nutraceuticals, incorporation of glucosamine into food formulations was found to be an effective means to replace the supplementation pills which have lesser consumer demand. Currently, beverages containing glucosamine have taken part of food market of many countries. Glucosamine containing orange juice was the very first commercial product of glucosamine beverages. Following the concept, various kinds of beverages containing glucosamine as functional ingredient entered into food market and GRAS certification for glucosamine overcame one of the main barriers of the mass marketing. Hence, glucosamine-enriched beverage has become a more successful approach than other product such as cereals products [43.58]. Further, glucosamine become a very labile formulation when processed in a milk beverage which has a pH of around 6.5 even though neutraceutical manufactures prefer pH 4.5. It has been found that glucosamine-enriched milk can be developed as pasteurized product as a heat treatment of 100 °C or higher destabilize the milk protein [43.59]. High-performance liquid chromatography (HPLC) equipped with refractive index detector has developed as a simple method to determine the concentration of glucosamine in milk-based products [43.60].

43.2.4 Sulfated Polysaccharides

Sulfated polysaccharides (SPs) comprise a complex group of macromolecules which have high degree of structural diversity due to differences in molecular weight, disaccharide construction, and sulfation. It is believed that the structural heterogeneity and the strong negative charges are responsible for the affinity of the SPs to positively charged biological macromolecules. Therefore, SPs show a wide range of biological activities. This has resulted in opening up a broad discussion on structural diversity and functions of SPs. Marine algae are a well-known source of biologically active SPs. A variety of sulfated fucans and sulfated galactans present in marine algae have been extensively researched as therapeutic agents as well as potential candidates for the development of functional food and nutraceuticals [43.61, 62]. Large number of research outcome support that sulfated polysaccharides can enhance the innate immune response and thereby promote the tumoricidal activities of macrophages and natural killer cells [43.63]. Sulfated polysaccharides can enhance the adaptive immune response by promoting the migration of antigen presenting cells into the tumor cells and thereby presenting tumor antigen to be detected by T-helper cells. The T-helper cells stimulate the activation of T-cells which can exert a cytotoxic activity toward tumor cells [43.64]. Moreover, sulfated polysaccharides enhance the proliferation of T lymphocytes by binding to CD2, CD3, and CD4 receptors in T lymphocytes [43.65]. This mechanism of action is responsible for the anticancer activity of marine-sulfated polysaccharides such as fucoidan, heparin, pentosan polysulfate, chondroitin-4-sulfate, and

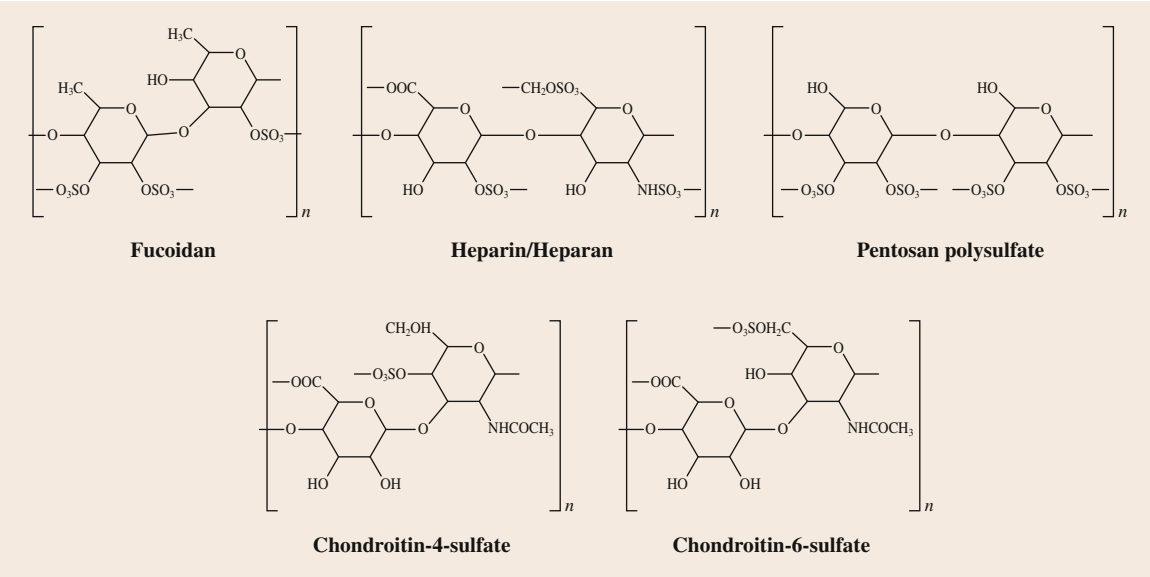

Fig. 43.3 Structures of anticancer sulfated polysaccharides

chondroitin-6-sulfate (Fig. 43.3). The potent of marine-derived sulfated polysaccharides as anticancer agents could be well adapted in cancer therapy.

Fucoidan (Fig. 43.3) is the most common and representative marine-sulfated polysaccharide. It is commonly extracted from the marine brown algae cell wall, and accounts for more than 40% of the dry weight of the cell wall. Small amount of fucoidan is also being extracted from echinoderms. Often fucoidan is consist of *O*-sulfated α-L-fucopyranose residues linked through (1−2), (1−3), and (1−4) linkages arranged in branched structures. The molecular weights of fucoidans vary from 100 to 1600 kDa [43.66]. Fucoidan is soluble in water and acidic solutions. The molecular characteristics such as sulfation, molecular weight, and confirmation of the sugar residues vary with the mother source [43.67]. Generally seaweed-derived fucoidans are highly branched and echinoderms, especially sea cucumber derived fucoidans are comparatively linear. Fucoidan is popularly extracted from edible seaweed species such as *Fucus vesiculosis*, *Cladosiphon okamuranus*, *Laminaria japonica*, and *Undaria pinnatifida*.

Diverse interesting biological functions of fucoidan have been extensively reviewed in the last decade. The activities are ranging from antioxidant activity [43.68], antivirus activity [43.69], cardioprotective activity (*Thomas* et al. [43.70]), anti-inflammatory activity [43.71], anticancer activity [43.72, 73], anticogulatory activity [43.74], and neuroprotective activity [43.75]. Among identified functional sulfated polysaccharides fucoidans have been extensively studied for their potential in developing drugs and nutraceuticals. There are large number of companies all over the world marketing nutraceuticals prepared from fucoidan (Fig. 43.4). The biological activities of fucoidan are dependent on the source, molecular weight, composition, structural arrangement, and the mode of administration [43.76]. Sulfation is a critical factor for its functionality and it was found that desulfated fucoidan

Fig. 43.4 Fucoidan-based nutraceutical products in the market

moieties fail to perform as bioactive molecules [43.77, 78]. The structural heterogeneity and high molecular weight are challenges faced by fucoidan as a therapeutic agent. Currently scientists are extensively working on identification of active fragments of fucoidan. This process will also direct to the identification of structure activity relationships of fucoidan and biological molecules, which is still an unresolved issue. Moreover, this will increase the therapeutic applications of fucoidan.

43.3 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) especially omega-3 fatty acids are for the metabolism and cannot be synthesized by the human body as human body do not synthesize the precursor for omega-3 fatty acids, α-linolenic acid (ALA). Therefore, external supplement of PUFA is required to maintain the physiological functions of the body. Besides its functions in regulating the normal metabolism, several PUFA such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha linolenic acid (ALA) (Fig. 43.5) have proven to possess vast array of health benefits such as prevention of coronary heart diseases, hypertriglyceridemia, blood platelet aggregation, atherosclerosis, general inflammation, hypertension, type-II diabetes, ocular diseases, arthritis, cystic fibrosis, and several carcinomas [43.79–81]. PUFA are popularly consumed as nutraceuticals to protect against heart diseases. Findings suggest that high levels of EPA and DHA in blood which in turn reduce the rate of coronary heart diseases have an association with inhibition of lipid-rich atherosclerotic plaques growth, reduction in formation of thrombus, improving vascular endothelial function and lowering blood pressure [43.80]. In 2004, US Food and Drug Administration has declared a *qualified health claim* status to EPA and DHA [43.82]. Therefore, both EPA and DHA have been extensively used as therapeutics to prevent above mentioned adverse health conditions [43.83, 84]. In consideration to the promising therapeutic properties, worldwide demand for the fish-oil-based nutraceuticals is rapidly increasing.

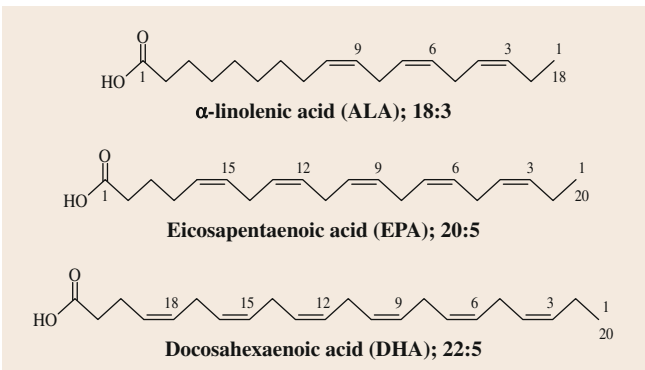

Fig. 43.5 Structures of polyunsaturated fatty acids

The principal dietary source of DHA and EPA is fish and fish by-products. Cold water oily fish such as salmon, herring, mackerel, anchovies, sardines, and fish oil derived from these fish have been recognized as well balance sources of omega-3 fatty acids, especially DHA and EPA. Due to highlighted health benefits of omega-3 fatty acids, world fish consumption reach to a level of which threaten the marine fish sources and thus fishing for oil extraction is not encouraged. Therefore, fish processing by-products have been identified as an ideal candidate for extraction of fish oil rich in omega-3 fatty acids while giving a positive insight on sustainable marine fisheries [43.85]. The processing leftovers head, skin, and internal organs are rich sources of omega-3 fatty acids and method and conditions for the extraction are determined based on the nature of fish source. Presently, several methods such as high-speed centrifugation, Soxhlet extraction, low temperature solvent, and supercritical fluid extraction are employed to extract fish oil [43.86]. Purification of omega-3 fatty acids form extracted fish oil seems as a challenge due to the presence of complex mixture of triacylglycerols and vulnerability of free fatty acids EPA and DHA to oxidize into hydro-peroxides. A recent study shows that enzymatic deacidification of high-acid crude fish oils is an effective approach to extract high amount of *n*-3 fatty acids [43.87]. These extracted fish oils are available in the market as nutraceutical pills.

However, due to unpleasant fishy odor and being a declining resource; there is a serious commercial and environmental issue related to the continued exploitation of fish in order to meet the demands of the expanding market [43.88, 89]. And also concerns have arisen on using nutraceuticals derived from fish oil as it can be contaminated with trace pollutants such as mercury, polychlorinated biphenyls, and dioxins from their habitats [43.90]. More importantly, the realization of the fact that fish itself do not have the ability to synthesize DHA and EPA, and microalgae in their diet is the

primary source of fatty acids for fish [43.91] arises the interest to explore marine microalgae as an alternative safe source of PUFA-based nutraceuticals and supplements, especially EPA and DHA.

Microalgae have been identified as a rich source of long chain polyunsaturated fatty acids where it accounts for 10–20% of cell weight in some species. Biosynthesis of PUFA in microalgae has been described by *Tonon* et al. (2004) which involves a series of desaturation and elongation steps starting with oleic acid [43.92]. As a commercially demanding source of PUFA, design of optimum culture conditions to yield maximum amount of PUFA in a cost-effective manner is essential. Environmental conditions have a significant influence on the PUFA compositions of microalgae and therefore the external factors such as the medium composition, nitrogen source, pH, incident light intensity, degree of aeration, and temperature should be optimally manipulated depending on the species [43.89, 93]. Use of ammonium or nitrate as the nitrogen source has produced the highest level of PUFA in *Chlorella minutissima* (44.4% of the total fatty acids) and *Tetraselmis gracilis* (46.6% of the total fatty acids) [43.94].

Studies on the EPA and DHA content of microalgae have reported an immense potential of several microalgal species as a primary source of ALA, EPA, and DHA. *Nannochloropsis* spp. (*Fuentes* et al. [43.95]), *Porphyridium cruentum* [43.95, 96], *Phaeodactylum tricornutum* [43.97, 98], and *Chaetoceros calcitrans* [43.99] are identified as the best EPA producing microalgae, while *Isochrysis galbana* [43.100], *Cryptecodinium* spp. [43.101], and *Schyzotrichium* spp. [43.102] have been reported as promising sources of DHA. Furthermore, *Pavalova lutheri* and *Thalassiosira pseudonana* have been identified as a rich source of both EPA and DHA [43.103–105]. Commercialization of these species as sources of EPA and DHA should be followed by extensive studies on the economically feasible culture and extraction conditions.

Fig. 43.6 Omega-3-based nutraceuticals derived from fish oil and marine microalgae in the market

The demand for the PUFA-based nutraceuticals and PUFA fortified functional foods are increasing at a high pace. According to the World Health Organization the daily recommendation for EPA and DHA per capita consumption is 0.3–0.5 g d^{-1} [43.106]. The average per capita consumption of EPA and DHA in the world is 0.38 g/d [43.107, 108]. To meet with this demand, large number of nutraceutical companies are producing and marketing omega-3-fatty acid encapsulated pills worldwide (Fig. 43.6). According to the global business of nutraceutical reports on 2012, the market demand for fish oils were around 3.08 billion USD in 2011. Encapsulation is highly necessary as the omega-3-fatty acids are highly susceptible for oxidation due to their higher degree of unsaturation [43.108]. Encapsulation protects the fatty acids from light and heat-mediated oxidation and thereby ensures longer storage times.

43.4 Carotenoids

Carotenoids are a class of terpenoid pigments, derived from a 40-carbon polyene chain, which provides distinctive molecular structures of carotenoids [43.109]. This polyene chain can be substituted with cyclic groups and oxygen containing functional groups in the biosynthesis of different carotenoid compounds. The oxygenated derivatives of carotenoids are denoted as xanthophylls. Xanthophylls are again classified depending on the nature of oxygen present, such as lutein (oxygen is present as –OH), cantaxanthin (as oxy-groups) and astaxanthin (combination of both –OH and oxy-groups) [43.109, 110].

There are number of carotenoids that have been identified from marine resources which bears pharmaceutical potential such as β-carotene, lycopene, zeaxanthin, fucoxanthin, astaxanthin, cantaxanthin, lutein, and violaxanthin (Fig. 43.7) [43.111, 112]. These carotenoids are potent antioxidants and this activity is well described by relating to its complex ring structure which has the ability to absorb the energy of oxida-

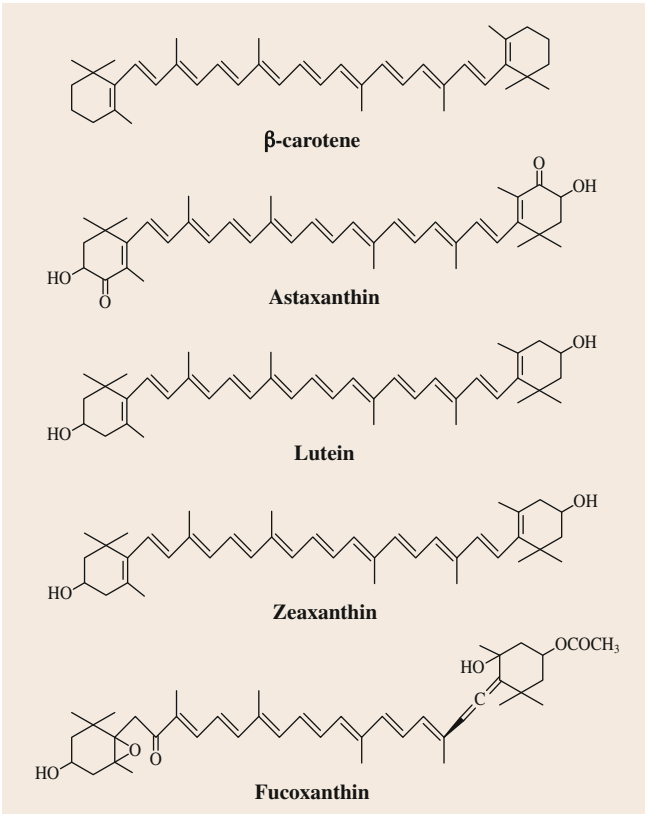

Fig. 43.7 Structures of carotenoids commonly isolated from marine sources

tive radical species [43.113]. And also it has been reported that carotenoids enhance the immunity via facilitating the monocyte function to increase the number of surface molecules expressed [43.114]. Based on this antioxidative and immune modulatory power of carotenoids, they can extend their activity in several disease conditions which have a direct relation to oxidative stress and chronic inflammation such as cancer, cardiovascular disease, eye disorders, rheumatoid arthritis, and several neurodegenerative diseases [43.115, 116]. Moreover, the use of fucoxanthin to reduce body fat has been well investigated and is popularly used as a nutraceutical to control body weight [43.117]. Some of the findings related to the biological activities of carotenoids are listed in Table 43.2.

The market for carotenoid-based nutraceuticals is booming with the understanding of their therapeutic potentials. Carotenoid nutraceuticals are marketed in different forms such as cold water soluble powders, oil emulsions and beadles of single pigments or blend of few [43.130]. The carotenoid amount in these products is ranging from 0.2 to 100 [43.116]. Until recent times synthetic carotenoids mimicking the properties of natural pigments are ruling the market. However, with the development of customer understanding on the beneficial use of the natural pigments, the demand for extracted natural pigments increased rapidly. Among the natural sources of carotenoids marine microalgae has gained much attention due to the higher content and possibility of sustainable production of carotenoids [43.131].

Table 43.2 Biological activities of carotenoids found in marine sources

Carotenoid	Bioactivity	References
β-carotene	Protect against heart diseases	[43.118–121]
	Provitamin A activity	[43.122]
	Anticancer activity	[43.123–125]
	Protection of skin against UV damage	[43.126]
Astaxanthin	Anticancer activity against prostate and liver cancers	[43.126, 127]
	Anti-inflammatory activity	[43.128]
Zeaxanthin	Liver protection	[43.123]
	Protects against heart diseases	[43.120]
	Helps in preserving normal visual functions	[43.129]
Lutein	Protect against heart diseases	[43.120]
	Helps in preserving normal visual functions	[43.129]

Green unicellular marine microalgae *Dunaliella salina* has been identified as one of the most promising source of β-carotene with 14% of dry weight being β-carotene at appropriate culture conditions [43.132]. The particular growth conditions of *D. salina* which yield the highest amount β-carotene at industrial scale ($100\,g\,kg^{-1}$ dry weight) and specific extraction methods of β-carotene has been extensively studied [43.133, 134]. With the development of optimum culture and extraction conditions, *D. salina* is commercially cultured as a source of β-carotene [43.91]. SFE of metabolites from *Haematococcus pluvialis* has also produced β-carotene and astaxanthin where astaxanthin was the main product (about 75%) [43.135]. Up to date *H. pluvialis* is considered the highest astaxanthin producing microalgae with approximately 1.5–3% by weight and therefore, it is been exploited commercially [43.136]. Interestingly, it has been reported that astaxanthin bears the highest antioxidative potential compared to other carotenoids and commercially available antioxidative

agents such as vitamin E [43.137]. Lutein is a highly polar carotene compound corresponding to the presence of hydroxyl groups on the cyclic ring structure and as a result it bears a higher antioxidative potential which could be effectively harvested to promote health and protect against chronic disease conditions. Specifically lutein has the ability to protect the aging eye-related complications such as cataract and macular degeneration via retarding the pathological mechanisms underlying these diseases [43.138]. Marigold is the most widely used source of lutein. However, mass plantation of marigold requires large areas and also it is climate dependent, and hence, the cultivation of *Chlorella pyrenoidosa* was discovered as the most promising alternative source of lutein with 2–4 mg g^{-1} dry weight which is comparable to that of marigold. *Wu* et al. (2007) has published SPF as the most appropriate method for optimal extraction of lutein which resulted in 87.0% extractive of lutein under the optimized conditions of 25 MPa and modified CO_2 with 50% ethanol [43.139]. In addition, it was found later that *Muriellopsis* sp., could also be economically employed for the extraction of high lutein content up to 35 mg L^{-1} [43.110]. Corresponding to this high growth rate and higher cell density, the culture of *Muriellopsis* sp. as the best source of lutein is practiced commercially [43.140].

The market value of carotenoids is in a continuous increasing phase. According to the business communications, the market value for carotenoids will raise from 766 million dollars in 2008 to 919 million dollars in 2015. The most demanded carotenoids includes beta-carotene, lycophene, astaxanthin, lutein, and cantaxanthin. The expected market demand value for beta-carotene will be 285 million dollar by 2015. Currently,

Fig. 43.8 Carotenoid-based nutraceuticals in the market

Chlorella vulgaricus derived nutraceuticals are sold to a price of 30–100 USD kg^{-1}. And the dry powder or tablets of *Isochrysis galbana*, *Phaeodactylum tricornutum*, *Arthrospira*, *Dunella salina* is marketed to price of more than 200 USD kg^{-1}, where the dry powder of *H. pluvialis* leads the group with a market price of more than 600 USD kg^{-1} [43.116]. Large number of companies in the world is marketing the carotenoid-based nutraceuticals in the form of tablets, pills, and capsules produced with carotenoid rich microalgae, *Arthrospira*, *Chlorella*, *Dunaliella*, *Spirulina* and *Aphanizomenon* (Fig. 43.8). Moreover, the dried powders of carotenoid rich microalgae are being used in food formulations such as pasta, snacks, drinks, etc.

43.5 Soluble Calcium

Calcium is an essential mineral and is acquired through diet. It has been found that calcium is necessary to maintain good health as it provides diverse biological functions. It is commonly known that calcium is needed to strengthen and maintain the bones and teeth; however it is also required for other physiological functions such as blood coagulation, nerve function, production of energy, muscle contraction, maintain heart function, and proper immunity [43.141]. Due to its role in bone strengthening, calcium also plays an important role in preventing the onset of bone-related diseases such as osteoporosis, osteopenia, osteomalacia, and rickets [43.142]. Even though, calcium is readily available in most of the dietary sources, the bioavailability of calcium of such sources are low, where milk and fish being the most common sources of soluble calcium [43.143].

Fish frame, account for approximately 10–15% of total fish biomass, is discarded as a waste from commercial fish fileting lines. But close observation on chemical composition of fish frame revealed that bones mainly composed of calcium phosphate and collagen protein with some special carbohydrates and lipids. The calcium and phosphorus content in the whole fish ac-

Fig. 43.9 Fish bone derived calcium supplements in the market

counts for 2% of the dry weight. Thus, the waste could be used as mineral source to obtain soluble calcium to be used in nutraceutical formulations. Even though the bioavailability of calcium of fish bones is not deeply studied, it is well-known fact that small fish are good source of balance calcium source and readily absorbed into human body. Moreover, in a recent study it was found that the calcium from salmon and cod bone are well absorbed in young healthy men [43.144]. Thus bones from fish processing waste can be used to produce fortified products with high biological value and several convenient methods have been developed to soften the fish bone to convert it into an edible form. With these findings on the beneficial and potential use of fish bone calcium, number of nutraceutical products has been introduced to market worldwide (Fig. 43.9). According to the market report of nutraceuticals published in the nutraceuticals world magazine calcium supplements have been identified as one of the demanding nutraceuticals in 2011 with 5.19 billion USD market value.

43.6 Fish Collagen and Gelatin

Collagen is the most abundant protein of animal origin, and there are 28 types of collagen have been identified. Type I and III collagen are the most naturally abundant collagen in animal and found in skin, tendon, vascular ligature, organs, and bone. Collagen has earned much interest as a biomaterial in medical applications due to its biodegradability and weak antigenicity [43.145]. It is generally composed of three similarly sized triple helix polypeptide chains which consist of around 1000 amino acids residues. Gelatin is structurally different form of the same macromolecules which make collagen and particularly a hydrolyzed form of collagen. The popularity of common source of collagen and gelatin such as bovine hide, pig skin, or chicken waste are rapidly decreasing due to biological contaminants and religious issues [43.146]. Fish waste has been identified as the best alternative source for collagen and gelatins for commercial purposes due to its unique features. Fish skin and bones are mainly used for collagen extraction and popular extraction methods include neutral salt solubilization, acid solubilization, and enzyme solubilization. During extraction of collagen triple helix structure of collagen which contributes its unique properties has to be secured. Generally, extracted collagen is solubilized with hot water treatment by breaking down the hydrogen and covalent bonds of the triple-helix, resulting in helix-to-coil transition, and conversion into soluble gelatin [43.16]. Collagen is mainly extracted from skin of cod, haddock, and salmon. Moreover, tilapia fish scales are also being used as a source of low molecular weight collagen (around 1000 kDa) [43.4].

Fig. 43.10 Fish collagen-based nutraceuticals in the market

Fish collagen is widely gaining popularity as an ingredient in cosmetics. Oral supplementation of collagen is also popularly used to maintain a healthy skin. Evidences of clinical investigations suggest that ingestion of collagen/gelatin hydrolysates are capable of pain re-

Table 43.3 Biologically active peptides isolated from fish skin

Source	Peptide sequence	Activity	Reference
Alaska pollack skin gelatin	Gly–Pro–Leu	ACE inhibitory activity	[43.147]
Alaska pollack skin gelatin	Gly–Pro–Hyp	Antioxidant activity	[43.148]
Jambo Squid skin gelatin	Phe–Asp–Ser–Gly–Pro–Ala–Gly–Val–Leu Asn–Gly–Pro–Leu–Gln–Ala–Gly–Gln–Pro–Gly–Glu–Arg	Antioxidant activity	[43.149]
Hoki skin gelatin	His–Gly–Pro–Leu-Gly–Pro–Leu	Antioxidant activity	[43.150]
Japanese Flounder Skin Gelatin	Gly–Gly–Phe–Asp–Met–Gly	Antioxidant activity	[43.151]
Pcific cod skin gelatin	Thr–Cys–Ser–Pro	Antioxidant and ACE inhibitory activity	[43.152]
	Thr–Gly–Gly–Gly–Asn–Val		
Pacific cod skin gelatin	Leu–Leu–Met–Leu–Asp–Asn–Asp–Leu–Pro–Pro	Antioxidant and ACE inhibitory activity	[43.153]

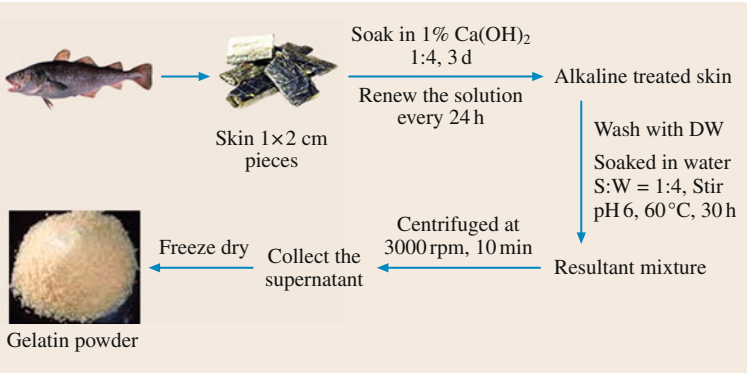

Fig. 43.11 Process of extracting gelatin from fish skin

lieving of the patients suffering from osteoarthritis and hydrolyzed collagen have been involved in cartilage matrix synthesis [43.154]. Also hydrolyzed collagen and gelatin are currently marketed as collagen peptide supplements to maintain of normal bone integrity, as a treatment for brittle nails and for the nourishment of scalp hair (Fig. 43.10) [43.16]. Fish-skin-derived collagen and gelatin were used as potential sources to isolate bioactive peptides. These peptides act as effective antioxidant and antihypertensive agents both in in vitro and in vivo models (Table 43.3). The biological activities of these peptides are attributed to their unique amino acid composition. The procedure of gelatin extraction from fish skin is shown in Fig. 43.11.

43.7 Marine Probiotics

The gastrointestinal tract is a harbor for large number of microbial species such as, *Bacteroides, Prevotella, Eubacterium, Clostridium, Bifidobacterium, Lactobacillus, Staphylococcus, Enterococcus, Streptococcus, Enterobacter, and Escherichia*. Gastrointestinal microbes could be both beneficial as well as harmful to the human health. Thus reducing the amount of potentially harmful or pathogenic species and promoting the growth of beneficial species are vital for wellbeing. A live microorganism which is beneficial for gut microflora and restricting the growth of disease causing bacteria is referred as a probiotic strain [43.155]. The applications of probiotics and prebiotics were boosted with modern nutraceutical and functional food market. Probiotics are considered as one of the highly demanded nutraceutical and food supplement in the world. Strains from the genera *Lactobacillus* and *Bifidobacterium* are predominantly used as probiotics [43.156]. Fermented

dairy products especially yoghurts, drinks, and capsules with freeze-dried bacteria are the most popular vehicles for delivering these organisms to the gastrointestinal tract [43.157]. Few strains of probiotic *lactobacillus* are identified from marine environments and they have shown unique characteristics in in vivo experiments. These findings revealed that marine-derived *Lactobacillus* species have advantages over conventional terrestrial species to use them as probiotics with advanced health benefits. Among the identified lactic acid bacterial species of marine origin, majority has been isolated form Pacific Ocean region of Japan and strains belong to genus *Caranobacterium*, *Marinilactobacillus*, *Halolactobacillus* [43.158].

43.8 Nutraceutical Market Trends and Quality Control

With the consumer knowledge on the health benefits of the foods increasing the use of foods or supplements with health promoting ability has gained alarming popularity. The ready-use supplements and attractive product range with beneficial effects have further enhanced the popularity of nutraceuticals and functional foods. Most people consume nutraceuticals to prevent the onset of life style related diseases such as heart disease, cancer, obesity, diabetes, etc. And also to delay the aging process by using antioxidants or skin regenerating supplements. The popularity of the nutraceuticals is also affected by the increase research on the identification of health benefits and constant communication of the benefits to the mass population via various marketing procedures. With the increasing popularity toward nutraceuticals, the gap between foods and drugs are getting narrower. Most nutraceuticals do not fall into both categories either a food or a drug; instead they reside in between the two. The nutraceutical market is mainly composed of two main categories including, functional foods and dietary supplements and herbal/natural products [43.159].

According to the market reports published worldwide, the global nutraceutical market has seen the maximum growth in the last decade from the emergence of the market in 1990. The annual average growth rate of the nutraceutical market from 2002 to 2010 is 14.7%, compared to that of 7.3% from 1999 to 2002. With the expansion of the nutraceutical market the growth is expected to rise till 2015. The United States also contributed to a market share of 140.1 billion USD by 2010. Among the marine nutraceuticals fish oil, carotenoids, calcium, and collagen peptides are highly demanded.

However, to maintain the market popularity, implementation of strict quality control and regulatory systems is of utmost importance. Even though there are no global regulations have been implemented to control the safety and quality of nutraceutical products till date, the regional regulatory agencies are controlling the key markets such as United States, European Union, China, Japan, and India. The regulatory bodies for these countries are; food and drug authority (USA), European food safety authority (EU), state food and drug administration (China), Japan health and nutrition food association (Japan), and food safety standards act (India).

43.9 R&D for Facing the Challenges and Supply for the Demand

Research and development on the marine nutraceuticals should be started out with improving the quality of existing products. For example, fishy odor in most of marine nutraceuticals such as fish oil, collagen, and peptides is a main drawback in expanding the market. Research on favor and odor masking of the products would be promising avenue for increasing the popularity for these products. And also development of novel concepts in the delivery systems instead of pills and capsules would also enhance the market popularity.

There are thousands of potential marine products which could be developed as nutraceuticals which are reported in the last few decades. However, only few have made their way up as nutraceuticals in the market. These potential products should be tested and developed as marine nutraceuticals as marine-active metabolites bears a promising capacity as nutraceuticals. The sustainable production of nutraceuticals is a major concern in the modern nutraceutical market. As most of the natural resources are declining, the investigation of marine microalgae and marine microorganisms as potential producers of active ingredients should be highly encouraged.

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44. Cosmetics from Marine Sources

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The application of marine resources for the formulation of cosmetics has been known for centuries. Marine organisms produce unique compounds, which are not found in terrestrial sources, to provide protection against hard environmental conditions. They have been used both to confer:

- Physicochemical *functional properties* to the cosmetic product, such as texture, emulsifying properties or color,
- *Bioactive properties*, including remineralizing, emollient, hydrating, antioxidant, sunscreening among others.

In this chapter, the major functional and biological activities of components isolated from marines sources, including micro and macroorganisms and with special emphasis on algae, are reviewed in relation to their application to cosmetics. Both the traditionally used compounds and fractions and those isolated and characterized in recent years are presented.

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44.1 Scenario of Marine Sources in the Cosmetic Industry

In recent years, health concerns and the demand for natural products have provided incentive for research on the abundant and alternative sources for novel ingredients and additives. Cosmetic industries, also influenced by consumers' preferences, have been increasingly incorporating natural ingredients into different products. However, the natural cosmetic market still represents a smaller fraction in relation to conventional cosmetics [44.1, 2].

Terrestrial plant-derived constituents are gaining popularity [44.3], but the marine environment is a richer source of both biological and chemical diversity [44.4]. Probably induced by the environmentally extreme conditions, marine organisms possess the capacity to produce molecules with unique chemical and structural features, which warrant a variety of potent and selective biological actions. Marine resources represent an underexploited source of highly diverse valuable

compounds [44.4–6] with potential applications in nutraceuticals and cosmetics [44.7–9].

Micro and macroalgae are established on the market for face and skin care products, such as anti-aging and regenerant creams, refreshing products, emollients, anti-irritants, sun protection, and hair care products. Components from cultivated microalgae are commercially available [44.10]. Seaweed resources have undergone successive periods of over-exploitation and neglect, but macroalgae have traditionally been used as a source of food and medicines, have been included in cosmetic formulations, and have played a major role in the development of spa products [44.11]. However, less than 1% of the identified species are used in pharmacy, food, and in cosmetology.

In addition to macro and microalgae, providing vitamins, minerals, proteins, and amino acids, sugars, lipids, terpenoids, polyphenols, polysaccharides, pigments, and enzymes, different marine sources have been considered for their potential applications in cosmetics and cosmeceuticals. Fish is an excellent source of gelatin and collagen; corals and crustaceans can provide diguanosine tetraphosphate, chitin, chitosan, and astaxanthin, and sea mud and sea salts have cosmetic applications [44.12]. Most of these active agents can be found in already marketed products [44.4] with a variety of functions [44.13]. Recent progress in cosmeceutical applications of marine- derived bioactive substances, including antioxidants, growth factors, peptides, anti-inflammatory, and pigment lightening agents, has been published [44.12].

This chapter presents general aspects on cosmetics, emphasizing on the potential of marine sources to provide components with a variety of functions, including technological and biological actions. The most recent innovation in this field, i. e., active cosmetics or cosmeceutical products, are also presented.

44.2 Cosmetics: Definition and Regulations

Cosmetics have been applied from ancient times in many civilizations for artistic, beautifying, protective, cleansing, and ceremonial purposes [44.14]. In order to avoid negative impacts on the consumers' health and to warrant the safety of the products, regulatory controls are established on the different markets, although the legislation applied to cosmetic products differs among countries. The major normative obstacles faced by the cosmetic industries include the different restrictions in the use of the ingredients, the distinct classifications of the cosmetics, and the varied labeling requirements [44.15]. The International Nomenclature of Cosmetic Ingredients (INCI) tries to unify the nomenclature in order to facilitate the adaptation of the products to different markets.

The need for an international harmonization on the status and safety requirements of cosmetics products and their ingredients has been claimed since their safety assessment is based on scientific and epidemiologic knowledge and also on their regulatory status [44.16]. Comparative studies of the regulatory features in different countries impacting on the manufacture and sale of cosmetic products, such as: cosmetic definition, licensing, labeling, safety substantiation, stability studies, and legal authority, are available [44.17–19].

In the major world markets, cosmetics are regulated according to two general models:

i) A wide definition of cosmetics and the safety of the products is based on a few lists of ingredients (positive, prohibited, or restricted), followed in the European Union and Japan, although in this latter area another category of cosmetics (quasi-drugs) has been established.
ii) A more limited definition of cosmetics, with few restrictions on ingredients and safety tests carried out by the manufacturers [44.17], followed in the USA and Canada approaches this model, including a list of prohibited or restricted ingredients.

The Cosmetic Products Regulation (EC) No. 1223/2009 [44.20] has replaced the European Directive 76/768/EEC [44.21]. It was dictated with the purpose of guaranteeing the safety of the cosmetic products and to facilitate their marketing. This regulation is applicable with full effect since July 2013 and reinforces product safety considering the latest technological developments. The term cosmetic product refers to any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity

with an exclusively or mainly purpose of cleaning perfuming, changing their appearance, protecting, keeping them in good condition, or correcting body odors. This regulation includes skin whitening-products, anti-wrinkle products, and products with nanomaterials.

In the USA cosmetics are regulated by the Food and Drug Administration (FDA), and the two most important laws pertaining to cosmetics are the Federal Food, Drug, and Cosmetic Act (FD&C) and the Fair Packaging and Labeling Act (FP&LA). The FD&C Act defines two main categories of products: cosmetics and drugs, including the specific subcategory of over-the-counter (OTC) drugs, which can be sold without prescription. The definition of products as cosmetics or drugs depends on their intended use, which is established on the basis of claims made about the product. Cosmetic products and ingredients are not subject to FDA premarket approval authority, with the exception of color additives, and cosmetic firms are responsible for substantiating the safety of their products and ingredients before marketing. The FD&C Act defines cosmetics as articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body for cleansing, beautifying, promoting attractiveness, or altering the appearance without affecting the body's structure or functions. Among the products included are skin creams, lotions, perfumes, lipsticks, fingernail polishes, eye and facial makeup preparations, shampoos, permanent waves, hair colors, and deodorants, as well as any substance intended for use as a component of a cosmetic product. The products not included in this definition are regulated as medicines. The intermediate category of cosmetics intended to treat, cure, mitigate, treat or prevent disease, or those affecting the structure or functions of the human body, are not specifically regulated. Among the products that are drugs and cosmetics are anticaries toothpastes, suntanning lotions to protect against sunburn, antiperspirants that are also deodorants, and antidandruff shampoos [44.17].

44.3 Cosmeceuticals

Like cosmetics, cosmeceuticals are applied topically but differ in that they contain potent ingredients such as vitamins, phytochemicals, enzymes, antioxidants, essential oils, ... that can deliver nutrients and influence biological functions to promote healthy skin [44.12, 13]. Cosmeceutical science is a new branch aimed at utilizing the resources of the natural environment to obtain efficient products [44.22]. The term cosmeceutical, formed from the words *cosmetic* and *pharmaceutical*, refers to cosmetic products that have drug-like benefits that enhance or protect the appearance of the human body [44.23–25]. These products lie between cosmetics and pharmaceuticals and they are medium-volume/medium-value products that offer lower risk and a quicker potential return on investment than the high-risk high-reward pharmaceutical market. The value of the cosmeceutical market has been increasing, and it is likely that this trend will continue [44.8]. Cosmeceuticals meet consumer demands for higher efficacy and have become the fastest-growing segment of the personal care industry [44.13, 23, 26], converting it in the area of dermatology with a more rapid commercial expansion; the number of new products introduced yearly onto the market is superior than that of pharmaceuticals [44.23]. The global market value for marine skin care products was estimated in 15% of the cosmetics industry. For marine resources the world market value for cosmetics was one third of that for pharmaceuticals [44.7].

Despite the relatively recent appearance of this concept, over 400 suppliers and manufacturers of cosmeceutical products have been estimated. *Amer* and *Maged* [44.27] classified the products in the cosmeceutical market into four major categories: nonbleaching agents, antioxidants, peptides, and growth factors. The market estimations project increases in the demand of cosmeceutical ingredients. Annual growth rates ranging from 4–25% and 4–10% have been reported for the periods 2002–2007 and 2007–2012, the injectables and age-defying products being the most dynamic segments, especially in emerging economies [44.13].

Because of their unique position as neither cosmetics nor pharmaceuticals, no specific regulation exists for these products [44.13]. Depending on the regulations of different countries, a group of products can be considered as cosmetic, therapeutic goods, or drug [44.28]. Since the main concern for functional products is safety and efficacy, it is likely that legislation covering pharmaceuticals will change to include nutraceuticals and cosmeceuticals to make efficacy claims. Consumers are often exposed to product information that is not scientifically sound or backed by rigorous clinical stud-

ies since the multitude of cosmeceutical products are not specifically regulated [44.17, 23, 28]. Appropriate controls on the safety of products, consumer protection, the extent of regulatory definition, market fairness, and inconsistency between countries have been suggested [44.28]. Dermatologists should familiarize themselves with the available products and assess their quality before recommending cosmeceuticals to their patients [44.13, 29, 30]. For practical applications and to effectively bring a new material to the marketplace, the viewpoints of many different agents should be considered, including: environmentally sustainable production, performance of the ingredients, confirmation of the mechanisms of action, quality assurance, regulations in markets around the world; and adequacy of supply to fulfill market demand with competitive pricing [44.31].

Recent trends in cosmeceutical development include skin protection from radiation and oxidative damage and the discovery of bioactives with the ability to scavenge free radicals and to prevent aging [44.32]. Particular focus is given to nonirritating ingredients to develop safer naturally-derived products [44.27], with components from plants and marine organisms, particularly those pure and uncontaminated. One example are the Antarctic and Southern Ocean seaweeds and marine microalgae, which show great potential due to their adaptation to survive under stressing osmotic pressure and temperature conditions, resulting in increased phycocolloid and polyunsaturated fatty acids contents [44.33]. Recent studies have addressed the screening of bioactive compounds from cold [44.34] and tropical waters [44.9, 35]. The complex matrix of cosmetics usually contains a high number of ingredients and emulsions. Nanoemulsions have attracted considerable attention in recent years as potential vehicles for the controlled delivery of bioactives because of their technological advantages to improve the availability and efficacy of lipophilic bioactives and to avoid changes during storage [44.36]. Nutricosmetics are another type of product than combine the cosmetic effect with nutritional supplements that can support the function and the structure of the skin, but they are not considered in the present chapter.

44.4 Target Organs and Cosmetics Delivery Systems

The skin is the main objective of cosmetic treatments, the other target organs for cosmetic products are hair and nails. Skin forms the interface between the human body and the environment, and is made up of three distinct layers: the epidermis, the dermis, and the hypodermis. The epidermis represents a physical, biochemical, and immunological barrier. The outermost layer of the epidermis is the stratum corneum, a continuous layer of protein-enriched cells embedded in an intercellular matrix enriched in non-polar lipids and organized as lamellar lipid layers [44.37]. It is responsible for the prevention of the loss of water and electrolytes, immune defense, and protection against ultraviolete (UV) radiation and oxidative damage and it is also capable of withstanding mechanical forces and preventing toxic substances from penetrating the skin.

The changes in this epidermal barrier can alter the appearance and the functions of the skin. Skin undergoes aging mainly as a consequence of genetic and external factors; UV irradiation from the sun causes oxidative damage and is the primary environmental factor causing skin aging and increasing the risk of cutaneous neoplasms [44.38, 39]. Whereas some symptoms, such as skin laxity or dyschromia, are observed on the surface, the origin is in deeper layers: the dermis and subcutaneous tissues. The penetration routes of drugs into the skin include the hair follicles, interfollicular sites, and through corneocytes and lipid bilayer membranes of the stratum corneum [44.40, 41].

Traditionally, most skin treatments are applied topically. Semisolid ointments or emulsions are the primary active delivery systems and the biphasic nature of emulsions allows the placement of actives based on solubility and stability [44.42]. Novel skin care methods target multiple aging mechanisms by utilizing functional active ingredients in combination with innovative delivery systems to increase stratum corneum permeability for cosmetic formulations and novel delivery systems, such as lipid systems, nanoparticles, microcapsules, . . . [44.43].

44.5 Components of Cosmetics

The general composition of a cosmetic includes an active ingredient, some excipients, thickening agents, additives, preservatives, colorants, and perfumes.

44.5.1 Active Compounds in Cosmetics

The active ingredient is the main substance, or substances, which confer the required activity to the cosmetic, i. e., the tensioactive in a shampoo, the colorant or pigment in a nail polish, or the moisturizers (glycerin, lactic acid, or urea) in a hand cream. It will determine the function of the cosmetic and its classification.

44.5.2 Excipients

Excipients are the vehicle ingredients with the function of dissolving or dispersing the active agents and other cosmetic ingredients. It is essential to choose them correctly, since they determine and control the delivery and effectiveness of the cosmetic in the targeted area, as well as the cosmetic form and presentation to the end user. The same cosmetic could be presented in different forms if different excipients are used. The most used excipients are water, alcohol, glycerin, acetone, vaseline, and lanoline.

The main purpose of stabilizers is to disperse the additives, thicken the fluid, and maintain the stability of the cosmetic during its lifetime. Thickeners, such as polymers and methyl cellulose, increase the viscosity of the formula. Humectants avoid dehydration by binding water; among the most frequent are glycerine, propylene glycol, and sorbitol. Chelating agents form complexes by binding with undesirable ions present in the formula, which could interfere with the properties of the cosmetic. pH modifiers are added to adjust the pH level close to the values of the skin. Citric, tartaric, or lactic acids are used to acidify the media and triethanolamine to raise the pH. Suspending agents, such as tensioactives, are used to improve the solubilization of some active ingredients, i. e., the dispersion of oily perfumes in aqueous solutions.

44.5.3 Additives

Preservatives

Preservatives are added to prevent or delay alterations in cosmetics, at least until the expiration date. According to the modifications to be prevented, these agents can be classified into antioxidants and antimicrobials.

Antioxidants

Their function is to prevent oxidation of fats and oils and the active principles present in the cosmetic formulations. Oxidative rancidity of fats and oils is caused by the degradation by oxygen in the air and occurs in a series of chain reactions involving free radicals. Oxidized fats become yellowish in color and the undesirable odors produced impair the cosmetic. Depending on their mechanism of action, different categories of antioxidants are established:

- Reducing agents, they become readily oxidized, preventing the oxidation of the active compounds; ascorbic acid and thiourea are examples of this type.
- Blocking agents that stop the oxidation chain without being consumed, i. e., butylated hydroxytoluene (BHT) and tocopherols (vitamin E).
- Synergistic agents increase the effectiveness of some antioxidants, i. e., citric acid and tartaric acid.
- Chelating agents form complexes with metal ions that can act as catalysts for the oxidative processes; ethylenediaminetetraacetic acid (EDTA) is one of the most used.

Antimicrobials

Antimicrobials prevent and protect the cosmetic product from microbial contamination (bacteria and fungi). Their addition is required to avoid contamination and growth of pathogenic germs over some threshold values that could lead to both deterioration of the product (turbidity, presence of molds, ...) and to health damage (skin infections). Among those frequently used are: nipasol (propyl *p*-hydroxybenzoate), nipagyn (methyl *p*-hydroxybenzoate), triclosan (2,4,4′-trichloro-2′-hydroxy diphenyl ether), dowicyl 200 (Quaternium-15), and imidazolidinyl-urea (1,1′-methylenebis and alkyldimethylbenzylammonium chloride (ADBAC).

Some cosmetics do not require additional preservatives, such as those containing an antiseptic ingredient, i. e., ethyl alcohol, and those sold in sealed ampoules, in this case the protection is restricted to the period when the cosmetic is closed.

Perfumes and Colorants

Perfume and colorant compounds are added with the function of providing a pleasant odor and color to the

final cosmetic, thus increasing the consumer interest in the product. In some cases, they can also serve to mask unpleasant odors and color resulting from mixing and processing the components of the cosmetic. Usually perfumes and dyes are associated with certain natural products, i. e., pink with the scent of strawberries or roses, green with mint, yellow with lemon, blue with the sea, ... Due to the low amount of dye and perfume added to cosmetics, in many formulations, information on their content is not assigned or provided, leaving this amount to the criteria of the manufacturer.

Water

Since ancient times, water has been used for both hygienic and curative purposes. It has been suggested that cavemen used it for this latter purpose, since they observed that injured animals flocked to warmer springs and to the sea to relieve their injuries.

44.6 Major Functions of Some Marine Components in Cosmetics and Cosmeceuticals

In this section, a survey of the major activities provided by marine-derived components with application in cosmetics and cosmeceuticals is presented. The traditional classification into both additives or ingredients and active agents was followed. However, recent studies have evidenced the biological actions of some fractions, i. e., hydrocolloids or pigments, conventionally used for thickening or for coloring purposes, respectively, and they could also provide other beneficial actions.

44.6.1 Physicochemical and Technological Properties

Surfactants, Emulsifiers, Thickeners, Stabilizers, Moisturizers, and Gelling Agents

Surfactants and emulsifiers are amphiphilic compounds containing both hydrophilic and hydrophobic moieties with many potential applications related to emulsification foaming, wetting, dispersion, and solubilization of hydrophobic compounds [44.44]. Marine microorganisms have been studied for the production of biosurfactants and bioemulsifiers [44.45], including protein polysaccharide complexes from *Acinetobacter* [44.46], *Pseudomonas* [44.47], *Myroides* [44.48], *Streptomyces* [44.49], *Yarrowia* [44.50], *Rhodotorula* [44.51], and *Halomonas* [44.52]; glycolipids from *Halomonas* [44.53], *Rhodococcus* [44.54], and *Alcanivorax* sp. [44.55]; lipopeptides from *Bacillus* sp. [44.56, 57] and from the sponge-associated marine actinomycetes *Nocardiopsis* [44.58, 59]; bile from *Myroides* bacterial strains [44.60]; and exopolysaccharides from *Bacillus* [44.61], *Planococcus* sp. [44.62], and *Cyanothece* sp. [44.63]. Table 44.1 shows some examples of marine ingredients with cosmetic application and their major technological properties. Marine lecithins, a mixture of phospholipids comprising predominantly long-chain and highly unsaturated fatty acids esterified on the glycerol backbone, are natural emulsifiers with traditional cosmetic use. Lecithins show excellent gelation in non-polar solvents when combined with water [44.64] and serve as an organic medium to enhance dermal penetration of poorly permeable drugs by effectively partitioning into the skin.

Seaweeds are the major source for thickeners, stabilizers, and gelling agents in the cosmetic and pharmaceutical industries, the so-called phycocolloids that include alginate, carrageenan, and agar. Alginates, cell-wall polyuronic acids from brown seaweeds, exhibit gelling properties resulting from their anionic properties and differing ratios of β-D-mannuronic and α-L-guluronic acids. They act also as wound healers and as excipients absorbable by the epidermis. Carrageenans, isolated from marine red algae, are widely used as emulsifiers, gelling, stabilizers, or thickeners in toothpastes, lotions, sun ray filterers, shaving creams, shampoos, hair conditioners, and deodorants. In toothpaste, carrageenan is used as a stabilizer to prevent ingredients from separating. Shampoos and cosmetics creams contain carrageenans as thickening agent [44.65, 66].

Chitosan is a multifunctional bioactive compound and is widely used in food, biotechnology, cosmetics, and medicine. Chitosan, which is soluble in acidic media, is a natural polysaccharide that is obtained from the polysaccharide chitin [44.67]. Chitin (poly(β-(1 $\rightarrow$ 4)-*N*-acetyl-D-glucosamine)) is one of the most abundant biopolymers in nature after cellulose. Chitins are synthesized by sea animals, such as annelida, mollusca, previously named coelenterate and crustaceans (lobster, crab, shrimp, prawn, and krill), microorganisms such as green algae, yeast (β-type), fungi (cell walls), brown algae, spores, chytridiaceae, ascomydes, and blastocladiaceae [44.68]. Chitosan can be obtained from shrimp

Table 44.1 Technological properties of some ingredients from marine sources with cosmetic use

Active component	Source	References
Surfactants, emulsifiers, thickeners, stabilizers, moisturizers, and gelling agents		
Acid- and/or pepsin-soluble collagen	Fish species, *Illex coindetii* (squid), deep-sea redfish, threadfin bream, walleye pollock, brownstripe red snapper, or unicorn leatherjacket	[44.69]
Alginates	Brown algae	[44.65]
Bile acids	*Myroides*	[44.60]
Carrageenans	Red algae	[44.65]
	Myroides, Streptomyces, Yarrowia, Rhodotorula, Halomonas	[44.48–52]
Chitosan and chitin	Sea animals as annelida, mollusca, coelenterata, and crustaceans (lobster, crab, shrimp, prawn, and krill), microorganisms such as green algae, yeast (β-type), fungi (cell walls), brown algae, spores, chytridiaceae, ascomydes, and blastocladiaceae	[44.68]
Collagen	*Scylla serrata*; *Chondrosia reniformis*; bivalve molluscs; *Ircina fusca, Takifugu rubripes* skin	[44.70–74]
Exopolysaccharides	*Bacillus, Planococcus, Cyanothece*	[44.61–63]
Gelatin	Fish species (cod, Atlantic salmon, megrim, squid, Nile perch, hake, skate, grass carp, yellowfin tuna, and channel catfish)	[44.75, 76]
Glycolipids	*Halomonas*	[44.53]
Lipopeptides	*Bacillus, Nocardiopsis alba*	[44.56–58]
Colorants		
Phycocyanin	Cyanobacteria	[44.77]
β-phycoerythrin	*Porphyridium cruentum*	[44.78]
R-phycoerythrin	*Corallina elongata*	[44.79]
Astaxanthin	*Penaeus semisulcatus*	[44.80]

by-products, which are annually produced in high amounts [44.81]. Chitosan and its derivatives are ingredients in various cosmetics, nail lacquers, toothpaste, lotions, hand and body creams, and hair care products, where they are used as emulsifiers, surfactants, gelling, stabilizers, thickeners, film-former, and also to encapsulate active compounds [44.82]. Chitin-related products, particularly chitosan and its derivatives, are used in several fields as a new type of functional material, based on their diverse biological and physicochemical characteristics [44.83]. They promote wound healing and are suited for cosmetic restoration. The combination with other active agents has been proposed, i. e., with taurine, which has effects on cell proliferation, inflammation, and collagen synthesis, and exhibits antioxidant effects [44.84]. The cosmetic use of chitosan accounts for 5% in the global market, although at the European level this application represents 20% [44.83].

Gelatin is a soluble protein compound obtained by partial hydrolysis of collagen. The most important properties of collagen and gelatin are associated with their gelling and water binding behavior (gel formation, texturizing and thickening) and their surface behavior (emulsion formation and stabilization, adhesion and cohesion, and protective colloid function). Traditionally the cosmetic application of gelatin was based on its gel-forming and viscoelastic properties, but more recently it has been used as a moisturizer in cosmetic creams for dry skin and has high potency in tissue regeneration [44.69]. Collagen from sea animals, such as the marine sponges *Ircina fusca* [44.74] and *Chondrosia reniformis* [44.72], sea urchins [44.85], the marine crab *Scylla serrata* [44.70], or bivalve molluscs [44.73] is a safer alternative to those of terrestrial origin [44.71, 72]. The number of fish or marine species studied for gelatin extraction is continually growing due to the interest in valorization of by-products from the fish industry [44.69, 75, 76].

Moisturizers have beneficial effects in treating dry skin. Orange roughy (*Hoplostethus atlanticus*) oil, a marine-derived wax ester, performed comparably to a petrolatum-based moisturizer (vaseline) in clinical trials in subjects with moderate to severe skin dryness [44.86].

Colorants

A wide variety of pigments, like chlorophylls, carotenoids, and phycobiliproteins, which exhibit colors ranging from green, yellow, and brown to red are present in algae and in other marine organisms.

The carotenoid pigments have significant application in the cosmetic industry, especially astaxanthin, which is found in salmon, rainbow trout, sea bream, lobster and caviar, crustaceans, the marine bacterium *Agrobacterium aurantiacum*, and the green microalga *Haematococcus pluvialis* [44.80].

Phycobiliproteins, including phycoerythrin, phycocyanin, allophycocyanin, and phycoerythrocyanin, are a group of colored proteins commonly present in cyanobacteria (blue–green algae), in a class of biflagellate unicellular eukaryotic algae (cryptomonads), and in Rhodophyta (red algae). They are used in the cosmetic industry to replace synthetic dyes that may be toxic, carcinogenic, or otherwise unsafe [44.87, 88]. In Japan, where algal cultivation is a well-developed industry, some natural pigments from phycobiliproteins were have patented early [44.89]. β-phycoerythrin is the most valuable of the phycobiliproteins due to its intense and unique pink color [44.90], heat stability, and pH tolerant characteristics, and has been applied as natural pink and purple colorants for lipsticks, eyeliners, and also formulations for cosmetic products [44.91]. Phycocyanin, a blue pigment, is also used as a colorant in eyeliners [44.77].

Carotenoids are isoprenoid molecules synthesized *de novo* by photosynthetic plants, fungi, and algae. Carotenoids are lipophilic compounds, some of which act as provitamin A. These compounds are classified into two major groups based on their structural elements; carotenes, constituted by carbon and hydrogen (e.g., β-carotene, α-carotene and lycopene), and xanthophylls, constituted by carbon, hydrogen, and additionally oxygen (for example, lutein, β-cryptoxanthin, zeaxanthin, astaxanthin, and fucoxanthin). Industrially, these carotenoids are used as pigments in food, feed, cosmetics, and pharmaceuticals. They can be produced by chemical synthesis, fermentation, or isolation from natural sources; most commercially used carotenoids (for example, β-carotene, astaxanthin, and canthaxanthin) are obtained by chemical synthesis [44.92].

Many macroalgae and microalgae are rich in carotenoids, which aid in the absorption of sunlight. Compared to other sources, the production of carotenoids from algae has many advantages, including lower costs, easy and environmentally friendly extraction, and the possibility of supplying materials without limitations or seasonal variations [44.93]. Recently, there has been considerable interest in carotenoids associated to their antioxidant properties [44.94–96] and because of their antiobesity and anabolic effects [44.97–99].

Antioxidants

The antioxidant activity of many marine-derived substances has been studied extensively [44.100–102]. The interesting aspect of this property is not only in relation to the increase in shelf life of the products, by delaying or avoiding the oxidation of fats and oils, but also due to other biological actions. The imbalance between generation of the reactive oxygen species (ROS) and the scavenging or detoxification by the organisms causes oxidative stress, which could lead to disruptions in normal mechanisms of cellular signaling and be involved in the development of aging and chronic diseases. Different marine-derived compounds have been confirmed as potent antioxidants, including oligosaccharides, peptides, phlorotannins, carotenoids, and vitamins. Table 44.2 shows some examples of the major components obtained from marine sources, which could be responsible for antioxidant properties. Due to the extensive and growing number of publications, only review papers are mentioned to illustrate the potential of some fractions from macroalgae. In most studies crude extracts and fractions are evaluated, and the presence of a mixture of compounds can provide a more potent antioxidant action because of the synergistic interaction between them [44.100].

Mixtures of different compounds have also been proposed; one example is the combination of isolated and purified marine oligosaccharides with collagen peptides derived from tilapia fish skin. This preparation showed a higher radical scavenging activity than the individual components [44.103].

Algae are one of the most studied sources of marine antioxidants. Extracts from brown algae have been observed to be more effective than those from red and green algae in terms of antioxidant activity [44.102, 122]. One of the suggested reasons for this is the phlorotannin content, and the characterization of some phloroglucinol derivatives has been published [44.123–126]. However, other components, from the polysaccharide and carotenoid fraction [44.96], are efficient antioxidants and could contribute to this activity. Aqueous and solvent extracts are the most used, but alternative processes have been proposed, i. e., fermentation of the *Ecklonia cava* processing byproduct [44.127]. Other relevant sources include the microalgae to produce carotenoids, the fish protein fraction to obtain peptides [44.94, 128, 129], and fish roe as a source of gadusol [44.130]. Less explored organisms have been studied, i. e., the marine phanerogam *Syringodium filiforme*, growing associated to *Thalassia testudinum* [44.131] or less conventional agents, such as

Table 44.2 Major families of compounds from marine sources showing in vitro antioxidant activity

Component	Source	Antioxidant activity	References
Carotenoids and other terpenoids	Microalgae, macroalgae marine sponges	Radical scavenging: DPPH, 12-DS, NB-L, AAPH, ABTS, ABAP, superoxide anion, TRAP Cu–Zn SOD and activity catalase activity in rat plasma and erythrocytes	
		Protection against oxidation	[44.95, 104–106]
Peptides	Enzymatic hydrolysis of protein from fish by-products: skin, head, viscera, trimmings, liver, frames, bones, and roes	Reducing activity, scavenging activity against DPPH radical, ABTS radical superoxide anion, hydroxyl radical, hydrogen peroxide, lipid peroxidation inhibition activity, ferrous and cuprous ion chelating activity, prevention of oxidation in emulsion of β-carotene linoleic acid and in lecithin liposomes	[44.107–110]
Phlorotannins	Extracts from brown algae	Scavenging activity against DPPH radical, hydroxyl radical and superoxide anions, protection against oxidation, ferrous and cuprous ion chelating activity, reducing activity, protection against oxidative stress in cells, protection of membrane oxidation in cells	[44.102, 105, 111–113]
Vitamin C and E	Macroalgae and seagrasses; tropical, temperate, and polar fish	Scavenging activity against DPPH radical, reducing power, endogenous enzyme activity of superoxide dismutase and glutathione peroxidase	[44.12, 114, 115]
Low molecular weight fractions from algal polysaccharides	Aqueous extracts and fractions from seaweed further subjected to chemical and/or enzymatic hydrolysis	Scavenging activity against DPPH radical, hydroxyl radical and superoxide anions. Inhibition of H_2O_2 induced hemolysis of rat erythrocytes, protection on lipid peroxidation of liver homogenate, prevention of oxidation in emulsion of β-carotene linoleic acid, in phosphatidylcholine liposomal suspension, restoration of endogenous antioxidant enzymes: superoxide dismutase and glutathione peroxidase; ferrous ion chelating activity	[44.105, 116–121]

DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABAP: 2,2′-azo-bis-2-amidinopropane; ABTS: 2′azinobis(3-ethylbenzothiazoline-6-sulfonic acid); SOD: superoxide dismutase; TRAP: total radical antioxidant parameter; AAPH: 2,2′-azobis(2-amidino-propane) dihydrochloride; 12-DS: 12-doxylstearic acid; NB-L: nitrobenzene with linoleic acid

5-hydroxymethyl- 2-furfural isolated from the red algae *Laurencia undulata* [44.132].

44.6.2 Biological Activities

Photoprotective and Anti-Photoaging

Skin is the largest part exposed in the human body, and it is well established that overexposure to UV radiation provokes acute sunburn reaction. Sun overexposure is clinically manifested as erythema, and chronically irradiated skin is associated with abnormal cutaneous reactions such as epidermal hyperplasia, accelerated breakdown of collagen, and inflammatory responses [44.133]. The use of photoprotective agents mainly determines the delay of the effects of photoaging by decreasing the adverse effects of free radicals. The increased public awareness towards the importance of skin care has fostered the study and the demand of compounds or extracts aimed at reducing the effects of UV irradiation as preventive or palliative agents. Recent studies have focused on marine organisms as a source of natural bioactive molecules and some UV-absorbing algal compounds are under investigation as candidates for new natural sunscreens, photoprotective, and antiphotoaging agents [44.134, 135]. Mycosporines and mycosporine-like amino acids (MAAs), which are accumulated by a wide range of microorganisms, prokaryotic (cyanobacteria), as well as eukaryotic (microalgae,

yeasts, and fungi), and a variety of marine macroalgae, corals, and other marine life forms, can be used as sunscreen compounds [44.136–138].

Solvent extracts from algae [44.135, 139, 140] and from other organisms, such as fungi, lichens, bacteria, cyanobacteria, and marine animals [44.141, 142] have been explored as a source of novel photoprotective compounds for the formulation of pharmaceutical and cosmeceutical products. Several pure algal compounds have shown this activity, for example, sargaquinoic acid and sargachromenol from *Sargassum sagamianum* [44.143], dieckol from *Ecklonia cava* [44.133], or fucoxanthin from *Sargassum siliquastrum* [44.144]. Bacterial exopolysaccharides, i. e., from the bacterium *Alteromonas macleodii*, have also been proposed to alleviate ultraviolet B radiation (UVB) aggression [44.145].

Anti-Wrinkling and Skin Regeneration

Skin aging involves changes in skin physical properties and visible signs on the surface due to the degradation of the extracellular matrix in both the epidermal and dermal layers, producing visible signs such as irregular dryness and pigmentation, sallowness, telangiectases, premalignant lesions, laxity, and wrinkling. Some anti-aging cosmetics are designed to treat premature aging caused by environmental factors [44.146]. The skin regeneration properties of some red algae, such *as Porphyra atropurpurea* and *Chondrus crispus*, have been used traditionally to treat wounds and burns [44.147]. The methanol extracts of the alga *Corallina pilulifera* reduced the expression of human matrix metalloproteinases MMP-2 and -9 induced by UV irradiation in fibroblasts [44.148]. *Joe* et al. [44.149] demonstrated the inhibitory effect of eckol and dieckol from *Ecklonia* species on MMP-1 expression in human dermal fibroblasts and suggested their use for the prevention and treatment of skin aging.

Depigmenting or Whitening

Tyrosinase, also referred to as polyphenol oxidase, is a metalloenzyme oxidase that catalyzes two distinct reactions of melanin synthesis in which l-tyrosine is hydroxylated to L-3,4-dihydroxyphenylalanine L-DOPA (monophenolase activity). The latter is subsequently oxidized to dopaquinone (diphenolase activity). Tyrosinase is the rate-limiting enzyme in melanin production, which occurs in melanocytes that are located within the basal epidermis [44.150]. Melanin has a photoprotective function in human skin, but its abnormal accumulation can result in hyperpigmentation with undesirable esthetic implications [44.151]. The downregulation of tyrosinase is an efficient method for the inhibition of melanogenesis, addressed by a number of approaches, including the direct inhibition of tyrosinase, the acceleration of tyrosinase degradation, the inhibition of tyrosinase mRNA (messenger ribonucleic acid) transcription, aberration of tyrosinase glycosylation or interference with melanosome maturation and transfer [44.151]. Accordingly, a huge number of compounds acting by alternative approaches have been identified successfully. The number of studies searching for potent melanogenesis inhibitors from natural sources for cosmetic uses is increasing, as there is a demand for tyrosinase inhibitors in the cosmetic industry due to their skin-whitening effect [44.152].

In a screening of 43 marine algae for antibrowning effects, *Endarachne binghamiae*, *Schizymenia dubyi*, *Ecklonia cava*, and *Sargassum siliquastrum* inhibited cellular melanin synthesis and tyrosinase activity similarly to kojic acid. Furthermore, whereas toxicity was observed in the positive controls, no toxicity was observed in the algal species [44.153]. Kojic acid was identified as the active compound isolated from the acetone extracts produced from selected strains in a screening study with 600 organisms of marine fungi [44.154]. Phlorotannins from marine brown algae are effective tyrosinase inhibitors and are potential ingredients for treating dermatological disorders associated with melanin [44.155]. Phloroglucinol, eckol, and dieckol from *Ecklonia cava* showed effects on melanogenesis via the inhibitory effect on tyrosinase and reduction of melanin synthesis, among them, dieckol showed higher activity than kojic acid [44.133] and also showed more effective melanin reducing activities than arbutin in B16F10 melanoma cells, without apparent cytotoxicity [44.156]. 7-phloroeckol from *Ecklonia cava* inhibited melanin production in melanoma cells more potently than arbutin and kojic acid [44.157]. In increasing order phlorofucofuroeckol A, eckstolonol, phloroglucinol, eckol, and dieckol from *Ecklonia stolonifera* inhibited tyrosinase [44.158]. Also diphlorethohydroxycarmalol demonstrated protective effects on melanogenesis [44.126]. Solvent extracts from bacteria of the *Pseudomonas* genus, associated with marine invertebrates, were found to be effective whitening agents in assays with cultured melanocytes, cultured skin, and in vivo zebrafish [44.156]. Other antimelanogenic compounds are fucoxanthin [44.144, 159], and geoditin A, an isomalabaricane triterpene isolated from the marine sponge *Geodia japonica*, with potent activity and relatively low cytotoxicity [44.160].

Human Skin Melanoma

Cutaneous melanoma is one of the most aggressive forms of skin cancer with high metastatic potential and strong resistance to radiation, immunotherapy, and chemotherapy [44.161]. The incidence of melanoma is rising at an alarming rate, possibly associated with the increased exposure to ultraviolet radiation, and has become a major public health concern in many countries [44.162].

The carotenoid fucoxanthin has antiproliferative effects in vitro and in vivo on melanoma B16F10 cells by inducing apoptosis and cell-cycle arrest [44.163]. A low molecular weight fraction of the water-soluble extract of *Porphyra yezoensis* inhibited proliferation of mouse B16 melanoma cells [44.164]. Several polysaccharide fractions, especially those from brown marine alga (*Ecklonia cava*, *Sargassum stenophyllum*, *S. hornery*, *Costaria costata*) decreased melanoma cell tumor growth [44.165–167]. Marine peptides and depsipeptides have shown antimelanoma activity. Among them aplidine, a cyclodepsipeptide isolated from the tunicate *Aplidium albicans*, and kahalalide F, a depsipeptide isolated from the herbivorous marine mollusk *Elysia rufescens*, *E. ornata*, or *E. grandifolia* has undergone phase III clinical study in patients with advanced malignant melanoma [44.129, 168].

Jaspine B, an anhydrophytosphingosine derivative isolated from the marine sponge *Jaspis* sp., decreases the viability of murine B16 and human SK-MEL-28 melanoma cells in a dose-dependent manner. Jaspine B is able to kill melanoma cells by acting on sphingomyelin synthase, an enzyme that converts de novo ceramide into the membrane lipid sphingomyelin, and may represent a new class of cytotoxic compounds [44.169]. Exposure of SK-MEL-2 cells to dideoxypetrosynol A, a polyacetylene from the sponge *Petrosia* sp., resulted in growth inhibition and induction of apoptosis in a dose-dependent manner [44.170]. Marinomycin A from a marine actinomycete of the genus *Marinispora* showed remarkable selectivity for melanoma cell lines in the US National Cancer Institute's (NCI) 60-cell panel [44.171]. A biindole, halichrome A, from the marine sponge *Halichondria okadai*, exhibited cytotoxicity against melanoma cells [44.172]. Also unusual polyunsaturated fatty acids (PUFAs), such as several $\omega 3$ fatty acids and the $\omega 7$ heneicosa-5,8,11,14-tetraenoic acid (21 : 4 n-7) from the marine opisthobranch *Scaphander lignarius*, were active against human cancer cell lines of melanoma [44.173]. Elatol, a sesquiterpene isolated from algae *Laurencia microcladia*, showed in vitro and in vivo antitumor properties [44.174]. Synthetic derivatives of marine natural products have also been proposed, i. e., sarcodiol, a derivative of sarcophine that inhibits melanoma cell proliferation [44.175].

Anti-Pruritic, Anti-Inflammatory Activity, and Antiallergic Activity

Inflammatory skin diseases such as contact dermatitis, atopic dermatitis and psoriasis are skin disorders that constitute a major health problem worldwide. One of the most challenging pruritic skin inflammatory diseases needing a better therapeutic approach is atopic dermatitis. This skin inflammatory disease can occur at any age and is characterized exclusively by the elevated serum immunoglobulin E (IgE) levels and peripheral eosinophilia. Inhibition of IgE production or reduction in the concentration of IgE would be the optimal therapeutic approach in treating inflammatory skin diseases [44.176].

Allergic diseases are caused by chemical or immunological activation of mast cells leading to a massive release of endogenous mediators, such as histamine, as well as a wide variety of other inflammatory mediators, such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines. Among the inflammatory substances released from the effector cells, histamine remains the best characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity. An allergic reaction that produces mild to moderate symptoms can be easily treated with antihistamine compounds [44.177].

Many natural compounds from natural marine organisms exhibit antiallergic and anti-inflammatory properties and hyaluronidase inhibitory activities [44.178]. Hyaluronidase is an enzyme that depolymerizes the polysaccharide hyaluronic acid in the skin and is known to be involved in allergic effects, migration of cancers, and inflammation. Mainly marine algae have received much attention because they are a valuable source of chemically diverse bioactive compounds with numerous health benefits in the treatment of allergic disorders [44.177].

The levels of serum IgE and histamine were suppressed in rats fed a diet supplemented with a dried *Eisenia arborea* extract [44.179, 180]. Phlorotannins are the most studied group of compounds that have shown potential pharmacological applications as antipruritic, anti-inflammatory, and antiallergic agents [44.112, 155]. Several phlorotannins exhibit potential antiallergic action on human and rats

basophilic leukemia cultured cell lines, 6,6′-bieckol, 1-(3′,5′-dihydroxyphenoxy)-7 (2″,4″,6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin, and dieckol suppressed binding activity between immunoglobulin E (IgE) and high-affinity receptor for the Fc region of immunoglobulin E (FcεRI) receptors [44.181], and fucodiphloroethol and phlorofucofuroeckol A reduced histamine release [44.182, 183]. Phlorotannins such as eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol isolated from *Eisenia bicyclis* and *Ecklonia kurome* have shown a stronger inhibition effect against hyaluronidase than well-known inhibitors such as catechin and sodium cromoglycate [44.184]. From these results, it is understood that phlorotannins can be useful in the management of allergic and inflammatory skin diseases through the reduction of IgE concentration and their histamine inhibitory activities. The carotenoid fucoxanthin was found to exhibit anti-inflammatory effects [44.185].

The polysaccharidic fractions of marine algae have promising anti-inflammatory activities [44.186]. Alginic acid exhibited an inhibitory effect on hyaluronidase and on histamine release from mast cells [44.187], suppressed antigen-induced Th2 development by inducing interleukin IL-12 production, and inhibited in vivo IgE production, suggesting its potential as an antiallergic agent [44.188]. Fucoidan significantly reduced IgE production in human peripheral blood mononuclear cells of patients and healthy donors, even after the onset of atopic dermatitis [44.189]. Oral administration of porphyran, a major component of the red algae *Porphyra tenera* and *P. yezoensis*, suppressed the contact hypersensitivity reaction (ear edema) induced by 2,4,6-trinitrochlorobenzene [44.190]. Epicutaneous application of sacran, a sulfated polysaccharide extracted from the alga *Aphanothece sacrum*, inhibited the development of allergic dermatitis skin lesions in mice [44.191].

PUFAs are metabolized by skin epidermal enzymes into anti-inflammatory and antiproliferative metabolites that are associated with a variety of benefits regarding inflammatory skin disorders [44.192, 193]. PUFAs can be recovered from marine protists and microalgae [44.194], Antarctic krill [44.195], marine and fish products, and by-products [44.196, 197]. Among the fatty acids, the ω3-PUFA, eicosapentaenoic acid (EPA, 20 : 5n-3), and docosahexaenoic acid (DHA, 22 : 6n-3), possess potent immunomodulatory activities [44.198] and can reduce the sensitivity of human skin to sunburn [44.199]. Animal experiments and clinical intervention studies indicate that diseases such as lupus erythematosis and psoriasis, characterized by a high level of IL-1 and the proinflammatory leukotriene LTB4 produced by ω-6 fatty acids, can be treated with ω-3 fish oil [44.198]. In this context, marinosomes, i. e., liposomes made of a natural marine lipid extract, were envisaged for the prevention and treatment of skin diseases [44.200].

Antimicrobial Activity

Bacterial skin infections are very common and can range from being merely annoying to deadly. Most bacterial infections of the skin are caused by two bacteria, *Staphylococcus aureus* and a form of *Streptococcus*. Viral infections of the skin are common and include warts, cold sores, chicken pox, shingles, molluscum contagiosum; and hand, foot, and mouth disease. The three main types of viruses that cause most viral skin infections are the human papilloma virus, the herpes simplex virus type 1 (HSV-1, HSV-2) and the pox virus. Whereas these viruses cannot be cured, their effects on the skin can be prevented or minimized through proper medical treatment. Examples of compounds from marine origin showing antimicrobial and antiviral action are listed in Table 44.3. This activity is also interesting with respect to the protection of cosmetic products from microbial contamination, thus having the role of preservatives.

A number of studies has been performed concerning the antimicrobial activity of phenolic compounds isolated from marine sponges. 2-(2′,4′-dibromophenoxy)-4,6-dibromophenol from the marine sponge *Dysidea granulosa* exhibited potent and broad spectrum in vitro antibacterial activity, especially against methicillin-resistant and sensitive *Staphylococcus aureus*, vancomycin-resistant and -sensitive *Enterococci*, and *Bacillus* sp. [44.198]. Marinomycin A from a marine actinomycete of the genus *Marinispora* [44.171] and dehydroxychlorofusarielin B, a diterpene from the marine-derived fungus *Aspergillus* sp., exhibited antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* [44.207]. Parabens, widely used in cosmetics, can be biosynthesized by a marine bacterial strain belonging to the genus *Microbulbifer* [44.216]. To illustrate the potential of algae, examples related to recent studies are summarized, either evaluated as extracts [44.217] or as pure compounds and fractions. Phlorotannins (phloroglucinol, eckol, fucofuroeckol-A, phlorofucofuroeckol-A, dioxinodehydroeckol, 8,8′-bieckol, 7-phloroeckol, and dieckol; and triphloroethol A, 6,6′-bieckol and 8,4‴-dieckol) from brown algae were active against *Campylobacter jejuni*, *Escherichia coli*, *Klebsiella pneumonia*,

Table 44.3 Some active components and their bioactive properties

Active component	Source	Reference
Photoprotective and antiphotoaging		
Aqueous ethanolic extract: thalassiolin B	*Thalassia testudinum*	[44.141]
Carotenoids	Cyanobacteria, bacteria, fungi, phytoplankton, macroalgae, plants, and animals	[44.201]
Methanol extracts	*Corallina pilulifera*	[44.148]
Mycosporine-like amino acids (MAAs)	Cyanobacteria, microalgae, yeasts, fungi, macroalgae, corals Arthropods, rotifers, molluscs, fishes, cnidarians, tunicates, eubacteriobionts, poriferans, nemertineans, echinodermates, platythelminthes, polychaetes, bryozoans, and protozoans *Gracilaria birdiae, G. domingensis*	[44.136–138, 201–203]
Phenolic extracts	*Macrocystis pyrifera, Porphyra columbina Sarcothalia radula, Gigartina skottsbergii*	[44.139]
Phlorotannins (mainly dieckol)	*Ecklonia cava*	[44.133]
Polypeptide	*Chlamys farreri*	[44.204]
Sargaquinoic acid and sargachromenol	*Sargassum sagamianum*	[44.143]
Antimelanogenesis (antityrosinase and melanin synthesis)		
Anhydrophytosphingosine derivative Jaspine B	*Jaspis sp.*	[44.169]
Dieckol	*Ecklonia cava*	[44.150]
Diphlorethohydroxycarmalol	*Ecklonia cava*	[44.126]
Fucoxanthin	*Ecklonia cava*	[44.144, 159]
Geotidin A	*Geodia japonica*	[44.160]
Kojic acid	*Marine fungi*	[44.154]
Marinomycin A	*Marinispora*	[44.171]
7-Phloroeckol	*Ecklonia cava*	[44.157]
Phloroglucinol, eckol, and dieckol	*Ecklonia cava*	[44.133]
Phlorotannins	Brown algae	[44.155, 205]
Phloroglucinol, eckstolonol, eckol, phlorofucofuroeckol A, and dieckol	*Ecklonia stolonifera*	[44.158]
	Endarachne binghamiae, Schizymenia dubyi, Ecklonia cava and Sargassum siliquastrum	[44.153]
Polyacetylene dideoxypetrosynol A	*Petrosia* sp.	[44.170]
Sulfated polysaccharide: fucoidan	*Sargassum* sp., *Fucus vesiculosus*	[44.189]
Solvent extracts	*Pseudomonas*	[44.156]
Antiallergic and anti-inflammatory skin activity		
6,6′-bieckol, 1-(3′,5′-dihydroxyphenoxy)-7 (2″,4″,6 trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin and dieckol	*Ecklonia cava*	[44.181]
Eckol and dieckol	*Ecklonia stolonifera*	[44.149]
Eckol, phlorofucofuroeckol A, dieckol, and 8,8′ -bieckol	*Ecklonia bicyclis* and *E. kurome*	[44.184]
Phlorofucofuroeckol-B	*Eisenia arborea*	[44.182]
Polysaccharide alginic acid	Brown algae	[44.187]
Sulfated polysaccharide fucoidan	Brown algae	[44.189]
Sulfated polysaccharide porphyran	*Porphyra tenera, P. yezoensis*	[44.190]
Sulfated polysaccharide sacran	*Aphanothece sacrum*	[44.191]

Table 44.3 (continued)

Active component	Source	References
Antimicrobial, antibacterial, and antiviral activity		
Antibacterial activity		
2-(2′,4′-dibromophenoxy)-4,6-dibromophenol	*Dysidea granulosa*	[44.206]
Diterpene dehydroxychlorofusarielin B	*Aspergillus* sp.	[44.207]
Diterpene sargafuran	*Sargassum macrocarpum*	[44.208]
Marinomycin A	*Marinispora*	[44.171]
HSV-1 inhibitory activity		
Sulfated polysaccharide A1	*Cochlodinium polykrikoides*	[44.209]
Sulfated polysaccharide SP-2a	*Sargassum patens*	[44.210]
HSV-1 and HSV-2 inhibitory activity		
DL-galactan hybrids	*Gymnogongrus torulosus*	[44.211]
Galactofucan	*Adenocystis utricularis, Undaria pinnatifida*	[44.212, 213]
Novel linear peptides halovirs A–E	*Scytalidium* sp.	[44.214]
Sulfated polysaccharide naviculan	*Navicula directa*	[44.215]

Staphylococcus aureus, and *Vibrio parahaemolyticus*. Dieckol was also active against *Trichophyton rubrum*, a human tinea pedis fungus [44.111]. Methanol extracts of *Ulva fasciata* showed potent inhibition of the oral pathogenic bacteria *Actinomyces viscosus*, *Streptococcus mitis*, and *S. mutans*, suggesting possible uses in preventing and treating dental caries [44.218]. Laurene-type sesquiterpenes from the red alga *Laurencia obtusa* exhibited potent activity against the Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, and *Candida albicans* [44.219]. Diterpene sargafuran from *Sargassum macrocarpum* [44.208] and solvent extracts from *Ecklonia cava*, *E. kurome*, *Ishige sinicola*, and *Symphyocladia latiuscula* exhibited potent inhibition against *Propionibacterium acnes* [44.220], showing potential to be developed into new skin care cosmetics to prevent or treat acne. A crude sulfated polysaccharide from the red alga *Gracilaria ornata* inhibited the growth of *Escherichia coli* [44.221]. A polysaccharide from the green algae *Chaetomorpha aerea* exhibited selective antibacterial activities against Gram-positive bacteria [44.222]. Methanolic extracts of some cyanobacteria inhibited the growth of *Pseudomonas* [44.223]. Sea cucumber extracts were an efficient antibacterial and antifungal agent against *Escherichia coli*, *Aeromonas hydrophila*, *Enterococcus* sp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Aspergillus fumigatus, Fusarium oxysporum*, *Cladosporium fulvum*, and *Monilia* sp. and *Vibrio harveyi*. The antimicrobial action has been ascribed to the presence of steroidal sapogenins, peptides, and triterpene glycosides [44.224].

Recently, great interest has been expressed regarding marine organisms such as algae, sponges, tunicates, echinoderms, mollusks, shrimp, bacteria, and fungi as promising anti-HSV agents [44.225]. Several researchers have investigated the inhibitory effects of sulfated polysaccharides on the herpes simplex virus strains HSV-1 and HSV-2 [44.186]. A sulfated polysaccharide from *Sargassum patens* significantly inhibits the in vitro replication of both the acyclovir-sensitive and resistant strains of HSV-1, in dose-dependent manners [44.210]. Galactofucan from *Undaria pinnatifida* [44.213] and *Adenocystis utricularis* [44.212], and a novel series of DL-galactan hybrids extracted from the red seaweed *Gymnogongrus torulosus* [44.211] showed a high inhibitory activity against herpes HSV-1 and HSV-2, with no cytotoxicity, whereas the uronofucoidans from *Adenocystis utricularis* carry no antiviral activity [44.212]. A sulfated polysaccharide from the marine microalga *Cochlodinium polykrikoides* showed antiviral activity against herpes HSV-1 [44.209]. A sulfated polysaccharide named naviculan was isolated from a diatom, *Navicula directa*, and showed antiviral activities against HSV-1 and HSV-2 [44.215]. A series of novel linear peptides, halovirs A–E, which are produced during the saline fermentation of the marine fungus *Scytalidium* sp., were potentin vitro against HSV-1 and HSV-2 [44.214].

44.7 Treatments Based on Marine Resources

Marine resources have traditionally been the basis of effective and safe therapies associated with well-being. Some aspects of thalassotherapy and, particularly, of algotherapy are discussed here.

The term thalassotherapy, from the Greek word for sea (thalasso), refers to the therapeutic use of seawater, sea products, and shore climate for their beneficial effects on the skin [44.226]. Standard procedures include hot seawater baths, underwater massage, jet showers, and algal and peloid therapy. Thalassotherapy is used as an alternative treatment for medical conditions and has become a popular tourist attraction for relaxation and stress reduction, and spa therapy is offered claiming prevention and treatment of dermatological diseases [44.227].

The term *peloid*, which was first defined in 1949 by the International Society of Medical Hydrology, during its 6th Conference held in Dax (France), refers to the therapeutic use in mask, cataplasm, or bath forms of naturally occurring clays and minerals mixed with organic matter after physical, chemical, biological, and geological modifications or maturation [44.228–230].

In thalassotherapy, trace elements found in seawater, such as magnesium, potassium, calcium, sodium, and iodine are believed to be absorbed through the skin. The marine water composition, with variable salinity content (usually 3.5%) is made up by the elements shown in Table 44.4 [44.231]. Filtered and processed by reverse osmosis membrane and electrodialysis seawater has shown antibacterial action, anti-inflammation potential, and superoxide anion radical scavenging capacity. It was proposed that magnesium, zinc, potassium, and calcium ions could be the bio-functional agents responsible for those effects. Acidic cosmetic water isolated from seawater decreased tyrosinase and melanin activities in human epidermal melanocytes (HEMn-MP) and presented anti-growth and antimigration effects on human skin melanoma cells (A375.S2) [44.235].

In contrast to other cosmetic ingredients, the benefits of topically appliedingredients, the benefits of topically applied mineral salts have been largely ignored and unexploited. The most famous example for balneotherapy is the Dead Sea minerals, reported to be safe and effective for dermatological disorders in both in vitro and in vivo studies [44.227, 236–238]. Magnesium, sulfur, sodium, bromine, and iodine are known for their healing properties for psoriasis sufferers and patients with rheumatic complaints, and appear to be the prominent actors in rheumatology and dermatology when incorporated in cream or mud [44.239]. Because of its effect in retention of water in the skin, regulation of the pH of the skin, acne repair and prevention, enhancing of blood circulation, and antiaging effect, sea mud has been added to cosmetic formulae, cosmetics, and therapeutic treatments [44.240].

Heated seawater baths have been recommended to stimulate a dilatation of cutaneous vessels. Baths at temperatures below 27 °C and between 28 °C and 32 °C can stimulate the individual and increase the amount of blood evacuated from the heart to the large vessels, decongest and smoothen articulation, and have been prescribed for the treatment of chronic evolutive polyarthritis. Showers prior to and after baths exert a dual thermal and mechanical action on vessels and nerve endings; alternation of short cold and warm water sprays may have the same tonifying ef-

Table 44.4 Chemical composition ($g\,kg^{-1}$) of seawater [44.228, 231–234]

Seawater chemical composition					
Cations	**($g\,kg^{-1}$)**	**Anions**	**($g\,kg^{-1}$)**	**Neutral compounds**	**($g\,kg^{-1}$)**
Na^{2+}	10.35	Cl^-	14.72–19.60	$B(OH)_3$	0.0197
Na^+	0.35–10.98	SO_4^{2-}	2.67–2.79	CO_2	0.0004
Mg^{2+}	0.56–1.37	HCO_3^-	0.1061–0.15		
Ca^{2+}	0.36–1.98	Br^-	0.05–0.0681		
K^+	0.36–0.4042	CO_3^{2-}	0.0145–0.02		
Sr^{2+}	< 0.0024–0.0080	$B(OH)_4^-$	0.0080		
Fe^{2+}	0.01–0.057	F^-	0.0012–0.0013		
		OH^-	0.0001		

fect as the Finnish sauna [44.66]. There are another treatments as such as ammotherapy or psammotherapy (partial or full-body warm sand baths), methodical exposure to the sun (heliotherapy), and marine climatotherapy (atmosphere, temperature, humidity, wind, barometric pressure, etc.) with beneficial effects to skin health [44.241].

Among the variety of marine resources available, algal extracts are being extensively demanded and used. Phytotherapy, and its component algotherapy, were officially recognized in 1986 by the French Ministry of Health as a reimbursable medical service by the National Health Scheme. However, this recognition is not found in other countries, and some of the claimed beneficial effects of these therapies have not been sufficiently contrasted with scientific and medical evidences.

Some examples of traditional algal treatments should be mentioned. *Lithothamnium calcareum* is a marine alga whose thallium is used in the treatment of decalcification, osteoporosis, painful joints, chronic fatigue, rheumatism, gingivitis, and stomach pains. A rather wide range of marine algae enter in the packs used in thalassotherapy, and algal flours or algal salts are sold for use in home bath therapy. Poultices of *Fucus*, *Laminaria*, *Ulva*, and *Ascophyllum* (with or without *Lithothamnium* powder) are heated to 40–50 °C and applied at thalassotherapy clinics to limbs to relieve rheumatism and arthritis pains. Other algae, such as the red *Porphyra* and *Eucheuma*, and the brown *Laminaria* and *Undaria* are frequently employed in algotherapy [44.66].

Cosmetic treatments with algae have stimulating and regenerating properties, associated with minerals, vitamins, and amino acids [44.242]. Other reported actions include tonifying, antiseborrheic, conditioning, moisturizing, bacteriostatic, UV-radiation blocking, and antioxidant [44.243–245]. Table 44.5 summarizes some studies confirming these actions. Seaweeds are particularly effective in slimming, firming, anticellulite, and the treatment of hair disorders and diseases.

44.7.1 Firming

These extracts are intended to restore the elasticity of the skin, contributing to its rejuvenation. They improve intercellular exchanges and also facilitate and potentiate the action of other drugs. Brown algal sulfated polysaccharide fucoidan is helpful in the maintenance of the skin's elasticity by increasing hydration and, thereby, improving the skin's elasticity [44.254].

Seaweed extracts, rich in oligo-elements, vitamins, marine plankton, amino acids, and other components are sold as potent lipolytic reducers. This action has been ascribed to the ability of releasing heat energy, increasing the metabolism of fat cells (adipocytes) and triggering combustion of triglycerides [44.66]. Fucoxanthin, which is characteristic of brown algae, has unique mechanisms of antiobesity properties [44.8]. The potential effect of the seaweed algae on weight reduction is associated with well-being, sleep amelioration, and an improvement of skin tonicity [44.255].

44.7.2 Cellulite

Cellulite is characterized by alterations on the skin surface of the buttocks and posterior and lateral thighs, and mainly affects women. Its pathogenesis is not completely understood, but it could be due to structural, inflammatory, morphological, and biochemical alterations of the subcutaneous tissue. Topical treatments for cellulite include activities related to increased microcirculation flow, reduced lipogenesis and promoted lipolysis, restoration of dermal and subcutaneous tissue, and free radical scavenging [44.256].

Table 44.5 Some studies about cosmetic treatments with algae

Active component	Source	Reference
Adipolysis and skin appearance enhancer		
Fucoxanthin	Brown algae	[44.246]
	Undaria pinnatifida	[44.8]
Sulfated polysaccharide: fucoidan	*Fucus vesiculosus*	[44.247]
Aqueous extract	*Fucus vesiculosus*, *F. lumbricalis*	[44.248]
Hydroglyceric extract	*Laminaria digitata*	[44.249]
Hydroglycolic extract	*Pelvetia canaliculata*	[44.249]
Oily extract rich in rhodysterol	*Gelidium cartilagineum*	[44.249]
Sea-salt	Seawater	[44.249]
Prevention of hair disorders		
Dieckol	*Ecklonia cava*	[44.250]
	Fucus vesiculosus	[44.66]
Diguanosine-tetra-phosphate	*Artemia salina*	[44.251]
Extract	*Grateloupia elliptica*	[44.252]
Burn shell	*Paracentrotus lividus*	[44.251]
Ethanolic extract	*Euchema cottonii*	[44.253]
Shell, ink	*Sepia officinalis*	[44.251]

Table 44.6 Examples of cosmetic products with marine ingredients in their formula classified by cosmetic uses

Products and action	
Face masks and creams, emulsions, lotions, gels, and oils for the skin	***Action***
Marine oligosaccharides and zinc	Antishine, antibacterial, anti-inflammatory
Exopolysaccharides from *Alteromonas* sp.	Soothing and reducing irritation of sensitive skin against chemical, mechanical, and UVB aggression
Polysaccharides from *Laminaria digitata* and proteins from *Enteromorpha* sp.	Calming, reduction of itching
Fiber from *Ascophyllum nodosum*	Skin protective action against environmental stress and extreme climates, moisturizer
Dictyopteris oil	Lips volume enhancer
Palmaria palmata extract	Microcirculation stimulator
Flavonoids from *Monostroma obscurum*	Antioxidant action
Corallina officinalis extract with high Ca, Mg content	Skin strength and brightness enhancer, vasoconstrictor, calming, soothing
Chlorella vulgaris extract and procollagen peptides	Circulation activation
Delesseria sanguinea extract	Microcirculation activation and eye contour decongestion
Asparagopsis armata extract	Skin purification and anti-imperfections
Laminaria ochroleuca and *Himanthalia elongata* extracts	Moisturizers
Proteins from caviar (Family Acipenseridae)	Moisturizing action
Lola implexa extract with high Mg and K content	Collagen synthesis stimulation, moisturizing action and skin protection against free radicals
Dead Sea mud (maris limus) with a high concentration of mineral salts	Absorption of liquids, skin protection
Collagen from salmon skin	Skin revitalization
Pearl powder	Skin brightening
α-hydroxy acids (AHAs) from *Porphyra yezoensis*, *P. umbilicalis*, *Lithothamnium calcareum*	Exfoliating action
Salt crystals	Exfoliant
Oily extract of *Chondrus crispus*	Moisturizing, antioxidant, and exfoliating action
Whitening	
Dictyopteris extract	
Slimming and anti-cellulite creams, gels and oils	
Palmaria palmata and Spirulina sp.	
Seawater	
Eicosanoic acid from *Odontella aurita*	
Fibers and vitamins B_3, B_5 and B_6 from *Laminaria digitata*	
Phyllacantha fibrosa, Durvillaea antarctica, *Halopteris scoparia* extracts	
Sterols from *Fucus vesiculosus*	
Ulva lactuca, *Chlorella pyrenoidosa* extracts	
Carrageenan and extract from *Chondrus crispus*	
Dead Sea mud (maris limus)	
Extract from sea cucumber, *Apostichopus japonicus* or *Stichopus japonicas*	
Sunbathing products	
Laminaria digitata, *Halopteris scoparia*, *Palmaria palmata* extracts	
Coral extract	
Polysaccharides from *Laminaria digitata*	

Table 44.6 (continued)

Products and action
Antiwrinkle, antiaging, and lifting products
Arthrospira and *Spirulina platensis* extracts
Polysaccharide from *Nannochloropsis oculata*
Extract and procollagen peptides from *Chlorella vulgaris*
Mannitol and extract from *Laminaria digitata*
Calliblepharis ciliata, Delesseria sanguinea, and *Dilsea carnosa* extracts
Fucoidan from *Undaria pinnatifida*
Phormidium persicinum extract
Exopolysaccharides produced by plankton
Fatty acids, vitamins, proteins, peptides, minerals, and free amino acids from salmon roe
Thalassiosira sp., *Codium* sp., *Ulva* sp.
Hydrating agents
Chondrus sp., *Asparagopsis* sp.
Ceramium rubrum extract
Sea urchin
Thalasso and algotherapy
Fucus sp., *Laminaria* sp.

44.7.3 Hair Growth Disorders

The demand for products that alter hair growth is a billionaire's market, but few natural effective products are available. Ethanol and aqueous extracts from *Eucheuma cottonii* enhances hair growth by higher proliferative activity [44.253]. The beneficial effect of *Ecklonia cava* extracts on the promotion of hair growth of immortalized vibrissa dermal papilla cells was reported [44.250].

44.8 Products Based on Marine Resources

Currently, a diversity of products based on marine-derived ingredients are marketed for different purposes, including: antiaging, whitening, moisturizers, UV-blocking, and skin or hair specific treatments. These formulations are sold as creams, gels, and lotions, depending on their penetration ability and efficiency and acceptance by consumers. Cosmetic companies are increasingly interested in the development of novel products with marine-based bioactives. Table 44.6 summarizes some representative actions and the active marine-derived ingredients that are found in some commercial formulae. Most of them are crude or refined extracts, but also purified fractions and compounds are used. Aqueous, solvent, and lipid extracts are available. Marine lipid extracts contain a large amount of PUFAs, such as (EPA, 20 : 5n-3) and (DHA, 22 : 6n-3), associated with a variety of benefits in skin disorders treatment. Their encapsulation in liposomes, good vehicles for cosmetic applications, has proved their suitability to improve storage stability of bioactives [44.200].

Some components are recognized ingredients with skin protecting and conditioning actions, or also provide some technological properties, such a viscosity controlling effect, a coloring effect, etc. On the other hand, some components traditionally used for conferring technological properties can be of interest as active agents. Furthermore, some compound used externally may also be effective when proposed for oral use, as nutraceuticals, fitting into the recent trend to provide beauty from within [44.254, 257, 258].

44.9 Conclusions

A great research effort related to the chemical structures, and physical and biological properties of marine natural products has emerged in recent years due to the fact that these resources are still largely unexplored and may have potential for further development of food, cosmetic, and pharmaceutical products. A variety of marine sources have been considered: microorganisms, fungi, macro and microalgae, and by-products from the food industry, such as fish skin, crustacean skeletons, or cartilages. Seawater and minerals are also sources of effective products. A continuous growth in the market of cosmetic products formulated with marine bioactive substances is expected. Both traditional treatments and innovative formulations with novel marine-derived components coexist in commercial skin and hair care products.

The research on bioactive compounds, particularly those that are found exclusively in marine media, is highly attractive due to the unique structures, which are not found in terrestrial sources, and their potent properties. In the next years, the research and development effort should be focused on the identification of the active metabolites, their detailed mechanisms of action, the design of clean extraction and purification technologies, and the development of efficient vehicles and products with enhanced activity, safety, and prolonged shelf-life. To succeed in this area with impressive potential, a close collaboration of different scientific and technical disciplines is encouraged.

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45. Omega-3 Fatty Acids Produced from Microalgae

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The applications of Omega-3 fatty acids for human health are rapidly expanding, which necessitates exploring alternative sources to fish. Many marine microorganisms across different kingdoms exhibit the ability to store a significant oil content, however are difficult to cultivate. Out of all marine microbes, thraustochytrids are considered a good source for the production of high value compounds such as polyunsaturated fatty acids (PUFAs). Optimization of culture conditions will be helpful in further enhancing cellular lipid content to suit fatty acid synthesis. This chapter describes some recent advances in the development of marine microbes for fatty acid production with a special emphasis upon thraustochytrids for biotechnological applications, focussing particularly on methods to enhanced docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) production.

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45.1 Importance of Unsaturated Fatty Acids

The wealth of the ocean is infinite in terms of the discovery of nutraceuticals and biological compounds. One of the major components of interest among them is omega-3 fatty acids. Omega-3 fatty acids are naturally occurring polyunsaturated fatty acids (PUFA) that include mainly alpha-linolenic acid (ALA), eicosapentaenoic acid [45.1] docosahexaenoic acid (DHA). PUFAs play multiple roles in cell function and biology; they regulate various regulatory molecules such as prostaglandins and thromboxanes. Some of the recent updates on biological functions of omega-3 fatty acids are presented in Table 45.1.

The awareness of consumers with regard to the health benefits of the consumption of omega-3 fatty acids has made them an important functional food component on the market. Global finished products of PUFA sales for 2011 reached US$24.5 billion and is expected to reach US$34.5 billion by 2016 conferring to package facts. The target market for omega-3 fatty acids is diverse in terms of the various types of products, ranging from fortified foods, beverages, and dietary supplements to infant food and pet foods [45.14]. Global aquaculture production was 547 million tonnes in 2003 and it is increasing by an average of 9% ev-

Table 45.1 Biological benefits of Omega-3 fatty acids

Biological benefits of omega-3 fatty acids	Reference
Helps in bone formation and prevents chronic inflammatory diseases, rheumatoid arthritis, and inflammatory bowel disease	[45.2]
DHA plays a significant role in the development of the neural and visual system in infants by aiding in the development of the central nervous system	[45.3]
Prevents primary and secondary cardiovascular (CV) diseases	[45.4]
Dietary intake helps prevent Alzheimer's disease	[45.5]
Reduces bronchial allergic inflammation that causes asthma	[45.6]
Recommended in treatment of schizophrenia; clinical data suggests that patient DHA and ALA levels are lower than normal	[45.7]
Reduces osteoarthritis and helps prevent disease when taken in diet	[45.8]
Prevents gastrointestinal tract cancer and has been proved as a chemopreventive agent with respect to colorectal cancer	[45.9]
Supplement to patients with severe hypertriglyceridemia, reported decrease in triglyceride levels and cardiovascular disease risks	[45.10]
Supplement can decrease plasma homocystine, an independent risk factor for cardiovascular diseases	[45.11]
Supplement of EPA and DHA are reported to be beneficial in decreasing the risk of depression leading to suicide and delaying the onset of neurological degeneration of aging	[45.12]
PUFAs can help to reduce inflammation in overweight, middle-aged, and older adults, and thus promote broad health benefits	[45.13]

ery year. Although the global demand is growing, fish is a declining resource because of environmental issues due to the continuous exploitation of fish stock to meet the demands of market. A lack of environmental sustainability and the increasing global demand has driven us to search for alternative sources for the future [45.15].

The main focus of the current research is screening efficient microbial sources that may produce both EPA and DHA. Thus, chapter includes the origin of omega-3 fatty acids, biosynthesis, and fermentation optimization in marine microbes.

45.1.1 Unsaturated Fatty Acids – Focus on Marine Origin

ALA is also regarded as the parent PUFA that is converted into EPA and DHA. The rate of conversion from ALA in to EPA and DHA is low in humans, and there are no other means of PUFA synthesis in the human body; hence EPA and DHA are classified as essential nutrients. Vegetable oil is considered as a main source of ALA, whereas DHA and EPA are predominantly found in fish oil [45.9, 16, 17]. Among the various sources, oily fish such as sardines, salmon, tuna, and herring are considered to be the best dietary sources of omega-3 fatty acids. In many of the oily fish, these oils are derived from microorganisms through their food web (Fig. 45.1) [45.4]. Marine phytoplanktons are considered ocean factories of omega-3 fatty acids, as they are the primary producers in the marine food web [45.18]. A major source of PUFAs are microorganisms, as they are easy to grow and more eco-friendly in terms of extraction and refining when compared to animal fatty acids, which are associated with odor and the presence of cholesterol [45.19].

Microorganisms have an advantage over traditional energy crops because they have a higher growth rate, produce more biomass in a shorter time span, and do not compete for land space [45.20]. Biotechnology may contribute towards the industrial production of fatty acids to serve the nutritional demands of DHA and EPA by producing feed enriched with PUFAs, which in turn would increase the content of essential fatty acids in meat, milk, and eggs [45.21, 22].

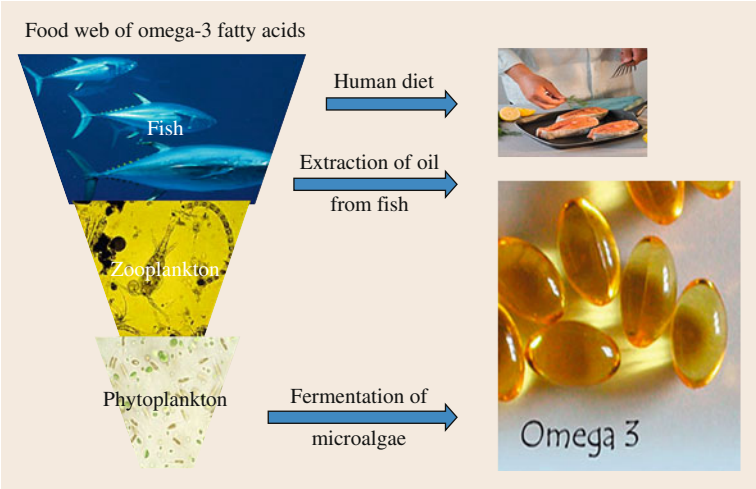

Fig. 45.1 Marine food web associated with the production of omega-3 fatty acids

45.2 Potential Alternative Sources for PUFA Production

Industrially, omega-3 fatty acids are extracted mainly from fish oil; various fish sources and their DHA and EPA contents are represented in Table 45.2. There are limitations in the use of fish oil due to the contamination of heavy metals by environmental pollutants [45.23]. In oily fish these oils were derived from microorganisms through their food web [45.18]. A few strains of bacteria and yeast were identified from the intestine of fish from the deep sea. Bacterial PUFAs are composed of phospholipids, which are 10–15% of the dry weight. Whereas in microalgae PUFAs in triacylglycerol constitutes to 80% of the dry weight [45.14]. Among phytoplankton, microalgae and marine diatoms are considered to play a significant role in the food web. In aquaculture, microalgae serve as feed for zooplankton and molluscs, which in turn serve as feed for fish and fish-eating sea animals. Especially the microalgae *Isochrysis* and *Nannochloropsis* are used as feed for rotifers, which, in turn, are supplied to fish to enrich their diet with EPA and DHA [45.24]. Food webs associated with omega-3 PUFAs are interrelated [45.25]. Humans mainly obtain DHA and EPA by consuming fish, whereas fish, in turn, obtain PUFAs from microalgae (Fig. 45.1). Microalgae-derived DHA and EPA can be used as a supplement for people who do not consume fish and seafood [45.26]. The food web association has led to the exploitation of marine microalgae to act as major reservoirs of PUFAs. PUFAs from microalgae are eco-friendly and more stable because of the presence of natural antioxidant carotenoids and vitamins that are bioencapsulated in the algal cell wall [45.27, 28]. Thus, microalgae can be considered as a potential source of production of PUFAs.

45.3 Marine Microalgae

Algae are spread in diversified ecosystems that include marine, the freshwater environment, the desert and hot springs, along with snow and ice. Their diversity in habitat is achieved by their ability to grow in natural environments under inorganic conditions by fixing CO_2 with the process of photosynthesis.

They are mainly classified into multicellular large seaweeds (macroalgae) and unicellular microalgae [45.30]. Based on their pigment composition they are further classified into nine divisions. Some of the largest groups include Chlorophyceae (green algae), Phaeophyceae (brown algae), Pyrrophyceae (dinoflagellates), Chrysophyceae (golden brown algae), Bacillariophyceae (diatoms), and Rhodophyceae (red algae) [45.31].

50 000 microalgae species have been reported, but not more than 30 000 species have been studied. The market for microalgae is 5000 tonnes per year, and

Table 45.2 Fish and seafood sources of DHA and EPA (after [45.29])

Fish and seafood sources of DHA and EPA	
Fish source (100 g portion)	**DHA + EPA (g)**
Carp, cooked, dry heat	0.45
Catfish, channel, farmed, cooked, dry heat	0.17
Cod, Atlantic, cooked, dry heat	0.15
Eel, mixed species, cooked, dry heat	0.18
Flatfish (flounder and sole), cooked, dry heat	0.50
Haddock, cooked, dry heat	0.23
Halibut, Atlantic and Pacific, cooked, dry heat	0.46
Herring, Atlantic, cooked, dry heat	2.01
Mackerel, Pacific and jack, mixed species, cooked, dry heat	1.84
Mullet, striped, cooked, dry heat	0.32
Perch, mixed species, cooked, dry heat	0.32
Pike, northern, cooked, dry heat	0.13
Pollock, Atlantic, cooked, dry heat	0.54
Salmon, Atlantic, farmed, cooked, dry heat	2.14
Sardine, Atlantic, canned in oil, drained solids with bone	0.98
Sea bass, mixed species, cooked, dry heat	0.76
Shark, mixed species, raw	0.84
Snapper, mixed species, cooked, dry heat	0.32
Swordfish, cooked, dry heat	0.81
Trout, mixed species, cooked, dry heat	0.93
Tuna, skipjack, fresh, cooked, dry heat	0.32
Whiting, mixed species, cooked, dry heat	0.51
Crustaceans	
Crab, Alaska king, cooked, moist heat	0.41
Shrimp, mixed species, cooked, moist heat	0.31
Spiny lobster, mixed species, cooked, moist heat	0.48
Molluscs	
Clam, mixed species, cooked, moist heat	0.28
Conch, baked or broiled	0.12
Mussel, blue, cooked, moist heat	0.78
Octopus, common, cooked, moist heat	0.31
Oyster, eastern, farmed, cooked, dry heat	0.44
Scallop, mixed species, cooked, breaded and fried	0.18

the annual sale value is US\$6 $\times 10^9$ with a productivity of 7.5×10^6 tonnes per year [45.32]. Further, the market for marine microalgae-derived omega-3 has been growing recently, with US\$2 billion revenue in 2012, which is expected to increase to double digits by 2016 due to the potential target of the Asia-Pacific market [45.14].

Microalgae are effective in the reduction of carbon dioxide into biomolecules like carbohydrates, proteins, lipids and triglycerides. Microalgae have gained lot of industrial attention due to their wide product perspectives, which serve the needs of the human community. Various commercial applications of microalgae for the benefit of mankind are discussed in Table 45.3.

45.3.1 Cultivation of Marine Microalgae

Microalgae are divided into groups such as autotrophic, heterotrophic, mixotrophic, and photoheterotrophic algae based on the type of metabolism used to synthesize energy and the ability to utilize various forms of energy [45.56].

The autotrophic microalgae *Chlorella vulgaris* can convert solar energy to chemical energy through the process of photosynthesis. Photoheterotrophs require light for the utilization of organic carbon sources. In industry, photoautotrophs and photoheterotrophs are cultivated by open, closed, or semiclosed outdoor bioreactors [45.57].

The heterotrophic microalgae *Haemotococcus pluvialis, Chlamydomonas* sp. and *Phaeodactylum* sp. can utilize organic compounds to produce energy; they are commercially cultivated with the help of fermenters of various scaling up ranges based on the requirements. The mixotrophic microalgae *Arthrospira platensis* can produce the essential growth compounds either in the presence of light or organic compounds based on the available conditions [45.57]. Photo bioreactors and commercial fermenters share the common purpose of cultivation but the difference lies in the source of energy, oxygen supply, pH control, light illumination, and the process of sterilization [45.58].

45.3.2 Fermentation of Microalgae

Microalgae are exploited for the industrial production of various metabolites such as carbohydrates, lipids, enzymes, polymers, toxins, antioxidants, pigment biomass, and many more [45.59]. The cultivation of microalgae involves two basic processes, the autotrophic and heterotrophic modes of growth. Based on the requirement of microalgae and the demand of the process, autotrophic fermentation processes are further categorized as open system and closed system [45.31]. An open system is the simplest method of cultivation with the advantage of low production costs. The open

Table 45.3 Commercial applications of various marine microalgae

Microalgae	Application	References
Chlorella, *Dunaliella*, *Isochrysis*, *Nannochloris*, *Nannochloropsis*, *Neochloris*, *Nitzschia*, *Phaeodactylum*, *Porphyridium* spp.	Biofuel	[45.33]
Arthrospira, *Chlorella*, *Nannochloropsis oculata*, *Dunaliella salina*	Cosmetics	[45.34, 35]
Chlorella, *Scenedesmus*, *Phormidium*, *Botryococcus*, *Chlamydomonas*, *Spirulina*	Domestic water treatment	[45.36–39]
Chlorella, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema*, *Thalassiosira*	Fish feed	[45.34, 40–42]
Dunaliella salina, *Haematococcus pluvialis*	Food industry	[45.43, 44]
Arthrospira, *Chlorella*, *Dunaliella salina*, *Aphanizomenon flos-aquae*	Human and animal nutrition	[45.34, 45, 46]
Chlorella, *Ankistrodesmus*, *Scenedesmus* sp.	Phycoremediation	[45.47–49]
Arthrospira, *Rhodophyta*, *Porphyridium*	Phycobiliproteins	[45.50, 51]
Chlorella pyrenoidosa	Production of immunostimulatory polysaccharides	[45.52]
Arthrospira, *Porphyridium* (AA), *Nannochloropsis*, *Phaeodactylum*, *Nitzschia*, *Crypthecodinium*, *Schizochytrium* (DHA)	Production of PUFAs	[45.34, 53–55]

culture system requires a large surface area and shallow depth; various models of the open pond system are in use, which include natural or artificial ponds, raceways, tanks, circular ponds with impellers, rotating arms, or paddle wheels. In Western Australia, *Dunaliella* is used for the production of β-carotene using an artificial shallow pond system [45.56]. Growth in the open pond system can be enriched by supplying CO_2 and mixing when required. Limited control on cultivation conditions and contamination of other microorganisms means that the system is restricted for microalgae which are tolerant to extreme conditions. The open pond system mainly suits the cultivation of *Spirulina* and *Chlorella* and is applied in places such as Japan, Thailand, California, Hawaii, Taiwan, India, and China.

To overcome these disadvantages closed photo bioreactors are designed in various sizes and shapes to meet the requirements of microalgae growth and development. In this process, plexiglas, acrylic, glass tubes, and flexible plastic coils are used as solar collectors; where the microalgal suspension is circulated continuously through rows of connected transparent tubes or coils [45.60]. A horizontal tubular photo bioreactor in Florence, Italy, has led to high productivity for *Spirulina* [45.58].

Heterotrophic microalgae are very few and they require the addition of organic sources in the growth medium for energy generation, which leads to the production of various metabolites in the process. Growth is independent of light energy, which allows a simpler scale up process to overcome the disadvantage of large investments in infrastructure and it also supports the reproducible biomass yield in the heterotrophic mode of growth [45.56]. In Japan, *Chlorella* sp. produced by heterotrophic fermentation led to biomass of 500 ton, which amounts to 50% of the total Japanese production of this alga [45.60].

To make the process of fermentation cost effective, both the autotrophic and heterotrophic processes are mixed to achieve the maximum algal biomass and product yield. *Chlorella sorokiniana* is fermented in a two-stage heterotrophic and phototrophic culture strategy that allows heterotrophic seed culture in the autotrophic open algal culture system, resulting in higher growth rate, cell density, and productivity [45.61, 62]. The photoheterotrophic fermentation method of *Chlorella minutissima* led to an 11.9 times higher lipid content than the autotrophic condition reported earlier [45.63].

45.3.3 Isolation of Microalgae for the Production of PUFAs

Isolation of the desired microalgae for the production of PUFAs plays a major role in deciding various parameters of fermentation at lab scale that adds to the production cost during the industrial fermentation of PUFAs. In general, microalgae can be isolated from freshwater and marine water by using the traditional micropipette washing technique, the centrifuge washing technique, and the streak plating technique. Microalgae rich in PUFA content can be isolated with the help of high-throughput cell sorting coupled with flow cytometry. Photosynthetic pigments in microalgae

emit various kinds of fluorescence, this is applied as the principle behind the flow cytometry. Chlorophyll autofluorescence (CAF) of eukaryotic photosynthetic phytoplankton and green auto-fluorescence of dinoflagellates, along with red and orange autofluorescence to differentiate between algal strains are used in the process of identification and isolation of microalgae. Furthermore, the isolated microalgae are cultivated to screen the strains with respect to high biomass yield and lipid content [45.64].

Adoptability to diversified environmental conditions is well reflected with the composition of fatty acids in algae, very long-chain (VLC) (18C) fatty acids and PUFAs are noticed to be more in eukaryotic algae compared to cyanobacteria. PUFAs amount to 20% of lipids in algae and include arachidonic acid (ARA), eicosapentaenoic acid [45.1], and docosahexaenoic acid (DHA) hindered to exploit and increase the accumulated PUFAs with the help of biotechnology [45.65].

Thraustochytrids are microalgae like microorganisms that can accumulate 60% of their dry weight as lipids, of which more than 25% is normally DHA. A study of the ultrastructure with transmission electron microscopy [45.47] illustrated that cells are heterogeneous in size, approximately 6–21 μm in diameter with a granular cytoplasm, containing oil micelles [45.66]. The high lipid content lends thraustochytrids to the commercial production of omega-3 fatty acids. In general, they are isolated with the pine pollen technique [45.67, 68] and acriflavine direct detection (AfDD) techniques [45.69]. These two methods are reported as effective isolation techniques with respect to thraustochytrids.

45.4 Biosynthesis of Omega-3 Fatty Acids in Marine Algae

Microalgae are known to produce large amounts of C_20–C_22 PUFAs. PUFAs can be synthesized by aerobic and anaerobic pathways. The aerobic pathway involves the role of *desaturases* and *elongases* enzymes, whereas the anaerobic pathway uses polyketide synthase enzymes [45.70]. Biosynthesis of PUFAs starts with a double bond formation in the aliphatic chain of the fatty acid, and the elongation by two carbon units of the acyl chain, hence in this biosynthetic pathway the alternative action of the two enzymes *desaturase* and *elongases* contributes to the synthesis of PUFAs [45.71]. Various biochemical pathways for the production of DHA and EPA are represented in Fig. 45.2.

45.4.1 Synthesis of EPA

The red alga *Porphyridium cruentum* has been used extensively to study the biosynthetic pathway for the production of EPA with the help of exogenously supplied radiolabeled fatty acids, to reveal the omega-3 and omega-6 pathways for the conversion of EPA. In the omega-6 pathway with the action of desaturase, Υ-linolenate was formed from linoleic acid followed by elongation to dihomo Υ-linolenate, which was further de-saturated into arachidonate, followed with EPA formation. In the case of the omega-3 pathway, linoleate was de-saturated to α-linoleate, which was further converted to EPA from the intermediate fatty acids 18 : 4n-3 and 20 : 4n-3. Among the two pathways, the omega-6 pathway is considered to be the active route [45.65, 72].

45.4.2 Synthesis of DHA

Biosynthesis of DHA is assumed to involve $\Delta 6$ desaturation to form C18 : 4n-3 from α-linoleate, further elongated to C20 : 4n-3 which undergoes desaturation to form EPA, which is then converted to DPA followed by DHA formation. Conversion of DPA to DHA by $\Delta 4$ desaturase is identified in *Thraustochytrium* sp., this is noticed to be different from the mammalian pathway of DHA synthesis, the Sprecher pathway that involves two consecutive elongation steps [45.73].

In the case of *Aurantiochytrium* sp., (or *Schizochytrium* sp.) biosynthesis of DHA is reported by the alternative pathway known as polyketide synthase pathway (PKS). Biochemical experiments indicated that desaturase and elongase enzymes are not involved in the PKS pathway but showed the PKS protein complex on molecular-genetic analysis. Although the exact sequence of the PKS pathway remains to be determined, identification of the protein domain provides evidence for the mechanism of cis double bond insertion through the action of dehydratase/2-trans,3-cis isomerase (DH/2,3I) [45.74, 75].

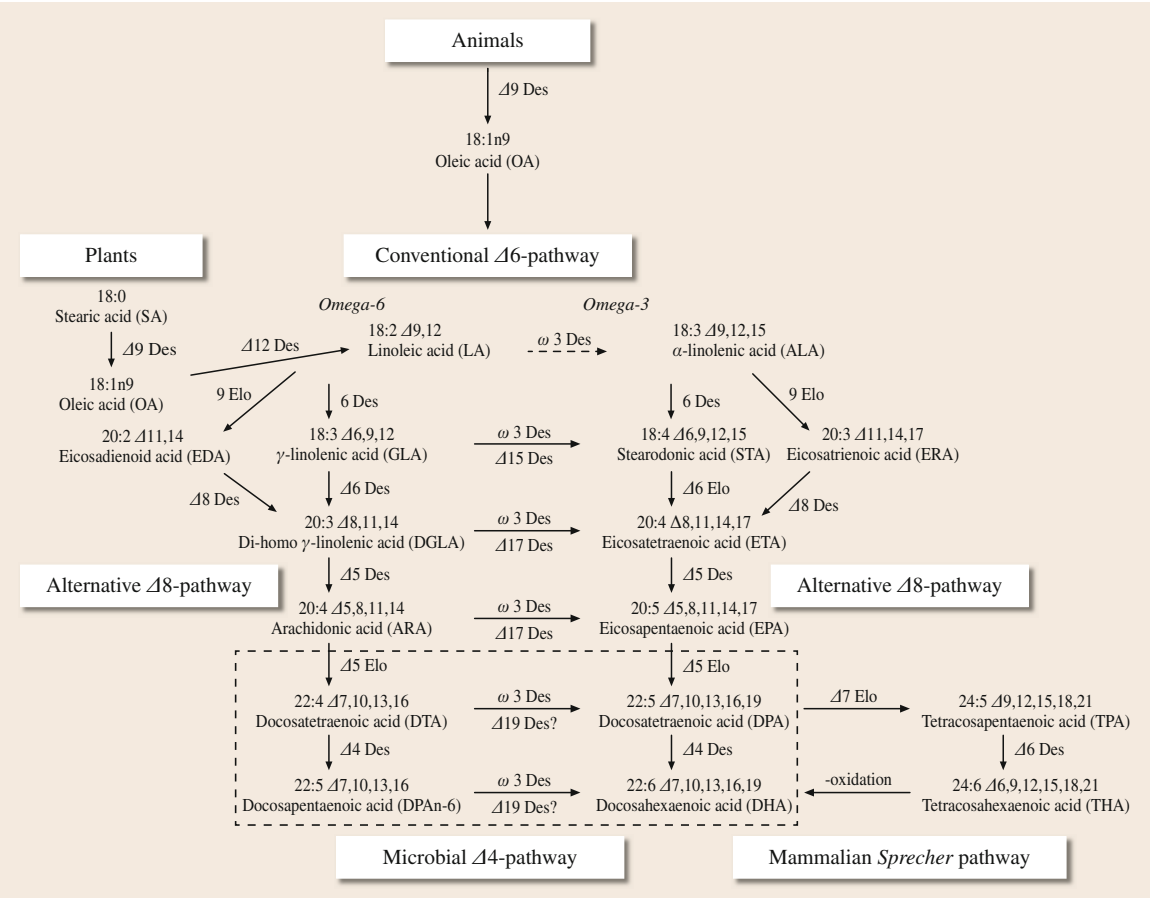

Fig. 45.2 Biochemical pathways for the synthesis of DHA and EPA (after [45.16])

45.5 Microalgae Fermentation for the Production of PUFAs

Microalgae are generally cultivated in natural lagoons or lakes to highly sophisticated bioreactors. Bioprocess engineering has contributed to process optimization of various parameters to ensure large-scale microalgae production [45.76]. Process optimization depends on the growth requirements of microalgae. Autotrophic, heterotrophic, or a mixture of both processes is applied based on the product yield aimed at low production cost. The heterotrophic-based microalgal production system favors a higher magnitude of PUFA production than that of outdoor autotrophic pond systems [45.60, 77].

Autotrophic growth of *Porphyridium cruentum* reported less total fatty acid accumulation when compared to heterotrophic growth with glucose and glycerol [45.83]. The microalgae *Pavlova lutheria*, grown under photoheterotrophic conditions of high and low light intensity produced significant results with the production of PUFAs, especially high light intensity favored high DHA yield and low light intensity reported high EPA yield [45.84]. Heterotrophically grown *Crypthecodinum* sp., *Chlorella* sp. are rich in DHA and ARA contents. Cells are broken by mechanical and enzyme hydrolysis methods to extract oil. The resulting algal biomass is further freeze dried and used as feed for rotifers and Artemia, which is further used in formulated brood stock diets of fish larvae [45.85]. A list of microalgae involved in PUFA production is given in Table 45.4. The mi-

Table 45.4 PUFA production by various microalgae

Microalgae	DHA[a] 22 : 6(n-3)	EPA[b] 20 : 5(n-3)	Reference
Amphora sp. NT13	0.3	13.9	[45.78]
Chaetoceros sp. CS256	0.8	16.7	
Fragilaria sp. GOC1	1	6.8	
Nitzschia spp. CS258	0.3	8.4	
Nitzschia spp. BC8	0.3	5	
Skeletonema spp. GOC27	1.4	12.9	
Skeletonema spp. GOC36	1.8	13	
Cryptomonas sp. CRFI01	6.6	12	
Rhodomonas sp. NT15	4.6	8.7	
Nephroselmis sp. GOC52	3	2.4	
Tetraselmis spp. NT18	0.1	4.3	
Tetraselmis spp. TEQL01	0.1	3.9	
Isochrysis sp. NT14	9.9	0.9	
Rhodosorus sp. CS249	0.4	7.8	
Navicula like diatom	0.4	29.6	[45.79]
Skeletonema sp.	4.2	20.2	
Thalassiosira pseudonana	1.3	10.2	
Naviculajeffreyi	2.1	20.1	
Proteomonas sulcata	5.3	3.3	
Rhodomonas salina	8.9	17.6	
Pavlova pinguis	10.9	27.1	
Heterocapsa	16.5	traces	
Amphidinium sp.	24.7	23.9	
Schizochytrium limacinum SR21	33.5	ND[c]	[45.80]
Aurantiochytrium mangrovei MP2	3	ND	[45.81]
Thruatochytrium sp. ONC-T18	1.77	36.05	[45.82]
Amphidinium sp. S1	26.33	17.07	[45.76]
Amphidinium sp. S2	24.58	17.41	
Amphidinium sp. S3	20.41	15.23	
Amphidinium sp. S4	22.31	15.29	
Asterionella sp. S1	8.65	25.01	
Asterionella sp. S2	8.89	26.43	
Prorocentrum minimum S1	20.87	2.91	
Prorocentrum minimum S2	20.06	3.66	
Prorocentrum triestinum S1	21.97	1.72	
Prorocentrum triestinum S2	20.39	1.92	
Prymnesium parvum S1	8.63	0.45	
Prymnesium parvum S2	11.18	0.52	
Amphora exigua	ND	4.98	[45.61]
Amphora bigibba	0.03	4.29	
Caloneis platycephala	ND	7.08	
Chaetoceros muelleri	0.37	2.96	
Cocconeis scutellum	0.06	10.1	
Cylindrotheca sp.	0.04	0.48	
Melosira nummuloides	0.39	1.69	
Navicula lyra	1.06	8.4	

Table 45.4 (continued)

Microalgae	DHA[a] 22 : 6(n-3)	EPA[b] 20 : 5(n-3)	Reference
Nitzschia panduriformis	0.05	6.52	
Nitzschia grossestriata	1.27	7	
Seminavis gracilenta	0.05	14.17	
Skeletonema costatum	2.68	7.47	

[a] DHA, [b] EPA figures are representative of the percentage of total fatty acid (% TFA), [c] ND – not detected

croalgae *Crypthecodinium cohinii* or microalgae like the microorganism *Schizochytrium* sp. are used in the industry for the fermentative production of PUFAs. They are currently being explored for the production of DHA, especially for the enrichment of food products and feed additives in aquaculture [45.15, 86].

Here we describe one example of a marine microbe that is used commercially for the production of omega-3 fatty acids such as DHA and EPA.

45.6 Thraustochytrid Fermentation

Various fermentation conditions including the usage of different sources and other physical parameters have been employed for the production of PUFAs, particularly DHA and carotenoids. They are discussed below.

45.6.1 Effect of Carbon and Nitrogen Sources and Other Promoters on PUFAs Production and Carotenoids

Monosaccharide such as glucose is the most frequently used carbon source due to its easy availability to the microbes. Disaccharides, polysaccharides, and oils like corn and canola oil have also been reported to be used as carbon source [45.87, 88]. The role of the carbon source has been demonstrated as an important constituent in the medium that may result in high DHA yield [45.88]. Apart from this, various other alternative carbon sources such as pure glycerol, crude glycerol (from biodiesel waste), coconut water, distillery wastewater, liquid residues from the food industry, soybean cake, beer and potato processing waste, sweet sorghum juice, cellulosic biomass, and spent yeast from breweries have been used to make the fermentation process economical [45.82, 89–98]. Glucose basal medium supplemented with empty palm fruit bunches yields a good amount of lipids and DHA at 12.5 and 5.4 g L^{-1} [45.89]. Moreover, *Crypthecodinium cohnii* was reported to yield better DHA output when carob pulp syrup was used as the carbon source rather than glucose [45.99].

Similarly, the use of nitrogen sources has been studied, showing the effect on lipid accumulation. *Goldstein* proposed the use of inorganic nitrogen sources glutamate, aspartate, their amides, and alpha alanine to achieve a good biomass yield [45.100]. Thraustochytrids readily utilize organic nitrogen sources such as yeast extract, peptone, and corn steep liquor [45.101, 102]. Moreover, a higher carbon to nitrogen ratio (C:N) supports higher DHA production [45.81, 103]. However, the nitrogen depletion showed an increment in the lipid content [45.68]. In addition to this, various modulators such as Tween 80, acetyl co-enzyme A, and nicotinamide adenine dinucleotide phosphate (NADPH) have been used to alter the biomass and lipid content on the higher end [45.104, 105]. The oleate present in Tween 80 might have triggered the metabolism resulting into high biomass and lipid accumulation. Similarly, acetyl Co-A (as ethanol) and NADPH (as malic acid) elevated the DHA content when introduced at the late lipid accumulation phase.

Thraustochytrids have been well documented with respect to the production of carotenoids such as beta-carotene and xanthophylls like astaxanthin, zeaxanthin, canthaxanthin, echinenone, and phoenicoxanthin [45.101, 106–108]. Carbon and nitrogen sources are very important nutrient factors for carotenoid production. Low glucose concentration resulted in high beta-carotene production, whereas a high glucose concentration produced a high canthaxanthin content [45.106]. Further, it was reported that with the use

of mutation strategy, involving N-methyl-N′-nitro-N-nitroso guanidine (NTG), carotenoid production was increased manifold [45.109]. Other substrates such as distillery waste as the only nitrogen source in the culture medium have been used for the production of DHA and xanthophylls, yielding 3.4 g L^{-1} and 7.7 mg L^{-1}, respectively [45.89].

45.6.2 Effect of Physical Parameters and Other Fermentation Strategies on PUFA Production

Like the chemical constituent of the fermentation medium, physical conditions also contribute to enhance the production of PUFAs. Thraustochytrids can utilize the 20–30 °C temperature range for its growth and lipid accumulation, which is considered an optimal environment [45.110]. A temperature shift study (30–20 °C) was designed to improve the DHA content in the fatty acid profile (≈ 52% of TFA) [45.111]. Although the dip in the temperature may not yield as high biomass, 30 °C was found to be the optimum temperature for the fermentation study. It is pointed out that low temperature has an effect on the lipid metabolism of low temperature marine microbes [45.112]. Also, the natural environments from which the strains have been isolated, may show a different lipid profile [45.113].

The biomass yield achieved for thraustochytrids was highest at neutral pH [45.114], although no significant change was observed in the fatty acid profile. Similarly, salinity plays an important role in thraustochytrid fermentation. Salinity up to 50% is known to yield high biomass [45.103]. Artificial seawater (ASW) has been extensively used for providing salinity in the medium. However, there was a report of using only sodium chloride as the salt source [45.115]. This salinity factor may depend on the type of habitat where the thraustochytrid was isolated. Moreover, it may significantly affect the lipid content, biomass, and fatty acid profile [45.110].

Other factors such as oxygen level, volume of the liquid medium, and statistical design have shown their respective effect on thraustochytrid oil production. At 4–8% of the saturation level of oxygen supply during biomass growth and less than 1% during DHA production, there was improvement in the DHA yield [45.116]. A low dissolved oxygen (DO) level was found to assist in lower DHA production [45.117], thus a low volume medium was recommended in shake flask fermentation. A two-stage oxygen supply system was introduced based on the oxygen transfer coefficient yielding a high concentration of DHA and its productivity [45.118]. Statistical tools such as central composite design, fractional factorial, and response surface methodology were found to be useful while targeting the optimization of various factors to enhance DHA production [45.119, 120].

Further, DHA production has been achieved in large-scale fermenters as reported in [45.68, 101, 121–123]. Several fermentation processes have been designed to elevate biomass and lipid production. A higher biomass growth rate was observed in fed-batch cultivation of *Schizochytrium* sp. [45.115]. Another two-stage system was employed to increase biomass and lipid production [45.122].

A continuous fermentation process has been used, which resulted in high lipid accumulation when compared to batch cultivation [45.124]. *Aurantiochytrium* sp. KRS101 was used in another fed batch fermentation with DHA production up to 40% of total lipids [45.123]. Further, a modified fed-batch strategy using *Aurantiochytrium* sp. KRS101 has been reported to increase the palmitic acid (with respect to biodiesel production) and DHA yield (towards PUFA production) where distinctive yield coefficients were used [45.125].

A recurrent oxygen feeding method was employed to maintain a 50% DO level in the fermentation environment, which achieved a high biomass of 61.76 g L^{-1} and 65.2% of the total lipid content, which also improved the DHA proportion up to 70% of the total fatty acids in the fatty acid profile [45.126]. A recent study involved *Aurantiochytrium* sp. KRS101 in a stepwise fermentation strategy using spent yeast as the sole nutrient source, which supported good biomass growth and lipid productivity [45.98]. With respect to downstream processing of microbial lipids, the direct transesterification method was found to be more effective in achieving high fatty acid yields [45.127].

45.7 Conclusions

The importance of PUFAs, particularly DHA and EPA, is gaining considerable attention because of the increasing list of health benefits. Health-conscious consumers are willing to spend more to ensure its availability for the well-being of their families. Omega-3 fatty acids of marine origin (e.g., fish oil) are already available

on the market and are being consumed. Due to the many shortcomings of fish-derived oil, including undesirable taste and odor, its chemical processing methods, and the presence of contaminants such as mercury, research has been diverted towards the exploitation of other marine species for the development of suitable alternatives. Thus, marine microorganisms are the best alternative at present for the production of these high-value fatty acids. Marine omega-3 fatty acids are making their way onto the market and the estimation of the growth of nutraceutical industry is certainly expanding. Marine microalgae (e.g., thraustochytrids) have been recorded for high PUFA accumulation with a good amount of omega-3 fatty acids. Researchers are trying to increase the productivity by different means such as synthetic biology so that the process can be made economical based on the application of genetic tools.

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46. Selenoneine in Marine Organisms

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A novel selenium-containing compound, selenoneine, 2-selenyl-N_α,N_α,N_α-trimethyl-l-histidine, has been identified as the predominant form of organic selenium in the blood and tissues of tuna. This selenium compound has a selenium atom in the imidazole ring, and is a selenium analog of a thiol compound, ergothioneine. Selenoneine has radical scavenging activity and exerts an antioxidant effect by binding to hemoglobin and myoglobin, protecting them from iron auto-oxidation. In addition, selenoneine has detoxifying activity against methylmercury (MeHg). Selenoneine has been found to be a specific substrate for the organic cation/carnitine transporter OCTN1 (solute carrier family 22 member 4, SLC22A4), and mediated the excretion and demethylation of MeHg by exosomal small vesicle formation. The dietary intake of selenoneine through fish consumption is an important selenium source in the human diet. Since selenoneine and its related selenoproteins have strong antioxidant activities, disease protective functions, such as anticarcinogenesis and aging effects, may be expected.

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46.1 Biochemistry of Selenium

Selenium is an essential micronutrient and a constituent of the selenocysteine residues of antioxidant selenoproteins, such as glutathione peroxidases, thioredoxin reductases and selenoprotein P [46.1–4]. The selenoproteome contains 25 genes encoding selenoproteins in the human genome, indicating that selenium is essential for protein synthesis [46.5]. The genes encoding the selenoproteins contain an in-frame TGA codon within their coding sequence, which directs the incorporation of selenium, in the form of selenocysteine, into these proteins. The selenocysteine-transfer RNA (Sec-tRNA) has its own special translation factor, which delivers it to the translating ribosome [46.4]. Thus, organic and inorganic selenium are used as a selenium source for selenoprotein synthesis. The molecular mechanisms of selenium incorporation into selenocysteine from other selenium compounds should be elucidated.

Selenium bioavailability may depend on the food source and chemical forms of selenium. The major compounds in plant foods are selenite, selenomethionine, and γ-glutamyl methylselenocysteine [46.6]. Although data on the chemical forms of organic selenium in foods of animal origin are limited, the major chemical forms of selenium are considered to be selenocysteine and selenomethionine,

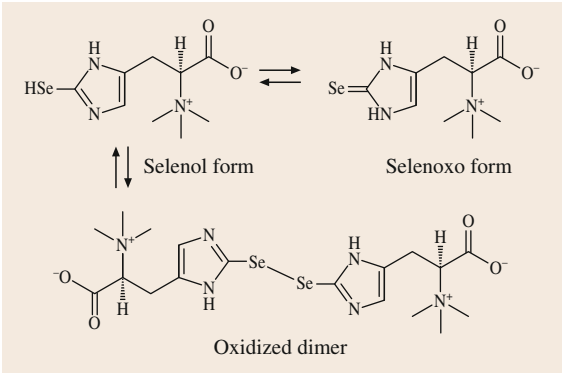

Fig. 46.1 Chemical structure of selenoneine. Selenoxo form bound to heme is present in vivo. A stable oxidized dimer is purified from fish tissues and is used for various bioassays

which is incorporated in muscle proteins. Dietary intake of selenium through fish consumption may be important for the enhancement of selenium redox functions. Fish are considered to be the major selenium source in Japan, contributing to approximately 35% of the total selenium consumed [46.1]. The selenium content and its chemical forms in the edible portions of fish and shellfish have been determined [46.7–9].

Recent studies showed that the muscles of tuna and other predatory fish contain high levels of the selenium-containing imidazole compound, 2-selenyl-N_α,N_α,N_α-trimethyl-l-histidine (selenoneine) (Fig. 46.1) [46.7]. This compound was identified as the major organic selenium source in the blood and muscle tissue of tuna. Selenoneine contains an imidazole ring with a unique selenoketone group and has radical scavenging activity [46.7]. Therefore, dietary intake of selenoneine through fish consumption is considered important for enhancing antioxidant effects in tissues and cells.

Selenium deficiency is associated with 50 human diseases, including prostate, lung, and colon cancers, as well as immunodeficiency, and heart diseases [46.4]. Diseases of domestic animals and birds, such as myopathy, exudative diathesis, and pancreatic degeneration, can be caused by selenium deficiency [46.1–4]. A selenium deficiency is often a result of eating food grown in soils deficient in selenium [46.1]. Also, some intestinal disorders are associated with a risk of developing selenium deficiency [46.10]. Therefore, the dietary intake of selenoneine through fish consumption might be important for free radical detoxification mechanisms in animal cells and tissues. Understanding the nutritional roles and biological significance of selenoneine may contribute to improving human diet and health.

46.2 Selenium and Selenoneine Determination

46.2.1 Selenium Analysis

Fluorometric Assay of Total Selenium

The total selenium concentration was measured using a fluorometric assay employing 2,3-diaminonaphthalene (DAN), according to the method described by *Hasunuma* et al. [46.11]. Each sample (0.1–0.2 g) was dissolved at 200–220 °C for 2–3 h in 1 ml of a 1 : 2 mixture of nitric acid and perchloric acid. The selenium(VI) binds with DAN and forms a fluorescent selenium-DAN heterocyclic compound. A linear relationship between fluorescence and selenium concentration was maintained within a 100 ng range of selenium.

Selenium Speciation Analysis

Thecomposition of selenium compounds in fish tissues was measured by high-performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS) [46.7, 9]. Chromatographic separation was performed using a 712 p HPLC pump (GL-Sciences, Tokyo, Japan) in conjunction with an Ultrahydrogel 120 (7.8 × 250 mm; Nihon Waters, Tokyo, Japan) analytic column equilibrated with 0.1 M ammonium formate buffer containing 0.1% (w/v) Igepal CA-630 (Sigma-Aldrich Japan, Tokyo, Japan). Each sample (0.1 g) was homogenized in 0.5 ml water, and 20 µl supernatant was analyzed after a two to fourfold dilution in the mobile phase. The injection volume was fixed at 20 µl and the mobile phase delivered isocratically at the rate of 1 ml/min. Selenium was detected through online HPLC-ICP-MS, performed using an ELAN DRC II mass spectrometer (Perkin Elmer, Waltham, MA, USA) in conjunction with a concentric quartz nebulizer (WE02-4371) and a quartz sample injector (2 mm id). During separation, glutathione peroxidase (GPx) and selenoneine were eluted at retention times of 5.4 and 10.1 min, respectively, and the selenium concentration was determined using the respec-

tive compounds as standards. To determine the tissue distribution of selenoneine and other organic selenium compounds, a speciation analysis method was used for organic selenium in animal tissues. This method was based on monitoring ^{82}Se using HPLC-ICP-MS with a gel permeation chromatography (GPC) column. Since selenoneine was associated with the gel matrix, it was eluted 10.1 min after the bed volume of the column (Fig. 46.2).

46.2.2 Selenium Contents in Seafood

The total selenium contents have been measured in the edible portions of various fish and shellfish in Japan [46.7, 9, 12]. The muscles of the alfonsino contain the highest level of selenium (16.1 nmol/g) among the fish muscles examined in this study. High levels of selenium (15.2–13.6 nmol/g) have also been found in the salted ovary products of mullet and Pacific herring. In other fish muscles, the selenium levels range between 1.5 and 9.8 nmol/g tissue.

Selenoneine concentrations in various tuna tissues have been determined [46.7]. Tuna red muscle contain selenoneine at 190 nmol/g and selenoproteins at 4.5 nmol/g. Almost all of the organic selenium 98% in the tuna muscle is present as selenoneine. Tuna and mackerel blood contain high levels of selenium as selenoneine (430–437 nmol/g). Other tissues, such as the spleen, hepatopancreas, heart, white muscle, and blood, also contain selenoneine at levels above 11.5 nmol/g. However, the data are limited to the cultured bluefin tuna in Japan. The selenoneine levels in the tissues may be dependent on fish size, stress, and nutritional conditions.

When the muscle contents of selenoneine were compared among various fish species, the selenoneine contents of bigeye tuna, Pacific bluefin tuna, albacore, yellowfin tuna, and alfonsino were 1.3–2.6 nmol/g tissue. In the muscles of these fish, the majority of organic selenium (9–42%) was present as selenoneine [46.9]. In other fish species, such as Pacific sardines, greeneye, skipjack, Pacific mackerel, horse mackerel, red sea bream, and Japanese barracuda, selenoneine levels were 0.1–1.4 nmol/g tissue, accounting for 3–34% of organic selenium [46.9]. In contrast, Japanese conger, Japanese anchovy, chum salmon, Pacific saury, white croaker, and marbled sole muscles contained levels of selenoneine below the detection level (0.05 nmol/g tissue) [46.9].

In comparison, tilapia blood, porcine kidney, chicken heart, gizzard and liver, and squid hepatopancreas contained low levels of selenoneine and selenoproteins [46.7]. Furthermore, porcine liver contained only selenoproteins and not selenoneine. Selenoneine

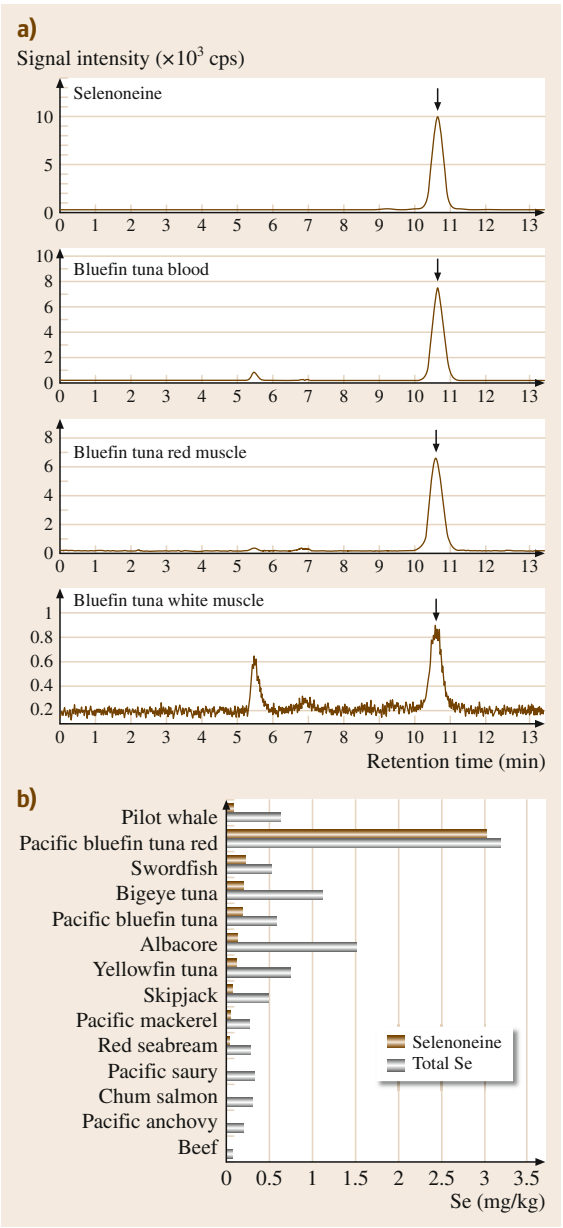

Fig. 46.2a,b Speciation analysis of organic selenium in the muscle of fishes by LC-ICP-MS. Water-soluble selenium compounds in fish muscle were speciated though LC-ICP-MS analysis. **(a)** Separation of selenoneine, **(b)** selenium concentration in muscles

was found to be distributed widely in various animal tissues and at especially high levels in tuna tissues [46.7]. These findings indicate that the chemical forms of organic selenium present in fish and terrestrial animals are very different. To determine selenoneine levels below the detection limit (0.05 nmol/g tissue), additional methods for extraction and concentration of selenoneine should be developed.

46.3 Biochemical Characterization of Selenoneine

46.3.1 Purification

Selenoneine can be purified from the blood and other tissues of tuna [46.7]. The red blood cells in tuna contain the highest levels of selenoneine, ranging from 188 to 638 nmol/g. Other tissues, such as red muscle, heart, gill, spleen, and hepatopancreas of ocean pelagic fish, including tuna, swordfish, yellowtail, and mackerel also contain high levels of selenoneine (> 10 nmol/g). The tissue concentration of selenoneine and selenium may depend on fish size and type of feed. Selenoneine is soluble in ultrapure water, methanol, and acetonitrile, and extractable in freshly prepared solvents. Because of its strong radical scavenging activity, selenoneine reacts with alcohols, oxidants, and metals. Selenoneine-oxidized dimer (yellow-green color) and selenoneine-heme complex (orange-red color) are extractable from the tissues. Selenoneine is monitored by detection by means of absorption at 260 and 430 nm wavelengths. A reverse-phase C18 column (Atlantis dC18, Waters) and GPC column (Ultrahydrogel 120, Waters) were used for purification. The selenoneine dimer was eluted in the flow-through fraction of the Atlantis dC18 column and eluted in the fractions after the bed volume in the GPC column. For large-scale preparation of selenoneine, a water soluble, extractable fraction after boiling, steam heating, or autoclaving of the tissues was used.

46.3.2 Radical Scavenging Activity

Strong antioxidant activity was assayed by radical scavenging activity with 1,1-diphenyl-2-picrylhydrazyl (DPPH). The activity of selenoneine was higher than ergothioneine and a water-soluble analog of vitamin E, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Table 46.1. The redox potential of selenoneine was −0.69 V (vs. Ag/AgCl/sat. KCl reference electrode, −0.49 V vs. normal hydrogen reference electrode (NHE)). This value was similar to the redox potential of selenocysteine/selenocystine (−0.488 V NHE), but significantly different from that of cysteine/cysteine (−0.233 V vs. NHE) [46.13]. Therefore, selenoneine and selenocysteine are more reactive than thiol compounds.

46.3.3 OCTN1 as a Selenoneine Transporter

Selenoneine is a selenium analog of ergothioneine with one selenium atom in its imidazole ring. OCTN1, transports ergothioneine and carnitine, and is involved in the selenoneine uptake and detoxification of methylmercury (MeHg) [46.14]. OCTN1 is ubiquitously distributed in mammalian cells and tissues [46.15–20]. HEK293 cells transfected with an OCTN1 cDNA (complimentary DNA) construct showed specific uptake of selenoneine. Given its role in heme metabolism, the transporter gene has also been identified and expressed in zebrafish blood cells and other cell types.

Selenoneine is the ideal, specific physiological substrate for OCTN1. K_m values of selenoneine uptake in HEK293 cells and zebrafish red blood cells have been estimated to be 13.0 and 9.5 μM, respectively. Previously, human OCTN1 was characterized to bind specifically ergothioneine (K_m 21 μM in HEK293 cells). Selenoneine was incorporated into human cells, zebrafish red blood cells, and embryos within 30 min. OCTN1 regulates selenoneine absorption and selenium metabolism in animal tissues. Since selenoneine has a strong radical scavenging activity, the distribution and expression of OCTN1 may be responsible for maintenance of the selenium redox status. In addition to cation absorption, mammalian OCTN1 is thought to be involved in the secretion of cations in renal proximal tubules and the small intestine [46.15–20].

Table 46.1 DPPH radical scavenging activity

Product	RS_{50} (μM)
Trolox[a]	880
l-Ergothioneine	1700
Selenoneine	1.9

[a] The water-soluble vitamin E-like antioxidant

Furthermore, OCTN1 mediated the incorporation of selenoneine and the excretion of MeHg in human cells and zebrafish embryos, indicating a role in MeHg detoxification.

46.4 Nutritional and Functional Properties of Dietary Organic Selenium

46.4.1 Metabolism and in vivo Antioxidant Activity

Selenoneine exists as two tautomers, the selenol and selenoxo-form monomers, and as an oxidized dimer [46.7]. For practical reasons, the stable oxidized dimer is used for bioassays and animal trials. The dimer can be dissociated to a monomer under reduced conditions in the presence of glutathione (GSH). When selenoneine was added to HEK293 culture medium, cell growth was enhanced and reactive oxygen species (ROS) levels were reduced. Treatment of human umbilical vein endothelial cells (HUVECs) with selenoneine induced cell growth and anti-apoptotic activity in the presence of 2 mM hydrogen peroxide. Rabbit red blood cells treated with selenoneine showed protection of hemoglobin from autoxidation and metohemoglobin formation. Therefore, the selenoneine oxidized dimer may be reduced by GSH, thus existing as a reduced monomer intracellularly and acting as a radical scavenger (Fig. 46.3).

To characterize the in vivo antioxidant activity of selenoneine, post-mortem meat color change and metmyoglobin (Mb) formation in the red muscle of yellowtails were examined after intravenous injection of selenoneine [46.21]. The selenoneine concentrations in the red blood cells and red muscle were higher in the selenoneine-injected fish than in the control fish. A close correlation was detected between the selenoneine concentration in the red blood cells and red muscle. In addition, a negative correlation was observed between the selenoneine concentration in the red muscle and the ROS level in the plasma. After the cold storage of sliced meat at 4 °C overnight, Mb formation and ROS production in the selenoneine-injected fish were delayed, and the a^* value of the red muscle was conserved compared with the control fish. Therefore, selenoneine was determined to be responsible for the color change in the meat and the prevention of Mb formation in the red muscle of yellowtails.

Dietary selenoneine intake in animal trials also induced antioxidant effects in vivo. Selenoneine was incorporated in the blood and muscle tissues, and serum ROS levels were reduced. Selenoneine intake also induced GPx1 activity in red blood cells. Selenoneine can enhance selenium antioxidant functions and selenoprotein synthesis, and act as an antioxidant in vivo.

In the Japanese fish-eating population, selenoneine was highly concentrated in the red blood cells, in a manner dependent on the frequency of fish consumption [46.22]. The red blood cells contained 6.38 nmol/g total selenium, 2.65 nmol/g selenoneine, and 3.28 nmol/g selenoproteins, whereas the serum contained 2.18 nmol/g total selenium. The highest concentrations of selenoneine, total selenium, and MeHg in the red blood cells were 2.38, 2.40, and 0.47 μg Hg/g, respectively. Selenoneine is the major chemical form of selenium in the red blood cells of the Japanese fish-eating population and is an important biomarker of the selenium redox status.

In human disease, OCTN1 and blood ergothioneine have been identified as involved in the etiology of Crohn's disease. The OCTN1/1672T mutation was associated with an increased risk of colorectal cancer [46.23]. The blood ergothioneine concentration was also significantly reduced in Japanese patients with Crohn's disease. This result was confirmed by the reduced distribution of ergothioneine in the small intestine and renal reabsorption of ergothioneine in OCTN1 (–/–) mice [46.15]. Selenium deficiency is common in patients with severe gastrointestinal disorders, including Crohn's disease and ulcerative colitis, and is related primarily to malabsorption. Crohn's disease patients had reduced concentrations of selenium and glutathione peroxidase in their plasma and red blood cells. Therefore, metabolism and uptake of selenoneine by OCTN1 may be essential for the prevention of gastrointestinal disorders and cancers. However, the relationship between selenoneine depletion and intestinal diseases has not been proven. Selenoneine intake and metabolism through fish consumption might be important determinants of the selenium redox status.

46.4.2 Bioavailability of Selenium

In the human diet, selenium bioavailability is evaluated by the level of induced expression of GPxs

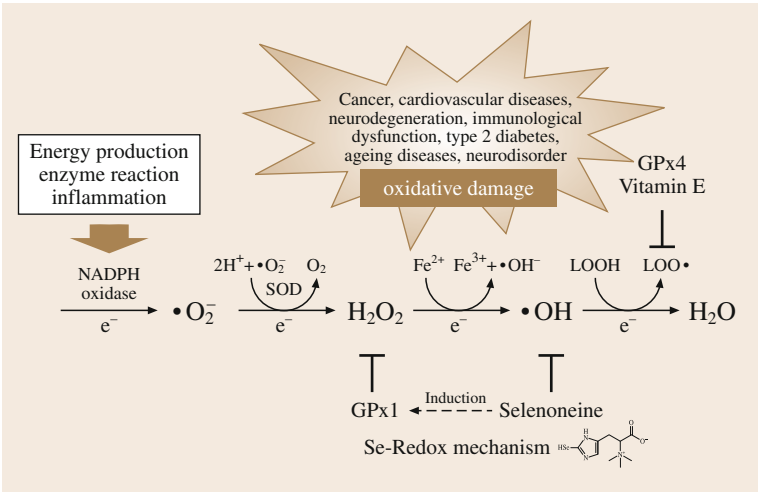

Fig. 46.3 Possible antioxidant mechanism of selenoneine. Selenoneine can react with hydroxyl radicals and block the radical production in the cells. Selenoneine induces expression of GPx1 and GPx4 at both transcriptional and translational levels

and other selenoproteins compared with the maximum expression level. The maximum selenoprotein expression is obtained by a daily dietary selenium intake of 0.1–0.2 mg/kg for animals and no more than 55 µg for humans [46.24]. The bioavailability of selenium depends on the food source due to the different chemical forms of selenium present. Plant foods contain selenite, selenomethionine, and γ-glutamyl methylselenocysteine, whereas terrestrial animal meat contains selenoproteins, including selenocysteine and selenomethionine [46.6, 25–31]. Ocean fish, such as tuna, mackerel, and swordfish were found to contain high levels of selenoneine [46.4, 7, 12]. Fish contain a highly bio available form of selenium. In Latvia, the number of fish meals consumed per month correlated with plasma selenium, selenoprotein P, and GPx levels [46.25]. The chemical form of selenium and the food source are considered key determinants of post-absorptive metabolism, and bioavailability evaluations should consider their metabolic retention and toxicity indices. Selenoneine is noncytotoxic and incorporated into cultured human and fish cells through the OCTN1 transporter. Selenoneine possesses a number of biological activities, including radical scavenging, cell growth promotion, and anti-apoptotic, as well as MeHg protection and demethylation functions. Since selenoneine induces the activities and gene expression of GPx1 in animal cultured cells, selenoneine can be used as the selenium source for selenoprotein synthesis. Therefore, the bioavailability of selenoneine should be compared with those of other selenium sources, such as sodium selenite, selenomethionine and selenomethionine-containing proteins, and selenoproteins.

46.4.3 Supernutritional Supplementation

Supplementation with inorganic and organic selenium compounds is considered to be anticarcinogenic at doses greater than those required to support the maximum expression of selenoproteins, which are generally regarded as wholly responsible for the nutritional effects of selenium [46.32–34]. In the rat model, the addition of sodium selenite to the diet at the 2–5 ppm level was effective in preventing chemical carcinogenesis using 7,12-dimethylbenzanthracene [46.35]. The effective doses of selenium for anticarcinogenic activity are close to the toxic dose (5 ppm) that causes a loss of body weight. *Combs* and *coworkers* [46.32–34] have suggested that antioxidant selenium compounds present in foods are metabolized to yield selenide or methylated selenides. Conversely, since selenoneine is nontoxic and shows powerful antioxidant activity, selenoneine supplementation can enhance antioxidant activity at concentrations of 10 pM to 1 mM, and induce the expression of GPx and other selenoproteins in cell culture and animal trials. DNA (deoxyribonucleic acid) damage induces GPx1 expression through the activation of the tumor suppressor protein p53 in cultured human cells [46.36]. Since selenoneine can activate GPx1 gene expression, it might also mediate activation of p53 and related DNA damage repair mechanisms. The antioxidant functions of selenoneine derived from seafood intake may mediate its anticarcinogenic activity; alternatively selenoneine may be biologically synthesized from other selenium sources in the cells and tissues. Therefore, the selenoneine de novo synthesis pathway in animal cells should be identified.

46.4.4 Burnt Meat

Selenoneine binds to the heme moieties of hemoglobin and myoglobin, protecting them from auto-oxidation by iron ions under hypoxic conditions. The selenoneine-heme complex is extracted in methanol from fish blood and muscles, and shows a bright red color, suggesting direct binding of selenium to the heme iron. Selenoneine levels in the tissues may be closely related to the low-oxygen adaptation mechanisms of marine animals, which prevent oxidation of heme proteins.

Burnt meat is a problem in aquaculture and fisheries of pelagic ocean fish, such as tuna, yellowtail, and mackerel in Japan [46.37]. The meat appears cooked, although it is raw, and the flesh lacks the typical bright red color of meat (Fig. 46.4). Burnt meat has a more watery, softer texture and is often seen in fish under stress conditions, for example those caught during the summer spawning period when the water temperature is high. The oxidative stress is caused by selenium deficiency and hypoxia. After being caught, extensive apoptosis and autophagy occur in the white muscle, and hemolysis occurs in fish that contain blood selenium levels of less than 1 μg/g tissue. The antioxidant effects of selenoneine may be essential in enabling fish to adapt and survive in low-oxygen marine environments. In rats, selenium deficiency was shown to induce hemolysis [46.38]. White muscle disease in selenium-deficient animals shows similar features [46.1]. Selenium levels

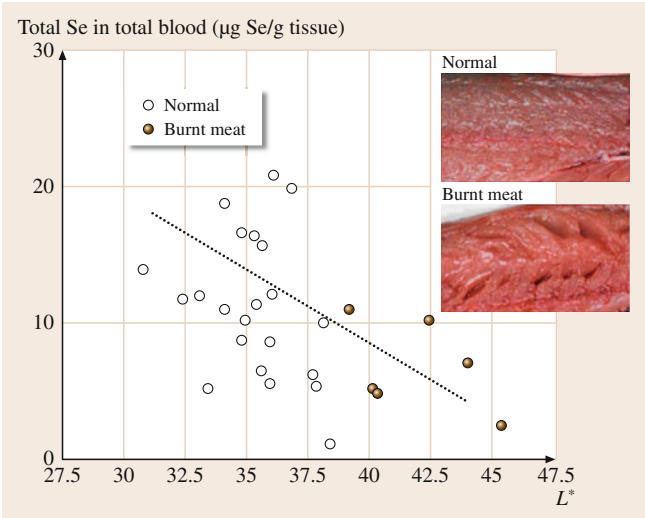

Fig. 46.4 Burnt meat of Pacific mackerel in spawning migration in summer. An index for burnt meat, L^* value, is negatively correlated to the selenium levels in the blood

were closely related to the DPPH radical scavenging activity against red blood cells in the blood of bluefin tuna [46.37]. Burnt meat in fish may be due to selenium deficiency. Therefore, selenium supplementation in cultured fish might reduce the burnt meat problem in aquaculture, and the nutritional selenium requirement of ocean fish species should be evaluated.

46.5 MeHg Detoxification

Dietary intake of selenium can reduce MeHg toxicity. In 1972, *Ganther* et al. [46.39] demonstrated that intake of canned tuna meat containing slightly elevated MeHg levels reduced the toxicity of Japanese quail and suggested a protective role of selenium. *Friedman* et al. [46.40] showed a protective effect of freeze-dried swordfish meat against MeHg toxicity in rats, and *Ohi* et al. [46.41] suggested that an unknown organic selenium compound in fish meat reduces MeHg neurotoxicity.

Recently, MeHg detoxification was shown to be mediated by selenoneine and its transporter OCTN1 in zebrafish embryos using morpholino antisense oligo (MO)-knockdown technology [46.14]. Upon exposure to 100–500 ppb MeHg-cysteine (MeHgCys) in culture medium, both selenium and mercury levels in normal control embryos decreased compared with OCTN1-deficient embryos. The addition of selenoneine to the culture medium (0.8–3.2 μM) reduced MeHg accumulation in the control embryos and resulted in the production of inorganic mercury. This result indicates that selenoneine mediates MeHg detoxification. In contrast, selenoneine had no effect on MeHg detoxification in OCTN1-deficient embryos. To further characterize MeHg detoxification mechanisms, MeHg was microinjected into the yolk, and MeHg accumulation in the embryos was examined (Fig. 46.5). In control embryos, the intracellular mercury level decreased to 0.7 ng/embryo following microinjection of MeHgCys (3 ng mercury), and inorganic mercury was produced (1.1 ng/embryo). This indicates that most of the MeHg introduced into the embryos was excreted. In contrast, the mercury level increased to 2.2 ng/embryo in OCTN1-deficient embryos, and a small quantity of inorganic mercury

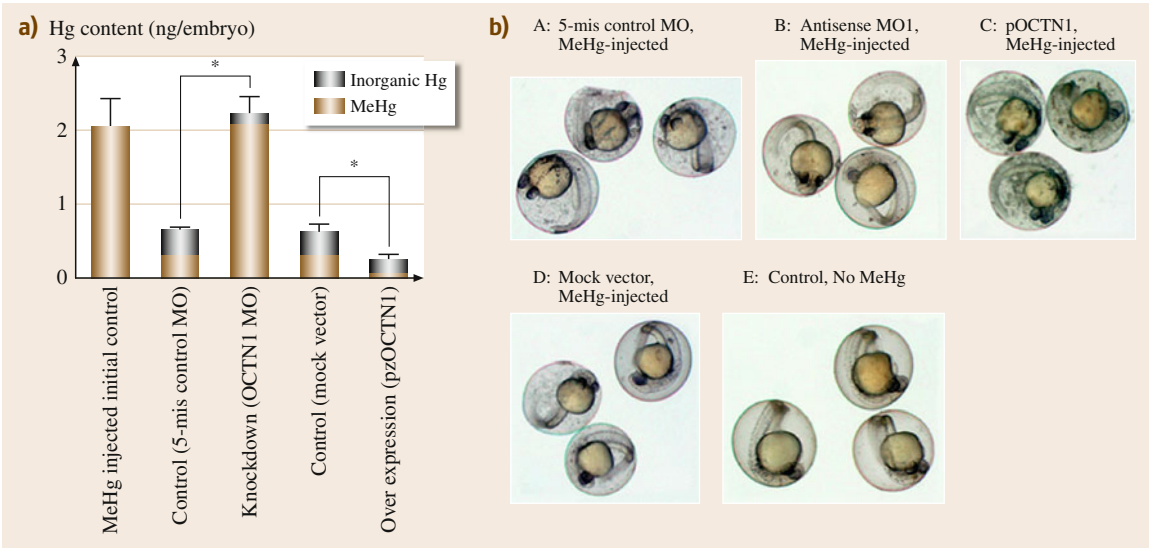

Fig. 46.5a,b Excretion of MeHg by the exosomal secretion pathway. **(a)** Mercury levels in embryos microinjected with MeHgCys (3 ng Hg/individual embryo) into the yolk sac. Embryos were cultured in Hank's medium at 28.5 °C for 12 h after MeHgCys microinjection at 12 hpf. MeHg and inorganic Hg contents of embryos were determined. **(b)** Production of gray granules by embryos after microinjection of MeHgCys

(0.1 ng/embryo) was produced. Bafilomycin A, an inhibitor of the vacuolar-type H^+-ATPase in lysosomes, inhibited the excretion of MeHg in the yolk and enhanced MeHg accumulation. Small vesicles, exosomes, were collected from the culture medium by ultracentrifugation. The exosomes contained high levels of mercury and lysosomal cathepsin L activity. Exosome production was induced by MeHg exposure, and inhibited by bafilomycin A1. These findings indicate that MeHg was excreted by the secretory lysosomal pathway through the function of OCTN1. The yolk exhibited strong excretion and MeHg demethylation mechanisms, mediated by the selenoneine-OCTN1 system. A similar MeHg detoxification function was identified in OCTN1-transfected HEK293 cells, which showed decreased selenium levels after MeHgCys treatment compared with the levels in mock vector-transfected cells, suggesting selenium transport by OCTN1. Mercury accumulation was decreased following selenoneine exposure in OCTN1-transfected cells, but not in mock

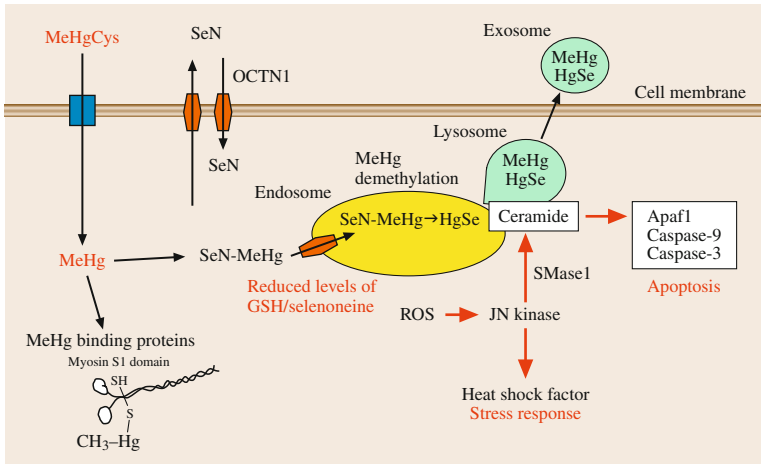

Fig. 46.6 Possible detoxification mechanism of MeHg. The OCTN1 and selenoneine (SeN) were essential for the MeHg detoxification. MeHg and selenoneine complex may be incorporated into the endosomes and lysosomes. The secretory lysosomal vesicle formation is stimulated by oxidative stress conditions. MeHg exposure induces ROS formation, c-Jun N-terminal kinases (JNK) activation, and ceramide formation. Demethylation reaction is accelerated in the lysosomes

vector-transfected cells, indicating that selenoneine and OCTN1 regulate MeHg detoxification in human cells.

From these recent findings, the mechanism of protection against MeHg toxicity is hypothesized to involve direct conjugation of selenoneine with MeHg (Fig. 46.6). The selenoneine–MeHg complex might be formed under reducing conditions in vivo and translocated into the lysosomal secretory vesicles by OCTN1. The MeHg complex may be decomposed to inorganic mercury, i. e., mercury selenide (HgSe). HgSe in the liver of marine mammals may be produced from the selenoneine–MeHg complex by this OCTN1-mediated secretory lysosomal mechanism. In the dolphin liver, the demethylation/accumulation of mercury is likely to involve the formation of HgSe and selenocompounds from selenium-containing residues of specific proteins [46.42–47]. In addition, the molar ratio of selenium and inorganic mercury was close to 1, suggesting that HgSe is the end product of MeHg detoxification [46.48, 49]. Therefore, the MeHg detoxification mechanism by which selenoneine–MeHg complexes are converted to HgSe by OCTN1 function may be commonly distributed in fish and mammals. Inorganic selenium and selenomethionine supplementation can reduce MeHg toxicity. Selenoneine may be biologically synthesized from other selenium sources in vivo and used in MeHg protection mechanisms.

Exosome vesicles are produced by ceramide stimulation under oxidative stress conditions [46.14]. Thus, MeHg exposure may induce an oxidative stress response and formation of exosome vesicles containing MeHg and inorganic mercury. Oxidative stress conditions activate c-Jun N-terminal kinases (JNK), which phosphorylate neutral sphingomyelinase 1 (SMase 1), a JNK substrate [46.50]. Ceramide generation by SMase 1 activation triggers the formation and release of exosomes, and the endosomal sorting complexes required for transport (ESCRT) machinery is thought to play important roles in cell–cell communication, antigen presentation, and pathogenesis [46.51, 52]. Thus, exosomal secretion mechanisms are also essential for MeHg detoxification. The detoxification of toxic chemicals and oxidants other than MeHg mediated by selenoneine appears to be a novel exosomal function in animal cells and tissues. Therefore, mercury and selenium in exosomes might be important biomarkers of detoxification of toxic chemicals. Selenium and mercury levels in serum exosomes should be measured in animal models and during medical checkups.

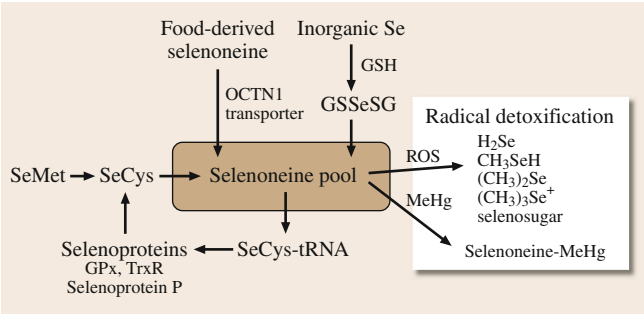

Fig. 46.7 Proposed model of selenium metabolism and radical detoxification. Selenoneine may be directly incorporated through OCTN1 transporter from fish consumption and biologically synthesized from other selenium sources. Selenoneine may be responsible for radical detoxification and used for selenoprotein synthesis

The findings presented here support the hypothesis that MeHg excretion is mediated by selenoneine and a specific OCTN1. Depletion of selenoneine and production of ROS after MeHg exposure may trigger ceramide generation and exosome formation. Although a MeHg demethylation enzyme, similar to bacterial organomercury lyases [46.53], could be present in the MeHg secretion pathway, MeHg can be converted to inorganic mercury directly and nonenzymatically in the presence of selenium. Further study is required to identify the demethylation reaction and mechanism of lysosomal vesicle formation.

46.6 Conclusion

To elucidate the nutritional and physiological significance of selenium as a micronutrient, which is linked to a variety of human and animal diseases, the organic selenium compounds responsible for the antioxidant and redox mechanisms must be identified and characterized. Selenoneine is related to a variety of biological activities as a strong radical scavenger in both fish and mammals (Fig. 46.7).

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47. Biological Activities of Marine-Derived Oligosaccharides

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Alginate is an acidic copolymer of α-L-guluronate (G) and its C5 epimer β-D-mannuronate (M), is arranged in homopolymer (polyguluronate or polymannuronate) or heteropolymer (a mixed sequence of these residues). Alginates extracted from brown algae are widely used in the food industry, biotechnology, and medical fields. In addition to the many interesting physicochemical properties of alginates, several studies have demonstrated that alginates have various bioactivities such as immunomodulation. Recent studies have demonstrated that alginate oligosaccharides prepared from alginate polymers show even many more improved bioactivities, not only in mammalian systems but also in plant systems. It is considered that the decreased viscosity and the loss of gel-forming properties of alginate oligosaccharides lead to an increase in biocompatibility.

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47.1 Overview

Alginate is an acidic linear polysaccharide that is usually extracted from brown seaweeds such as *Macrocystis pyrifera* and *Ascophyllum nodosum*. This polysaccharide is composed of two forms of uronic acids α-L-guluronate (G) and β-D-mannuronate (M), which form three types of polymer blocks: namely homopolymers of guluronate (PG), homopolymers of mannuronate (PM), or heteropolymers (a mixed sequence of these residues); these block structures are expressed as G-blocks, M-blocks, and MG-blocks (MG: heteropolymers of mannuronate and guluronate), respectively [47.1]. The differences in the molecular weight, the M/G ratio, and the entire sequence seem to be responsible for the diversity of physicochemical properties and bioactivities of alginates [47.2–7]. Alginates have been utilized in a wide range of commercial applications including thickening agents and dispersion stabilizers. Since alginates have gentle gelling properties in the presence of divalent cations such as calcium, they are also used for live cell encapsulation in vitro [47.8] and in vivo [47.9], and for several tissue engineering applications [47.10, 11]. Alginate oligosaccharides prepared by the enzymatic degradation of alginate polymers with relatively low molecular weight are also known to have many biological activities, including suppression of fibroblast proliferation and collagen synthesis in human skin [47.12], stimulation of endothelial cell growth and migration [47.13], stimulation of human keratinocyte growth [47.14], suppression of T helper 2 cells (Th2) development, and immunoglobulin E (IgE) secretion through inducing interleukin (IL)-12 secretion [47.15].

In addition to these mammalian models, it has also been reported that enzymatically depolymer-

ized alginates promote the growth of bifidobacteria, while the original alginate polymer has no such effect [47.16]. Furthermore, alginate oligomers seem to have some biological effects on plants as well. Alginate oligomers prepared with bacterial alginate lyase increase shoot elongation of komatsuna (*Brassica rapa* var. *pervidis*) seeds [47.17] and promote the elongation of barley roots [47.18]. Our recent studies have demonstrated that alginate polymers induce the tumor necrosis factor (TNF)-α secretion from the mouse macrophage cell line RAW264.7, and the activity is significantly influenced by the molecular size and M/G ratio [47.19].

Interestingly, a mixture of enzymatically depolymerized alginate oligomers showed more than ten times higher activity in terms of TNF-α secretion from RAW264.7 cells as compared to original alginate polymers [47.19]. Since the alginate oligomers have fairly low viscosity in aqueous solution even at quite high concentration and has no gel-forming property in the presence of calcium, it is considered that alginate oligomers are more applicable for in vivo systems [47.19, 20]. In fact, it was found that intraperitoneal (i.p.) administration of enzymatically depolymerized alginate oligomers to mice resulted in a significant increase in the serum levels of various cytokines such as TNF-α and G-CSF (granulocyte colony-stimulating factor) [47.21]. This finding indicates that alginate oligosaccharides even in mixture are capable of inducing certain biological activities. In addition to the biological activities of alginate polymers and their oligomers described above, recent studies have demonstrated that alginate oligomers [47.22], as well as polymers [47.23], show potent antioxidant activities.

To gain insight into the structure–activity relationship of alginate oligomers, it is important to use highly purified samples with a defined structure. Such purified alginate oligomers can be prepared from homopolymers of guluronate (PG) and homopolymers of mannuronate (PM) blocks prepared from alginate polysaccharide. In this chapter, various biological activities of alginate oligosaccharides are described as in the following five points. First, the methods of preparation of alginate oligosaccharides are discussed. Second, antioxidant activities of alginate oligosaccharides are treated. Third, cytokine-inducing activities of highly purified alginate oligosaccharides from macrophage cell line RAW264.7 cells in terms of structure and structure-activity relationship analysis are discussed. Fourth, the in vivo activity of alginate oligosaccharide mixtures to induce cytokines in the mice model are considered. Fifth, the growth-promoting effect of alginate oligosaccharides mixture on marine unicellular microalga is discussed.

47.2 Preparation of Alginate Oligosaccharide Mixture

47.2.1 Enzymatic Method

Various alginate polysaccharides are commercially available. Sodium alginate (1000 cps grade) purchased from Nacalai Tesque Inc. (Kyoto, Japan) was mainly used in our study. The method for the preparation of enzymatically depolymerized alginate oligosaccharide mixtures, which were found to have various biological activities, is described. Enzymatic digestion of alginates was conducted by basically the same procedure as described previously [47.21]. Alginate lyase required for the preparation of alginate oligosaccharide was purified from the culture medium of *Pseudoalteromonas* sp. strain number 272, as described previously [47.24]. This purified enzyme, which can recognize both polyguluronate and polymannuronate, produced unsaturated guluronate and mannuronate oligomers with various degree of polymerization [47.24]. In brief, 5% of alginate samples in aqueous solution was incubated with alginate lyase (final 1 μg/ml) at 40 °C for 3 days. The enzymatic reaction was stopped by heating the solution in boiling water for 10 min. No significant changes in the compositions of digestion products were observed after further enzymatic treatment. Gel-filtration analysis suggested that the enzymatically digested product contained several oligomers with a degree of polymerization (DP) of 3–9 (i. e., with a molecular weight of 529 $\approx$ 1585) [47.19]. Gel-filtration chromatography suggested that a similar composition of the oligomer mixture can be prepared by this procedure even from different alginate polymers with different molecular sizes and M/G ratios [47.19]. Before use, all samples were filtered through an endotoxin-removing filter (Zetapor Dispo filter) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). This filtration procedure reduced even the bioactivity of 1 μg/ml of lipopolysaccharide (LPS) to a negligible level.

47.2.2 Acid Hydrolysis

Another way to prepare alginate oligosaccharides is acid hydrolysis. Alginate polymer (1%) in 200 ml of acidic solution (0.3 M HCl, pH 4.0) was treated at 121 °C for 80 min. After cooling down to room temperature, the sample solution was neutralized with 1 M NaOH, and lyophilized in a vacuum freeze-dryer. By this procedure, saturated alginate oligosaccharide mixtures with free carboxyl groups were obtained [47.25].

47.2.3 Preparation of Purified Alginate Oligosaccharides

To prepare highly purified alginate oligosaccharides, it is necessary to obtain polyguluronate (PG) and polymannuronate (PM) first. PG and PM with 20–24 DP were prepared from sodium alginate by the method of *Haug* et al. [47.26]. The homogeneity of the prepared polyuronates was confirmed by circular dichroic spectral analysis with a Jasco spectropolarimeter J500A coupled with a data processor, based on the method of *Morris* et al. [47.27]. Guluronate and mannuronate oligosaccharides were prepared from PG or PM by enzymatic degradation or acid hydrolysis by basically the same methods as described before. Purification of each oligosaccharide with defined DP was conducted by gel-filtration chromatography with Bio Gel P-6 and subsequent re-chromatography with Bio Gel P-2, as described previously [47.28]. By this procedure, trimer to nonamer of G (G3–G9) and of M (M3–M9) oligosaccharides were obtained.

47.3 Antioxidant Activities of Alginate Oligosaccharides

From the viewpoint of the structure–activity relationship of alginate oligosaccharides as antioxidants, radical scavenging activities of PM and PG are described in this section [47.29]. The superoxide scavenging activities of PM and PG were examined by a luminol analog L-012-dependent chemiluminescence (CL) method [47.30]. As a source of superoxide, a hypoxanthine (HPX)-xanthine oxidase (XOD) system was employed. As shown in Fig. 47.1a, when XOD was added to the reaction mixture, a rapid CL response was observed, and the CL response was significantly reduced by the addition of superoxide dismutase (SOD), indicating that superoxide was generated in the system. By this system, the superoxide scavenging activities of PM and PG can be measured (Fig. 47.1b,c). Both PM and PG showed superoxide scavenging activity in a concentration-dependent manner. In the presence of 1000 μg/ml of PM or PG, the CL response was almost completely inhibited.

PG at 100 μg/ml showed a slightly stronger scavenging activity than PM, while neither oligosaccharide had significant activity at 10 μg/ml. Since PM and PG did not inhibit the production of uric acid from HPX with the catalytic reaction of XOD (data not shown), it was confirmed that the inhibition of the CL response was due to the direct superoxide scavenging activity of PM and PG, and not the results of the inhibition of XOD.

It is well known that hydroxyl radicals are an extremely reactive species that can react with various biological molecules, and their damaging action on various biological systems is the strongest among the reactive oxygen species (ROS) [47.31]. In the next experiment, the Fenton reaction was used as a hydroxyl radical generation system, in which Fe^{2+} and hydrogen peroxide are reacted to produce hydroxyl radicals. Although there are several methods for the detection of hydroxyl radicals, such as spectrophotometric or colorimetric methods, electron spin resonance (ESR) method has been used as the most reliable assay for monitoring free radicals because of its high sensitivity and rapidity. Thus, the scavenging activities of PM and PG on the hydroxyl radical were examined by the ESR method. When the spin-trapping agent 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was added to a solution of the Fenton reaction system, the typical 1 : 2 : 2 : 1 ESR signal of the DMPO-hydroxyl (DMPO-OH) adduct (an adduct from DMPO and the hydroxyl radical) was observed. Figure 47.2b,c show the representative ESR spectra of DMPO-OH obtained by the addition of the solvent alone and various concentrations of PM and PG. In the presence of PM or PG, the decreases in the height of the second peak of the spectrum, which represents relative amount of DMPO-OH adduct, were observed. These results clearly indicate that PM and PG have the ability to scavenge hydroxyl radicals. PM and PG showed similar concentration-dependent profiles in terms of scavenging efficiency, but they required higher concentrations to attain an effect sim-

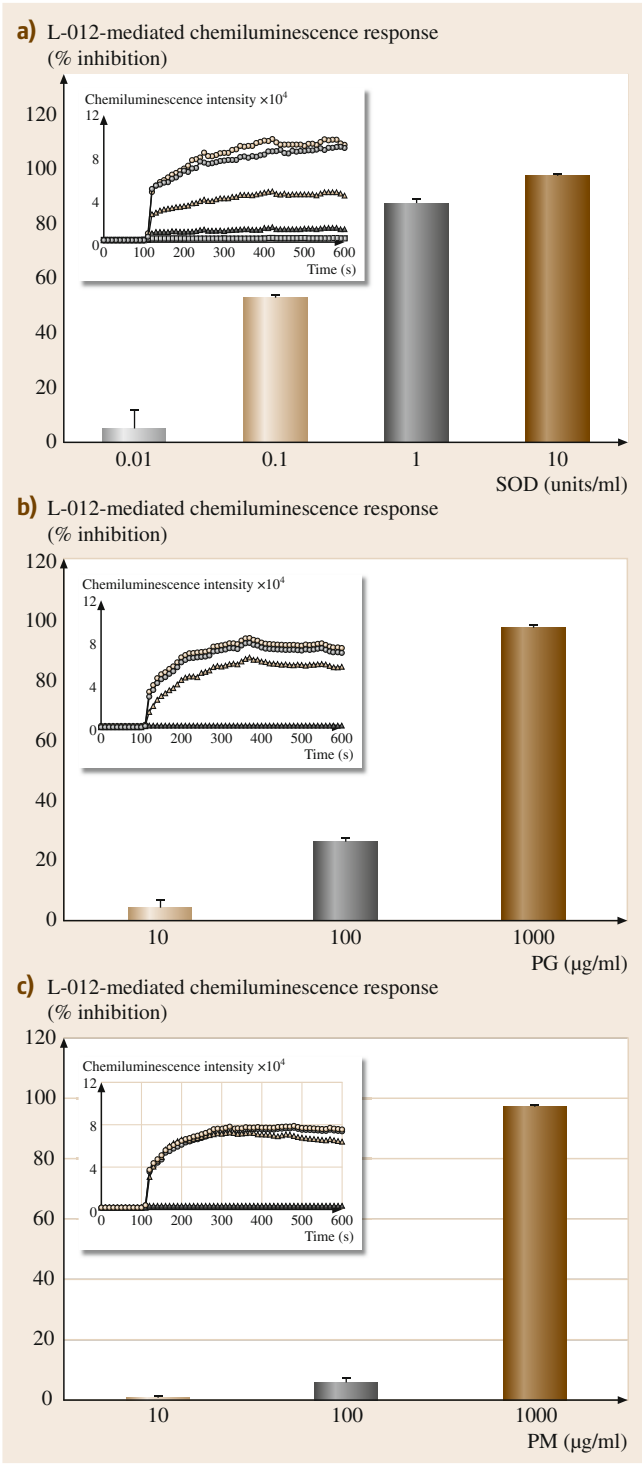
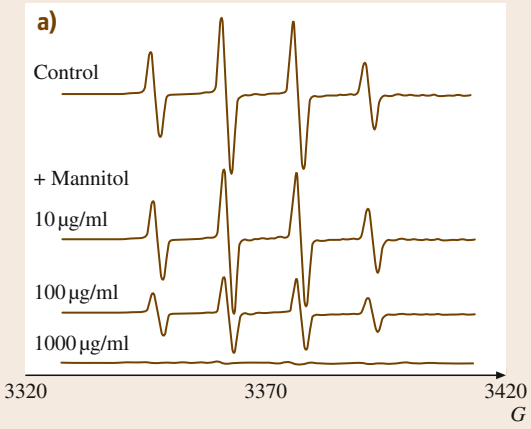
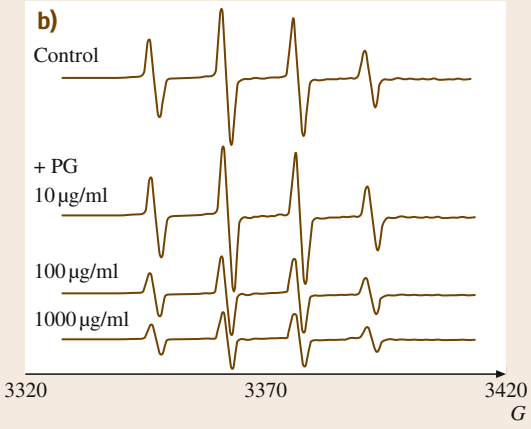


Fig. 47.1a–c Superoxide scavenging activity of PM and PG as measured by the L-012-dependent CL method. The effects of various concentrations of SOD **(a)**, PG **(b)**, and PM **(c)** on CL-responses in the HPX-XOD system were examined. *Insets* indicate the CL-response patterns during 10 min in the presence of 0 (●), 0.01 (○), 0.1 (▲), 1 (△), and 10 (■) units/ml of SOD **(a)**, or 0 (●), 10 (○), 100 (▲), and 1000 (△) μg/ml of PG **(b)**, or 0 (●), 10 (○), 100 (▲), and 1000 (△) μg/ml of PM **(c)**. *Columns* indicate percentage inhibition of the integrated CL-response intensity values during 10 min as compared to the control values. Each value represents the means ± standard deviation of triplicate measurements





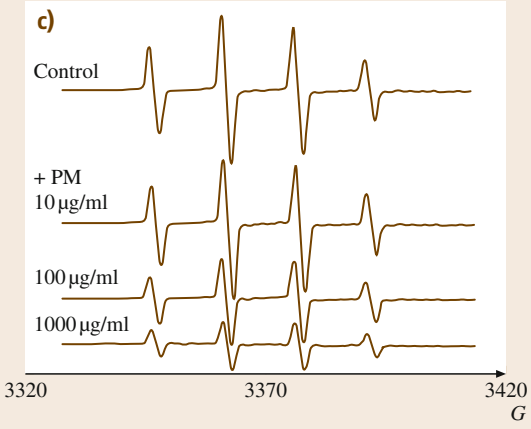


Fig. 47.2a–c Hydroxyl radical scavenging activity of PM and PG measured by the ESR-spin trapping method. The representative ESR spectra of DMPO-OH obtained by the addition of the indicated concentrations of mannitol **(a)**, PG **(b)**, and PM **(c)** to the Fenton reaction

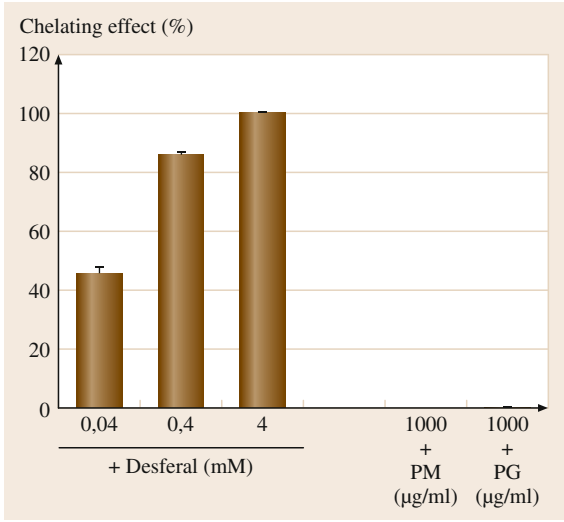


Fig. 47.3 Chelating activities of PM and PG on Fe^{2+}. *Columns* indicate Fe^{2+}-chelating effects of Desferal, PM, or PG as measured by the spectrophotometric method as described in the text. Each value represents the means ± standard deviation of triplicate measurements

ilar to mannitol as a known monosaccharide hydroxyl radical scavenger (Fig. 47.2a). Regarding the antioxidant activity of polysaccharides, it was proposed that polysaccharides can inhibit the formation of hydroxyl radicals, probably due to the hydrogen or electron abstraction mechanism [47.32]. It was also reported that the ease of abstraction of the anomeric hydrogen from the internal monosaccharide units made polysaccharides achieve the scavenging effect [47.33]. It is clear that further studies are required to clarify the molecular basis of the hydroxyl radical scavenging activities of PM and PG.

Regarding the hydroxyl radical scavenging mechanism of certain compounds, it has been pointed out that the scavenging activity was not due to direct scavenging but to inhibition of hydroxyl radical formation by chelating iron ions in the reaction system [47.34]. In fact, it was reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and inhibit the Fenton reaction [47.35]. Hence, the possible chelation of Fe^{2+} by PM and PG was estimated by the method of *Decker* and *Welch* [47.36]. As shown in Fig. 47.3, PM and PG had no significant chelating activity on Fe^{2+}, even at more than nearly 100 times the molar excess to Fe^{2+}, which was calculated by uronate bases, while 100 times mo-

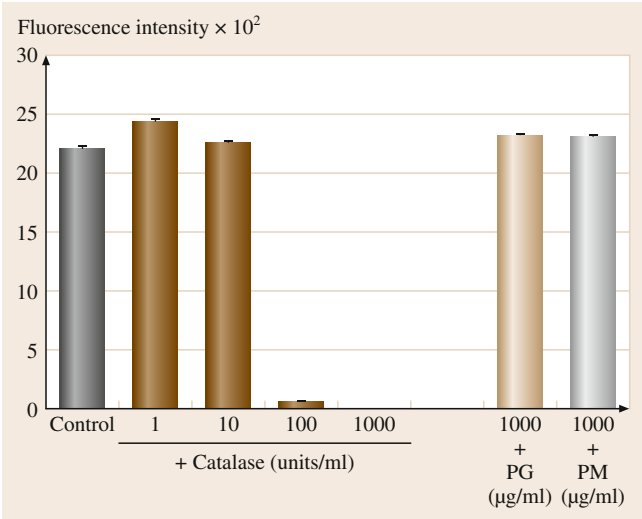


Fig. 47.4 Hydrogen peroxide scavenging activities of PM and PG measured by the PHPA-fluorescence method (PHPA: *p*-hydroxyphenyl acetic acid). *Columns* indicate fluorescence intensity obtained by the addition of indicated concentrations of catalase, PG, or PM to the PHPA-hydrogen peroxide system. Each value represents the means ± standard deviation of triplicate measurements

lar excess of Desferal, an Fe-specific chelater, completely inhibited color development in the reaction system. Similar to our results, it has been reported that alginate oligosaccharides showed no Fe^{2+} chelating activity, while chitosan oligosaccharides and fucoidan oligosaccharides exhibited a potent chelating activity on Fe^{2+} [47.22]. A comparative study on the radical scavenging activities of alginate oligosaccharides, chitosan oligosaccharides, and fucoidan oligosaccharides with similar molecular weight demonstrated that alginate oligosaccharides showed the highest hydroxyl radical scavenging activity among these marine oligosaccharides, whereas chitosan oligosaccharides had the highest superoxide radical scavenging activity [47.22]. Although the antioxidant mechanism of the oligosaccharides is not fully understood yet, these findings suggest that the chemical structure might be an important factor influencing the antioxidant activity.

Next, we examined the reactivity of PM and PG on hydrogen peroxide. Hydrogen peroxide is not a free radical, but it is cell-membrane permeable and can cause toxic effect on cells. In addition, it reacts with Fe^{2+} to form highly reactive hydroxyl radicals by the Fenton reaction, as mentioned above. As shown in Fig. 47.4, no significant scavenging activity of PM and PG toward hydrogen peroxide was observed up to 1000 μg/ml. Consistent with our results, it has been reported that the inhibitory effect of alginate oligosaccharides on hydrogen peroxide-mediated erythrocyte hemolysis is quite low as compared to that of chitosan oligosaccharides [47.22].

These results clearly indicate that PM and PG are capable of scavenging superoxide and hydroxyl radicals to a similar extent, but not hydrogen peroxide.

47.4 Cytokine-Inducing Activities of Alginate Oligosaccharides

As an immune stimulating activity of alginate oligosaccharides, their activities to induce cytokine secretion from macrophage cell line RAW264.7 cells are described in this section [47.20]. From the viewpoint of the structure–activity relationship, the results obtained by the studies conducted with purified alginate oligosaccharides are shown here. Figure 47.5 shows the structures of saturated or unsaturated mannuronate (M) and guluronate (G) oligosaccharides.

Pretreatment of RAW264.7 cells with polyguluronate (PG) and polymannuronate (PM) for 24 h did not result in any significant increase in the secretion of TNF-α into the medium. However, in the presence of enzymatically depolymerized unsaturated oligomers, the secreted levels of TNF-α markedly increased, depending on the oligomer structures. As shown in Fig. 47.6, similar structure-activity relationship profiles between guluronate oligomers and mannuronate oligomers were observed, although the maximum effect was found with G8 among guluronate oligomers and with M7 among mannuronate oligomers, respectively. On the other hand, the effects of saturated alginate oligomers, which were prepared by acid hydrolysis, on TNF-α secretion by RAW264.7 cells were fairly low or only trace levels as compared to those of unsaturated oligomers (Fig. 47.7). These results suggest that an unsaturated terminal structure with a double bond is important for the bioactivity of alginate oligomers regardless of the molecular size or structure (G or M).

Probably, RAW264.7 cells have a recognition mechanism that may be able to distinguish saturated and unsaturated terminal structures of alginate

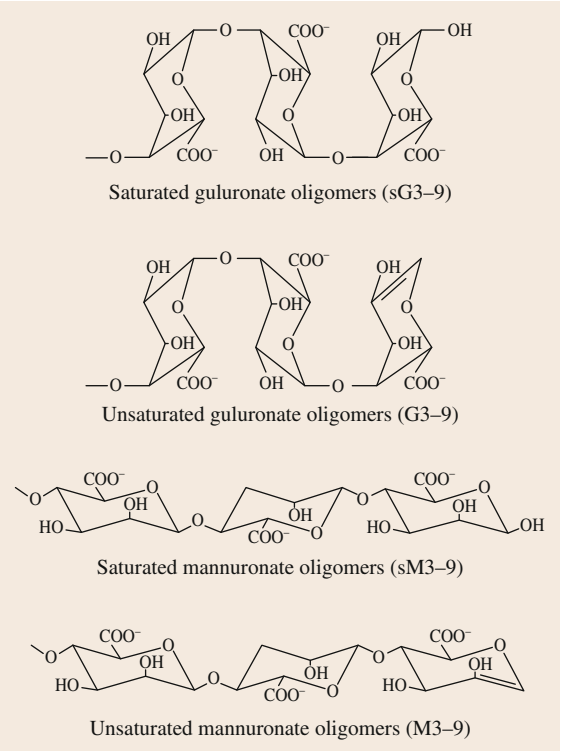


Fig. 47.5 Schematic representation of chemical structures of the saturated (sG3–9 and sM3–9) and unsaturated (G3–9 and M3–9) guluronate and mannuronate oligomers used in this study

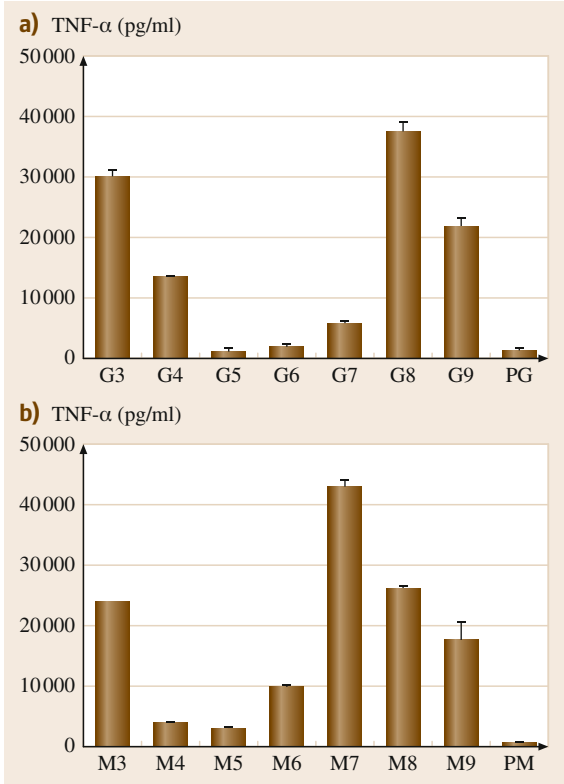


Fig. 47.6a,b Effects of unsaturated guluronate (G3–G9) **(a)** and mannuronate (M3–M9) **(b)** oligomers with various DPs on the production of TNF-α from RAW264.7 cells. Cells in 96-well plates (2×10^4 cells/well) were cultured in the growth medium in the presence of each purified alginate oligomer at a concentration of 1000 μg/ml at 37 °C. After 24 h, the supernatant was withdrawn from each well, and the amounts of TNF-α were measured by ELISA as described in the text. The effects of polymannuronate (PM) and polyguluronate (PG) were measured by the same way. Each value represents an average of triplicate measurements and each bar indicates the standard deviation

oligomers (Fig. 47.5). Since it has been reported that alginate polysaccharides stimulate human monocytes to produce IL-1 and IL-6 in addition to TNF-α [47.2], the effects of unsaturated alginate oligomers on the secretion of these cytokines from RAW264.7 cells were examined by the Bio-Plex assay system. The Bio-Plex assay is a suspension bead array technology that allows the simultaneous measurement of multiple cytokines in a single sample in a microplate well [47.21]. Similar to the results of enzyme-linked immunosorbent assay (ELISA), significantly high levels of TNF-α were detected in the cultured medium of RAW264.7 cells in the presence of some unsaturated oligomers, and the highest activities of G8 and M7 to induce TNF-α secretion were confirmed by this assay method (data not shown). As shown in Fig. 47.8, the secretion of IL-6 was also induced by unsaturated oligomers, whereas no significant effects of PG and PM were observed in this cytokine. The structure-activity relationship profiles of IL-6 were similar to those of TNF-α, although the induced levels of IL-6 were lower than those of TNF-α, and the activity of M7 was clearly higher than G8. Although the detected levels of IL-1α and IL-1β were lower than those of TNF-α, the patterns of the relationship between molecular structures of oligomers and the activities to induce secretion of IL-1α and IL-1β were similar to those of TNF-α (Fig. 47.9).

In a structure–activity relationship study of alginate oligomers, *Iwasaki* and *Matsubara* reported that an alginate oligosaccharide mixture had promoting ac-

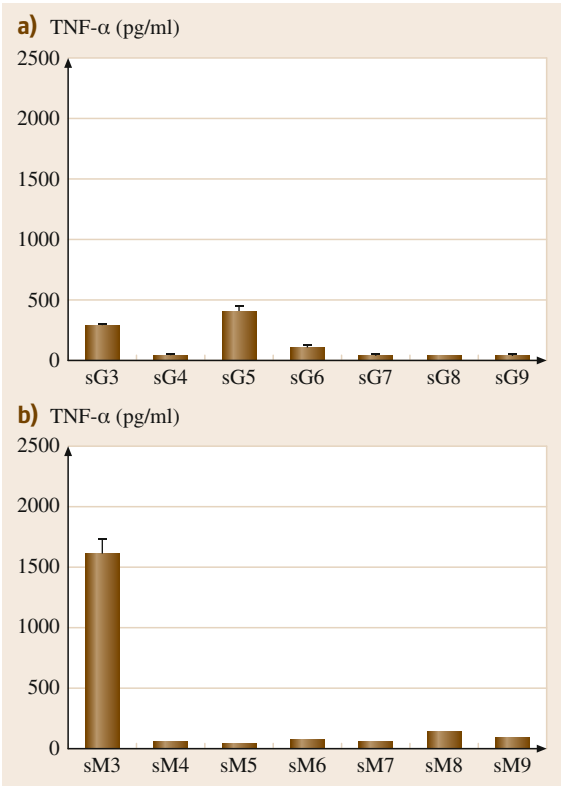


Fig. 47.7a,b Effects of saturated guluronate (sG3–sG9) **(a)** and mannuronate (sM3–sM9) **(b)** oligomers with various DPs on the production of TNF-α from RAW264.7 cells. The TNF-α inducing activities of saturated oligomers were measured as described in the legend to Fig. 47.6

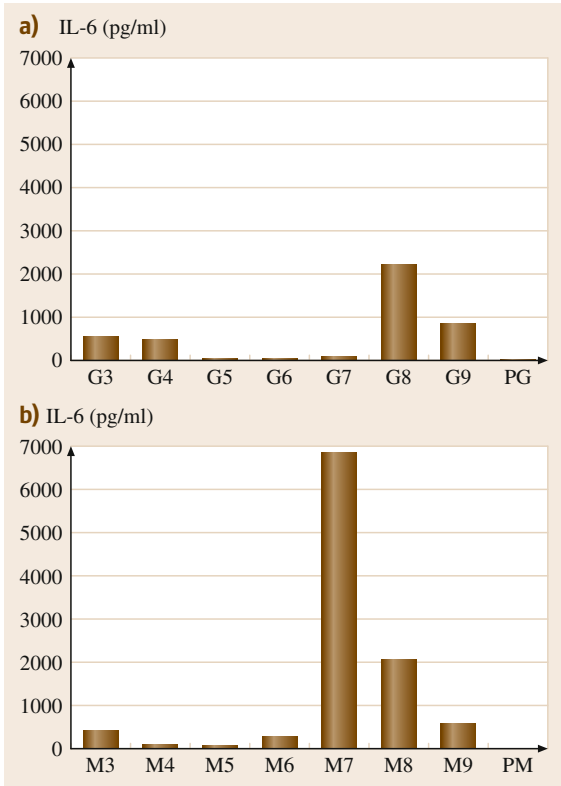


Fig. 47.8a,b Effects of unsaturated guluronate (G3–G9) **(a)** and mannuronate (M3–M9) **(b)** oligomers with various degree of polymerization DPs on the production of IL-6 from RAW264.7 cells. Cells in 96-well plates (2×10^4 cells/well) were treated with each oligomer as described in the legend of Fig. 47.2, and then the culture supernatants were subjected to Bio-Plex assay to determine IL-6 levels as described in the text. Each value represents an average of duplicate measurements. Differences between duplicate measurements for each value were within 5%

tivity toward the root growth of lettuce seedlings, and the tri, tetra, penta, and hexa-saccharides had especially strong activity [47.37]. In addition, it has been reported that penta-guluronate (G5) had the highest root growth-promoting activity on carrot and rice among the various alginate oligomers tested [47.28]. These findings together with the results described here suggest that there is the most effective structure or molecular size of alginate oligomer depending on each assay system in which the specific activities of oligomers are evaluated.

In the case of induction of cytokines from RAW264.7 cells, G8 and M7 showed the highest activity among each guluronate and mannuronate oligomer group, respectively. Previous structural studies have proposed that mannuronate and guluronate adopt different conformations, as shown in Fig. 47.5. Furthermore, X-ray diffraction analysis of the partial hydrolysis products of alginate has suggested that mannuronate blocks predominately form an extended ribbon structure, whereas guluronate blocks form a buckled chain [47.38]. Thus, such differences in the entire conformation between G8 and M7 may result in the fact that the most effective guluronate oligomer is one residue longer than the mannuronate oligomer. Since quite similar levels of TNF-α production were induced by G8 and M7, both oligomers may stimulate RAW264.7 cells with equal potency through a common recognition site on the cells.

Since the maximum activities of G8 and M7 were also observed in inductions of IL-1α, IL-1β, and IL-6

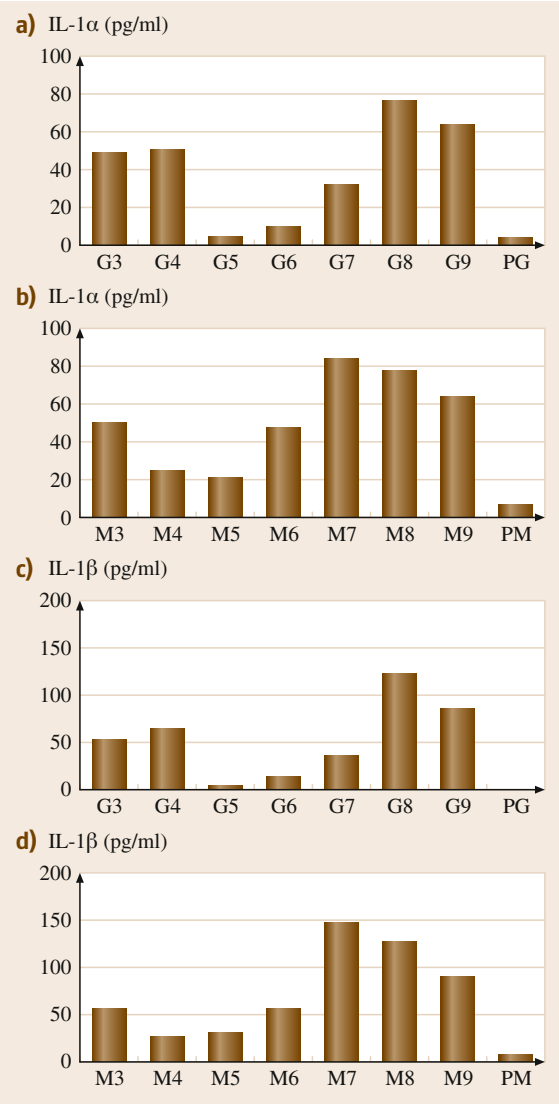


Fig. 47.9a–d Effects of unsaturated guluronate (G3–G9) **(a,c)** and mannuronate (M3–M9) **(b,d)** oligomers with various DPs on the production of IL-1α **(a,b)** and IL-1β **(c,d)** from RAW264.7 cells. Cells in 96-well plates (2×10^4 cells/well) were treated with each oligomer as described in the legend of Fig. 47.1, and then the culture supernatants were subjected to Bio-Plex assay to determine IL-1α and IL-1β levels as described in the text. Each value represents an average of duplicate measurements. Differences between duplicate measurements for each value were within 5% ◄

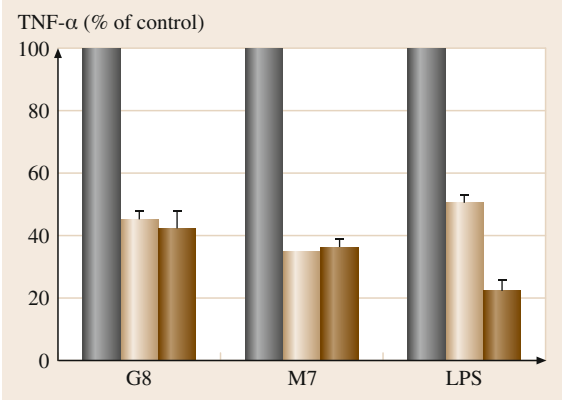


Fig. 47.10 Effects of anti-TLR2 and anti-TLR4 antibodies on the production of TNF-α by RAW264.7 cells stimulated with alginate oligomers (G8 and M7) and LPS. Cells in 96-well plates (2×10^4 cells/well) were pretreated with 10 μg/ml of anti-TLR2 (□) or anti-TLR4 (□) antibody for 1 h at 37°C. After washing with PBS, alginate oligomers (G8 or M7 at final concentration of 1000 μg/ml) or LPS (at final concentration of 1 ng/ml) were subsequently added, followed by incubation for further 24 h. Supernatants were analyzed for TNF-α levels as described in the legend of Fig. 47.2. Each value represents an average of triplicate measurements and each bar indicates the standard deviation. □: control without antibody

production, these cytokines may be induced simultaneously once stimulated by G8 and M7. Interestingly, G3 and M3, the smallest oligomers among the oligomers tested, also showed relatively high cytokine inducing activities, even higher than other larger oligomers. The reason for this is uncertain now, but the finding the cause may provide a clue to understanding the recognition site or mechanism on RAW264.7 cells by alginate oligomers.

Regarding the initial host defense against bacterial infection, it has been proposed that the recognition of microbial products such as LPS by the host defense system is mediated by members of the toll-like receptor (TLR) family [47.39]. TLRs are evolutionary conserved pattern recognition receptors that discriminate between self and non-self by recognition of pathogen-associated molecular patterns with no apparent structural similarity [47.39]. TLRs play important roles in signal transduction for the initiation of mammalian immunity responses, including cytokine production [47.40]. In addition to bacterial products, TLRs seem to be responsible for the polysaccharide-mediated stimulation processes. For instance, it has been shown that two

uronic acid-containing polysaccharides with different origins induce cell activation through TLR4 [47.41, 42]. Furthermore, it was recently reported that TLR2 and TLR4 are involved in the stimulation of cytokine production from human monocytes by mannuronate polymers isolated from mucoid produced by *Pseudomonas aeruginosa* [47.43]. To examine the possible involvement of TLR2 and TLR4 in G8 and M7-induced TNF-α secretion from RAW264.7 cells, the specific blocking antibodies were used. As shown in Fig. 47.10, both antibodies to TLR2 and TLR4 inhibited the activities of G8 and M7 to induce TNF-α secretion with similar efficiencies. On the other hand, LPS-induced TNF-α secretion was differently affected by these antibodies, and the influence of the antibody to TLR4 was greater than the antibody to TLR2 (Fig. 47.10). These results suggest that alginate oligomers with a molecular weight less than 2000 are also recognized by the innate immune system through TLRs, although the way of recognition might somehow be different from bacterial products.

This is the first report to indicate that alginate oligomers with defined chemical structures have cytokine-inducing activities in a structure-dependent manner. The highly purified sets of alginate oligomers obtained in this study are interesting agents, not only as potential therapeutic agents but also as a tool especially for analyzing pattern recognition mechanisms. Further study of the structure–activity relationship of alginate oligomers may provide insight into the TLR-mediated recognition mechanism as well as the signal transduction system leading to cytokine production.

47.5 Induction of Cytokines in Mice

It has been shown that enzymatically depolymerized alginate oligosaccharide mixture ((AOM) showed more than 10 times higher activity in terms of TNF-α secretion from RAW264.7 cells as compared to original alginate polymers [47.19]. In this section, the results of cytokine levels in mice serum after i.p. injection of AOM are described [47.21]. Since preliminary experiments indicated that G-CSF is a cytokine with the highest level among the cytokines detected in the mice serum after i.p. injection of AOM, time-course analysis focusing on G-CSF level in mice serum after i.p. administration of AOM was carried out. As shown in Fig. 47.11, the G-CSF level in the serum rapidly increased and reached the maximum level at 2 h after injection of 700 mg/kg body weight and the high level was sustained until 6 h, and then gradually decreased and returned to baseline level at 24 h. When the same dose (700 mg/kg) of original alginate polymer was injected, no significant G-CSF level was detected in the serum, at least during the time periods tested. These results suggest that the mixture of alginate oligomers is a superior cytokine inducer to the original polymer in vivo. In the in vivo system, the high viscosity as well as the gel-forming property of alginate polymers may prevent efficient transfer or even dispersion of the alginate polymer from the area injected to circulation system to reach the target organs or cells that may be involved in cytokine secretion. The improvement of these physicochemical drawbacks of alginate polymer by enzymatic degradation, as well as the increased cytokine-inducing activity at cellular level, may partly explain the reason for the potent cytokine-inducing activity of AOM in vivo.

A dose-response experiment was performed by i.p. injection of 0.07, 0,7, 7, 70, 700 mg/kg of the al-

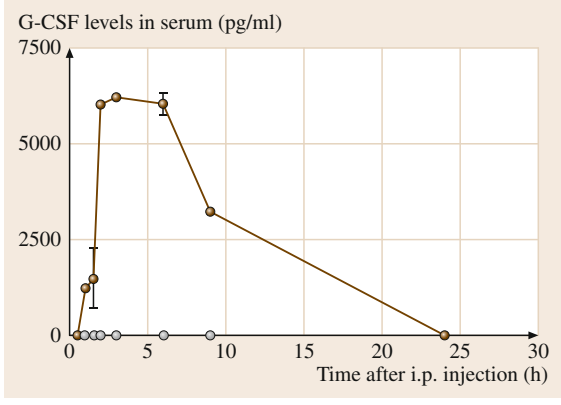


Fig. 47.11 G-CSF levels in the serum after i.p. injection of alginate oligomer mixture or alginate polymer. After single i.p. injection of alginate oligomer mixture (700 mg/kg bodyweight in 1 ml of PBS) or alginate polymer (700 mg/kg bodyweight in 1.0 ml of PBS), G-CSF levels in each serum collected at the indicated periods of time were measured by ELISA as described in the text. Each point represents an average of triplicate measurements and each bar indicates the standard deviation

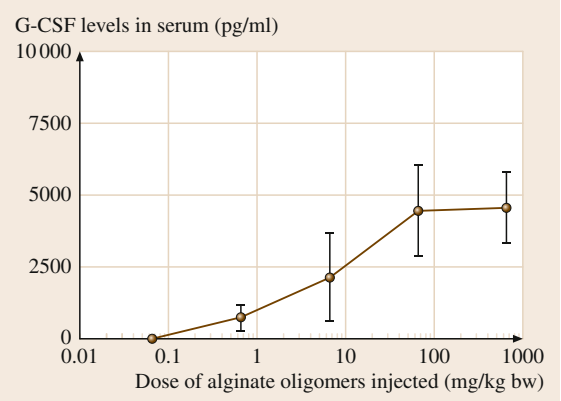


Fig. 47.12 G-CSF levels in the serum after i.p. injection of varying doses of alginate oligomer mixture. After single i.p. injection of varying doses of alginate oligomer mixture (0.07, 0.7, 7, 70, and 700 mg/kg bodyweight in 1 ml of PBS), G-CSF levels in each serum collected after 3 h were measured by ELISA as described in the text. Each point represents an average of triplicate measurements and each bar indicates the standard deviation

ginate oligomer mixture and examined G-CSF serum levels after 3 h. As shown in Fig. 47.12, G-CSF levels in the serum increased with the increased dose of the mixture and reached the plateau level at 70 mg/kg, which suggests that that i.p. injection of 70 mg/kg of the mixture is sufficient to induce the maximum serum level of G-CSF. Based on the result, it is calculated to be 4.2 g for 60 kg bodyweight, and this value is considered within applicable range for human beings.

As mentioned above, it has been generally considered that biological activities of alginate polymers are deeply influenced by the molecular size, M/G ratio, and entire molecular conformation. Therefore, it is necessary to select suitable alginate polymers or the appropriate origin of the polymer when it comes to the development of alginate polymers as certain therapeutic agents or food supplements that may require cytokine-inducing ability. However, because of the heterogeneous intramolecular arrangement of M-blocks and G-blocks, and the existence of the M/G random structure, the clarification of the structure–activity relationship of alginate polymers with a molecular weight more than 10 000 is almost impossible. Hence, enzymatic depolymerization may be a promising strategy to improve the bioactivities of alginate polymers regardless of their original activity. Although it is uncertain whether the cytokine-inducing activity is always responsible for the variety of bioactivities of alginates, the findings described here may suggest that alginate oligomers even in mixture are useful as bioactive agents in in vivo systems.

To investigate other cytokine levels in mouse serum after i.p. injection of alginate oligomer mixture, we employed the Bio-Plex bead assay that can detect 23 cytokines simultaneously. As shown is in Fig. 47.13, i.p. administration of alginate oligomers resulted in a significant increase in 20 cytokines in the serum with different levels and different kinetics depending on the cytokines. G-CSF, MCP-1 (monocyte chemoattractant protein), IL-6, KC (keratinocyte-derived chemokine), RANTES (regulated upon activation normal T cell expressed and secreted), and IL-12 (p40) levels were relatively high with an exceeded 5000 pg/ml serum level at the peak point. The serum level of TNF-α, interleukin (IL)-2, IL-3, IL-10, IL-12 (p70), IL-13, IL-17, GM-CSF (granulocyte-macrophage colony-stimulating factor), and interferon (INF)-γ clearly showed two peaks during the 24 h interval, whereas G-CSF, granulocyte macrophage (GM)-CSF, monocyte chemoattractant protein (MCP)-1, IL-1α, RANTES, KC, eotaxin, and IL-1α tended to show one peak. Although the exact reason for the biphasic kinetics of some cytokines is unclear now, resident cells in the intraperitoneal cavity may be responsible for the first peak of these cytokines, and then alginate oligomers transferred from the cavity to circulation may stimulate second target cells that, in turn, respond to the second peak of the cytokines. It was confirmed that among the cytokines induced by alginate oligomers mixture, the level of G-CSF was strikingly higher than others. To our knowledge, this is the first result indicating that alginate oligomers are capable of inducing G-CSF in vivo.

G-CSF is a hematopoietic factor that stimulates neutrophile production and release from bone marrow as well as activating mature neutrophilic function [47.44]. Since neutrophils are primary effectors in host defense against invading pathogens, the use of G-CSF has been tested in the treatment of bacterial infectious diseases [47.45], and even an antiviral effect of G-CSF on the hepatitis virus has also been reported [47.46]. Thus, the ability of the alginate oligomer mixture to induce G-CSF may be a useful bioactivity that should be taken into consideration in the application of alginate oligomers. This ability of alginate oligomers to induce G-CSF may partly explain the previous findings that i.p. administration of alginates with a high content of mannuronic acid enhanced the

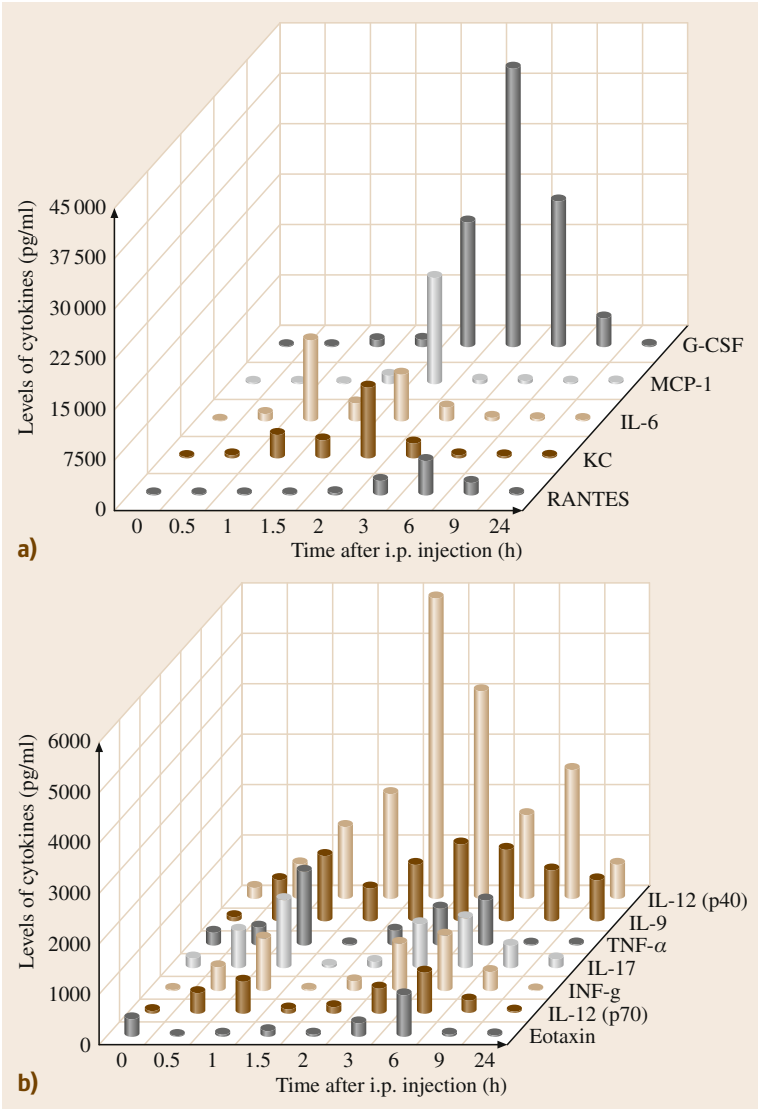


Fig. 47.13a–c Levels of various cytokines in the serum after i.p. injection of the alginate oligomer mixture. After a single i.p. injection of alginate oligomer mixture (700 mg/kg bodyweight in 1 ml of PBS), the levels of various cytokines in the serum collected after 3 h were measured by the Bio-Plex assay as described in the text. The results were summarized to relatively higher levels **(a)**, middle levels **(b)**, and lower levels **(c)** of cytokines. Each value represents an average of duplicate measurements. Differences between duplicate measurements for each value were within 5%. Abbreviations used in the figure: MIP – macrophage inflammatory protein

survival rate of lethally irradiated mice and stimulated murine hematopoiesis in vitro [47.47].

Regarding the biological effects of alginate oligomers in in vivo mammalian models, *Yoshida* et al. reported that i.p. injection of a mixture of alginate oligomers with an average degree of polymerization of 4.4 resulted in the suppression of both Th2 development and IgE production through IL-12 production [47.15]. Based on their results, *Yoshida* et al. proposed that alginate oligomers may be useful as a novel antiallergic agent.

In conclusion, the results clearly indicate that alginate oligomers in mixture are capable of inducing multiple cytokines, especially G-CSF, in vivo after i.p. administration. These findings may provide basic information about the potential activity of alginate oligomers as a potent cytokine inducer. Kinetics data of various cytokines obtained in this study may give a clue not only for the understanding of the underlying mechanism of the variety of biological activities of alginate oligomers reported so far but also for the study of future applications of alginate oligomers.

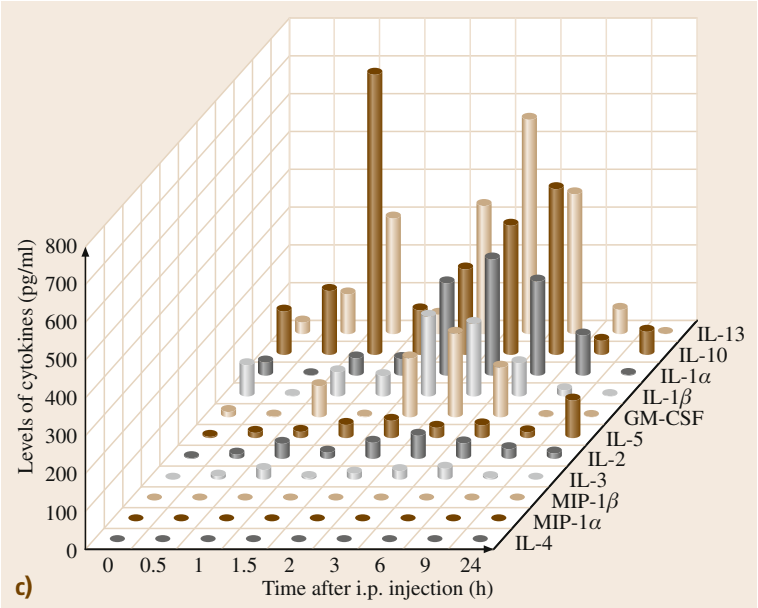


Fig. 47.13 (continued)

47.6 Growth-Promoting Effect of Alginate Oligosaccharides

In this section, the effects of AOM on the growth of unicellular marine microalga are described. In the aquaculture industry, microalgae are indispensable food sources for some species of bivalves, larvae of crustaceans, and fish species. They are also consumed by zooplankton used for feeding of larvae and juveniles of marine fish, as well as some crustaceans. *Nannochloropsis oculata* is a marine unicellular microalga classified in the class of Eustigmatophyceae and is frequently used in practical fish larval rearing in aquaculture industry.

Through the food chain initiated from microalgae, important nutrients such as vitamin E from microalgae are transferred to higher trophic levels via the intermediate zooplankton [47.48, 49]. It has been reported that *N. oculata* has a superior nutritional value with a relatively high level of vitamin E [47.50]. Thus, *N. oculata* is a highly important organism, not only as a direct food source for many aquatic animals but also as a feeding organism for zooplankton rotifers used as a second organism for fish larval rearing in aquaculture industry of marine fishes and crustaceans. Hence, the establishment of a stable and efficient culture procedure for useful microalgae is urgently required. It has been demonstrated that alginate oligosaccharides show growth-promoting activity in various higher plant systems. Since *N. oculata* is a unicellular marine phytoplankton with a eukaryotic plant cell type, it can easily be imagined that it has many functional and structural similarities to higher plant cells at cellular level and might be influenced by alginate oligomers. Under these circumstances, as a pilot study, the effects of AOM on the growth of *N. oculata* under normal conditions were examined. It was also investigated if AOM can improve Cu^{2+}-induced stress conditions [47.51].

N. oculata was supplied from the microalgae collection of the National Research Institute of the Aquaculture Fisheries Research Agency. *N. oculata* was cultured at 17 °C in 100 ml of modified Guillard's *f* medium in which $Na_2SiO_3 \cdot 9H_2O$, vitamin B_{12}, biotin and thiamine HCl were omitted from the originally reported constituents [47.52]. The culture was kept under illumination of fluorescent lamps at 30 $\mu mol/m^2$ s with a cycle of 12 h light and 12 h dark. All cultivations were done using sterilized instruments and were continuously stirred with a magnetic stirrer. Exponentially growing algal cells were used throughout the experi-

ments. The initial cell density of the algal cells was adjusted to about 10^5 cells/ml, and the culture started in the medium containing the indicated concentration of AOM under the conditions described above. To examine the effects of Cu^{2+} on the growth of *N. oculata*, the indicated final concentration of $CuSO_4 \cdot 5H_2O$ was added to the medium, which was cultured for a few days as described above. The algal cell densities were determined by haemocytometer counts, and the growth conditions were also monitored microscopically every day.

The effects of AOM with various concentrations on the cell growth of *N. oculata* are shown in Fig. 47.14. From the growth curve profiles, it is obvious that the algal growth was dramatically promoted by AOM in a concentration-dependent manner at the concentration range 1–20 mg/ml. Even with 1 mg/ml of AOM, a slight increase in the growth was observed. The maximum effect was attained at 20 mg/ml, after which the cell number reached a plateau level that was nearly five times higher than that of control without AOM. However, the growth-promoting effect of AOM was slightly decreased at 40 mg/ml to a level similar to that observed at 5 mg/ml. Thus, it seems likely that AOM has an optimum concentration to promote the growth of *N. oculata* at least under the conditions used. As judged from the patterns of growth of *N. oculata* in the presence of effective concentrations of AOM, the rate of cell division during the exponential growth phase also tended to be accelerated by AOM. Based on these data, it was suggested that AOM is capable of promoting the growth rate of *N. oculata* during the exponential phase and of increasing the maximum cell density at the stationary phase.

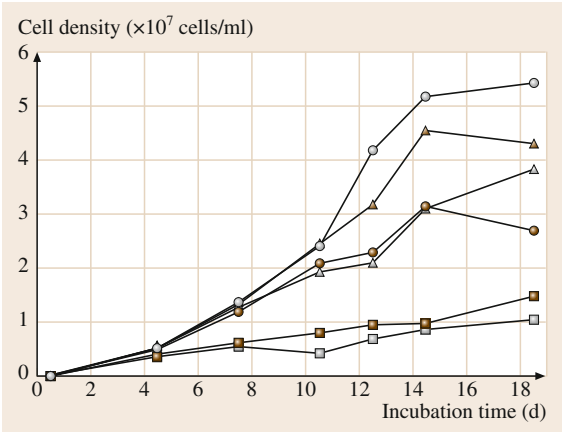


Fig. 47.14 Effects of AOM on the growth of *N. oculata*. *N. oculata* cells were inoculated into the medium (100 ml) containing 1 mg/ml (□), 5 mg/ml (▲), 10 mg/ml (△), 20 mg/ml (●), 40 mg/ml (○) of AOM or without AOM as a control (■), and cultured for the indicated periods of time under the conditions described in the text. The number of algal cells in each culture was counted daily as described in the text. Each value represents the average of duplicate measurements

The culture of microalga often suffers from various environmental stresses even under highly controlled conditions. Especially, trace amounts of contamination with heavy metals are known to cause suppression of the growth of microalgae [47.53]. Although Cu^{2+} is often used for extermination of parasites attached on cultured fish in aquaculture firms, this metal is also known to be toxic to microalga. Thus, Cu^{2+} was used as a representative heavy metal stressor for *N. oculata*. As shown in Fig. 47.15, the addition of 2.5 mg/ml of Cu^{2+} to the medium resulted in the complete inhibition of the growth of *N. oculata*. Even after 24 h incubation with Cu^{2+}, almost all cells were decolorized and became incapable of subsequent cell division. The potent toxic effect of Cu^{2+} on *N. oculata* disappeared almost completely with the addition of a semioptimal concentration of AOM (10 mg/ml), and the growth curve in the presence of AOM and Cu^{2+} together was similar to that of the control without AOM and Cu^{2+}. These results suggest that AOM is able to not only promote the growth of *N. oculata* under normal conditions but also to exert a beneficial effect on algal cells under stress-induced suppressive conditions. To our knowledge, this is the first demonstration that alginate oligosaccharide can promote the growth of marine microalga in addition to terrestrial higher plants. Since AOM was originally derived from seaweeds, it is a biologically nonhazardous and environmentally friendly material. Thus, AOM is one of the most promising candidates that can be introduced into actual aquaculture firms to establish a technique for stable mass culture of useful marine microalgae. Further studies are required to develop economically viable whole operations, including a low-cost AOM preparation procedure.

Although the underlying mechanisms of the beneficial effects of AOM on *N. oculata* is still unclear, it is possible to speculate that *N. oculata* is stimulated by AOM through the specific recognition and subsequent intracellular signal transduction, which may lead to the eventual growth promotion. Regarding this

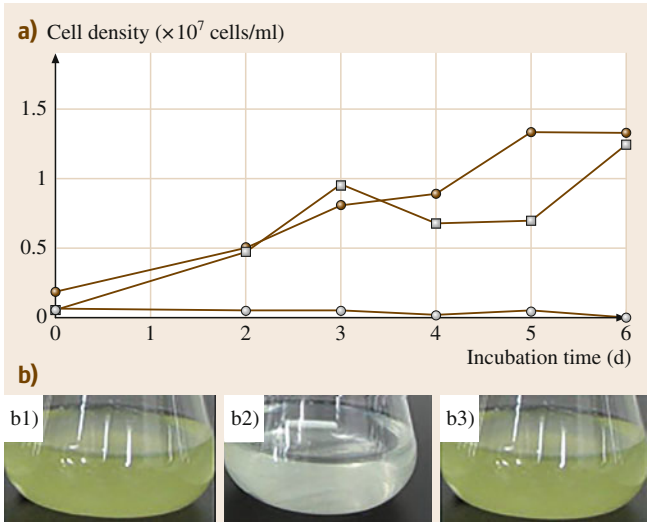


Fig. 47.15 Effects of AOM on the growth of *N. oculata* in the presence of Cu^{2+}. *N. oculata* cells were inoculated into the medium (100 ml) containing 2.5 mg/ml of Cu^{2+} and 10 mg/ml of AOM (●), 2.5 mg/ml of Cu^{2+} (○) or without these agents as a control (□), and cultured for the indicated periods of time under the conditions described in the text. The number of algal cells in each culture was counted daily as described in the text. During the incubation time, the algal cells were observed microscopically. Each value represents the average of quadruplicate measurements. **(b)** The pictures show the algal cells after 2 days incubation with 2.5 mg/ml of Cu^{2+} and 10 mg/ml of AOM (b1), 2.5 mg/ml of Cu^{2+} (b2) or without these agents as a control (b3)

point, there have been several reports on oligosaccharide molecules derived from the cell walls of plants or microbes that have various biological functions on plant cells [47.54]. Such biologically active oligosaccharides were first called oligosaccharins by *Albersheim* and *Darvill* [47.55]. Among such biologically active oligosaccharides, the capabilities of oligogalacturonides to regulate various physiological functions in plants have been well documented so far, and it has been suggested that oligosaccharin has a pleiotropic nature.

In conclusion, these results demonstrate that AOM not only promotes the growth of *N. oculata* but also overcomes Cu^{2+}-induced growth suppression of the alga. AOM is considered to be a useful and promising agent for the establishment of improved techniques for stable and efficient mass culture of *N. oculata*. Our recent study demonstrated that AOM prepared by enzymatic degradation shows a growth-promoting effect on the green alga *Chlamydomonas reinhardtii*, which is known as a model organism in fundamental molecular biology studies as well as a producer of biofuel. Interestingly, the fatty acid composition of *Chlamydomonas reinhardtii* was also influenced by the AOM; the content of C16 : 0, C18 : 2cis and C18 : 3*n-3* increased in AOM-treated cells. However, AOM prepared by acid hydrolysis had no effect on the growth of *Chlamydomonas reinhardtii* [47.56].

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48. Vector and Agricultural Pest Control

Venkateswara Rao Janapala

Many of the small-scale farmers in developing countries continue to use high volumes of pesticides for crop protection due to their illiteracy, and lack of adequate information. Such practices have resulted in many environmental problems such as effect on nontarget species, pesticide residue accumulation, development of resistance to pests, ecological imbalance by elimination of natural predators as well as environment pollution. The current critical situation is that many of the previously used and economically affordable insecticides are no longer effective and are also banned in many countries. Therefore, there is an urgent need to develop newer, more effective, target specific and less persistent insecticides. In the search for developing newer insecticides, several researches had paid attention toward marine natural products. It is a well-known fact that the marine environment is an exceptional reservoir of bioactive natural products, which produce several novel structures with unique biological properties which may not be found in terrestrial natural products. In recent years, a number of studies have suggested that some of the bioactive compounds isolated from marine organisms exhibit pesticidal properties. Such bioactive metabolites from marine organisms may be an alternative source for vector and agricultural pest control agents. It is anticipated that these bioactive metabolites may potentially replace the existing and highly toxic synthetic insecticides and will play an important role in the future insecticide development program. This chapter provides an overview of recent trends in the exploration of marine secondary metabolites for pest management system.

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48.1 Preamble

The ever-increasing global population presents an enormous challenge to existing agricultural infrastructure and practices. The global population will reach 9.1 billion by 2050, which is 34% higher than today. To meet the requirements the Global food production will need to jump by 70–100% to feed these people. Agricultural experts estimate that countrywide more than one-third crop yield is lost to insect pests, diseases, and weeds, both in the fields, and during storage and transport [48.1]. Much of the increase in agricultural productivity over the past half century has been due to the control of these pests with synthetic chemical pesticides [48.2]. Crop protection chemicals continue to be the major tools for protecting food and fiber crops from damaging pests.

Synthetic pesticides are well recognized as an economic approach to controlling pests; despite having several beneficial possessions to agriculture. These pesticides constitute some persistent problems in causing pollution to the environment [48.3]. Now there is growing concern worldwide over the indiscriminate use of such chemicals that results in environmental pollution and toxicity risk to nontarget organisms. Secondly, continuous usage of these pesticides results in developing resistance in pest organisms. To address environmental concerns, new agrochemicals developed from natural products may be perceived as more environmentally acceptable than that present scenario.

The investigation of several marine natural pesticides such as nereitoxin, bensultap, cartap, and thiocyclam derivatives have exhibited significant insecticidal properties against important insect pests [48.4]. According to *Peng* et al. [48.5], the macrolides, polypeptides, and alkaloids are the promising agents for insecticidal, herbicidal, and fungicidal, and they also noticed that manzamine F can be used as a new pesticide. In contrast, the plants, animals, and microorganisms in the marine environment, with their wide range of chemical and bioactive diversity, are still largely an unexplored resource for new agrochemical agents [48.6, 7].

The marine environment is an exceptional reservoir of bioactive natural products, which produced several novel structures with unique biological properties that may not be found in terrestrial natural products [48.7, 8]. These marine organisms have evolved chemical defence mechanisms to fight successfully against other invading organisms, which involve the production of secondary metabolites [48.9]. The current thrust of the investigations involves identifying newer drugs and other pharmaceuticals from marine origin, whereas comparatively little attention has been paid with respect to the discovery of pesticide molecules. The secondary metabolites isolated from marine organisms may be an alternative source for agrochemicals to replace the existing and highly toxic synthetic insecticides and will play an important role in the future insecticide development program. *Venkateswara Rao* et al. [48.10] suggested that the secondary metabolites isolated from the marine sponges may be an alternative source for vector control agents to replace the existing and highly toxic synthetic insecticides and will play an important role in future insecticide development program. Previously, *Bradford* et al. [48.11] described the marine potential natural products to serve as insect control agents via mechanisms of toxicity, interference with molting of metamorphosis and feeding deterrence. *Donia* and *Hamann*, [48.12], *Blunt* et al. [48.13], *Haefner* [48.14], and *Venkateshwara* et al. [48.7] demonstrated that the sponge consisting of sesquiterpenes and diterpenes – secondary metabolic compounds might be a source of new insecticides; it did not liberate compounds of commercially significant potency against important insect pests. Earlier studies have also suggested that some secondary metabolites isolated from marine organisms have been shown to exhibit larvicidal, insecticidal, and IGRs (insect growth regulators) activities [48.15–19].

This chapter aims to explore Indian marine biodiversity as a source of bioactive metabolites with potential application as agrochemicals. Such agrochemicals will act by new molecular modes of action and should be effective against vectors and pests that are resistant to current formulations.

48.2 Current Status of Research and Development in the Subject

Natural marine products have the potential to replace synthetic pesticides and other agents used to maximize crop yields and growth. The chapter in this area is likely to result in useful natural pesticides that would provide greater specificity and fewer harmful side effects than the conventional synthetic pesticides. Current biopes-

ticides from marine and other sources are expected to capture an estimated 10% of market [48.20].

An example of a marine biopesticide in use today is Padan, which was developed from a bait worm's toxin known to ancient Japanese fishermen. It was derived from nereistoxin, a naturally occurring insecticidal substance, isolated from marine segmented worms *(Lumbriconereis heteropoda)*. This insecticide was introduced globally as one of the major insecticides with a broad spectrum of activities. This natural pesticide has demonstrated activity against larvae of the rice stem borer, the rice plant skipper, and the citrus leaf miner, among other pests. It has strong stomach poison, contact, antifeed, and ovicide function. It is mainly used for preventing injurious insects such as Lepidoptera, Coleopteran, Hemiptrea, and Diptera.

48.2.1 Padan

Padan S,S'-(2-dimethylaminotrimethylene) bis(thiocabamate) hydrochloride; S,S'-[2-(dimethylamino)-1,3-propanediyl] dicarbamothioate hydrochloride.

Synonyms:
1,3-bis (carbamoylthio)-2-(*N*,*N*-dimethylamino) propane monohydrochloride, Cartap hydrochloride, and patapvegetox

In India, two formulations of this products were introduced by M/s. Coromandel Agrico Pvt Ltd., New Delhi (Padan 4G (4% Cartap hydrochloride in granule form and Padan-50SP (50% water soluble powder)). The formulation, Padan 4G is generally used in two major crops, i. e., rice and sugarcane. Padan 50SP has been found effective in the control of most of major pests on rice, potato, sweet potato, maize, cabbage, onion, ginger, grapes, tea, citrus, and other vegetables. The active ingredient is also effective against diamondback moth. These formulations are compatible with other insecticides and fungicides, for effective integrated pest management.

More recently, scientists in Montana discovered novel compounds in marine algae and marine sponges containing symbiotic microorganisms. A large number of compounds have been screened as newer pest control agent from marine flora. Sponges serve as sources of bioactive compounds and are capable of synthesizing many secondary metabolites and have been a prolific source of new molecules [48.21, 22]. Similarly, extracts derived from sponge (*Dysidea etheria*) and *Nudibranch* species also demonstrated powerful insecticidal activity against two species, grasshoppers and the tobacco hornworm [48.23]. The sponge, *D. etheria* and the *Nudibranch*, *Hypselodoris zebra* have yielded a number of sesquiterpenes, some of which exhibit feeding deterrent activity (antifeedant) in insects (Fig. 48.1). Furodysinin **(1)** was not toxic to advanced larval stage of tobacco hornworms at doses of 250 ppm in an artificial diet. Most of the insects were unable to shed their molts completely and two hornworms developed double head capsules [48.24]. The other two *Dysidea* metabolites tested, 5-acetoxy- and 5-hydroxynakafuran-8 **(2,3)**, exhibited a different activity profile against the grasshopper. The acetate **2** was toxic at the high dose and antifeedant at the lesser dose, while the alcohol **3** was antifeedant even at lower doses.

Recent past, some of the novel molecules isolated from sponges and soft corals with pesticide properties were identified. Among them, swinhoeiamide A **(4)** isolated from Guinean sponge *Theonella swinhoei* [48.16], exhibited insecticidal activity toward neonate larvae of the polyphagous pest insect, *Spodoptera littoralis* when incorporated in an artificial diet offered to the larvae in a chronic feeding bioassay (ED_{50} 2.11 ppm, LD_{50} 2.98 ppm).

Soft corals of the genus *Nephthea* (*Alcyonacea*, *Nephtheidae*) have yielded a variety of bioactivee sesquiterpenes and diterpenes. Among the seven isolated oxygenated sesquiterpenes, from the Indonesian soft coral, *Nephthea chabrolii*: hydroxycolorenone **(5)** exhibited insecticidal activity on a polyphagous pest *S. littoralis*, with an EC_{50} of 8.8 ppm and a LC_{50} of 453 ppm, when incorporated in artificial diet and offered to larvae in a chronic feeding bioassay [48.25]. Similarly, a new sesquiterpene, hydroxycolorenone **(6)** was isolated from the soft coral *N. chabrolii*, which showed strong insecticidal activity with an EC_{50} 35 μM and an LC_{50} 1.8 mM against neonate larvae of *S. littoralis* [48.25].

A tropical marine sponge, *Axinella carteri* afforded six unusual alkaloids, including the new brominated guanidine derivative, 3-bromo-hymenialdisine [48.19]. All alkaloids were screened for insecticidal activity against *S. littoralis*. The guanidine alkaloids hymenialdisine and debromohymenialdisine exhibited insecticidal activity at LD_{50} of 88 and 125 ppm, respectively, when incorporated into artificial diet and offered to the larvae in a chronic feeding bioassay. The marine-derived, alkaloid manzamine A **(7)** was reported to exhibit insecticidal activity toward neonate larvae of *S. littoralis* with an ED_{50} of 63 μM [48.26]. Pyriodacridine alkaloids are characterized by an 11H-pyrido (4,3,2-mn) acridine moiety. Kuanoniamin C **(8)** and kuaoniamine D **(9)** exhibited insecticidal activ-

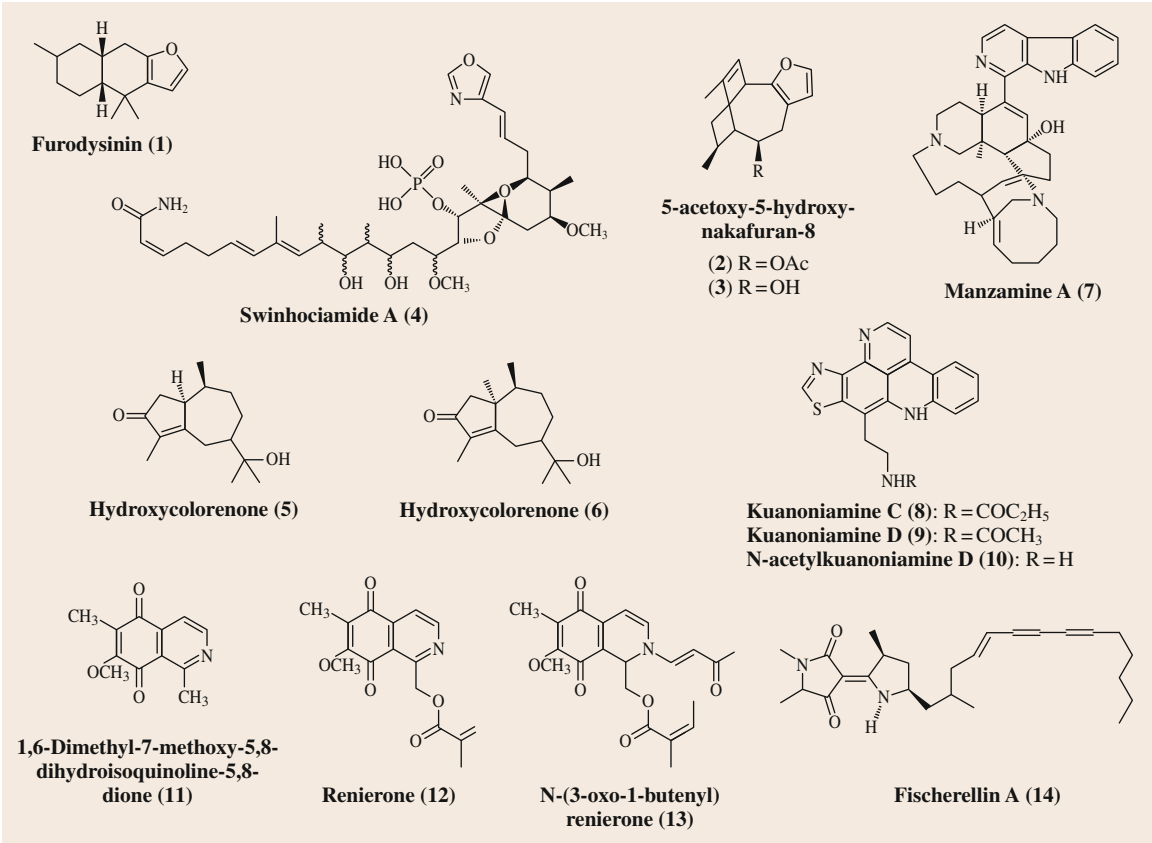


Fig. 48.1 Structures of the marine pesticidal molecules mentioned in the text

ity against *S. littoralis* with LC_{50} values of 0.42 and 0.16 mM, respectively, whereas *N*-acetylkuanoniamine D **(10)** was marginally active and inhibited the growth of the larvae with an ED_{50} of 0.44 mM [48.27]. The isoquinoline alkaloids isolated from the Philippine sponge of the genus *Xestospongia* also exhibited insecticidal activity [48.26]. 1,6-Dimethyl-7-methoxy-5,8-dihydroisoquinoline-5,8-dione **(11)** showed insecticidal activity with an EC_{50} of 35 ppm and an LC_{50} of 521 ppm against neonate larvae of *S. littoralis*. However, Renierone **(12)** and *N*-(3-oxo-1-butenyl) renierone **(13)** exhibited weak insecticidal activity, whereas 100% inhibition of the powdery mildew on barley (*Erysiphe graminis*) required a concentration of 2.4 mM. Fischerellin A **(14)** exhibited 80% inhibition of downy mildew (late blight, *Phytophthora infestanson* tomato) and rice blast (*Pyricularia oryzae*) at 2.4 mM. Less activity (30% growth inhibition at 2.4 mM) was observed against brown rot (blossom blight, *Monilinia fructigena*) and stem break (*Pseudocercosporella herpotrichoide*) grown on agar.

48.3 Research in India

The higher specificity, little or no toxicity to nontarget organisms and biodegradability combine to make natural compounds more suitable for insect control. It is well established that the plant kingdom is a rich source of chemicals with a potential, to be successful pest control agents, which could cause mortality or disrupt normal growth, development, and reproduction of insects [48.28–31]. Last few years, we have made an

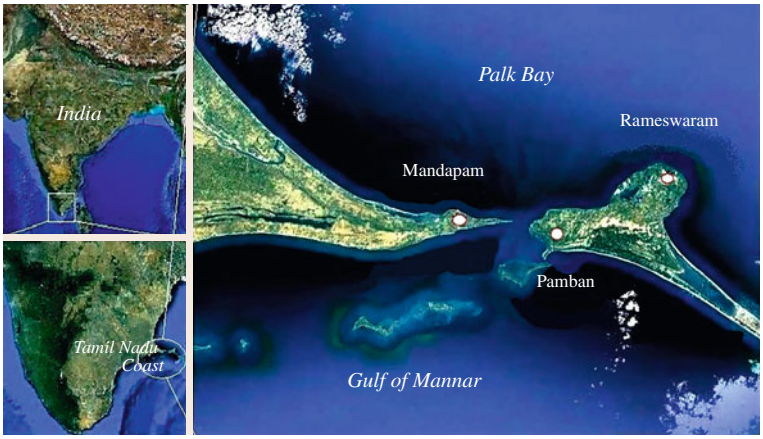


Fig. 48.2 Location map of the sampling sites (Mandapam, Pamban, and Rameswaram) in Gulf of Mannar, southeast coast of India

attempt at our institute, CSIR-Indian Institute of Chemical Technology, Hyderabad, to investigate the pesticidal activities of marine natural products of Gulf of Mannar (GoM), against some insect pest and vectors of public health importance. We found that there is a lot of scope to obtain new leads of pesticidal molecules especially new toxophoric groups from marine origin, which can be appropriately incorporated in molecules to obtain potent synthetic products with targeted features.

48.3.1 General Information on Gulf of Mannar

The GoM located in the Peninsular India and the Bay of Bengal, encompasses 21 coastal islands located between 8° 46′ – 9° 14′ N, latitude and 78° 9′ – 79° 14′ E longitude from Mandapam in the north to Tuticorin in the south (total distance 170 nautical miles) (Fig. 48.2). It is highly productive area endowed with 3600 species of flora and fauna, including 377 species that are endemic to the region [48.32, 33]. The Gulf, which has been declared as Gulf of Mannar Marine Biosphere Reserve GoMBR on February 18, 1989, is remarkable for its faunal and floral wealth, especially the world's most diverse ecosystems, the coral reefs and its associates.

48.3.2 Sample Collections and Identification

The marine flora and fauna were collected during low tide from the inter tidal zones at different localities (visually unpolluted and undisturbed areas) between 10–15 ft depth using skin diving, and deep water collections at different localities (30–40 ft) using self-contained under water breathing apparatus (SCUBA). The samples were collected from the following locations of Gulf of Mannar, Southeast coast of India.

Mandapam Coast

Situated between 09° 17.417′ N to 09° 17.479′ N, latitude and 079° 08.400 E to 079° 08.558′ E, longitude; on a narrow tongue of land projecting from the southern part of the east coast of India. To the north of the peninsular extension is Palk Bay and to the south is Gulf of Mannar. Sea grass beds have been abundantly disturbed along the coast.

Pambam Coast

Situated between 09° 17.523′ N to 09° 17.625′ N, latitude and 079° 13.164′ E to 079° 14.915′ E, longitude; at the western edge of Pamban Island. Most of Pamban Island is covered with white sand and hence is not suitable for cultivation. It is a fishing village with natural harbor.

Rameswaram Coast

The Rameswaram town is extending between 09°18.390′ N to 09° 18.853′ N, latitude and 079°19.662′ E to 079°20.076′ E, longitude; on the northeastern region of the Gulf of Mannar at the very tip of Indian peninsula. It is separated from mainland India by the Pamban channel and is connected to the mainland by the Pamban Bridge.

48.3.3 Marine Flora

Marine algae grow in the littoral and sub littoral regions of the sea on the rocks and other hard substrata. They are usually submerged. Winter season is the most favorable period for the rapid growth of marine algae, when

the atmospheric temperature is relatively low. The algal vegetation is well exposed along the collection sites when the tide is favorably low and collections were made by the hand-pick method, in littoral and sub littoral regions.

Large quantities of fresh macro algae (weighing approximately 50–100 kg for each species) belonging to different classes (Chlorophyceae, Rhodophyceae, and Pheophyceae) were collected from the intertidal zones and separated according to species based on external morphological characters. The collected algae were washed thoroughly with fresh water to get rid of the unwanted material. Then the algae were drip dried on nylon nets and were dried in the shade, with well aeration and intermediate mixings were carried out to avoid any fungal growth. The total dried material of each species was packed in separate bags, labeled, and transported to the laboratory for further work.

48.3.4 Marine Fauna

Various sponges and soft corals were collected during low tide from different localities by using skin and SCUBA divers. Specimens weighing approximately 10 kg of each species were collected and cut into small pieces. These pieces were placed in 5–15 L capacity corboyles containing methanol. Care is taken to properly dip or soak in the solvent so as to prevent putrefaction. Specimens of each species were separately preserved in 10% formalin for identification. The morphological description along with the date and site of collection of each specimen was recorded.

48.3.5 Identification of the Specimens

Marine Flora

The collected specimens were sent to the Central Marine Fisheries Research Institute (CMFRI), Mandapam, Tamil Nadu, India, Centre for Advanced Studies in Marine Biology, Parangipettai, Tamil Nadu, and also to Central Salt and Marine Chemicals Research Institute (CSMCRI), Bhavnagar, Gujarat, India, for identification.

Marine Fauna

All the intact sample specimens preserved separately, were sent with necessary details to the CMFRI, Trivandram, Kerala, India and National Institute of Oceanography (NIO), Goa, India, for their identification and repository purpose.

48.4 Preparation of Crude Extracts

48.4.1 Marine Flora

The respective washed and dried material of each species (macro algae belonging to different classes) was powdered thoroughly (0.5–5 kg) and extracted with dichloro methane and methanol (1 : 1 ratio) using Soxhlet apparatus. The pooled solvent extracts was filtered and the filtrate was concentrated under reduced pressure in rotavapor (Buchi rotavapor R 110) to obtain solvent-free crude extract. The extracts were stored in refrigerator until they were used for different bioassays against the insect pest and vectors of public health importance.

48.4.2 Marine Fauna

The methanol soaked cut pieces were separated and resoaked in dichloro methane and methanol in 1 : 1 ratio. The aqueous methanol extract was concentrated at reduced pressure and lyophilized for further extractions. The repeated dichloro methane and methanol (1 : 1 ratio) extracts were pooled and concentrated at 10 L capacity industrial rotavapor (Buchi rotavapor R 151) for obtaining solvent free crude extract. The lyophilized powder was once again extracted with the same solvents and the crudes were combined and used for studying their pesticidal activities against houseflies and mosquitoes.

48.5 Active Extracts of Marine Origin

The crude extracts prepared were subjected to their biological activities against public health important vectors like houseflies and mosquitoes. Though a good number of extracts exhibited pesticidal activities, but for the convenience of rapid screening the highest dose or concentration ranges were fixed for

200 µg/insect or 200 ppm as active extracts. The pesticidal properties (larvicidal and insecticidal activities) of active crude extracts are presented in Tables 48.1 and 48.2. As a first attempt, the active crude extract, *Dictyosphaeria favulosa* was subjected for fractionation to identify the active principle. The original crude extract of *D. favulosa*, a green alga was fractionated by using silica gel column chromatography. The details of the fractionation are as follows.

The 34 aliquots were pooled into 7 fractions (Table 48.3) based on thin layer chromatography (TLC). All the seven fractions were bioassayed and fraction 1 (DF1) has shown more activity. This fraction was crystallized from hexane to obtain heavy colorless cubes (700 mg, i. e., 0.14% on dry weight basis) and was analyzed for its characteristics. The structure of the compound was determined based on ^{1}H NMR spectrum, and mass and X-ray crystallography with the help of the chemists at Organic Chemistry Division, IICT, Hyderabad. The structure of the compound, ethylene bisisobutyl xanthate (1) [48.34] is shown below.

The compound was synthesized in good quantities to reconfirming its biological properties. It was tested against various aquatic stages of *Aedes aegypti* mosquito for larvicidal, and female adult housefly, *M. domestica* for insectidal properties. The synthesized compound exhibited larvicidal and insect growth regulatory activity in dose-dependent manner [48.35]. Around 35 analogs were synthesized and tested against vectors of human health importance and agricultural polyphagous lepidopteron pests. Among them six analogs have been identified as good antifeedant and insect growth regulators (IGRs). Three of the compounds from the six analogs are structurally known but the activity was identified for the first time (Table 48.5) and remaining three are totally new structures with pesticidal properties (Table 48.6).

Table 48.1 Larvicidal activity of marine crude extracts against fourth instar larvae of *Aedes aegypti*[a]

Crude extracts of marine organisms	Regression equation $Y = Y^{bar} + b(x - x^{bar})$	$LC_{50} \pm SE$ (µg mL^{-1})
Marine algal species		
Acanthophora spicifere	$Y = 1.36 + 1.81x$	103.13 ± 7.22
Chnoospora implexa	$Y = -0.50 + 2.43x$	184.72 ± 9.77
Cystosieria trinoides	$Y = 1.16 + 1.90x$	103.23 ± 6.84
Dictyosphaeria favulosas	$Y = 0.73 + 2.78x$	34.21 ± 8.56
Dictyota dichotama	$Y = 0.69 + 2.06x$	122.54 ± 12.01
Gracilaria corticata	$Y = 0.85 + 2.03x$	109.88 ± 6.81
Gracilaria crassa	$Y = 0.90 + 1.99x$	113.19 ± 12.69
Gracilaria folifera	$Y = 0.63 + 2.10x$	119.68 ± 11.83
Hypnea valentia	$Y = 0.005 + 2.21x$	181.78 ± 10.67
Laurentia obtuse	$Y = -0.76 + 2.68x$	139.24 ± 8.85
Sargassum ilicifolium	$Y = 0.11 + 2.33x$	126.45 ± 10.48
Sargassum myriocystum	$Y = 0.66 + 2.05x$	128.58 ± 11.93
Sesavieum portulicastum	$Y = 0.77 + 1.97x$	136.75 ± 12.28
Stoechospermum marginatum	$Y = -0.52 + 2.57x$	138.50 ± 9.25
Turbinaria conoides	$Y = -0.90 + 2.77x$	133.52 ± 8.62
Marine sponges		
Agelas ceylonica (*Dendy*)	$Y = -0.72 + 2.65x$	145.88 ± 8.94
Aulospongus cavernosa	$Y = -1.19 + 2.73x$	184.59 ± 8.72
Aurora globostellata (*Carter*)	$Y = -1.12 + 2.68x$	189.37 ± 8.92
Axinella andamanensis	$Y = 0.10 + 2.29x$	135.59 ± 10.48
Axinella donnani (*Bowerbank*)	$Y = -1.10 + 2.69x$	183.55 ± 8.82
Callyspongia reticutis	$Y = -0.62 + 3.69x$	33.46 ± 1.80
Cinachyra cavernosa (*Lamarck*)	$Y = 0.49 + 2.23x$	102.62 ± 5.78
Clathria frondifera (*Bowerbank*)	$Y = 0.14 + 2.32x$	123.18 ± 10.55
Clathria reinwardti (*Vosmaer*)	$Y = 1.18 + 2.75x$	24.56 ± 1.63
Damirinia schmidti	$Y = 0.56 + 2.17x$	110.82 ± 6.44

Table 48.1 (continued)

Crude extracts of marine organisms	Regression equation $Y = Y^{bar} + b(x - x^{bar})$	$LC_{50} \pm SE$ ($\mu g\,mL^{-1}$)
Marine sponges		
Dendrilla cactus (*Selenka*)	$Y = 0.54 + 2.22x$	100.71 ± 5.73
Dendrilla nigra (*Dendy*)	$Y = 0.61 + 2.43x$	63.45 ± 4.54
Dysidea fragilis (*Montagu*)	$Y = 0.74 + 2.4x$	60.16 ± 7.3
Dysidea herbacea	$Y = -0.14 + 2.36x$	151.11 ± 9.93
Echniodictyum gorgonides	$Y = -0.56 + 2.58x$	141.78 ± 9.18
Fasciospongia cavernosa (*Schmidt*)	$Y = 0.006 + 2.39x$	121.73 ± 10.24
Galliodes cellaria (*Rao*)	$Y = 0.73 + 2.06x$	116.05 ± 12.11
Haliclona cribricutis (*Dendy*)	$Y = 0.05 + 3.17x$	36.43 ± 2.81
Haliclona pigmentifera	$Y = 0.53 + 2.44x$	67.47 ± 4.69
Haliclona retiderma	$Y = 1.19 + 2.80x$	162.98 ± 15.34
Haliclona tenuiramosa (*Burton*)	$Y = -0.45 + 2.48x$	155.61 ± 9.42
Hyattella cribriformis (*Hyatt*)	$Y = 0.86 + 1.88x$	158.97 ± 12.55
Ircinia fasciculate (*Pallas*)	$Y = 0.56 + 2.15x$	116.93 ± 11.61
Ircinia fasciculate	$Y = -9.15 + 3.63x$	79.49 ± 3.02
Ircinia fusca (*Carter*)	$Y = -8.89 + 3.52x$	87.71 ± 3.45
Mycale grandis (*Gray*)	$Y = 0.41 + 2.27x$	103.61 ± 5.74
Mycale mytilorum (*Annandale*)	$Y = -0.03 + 2.25x$	170.74 ± 10.39
Myxilla arenaria (*Dendy*)	$Y = -1.19 + 2.86x$	144.54 ± 8.26
Petrosia nigricans (*Lindgren*)	$Y = 0.74 + 2.05x$	115.78 ± 12.18
Petrosia similis (*Ridley and Dendy*)	$Y = 0.12 + 2.70x$	63.97 ± 4.66
Petrosia testudinaria (*Lamarck*)	$Y = 0.3 + 2.75x$	51.56 ± 5.84
Phyllospongia folleascencis	$Y = 0.95 + 1.92x$	127.70 ± 12.90
Psammaplysilla officinalis	$Y = -0.22 + 2.51x$	121.33 ± 9.75
Psammaphysilla purpurea (*Carter*)	$Y = -1.40 + 4.62x$	24.35 ± 0.58
Psammplysilla purpurea (*Carter*)	$Y = 0.46 + 2.12x$	139.29 ± 11.38
Psammascus lamella (*Lendenfeld*)	$Y = 0.34 + 2.20x$	130.82 ± 11.04
Sarcophyton trocheliophorum	$Y = 1.28 + 1.83x$	106.12 ± 7.29
Sigmadocia cavernosa (*Dendy*)	$Y = -0.62 + 2.47x$	189.93 ± 9.67
Sigmadoca fibulata (*Schmidt*)	$Y = 0.42 + 2.66x$	52.66 ± 5.22
Sigmadocia pumila (*Lendenfeld*) *globosa*	$Y = -0.28 + 2.41x$	153.54 ± 9.70
Sinularia dissecta	$Y = -0.31 + 2.35x$	182.10 ± 16.76
Spirastrella inconstans var. globosa (*Dendy*)	$Y = 0.15 + 2.32x$	122.54 ± 10.58
Spirastrella inconstans (*Dendy*)	$Y = -0.11 + 2.27x$	176.80 ± 10.35
Spongia officinalis (*Dendy*)	$Y = -0.05 + 2.41x$	124.67 ± 10.11
Spongia officianalis L. Var.ceylonensis (*Dendy*)	$Y = -0.21 + 2.34x$	167.28 ± 9.99
Tedenia anhelans (*Lieberkuhn*)	$Y = -1.93 + 3.02x$	195.78 ± 8.11
Soft corals		
Axinella carteri (*Dendy*)	$Y = 0.07 + 2.27x$	146.73 ± 10.46
Lobophytum hirsutum	$Y = -0.67 + 2.49x$	187.17 ± 9.53
Nephthea sp.	$Y = 0.14 + 2.15x$	179.19 ± 10.91
Sarcophyton crassum	$Y = -0.33 + 2.35x$	185.35 ± 10.08
Sarcophyton ehrenbergi	$Y = -0.21 + 2.36x$	159.88 ± 9.88
Sarcophyton trocheliopharum	$Y = 0.07 + 2.21x$	170.27 ± 10.60
Sinularia brassica	$Y = -0.29 + 2.40x$	160.53 ± 9.75
Sinularia dissecta	$Y = 0.52 + 2.08x$	140.17 ± 11.53

Table 48.1 (continued)

Crude extracts of marine organisms	Regression equation $Y = Y^{bar} + b(x - x^{bar})$	$LC_{50} \pm SE$ ($\mu g\,mL^{-1}$)
Soft corals		
Sinularia hirta	$Y = -0.38 + 2.84x$	77.70 ± 4.18
Sinularia inacta (*Tixier-Durivalt*)	$Y = -0.22 + 2.38x$	156.07 ± 9.82
Sinularia kavarattiensis (*Alderslade and Shirwaiker*)	$Y = -0.24 + 2.35x$	168.88 ± 9.96
Sinularia manaarensis	$Y = -1.45 + 2.83x$	189.59 ± 8.50
Sinularia parulekari (*Alderslade and Shirwaiker*)	$Y = -0.17 + 2.31x$	173.85 ± 10.16
Subergorgia suberosa	$Y = 0.86 + 1.87x$	159.69 ± 12.55

[a] Fourth-instar larvae of *Aedes aegypti* were used as an experimental model to determine the larvicidal activity. The cyclic colony of *Aedes aegypti* (yellow fever mosquito vector) were reared in our insectary at $27 \pm 1\,^{\circ}C$ and $80 \pm 5\%$ RH with a photo period of 14 : 10 h light and dark cycles followed by the methods of [48.36] and [48.37], respectively, with little modifications. Fourth-instar larvae were treated with different concentrations of sponge extracts for 24 h and lethal concentration was investigated through probit analysis. Regression equation $Y = Y^{bar} + b(X - x^{bar})$ or $(Y^{bar} - bx^{bar}) + (bx)$. Y^{bar} = weighted average of working probit values, x^{bar} = average of $\log_{10}$ (concentration), b = slope of the line, $X = x$-scale concentration in log. Four replicates for each concentration and the control (without sponge extract), were tested for larval bio-efficacy and the data were used to calculate the probit regression equation. LC_{50} is defined as the concentration of extract that can cause 50% mortality in the exposed population.

Table 48.2 Insecticidal activity of marine crude extracts against 3–4-day-old female houseflies *Musca domestica*

Crude extracts of marine organisms	Regression equation $Y = Y^{bar} + b(x - x^{bar})$	$LD_{50} \pm SE$ (μg/insect)
Marine algal species		
Caulerpa scalpelliforum	$Y = -0.39 + 2.53x$	134.55 ± 9.47
Cystosieria trinoides	$Y = 1.21 + 1.89x$	101.03 ± 6.77
Dictyosphaeria favulosas	$Y = 1.27 + 2.66x$	25.36 ± 1.69
Dictyota dichotama	$Y = -1.30 + 2.74x$	200.30 ± 8.95
Gracilaria corticata	$Y = -0.58 + 2.43x$	200.61 ± 10.29
Gracilaria edulis	$Y = 0.24 + 2.31x$	114.97 ± 10.76
Hypnea valentia	$Y = -0.21 + 2.46x$	130.79 ± 9.79
Laurencia gracilus	$Y = 0.12 + 2.19x$	166.63 ± 10.66
Padina boergence	$Y = 0.22 + 2.26x$	128.94 ± 10.75
Sargassum wightii	$Y = 0.88 + 2.01x$	111.78 ± 12.59
Spatoglossum asperum	$Y = 0.99 + 1.86x$	138.57 ± 13.05
Ulva lactuca	$Y = -0.78 + 2.52x$	198.23 ± 9.63
Marine sponges		
Acanthella elongate	$Y = -0.10 + 2.33x$	153.19 ± 10.03
Aulenella foramimofera	$Y = -1.187 + 2.70x$	197.08 ± 9.02
Axinella donnani (*Bowerbank*)	$Y = -1.48 + 2.82x$	195.56 ± 8.60
Biemna fortis	$Y = -0.46 + 2.44x$	170.81 ± 9.58
Callyspongia reticutis	$Y = -0.78 + 2.60x$	164.28 ± 8.98
Damirinia schmidti	$Y = 0.47 + 2.12x$	136.59 ± 11.41
Dendrilla cactus (*Selenka*)	$Y = 0.89 + 1.87x$	156.45 ± 12.62
Dendrilla nigra (*Dendy*)	$Y = 0.69 + 2.44x$	58.96 ± 6.44
Echinodictym clathratum (*Dendy*)	$Y = -0.14 + 2.29x$	174.77 ± 10.24
Fasciospongia cavernosa (*Schmidt*)	$Y = 0.47 + 2.12x$	137.29 ± 11.39
Haliclona cribricutis (*Dendy*)	$Y = -1.40 + 4.62x$	24.35 ± 0.58
Haliclona retiderma	$Y = 1.23 + 1.85x$	109.05 ± 7.43
Heteronema erecta (*Keller*)	$Y = -0.83 + 2.55x$	195.80 ± 9.49
Hyattella cribriformis (*Hyatt*)	$Y = -1.58 + 2.87x$	195.06 ± 13.37
Itrochota baculifera (*Ridley*)	$Y = -0.59 + 2.43x$	198.19 ± 9.95

Table 48.2 (continued)

Crude extracts of marine organisms	Regression equation $Y = Y^{bar} + b(x - x^{bar})$	$LD_{50} \pm SE$ (µg/insect)
Marine sponges		
Ircinia fusca (*Carter*)	$Y = -0.99 + 2.62x$	193.67 ± 9.20
Mycale grandis (*Gray*)	$Y = 0.22 + 2.36x$	104.69 ± 5.58
Mycale tenuispiculata (*Dendy*)	$Y = -0.66 + 2.62x$	143.69 ± 9.04
Mycalecarmia monachorata (*Burton and Rao*)	$Y = -2.85 + 3.47x$	182.04 ± 16.23
Myrmekioderma granulata (*Esper*)	$Y = 0.019 + 2.24x$	168.09 ± 10.46
Petrosia nigricans (*Lindgren*)	$Y = -0.11 + 2.34x$	150.78 ± 10.00
Petrosia similis (*Ridley and Dendy*)	$Y = 0.48 + 2.17x$	119.57 ± 11.39
Petrosia testudinaria (*Lamarck*)	$Y = 0.67 + 2.11x$	112.07 ± 12.54
Phyllospongia folleascencis	$Y = 0.65 + 2.10x$	117.02 ± 11.87
Psammaphysilla purpurea (*Carter*)	$Y = -1.43 + 4.71x$	23.21 ± 0.52
Psammplysilla purpurea (*Carter*)	$Y = -0.59 + 2.59x$	144.41 ± 9.15
Sigmadocia cavernosa (*Dendy*)	$Y = 1.28 + 1.81x$	110.93 ± 7.74
Sigmadoca fibulata (*Schmidt*)	$Y = -0.40 + 2.38x$	184.95 ± 9.94
Sinularia dissecta	$Y = 1.15 + 1.89x$	108.44 ± 7.22
Spirastella vagabunda Ridley	$Y = 0.76 + 2.06x$	113.83 ± 12.22
Spirastrella inconstans var. globosa (*Dendy*)	$Y = -1.23 + 2.90x$	139.03 ± 8.18
Spirastrella inconstans (*Dendy*)	$Y = -1.15 + 2.89x$	133.65 ± 8.26
Spongia officinalis (*Dendy*)	$Y = 0.60 + 2.13x$	116.48 ± 11.73
Spongia officianalis L. Var.ceylonensis (*Dendy*)	$Y = 0.32 + 2.08x$	176.22 ± 11.28
Zygomycale parishii (*Bowserbank*)	$Y = -0.07 + 2.37x$	138.54 ± 10.09
Soft corals		
Lobophytum crassum (*von Marenzeller*)	$Y = -0.13 + 2.25x$	186.99 ± 10.51
Lobophytum microlobulatum	$Y = 0.002 + 2.22x$	177.35 ± 10.57
Lobophytum sarcophytoides (*Moser*)	$Y = -0.93 + 2.61x$	188.63 ± 9.16
Nephthea sp.	$Y = 0.19 + 2.16x$	168.02 ± 10.84
Sarcophyton crassum	$Y = 0.41 + 2.07x$	163.87 ± 11.31
Sarcophyton ehrenbergi (*von Marenzeller*)	$Y = 0.29 + 2.14x$	158.93 ± 10.96
Sinularia brassica	$Y = -1.59 + 2.96x$	168.22 ± 13.63
Sinularia dissecta	$Y = 0.41 + 2.11x$	148.11 ± 11.28
Sinularia inelegans (*Tixier-Durivalt*)	$Y = 0.35 + 2.21x$	125.73 ± 11.06
Sinularia kavarattiensis (*Alderslade and Shirwaiker*)	$Y = 0.54 + 2.02x$	160.46 ± 11.62
Sinularia ornate	$Y = -0.05 + 2.28x$	165.42 ± 10.27
Sinularia vrijanoethi	$Y = -0.58 + 2.59x$	141.42 ± 9.15

[a] Housefly, *M. domestica* was used as an experimental model to determine the insecticidal activity. The cyclic colony of houseflies were reared in our insectary at 27 ± 1 °C and 80 ± 5% RH with a photo period of 14 : 10 h light and dark cycles followed by the methods of [48.36] and [48.37] respectively, with little modifications. Three to four days old of female houseflies, *M. domestica* were treated with different concentrations of sponge extracts for 24 h and lethal dose was investigated through probit analysis. Regression equation $Y = Y^{bar} + b(X - x^{bar})$ or $(Y^{bar} - bx^{bar}) + (bx)$. Y^{bar} = weighted average of working probit values, x^{bar} = average of $\log_{10}$ (dose), b = slope of the line; X = x-scale dose in log. Four replicates for each dose and the control (without sponge extract), were tested for insecticidal bio-efficacy and the data was used to calculate the probit regression equation. *LD_{50} is defined as the dose of extract that can cause 50% mortality in the exposed population.

Table 48.3 Details of the fractionation of crude extract of *D. favulosa*

S. No.	Fraction	Eluent	Remarks
1	1–8	Hexane	Compound DF-1
2	9–15	Hexane:Ethyl acetate (10 : 1)	
3	16–20	Hexane:Ethyl acetate (9 : 1)	Green substance
4	21–25	Hexane:Ethyl acetate (3 : 1)	
5	26–30	Hexane:Ethyl acetate (1 : 1)	Gummy substance
6	31–32	Hexane:Ethyl acetate (1 : 3)	
7	33–34	Ethyl acetate	Fatty substance

48.6 Pesticidal Properties of Alkyl Xanthates

48.6.1 Larvicidal Activity of Alkyl Xanthates Against Mosquito

The toxicity of active alkyl xanthate analogs to *Aedes aegypti* larvae is listed in Table 48.4. The results indicated that about six compounds (out of 35) exhibited excellent activities against mosquito at less than 15 $\mu g\,mL^{-1}$ (ppm). In particular, compound **3** still maintains high larvicidal activity even at 3.77 $\mu g\,mL^{-1}$ (LC_{50} value). All the title compounds exhibited significant larvacidal activities against mosquito.

48.6.2 Developmental Inhibition Activity of Alkyl Xanthates Against Second Instar Larvae of *Aedes Aegypti*

The sublethal concentrations of all the analogs (LC_{10} to LC_{20} concentrations) have exhibited developmental inhibition on mosquito, *Aedes aegypti*. Abnormal pupal formation and pupal case attachment to emerged adults (leads to mortality) were quite common in most of the alkyl xanthates. Among them, compounds **4** and **5** have shown significant IGR properties. The activities of the compounds (**4** and **5**) were analyzed to compare with control experiments (with out treatment) and are presented in Table 48.7.

The majority of the abnormal pupae (> 60%) were appeared at high concentrations of both the compounds (Fig. 48.3a). The higher concentrations of compound **4** inhibited the adult emergence by attaching pupal casing to the abdominal region of emerging adults (Fig. 48.3b).

Similar effects were also observed in compound **5** in which majority of the adult wings and legs were attached to the puparium at the time of emergence (Fig. 48.3c). Although the significant number of adults emerged in the lower concentrations of compounds **4** and **5**, which appears to be normal but they were not in a position to take off even after few hours of emergence and led to mortality (Fig. 48.3d).

48.6.3 In Vivo Toxic Activity of Alkyl Xanthates Against Brine Shrimp, *Artemia Salina*

The in vivo toxicity of alkyl xanthates was evaluated against shrimp larvae. The assay was performed in triplicate vials at different doses (2.5–25 ppm) as

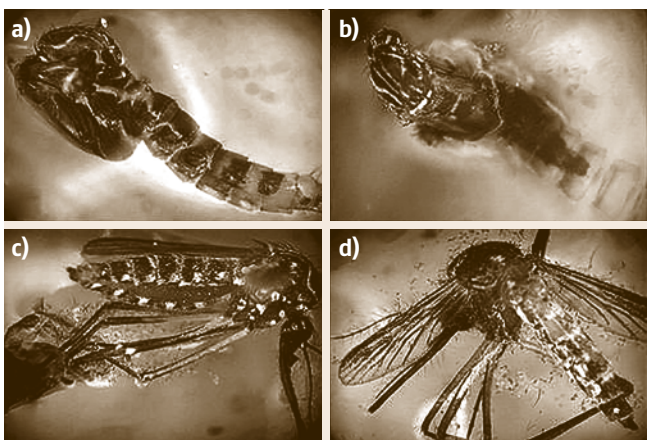
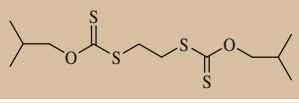
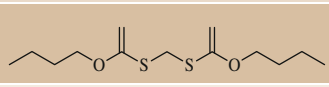
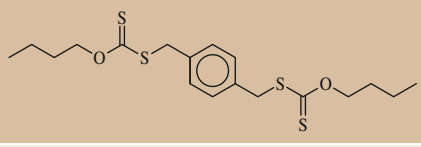


Fig. 48.3a–d Effect of alkyl xanthates on the formation of pupae and adult emergence after larval treatment. The digital photographs (at 50×) of deformed pupae and adults were computerized with a compound microscope (Polyvar, Reichert- Jung light microscope) attached to Ethovision – version 2.3 (Noldus Information Technology, The Netherlands) through a CCD camera (Sony CCD IRIS, Model No: SSC-M370CE). The treated larvae took unusually longer periods to enter into the pupal stage, compared with control larvae

Table 48.4 Larvicidal activity of alkyl xanthates and its analogs against fourth-instar larvae of *Aedes aegypti*

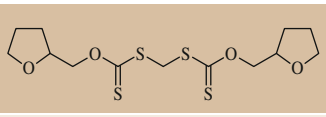
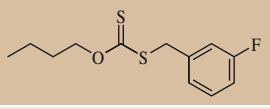
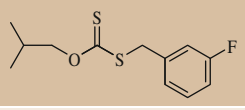
S. No	Compound	$LC_{50} \pm$ S.E. (mg L^{-1})
1	Ethylene bis(isobutylxanthate)	8.06 ± 0.22
2	Methylene bis(*n*-butylxanthate)	5.33 ± 0.97
3	Methylene bis(tetrahydrofurfurylxanthate)	3.77 ± 0.59
4	*p*-Xylene bis(*n*-butylxanthate)	7.35 ± 0.51
5	*m*-Fluorobenzyl *n*-butylxanthate	12.53 ± 1.24
6	*m*-Fluorobenzyl isobutylxanthate	11.78 ± 2.35

Table 48.5 The known structures, and their pesticidal properties were first observed

Ethylene bis(isobutylxanthate) 	
Physical state	Colorless solid
Molecular formula	$C_{12}H_{22}O_2S_4$
^{1}H NMR	(CDCl3, 200 MHz)δ1.0 (6 H, d, $J = 7.5$ Hz), 2.15 (1 H, m), 3.42 (2 H, m) and 4.35 (2 H, d, $J = 7.5$ Hz).
IR (Neat)	ν_{max} (KBr) 2950, 1465, 1235, 1200, and 1140 cm^{-1}
EIMS (70 eV)	*m/z* 326
UV (MeOH)	λ_{max} (MeOH) 217 (ε 12725), 232 (14766) and 278 nm (20048)
Methylene bis-n-butylxanthate 	
Physical state	Colorless solid
Molecular formula	$C_{11}H_{20}O_2S_4$
^{1}H NMR	(CDCl3, 200 MHz) δ 0.98 (3 H, t, $J = 7.5$ Hz), 1.45 (2 H, m), 1.78 (2 H, m), 4.58 (2 H, t, $J = 7.5$ Hz) and 4.80 (1 H, s).
IR (Neat)	ν_{max} (KBr) 2955, 1470, 1230, 1195 *and* 1130 cm^{-1}
EIMS (70 eV)	*m/z 312*
UV (MeOH)	λ_{max} (MeOH) 215 (ε 12000), 235 (15500), 275 nm (19260)
p-Xylene bis(n-butylxanthate) 	
Physical state	Colorless solid
Molecular formula	$C_{18}H_{26}O_2S_4$
^{1}H NMR	(CDCl3, 200 MHz) δ 0.98 (3 H, t, $J = 7.0$ Hz), 1.45 (2 H, m), 1.80 (2 H, m), 4.32 (2 H, s), 4.60 (2 H, t, $J =$ 7.0 Hz) and 7.30 (2 H, s).
IR (Neat)	ν_{max} (KBr) 3160, 2970, 1620, 1215 and 1060 cm^{-1}
EIMS (70 eV)	*m/z 402*
UV (MeOH)	λ_{max} (MeOH) 210 (ε 30150), 222 (26135), 270 nm (20140)

described by [48.38] and modified by [48.39]. All the 35 analogs were tested against brine shrimp, *Artimia salina*, and the lethal concentrations (LC_{50}) of active alkyl xanthates are listed in Table 48.8. Experimental data revealed that a new structure, methylene bis(tetrahydrofurfurylxanthate) with an LC_{50} values of 5.63 ± 0.62 (ppm) was the most toxic among the active compounds.

Table 48.6 The new structures with pesticidal properties

Methylene bis(tetrahydrofurfurylxanthate) 	
Physical state	Colorless liquid
Molecular formula	$C_{13}H_{20}O_4S_4$
^{1}H NMR	(CDCl3, 200 MHz) δ 1.70 (2 H, m), 1.95 (2 H, m), 3.71 (2 H, m), 4.20 (1 H, m), 4.55 (2 H, t) and 4.78 (1 H, s).
IR (Neat)	ν_{max} (KBr) 2920, 1470, 1235 and 1140 cm^{-1}
EIMS (70 eV)	*m/z 368*
UV (MeOH)	λ_{max} (MeOH) 217 (ε 11150), 242 (1650), 270 nm (19160)
m-Fluorobenzyl n-butylxanthate 	
Physical state	Pale yellow liquid
Molecular formula	$C_{12}H_{15}FOS_2$
^{1}H NMR	(CDCl3, 200 MHz)δ 1.0 (3 H, m), 1.42 (2 H, m), 1.75 (2 H, m), 4.60 (2 H, t, J = 7.0 Hz), 4.35 (2 H, s) and 6.95–7.20 (4 H, m).
IR (Neat)	ν_{max} 2960, 2931, 1615, 1590, 1460, 1240 *and* 1045 cm^{-1}
EIMS (70 eV)	*m/z 258*
UV (MeOH)	λ_{max} 210 (ε 37105), 218 (35660) and 280 nm (27354)
m-Fluorobenzyl isobutylxanthate 	
Physical state	Pale yellow liquid
Molecular formula	$C_{12}H_{15}FOS_2$
^{1}H NMR	(CDCl3, 200 MHz) δ 1.0 (6 H, d, J = 7.0 Hz), 2.12 (1 H, m), 4.34 (4 H, brs) and 6.92–7.22 (4 H, m).
IR (Neat)	ν_{max} 2964, 2937, 1620, 1585, 1472, 1230, 1165 and 1030 cm^{-1}
EIMS (70 eV)	*m/z 258*
UV (MeOH)	λ_{max} 214 (ε 37190), 220 (35674), and 274 nm (27378)

48.7 Antifeedant and IGR Activities of Xanthates

48.7.1 Antifeedant Activity

Antifeedant activity of the alkyl xanthates was studied using leaf disk no-choice method [48.40]. Fresh castor leaf (*Ricinus communis* L.) disks of 4 cm in diameter were punched using cork borer and their surfaces were painted with different concentrations ranging from 5–100 $\mu g\,cm^{-2}$, individually. The leaf disks treated with acetone were used as negative control and a natural product isolated from Neem seed kernel, Azadirachtin (5, 10, 15, and 20 $\mu g\,cm^{-2}$) was used as positive control. In each petri dish (1.5 × 9 cm^2) wet filter paper was placed to avoid early drying of the leaf disks and single third-instar larva was introduced into each petri dish. Progressive consumption of leaf area by the treated and control larvae after 12 and 24 h was recorded using leaf area meter (CI-203 Area meter, CID, Inc., USA). Based on the data, the percent protection of individual compound was calculated and compared with positive control. Control and test larvae were transferred on fresh leaves and monitored till adult emergence to study the growth and behavioral aspects of the insect.

Newly-synthesized alkyl xanthates exhibited a significant antifeedant activity in a dose dependent manner (Fig. 48.4). Data pertaining to the experiment clearly revealed that maximum antifeedant activity was recorded

Table 48.7 Effect of alkyl xanthates on growth and metamorphosis of second-instar larvae of *Aedes aegypti*

S. No	Activity		Control[a]	Methylene bis(tetrahydrofurfurylxanthate) Compound 4 Conc. in ppm				*m*-Fluorobenzyl *n*-butylxanthate Compound 5 Conc. in ppm			
				0.01	0.025	0.05	0.1	0.01	0.05	0.1	0.25
1	Percent mortality after 24 h		-0-	-4-	19	25	36	-7-	21	27	38
Pupal formation from the survival of larvae											
	Total no. of survived larvae		*100*	*96*	*81*	*75*	*64*	*93*	*79*	*73*	*62*
2	Pupation	% Normal	100 (-0-)	63.54 (61)	38.27 (31)	17.33 (13)	7.81 (-5-)	55.91 (52)	39.24 (31)	23.29 (17)	3.23 (-2-)
		% Abnormal	-0- (-0-)	34.38 (33)	54.32 (44)	68.00 (51)	67.18 (43)	39.78 (37)	51.89 (41)	64.38 (47)	69.35 (43)
		% Mortality	-0- (-0-)	2.08 (-2-)	7.41 (-6-)	14.67 (11)	25.00 (16)	4.30 (-4-)	8.86 (-7-)	12.32 (-9-)	27.41 (17)
Adult emergence based on the number of survived pupae											
	Total no. of pupae (formed)		*100*	*94*	*75*	*64*	*48*	*92*	*74*	*66*	*47*
3	Adult emergence	% Normal	100 (-0-)	59.57 (56)	49.33 (37)	14.06 (-9-)	6.25 (-3-)	66.30 (61)	41.89 (31)	19.70 (13)	4.26 (-2-)
		% Abnormal	-0- (-0-)	18.09 (17)	21.33 (16)	32.81 (21)	14.58 (-7-)	21.74 (20)	25.68 (19)	18.18 (12)	6.38 (-3-)
		% Nonemergence	-0- (-0-)	22.34 (21)	29.33 (22)	53.13 (34)	79.17 (38)	11.96 (11)	32.43 (24)	62.12 (41)	89.36 (42)

[a] Without toxicant, the initial sample size of each concentration is 100 numbers. Number in parentheses indicate the sample size

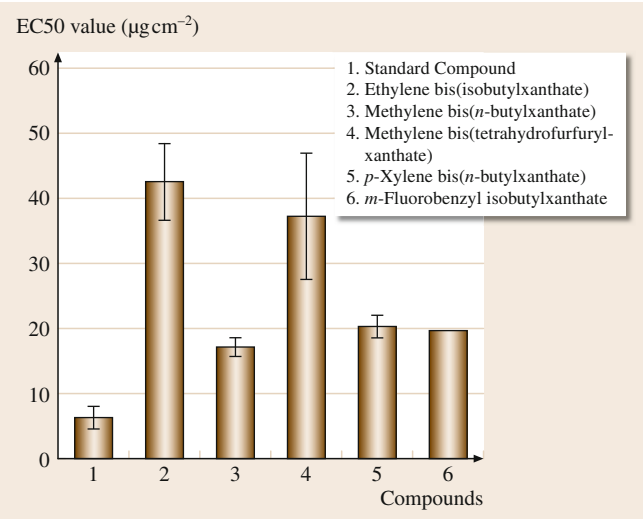


Fig. 48.4 Relative antifeedant activity of alkyl xanthates against *Spodoptera litura*

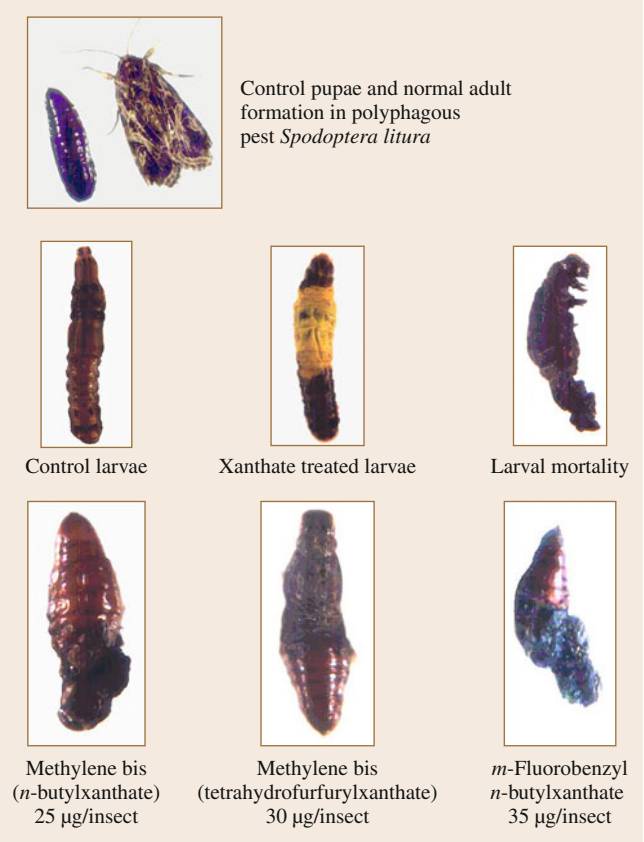


Fig. 48.5 Topical treatment of alkyl xanthates on third-instar larve of *S. litura*

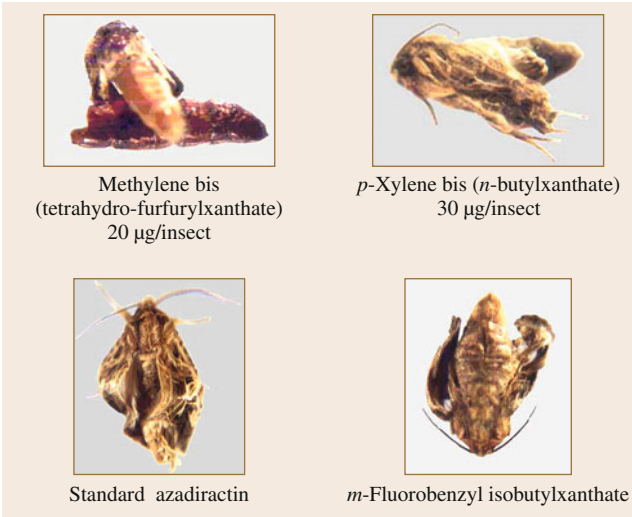


Fig. 48.6 Abnormal adult formation of *S. litura* after topical treatment of alkyl xanthates at third-instar larve

in methylene bis(*n*-butylxanthate) followed by *m*-fluorobenzyl isobutylxanthate, *p*-xylene bis(*n*-butylxanthate), methylene bis(tetrahydrofurfurylxanthate), and ethylene bis(isobutylxanthate). They are relatively three to seven times less active than positive control, Azadirachtin (Table 48.9).

48.7.2 Growth Inhibitory Activity

In continuation to anteefedant experiments, the larvae (leaf consumed along with compound-calculated) were individually monitored till adult emergence. Based on the formation of normal, abnormal pupae, normal adult emergence, and abnormal adult emergence were recorded and the growth regulatory activity of the compound in comparison with standard was calculated. Relative inhibition 50 values of alkyl xanthates on *S. litura* were statistically analyzed.

Table 48.8 Toxic activity of alkyl xanthates and its analogs against Brine shrimp, *Artemia salina*

S. No	Compound	$LC_{50} \pm$ S.E. (mg L^{-1})
1	Ethylene bis(isobutylxanthate)	25.78 ± 2.90
2	Methylene bis(*n*-butylxanthate)	34.65 ± 3.91
3	Methylene bis(tetrahydrofurfurylxanthate)	5.63 ± 0.62
4	*p*-Xylene bis(*n*-butylxanthate)	45.23 ± 5.63
5	*m*-Fluorobenzyl *n*-butylxanthate	19.00 ± 2.83
6	*m*-Fluorobenzyl isobutylxanthate	22.54 ± 3.26
7	Standered *Campothecin*	2.53 ± 0.43

IGR activity of alkyl xanthates against *S. litura:* Insect growth regulation properties of marine extracts are very interesting and unique in nature, since insect growth regulator works on juvenile hormone. The enzyme ecdysone plays a major role in shedding of old skin and the phenomenon is called ecdysis or molting. When the active marine compounds enter into the body of the larvae, the activity of ecdysone is suppressed and the larva fails to moult, remaining in the larval stage and ultimately dying [48.41]. Growth inhibitory activity of xanthates was compared with the activity of Azadirachtin and presented in Table 48.10. Further experiments were performed on *Helicoverpa armigera (Hubner)*, a lepidopteron pest was used as an experimental modal to reconfirm the IGR activities of alkyl xanthates (Table 48.11).

The topical application of alkyl xanthates analogues proved as IGR against two polyphagous lepidopteron pests and their mode of action is as similar to a natural product isolated from neem seed kernel, Azadiractin (standard compound). Among the alkyl xanthates, methylene bis(tetrahydrofurfurylxanthate) produced a maximum percentage of deformed larvae, pupae, and adults. The morphological deformities at larval, pupal, and adult stages are due to the toxic effects of alkyl xanthates on growth and development

Table 48.9 Antifeedant activity of alkyl xanthate and its analogs against late third instar of *Spodoptera litura*

Compound code	Conc. in $\mu g\,cm^{-1}$	Percent leaf consumption			Concentration required for 50% leaf protection
		12 h	24 h	A.I	EC_{50} in $\mu g\,cm^{-2} \pm$ S.E
Standard Azadiractin	20	4.2	12.7	76.42	6.34 ± 1.77
	15	16.4	27.6	54.98	
	10	18.6	33.3	48.09	
	5.0	33.7	57.2	24.84	
Ethylene bis(isobutylxanthate)	65	9.3	25.2	58.07	42.46 ± 5.76
	50	22.4	45.9	34.85	
	35	36.1	62.7	20.48	
	20	60.2	80.7	8.14	
Methylene bis(*n*-butylxanthate)	30	-0-	7.5	85.37	17.11 ± 1.36
	25	3.5	20.7	64.22	
	20	22.5	45.6	35.14	
	15	35.2	60.4	22.27	
	10	48.5	85.1	5.50	
Methylene bis(tetrahydrofurfurylxanthate)	50	22.1	45.5	35.23	37.21 ± 9.67
	35	25.9	47.3	33.52	
	25	31.5	60.2	22.42	
	12.5	48.6	80.6	8.20	
p-Xylene bis(*n*-butylxanthate)	25	20.5	24.6	58.86	20.45 ± 1.76
	20	35.2	57.6	24.51	
	15	62.4	84.3	5.97	
	10	57.3	90.2	2.59	
m-Fluorobenzyl isobutylxanthate	25	12.5	32.8	48.67	19.65 ± 2.99
	20	24.7	53.6	27.86	
	15	33.24	68.7	16.07	
	10	50.4	75.2	11.63	

Data represented as the mean of five replicates in three different sets of experiments.

$$\text{A.I} = \text{Anti-feedant index} = \frac{(\text{Control consumption}) - (\text{Treated consumption}) \times 100}{(\text{Control consumption}) + (\text{Treated consumption})}$$

Normal and control worms consumed 45–50% leaf in 12 h and 87–95% leaf consumption in 24 h

processes (Figs. 48.5–48.7). Since morphogenetic hormones regulate these processes, it can be suggested that these alkyl xanthates interfere with the hormones of the insect. These xanthates have simple structures unlike Azadiractin and possessing a similar kind of mode of action, which can be synthesized easily in the laboratory.

The present results clearly indicated that the marine originated molecules have a significant effect on agricultural pest and human health-related important vectors. Further extensive work is in progress to isolate many active substances as pesticide molecules for sustainable arthropod insect control agents.

Table 48.10 Growth inhibitory activity of alkyl xanthates on agricultural pest, *S. litura* (topical application)

Sample Code	Conc. (µg/ insect)	No. insects used for assay	Larval mortality (%)	*Pupal formation*			*Adult emergence*			
				Normal pupa	Abnormal pupa	Pupal Mortality	Normal adults	Abnormal adults	Intermediates	Non emergence
Methylene bis(*n*-butylxanthate)	100	20 × 5	86	-0-	14	14	-0-	-0-	-0-	14
	75	20 × 5	72	-0-	28	25	-0-	-1-	-2-	25
	50	20 × 5	57	-5-	38	24	-5-	-6-	-8-	-9-
	35	20 × 5	21	25	54	26	7	23	23	25
	20	20 × 5	-0-	47	53	17	24	27	32	-2-
	10	20 × 5	-0-	52	48	-8-	36	22	34	-1-
	5	20 × 5	-0-	59	41	-5-	41	26	28	-0-
m-Fluorobenzyl isobutylxanthate	100	20 × 5	96	-0-	-4-	-4-	-0-	-0-	-0-	-0-
	75	20 × 5	83	-9-	-8-	13	-0-	-3-	-1-	-2-
	50	20 × 5	70	13	15	-9-	-3-	11	-5-	-4-
	35	20 × 5	56	25	19	-7-	17	14	-6-	-0-
	20	20 × 5	27	43	30	-4-	39	22	-8-	-0-
	10	20 × 5	-10-	58	32	-8-	49	29	-4-	-1-
	5	20 × 5	-3-	73	24	-3-	60	32	-2-	-0-
Methylene bis(tetrahydro-furfurylxanthate)	100	25 × 4	98	-0-	-2-	-1-	-0-	-1-	-0-	-0-
	75	25 × 4	91	-2-	-7-	-5-	-2-	-2-	-0-	-2-
	50	25 × 4	80	-7-	13	-4-	-4-	-9-	-3-	-1-
	35	25 × 4	71	19	10	-7-	-9-	11	-2	-3-
	25	25 × 4	59	25	16	-7-	15	17	-2-	-2-
	10	25 × 4	46	44	10	-6-	28	19	-1-	-2-
	5	25 × 4	36	58	6	-5-	37	22	-0-	-3-
p-Xylene bis(*n*-butylxanthate)	100	20 × 5	95	-0-	-5-	-4-	-0-	-0-	-1-	-2-
	75	20 × 5	86	-3-	-11-	10	-0-	-2-	-2-	-2-
	50	20 × 5	71	-8-	21	14	-2-	13	-3-	-1-
	35	20 × 5	59	12	29	13	-9-	17	-2-	-4-
	20	20 × 5	20	59	21	-5-	25	36	-14-	-5-
	10	20 × 5	5	72	23	-3-	44	38	10	-1-
	5	20 × 5	-0-	85	15	-2-	59	29	10	-0-
Control	100	25 × 4	5	95	-0-	-0-	94	-0-	-0-	-1-
standard azadiractin	75	25 × 4	96	-0-	-4-	-4-	-0-	-0-	-0-	-0-
	50	25 × 4	88	-7-	-5-	-8-	-0-	-2-	-2-	-1-
	35	25 × 4	72	-10-	18	16	-2-	-6-	-4-	-2-
	20	25 × 4	52	19	29	10	10	17	11	-3-
	10	25 × 4	30	38	32	-7-	28	21	14	-1-
	5	25 × 4	-9-	45	34	11	38	19	20	-2-
	2.5	25 × 4	-0-	62	38	-6-	47	26	21	-6-

Table 48.11 Growth inhibitory activity of alkyl xanthates on agricultural pest, *Helicoverpa armigera* (topical application of xanthate analogs)

Sample Code	*Pupal formation*				*Adult emergence*					
	Conc. (µ g/insect)	No. insects used for assay	Larval mortality (%)	Normal pupa	Abnormal pupa	Pupal Mortality	Normal adults	Abnormal adults	Intermediates	Non emergence
Methylene bis(*n*-butylxanthate)	100	20 × 5	61	-0-	22	17	-0-	-0-	-2-	20
	75	20 × 5	49	-0-	27	24	-0-	-1-	-2-	24
	50	20 × 5	28	12	32	28	-8-	-9-	-6-	21
	35	20 × 5	12	27	28	33	17	11	18	-9-
	20	20 × 5	-7-	52	36	-5-	27	41	17	-3-
Methylene bis(tetrahydrofurfurylxanthate)	100	25 × 4	92	-0-	-6-	-3-	-0-	-1-	-0-	-5-
	75	25 × 4	85	-3-	-5-	-7-	-4-	-1-	-0-	-3-
	50	25 × 4	71	-5-	11	-9-	-5-	-7-	-2-	-2-
	35	25 × 4	62	12	15	11	-9-	16	-4-	-8-
	25	25 × 4	47	23	13	17	15	-7-	-5-	-9-
p-Xylene bis(*n*-butylxanthate)	100	20 × 5	85	-0-	-6-	15	-0-	-1-	-1-	-4-
	75	20 × 5	71	-5-	-7-	17	-0-	-2-	-2-	-8-
	50	20 × 5	56	10	13	21	-3-	13	-3-	-4-
	35	20 × 5	42	12	18	28	-9-	12	-3-	-6-
	20	20 × 5	18	39	27	16	33	19	-7-	-5-
Control	100	25 × 4	3	97	-0-	-0-	94	-0-	-0-	-3-
Azadiractin	50	25 × 4	91	-0-	-5-	-4-	-0-	-2-	-2-	-1-
	35	25 × 4	68	-8-	12	12	-2-	-6-	-4-	-3-
	20	25 × 4	47	13	27	13	10	17	-5-	-8-
	10	25 × 4	26	33	23	18	18	21	10	-7-
	5	25 × 4	11	47	31	11	38	18	20	-2-
	2.5	25 × 4	-2-	62	28	-8-	51	26	13	-0-

48.8 Conclusions

The results of the above studies reveal that the marine natural products acted as toxicants and insect growth regulators. Such extracts or compounds having varied properties are more attractive along with their low cost, biodegradability, and safety to the environment as vector/pest control agents. As an alternate method, natural products especially, marine natural products can support chemical control methods or can play an important role in integrated pest control programs (IPCP). From the current investigations, it has become apparent that alkyl xanthates could be used as a new lead for developing novel insect growth regulators. However, further investigations must be conducted for practical development of this class of compounds as new IGRs, i. e., more lipophilicity must be provided to the molecule to increase the penetration rate through the insect cuticle. It is hoped that the knowledge gained by studying these natural compounds especially marine natural products will be helpful for the development of newer vector/pest control programs. Their use in interference with insect's biochemistry and physiology has promising potential for the development as safe and biodegradable chemicals.

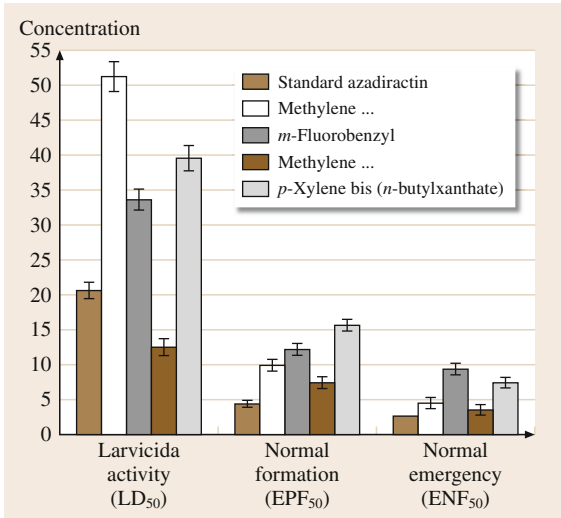


Fig. 48.7 Overall IGR activity of alkyl xanthates on *S. litura*

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Bioenerg

Part H

Part H Bioenergy and Biofuels

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49. Nanotechnology – from a Marine Discovery Perspective

Ramachandran S. Santhosh, Visamsetti Amarendra

Among the technologies reinvented from nature in the twentieth century, nanotechnology is an application of material science to improve the quality of human life. In this chapter, the authors mainly highlight how different nanoparticles (NP), nanocomposites, and nanodevices that exist in nature is supporting life on earth. Oceans harbor a large number of microorganisms; a few can synthesize NPs through green technology and are much superior in quality to the ones produced in the conventional way. The marine organisms themselves can detoxify nanomaterials, so a natural cycle is maintained to produce and detoxify nanomaterials in nature. The design and functioning of the organs of several marine organisms and their cellular organization can help to construct many nanodevices for medical treatment and daily life. Natural biological models are available in the ocean to design novel nanomachines, optical devices, sensors, filters, fuel cells, and acoustic machines. Seawater NPs can control the earth's climate. By incorporating biotechnological techniques into nanotechnology the wide scope for marine nanotechnology can be enhanced.

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49.1 Marine Nanotechnology

Nanotechnology is emerging with potential applications utilizing components with unique properties at the nanoscale level. There is a vast development in nanotechnology research all over the world, using resources either of organic/inorganic, or/and biodegradable/renewable nature. It is a broad and interdisciplinary science that is involving applications in physics, chemistry, electronics, biology, space, military, and environmental sciences (Fig. 49.1). The study of matter at nanoscale requires sophisticated instrumentation. It has created a huge impact on the materials utilizing in the day-to-day activities. The application of nanoscale components lies in its unique structural alignment and its precise interactions within the molecule. The modification of the arrangements of atoms/molecules is the most targeted prospects in nanotechnology research. However, in addition to its robust usage in various applications, the production of nanoscale components requires extreme conditions like temperature, pressure, acid/base, etc.

The biological process for the synthesis/development of nanomaterials has given new dimensions to the formulation of a product at low/ambient temperatures and pressures, in a precise and controlled

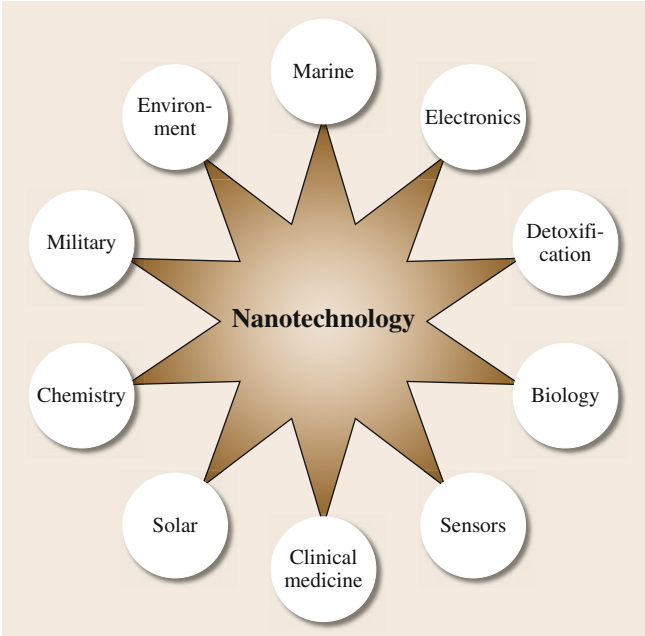

Fig. 49.1 Diverse applications of nanotechnology through marine biomaterials and biological sources

manner. The biological route of synthesis is a new trend in research for replacing all unfavorable conditions for the production of huge range of nanoscale components. Mostly microbial cell factories are vastly utilized for the manipulation and fabrication of submicrometer objects to nanometer-scaled products [49.1]. The synthesis of high surface area/volume ratio materials involves controlled and efficient processes and the reduced release of toxic by-products. Bacteria, algae, fungi, viruses at macromolecular level, and polypeptides, proteins, deoxyribonucleic acid (DNA) duplexes, ribonucleic acid (RNA) complexes, glycolipid components, and lipopeptides are natural biological elements utilized for the synthesis of nanomaterials for diverse applications. Biosynthesized materials can be used in the synthesis of metal NPs, as carriers in drug delivery applications, as therapeutics, in diagnosis, in imaging, as biosensors, electro-conductive transducers, in optics, nanorobotics/nanomachines, as water purifiers, water repellents, dust proof fabrics, and so on.

Marine resources can be used for the synthesis of nanostructured components. They mainly include marine organisms such as diatoms, sponges, echinoderms, mollusks, and its nonliving components, such as cellulose and carrageen of sea weeds, chitin of dead crustaceans, mollusk shells, silica shells of diatoms, siliceous spicules of sponges, etc. Biological materials such as diatom shells and sponge spicules from marine environments are extracted in the laboratory conditions, processed well, and replaced the molecular components with inorganic molecules to increase their mechanical/optical properties [49.2, 3].

In another case, the recombinant proteins of silicatein are synthesized, modifying its active site for synthesizing biomechanical structures [49.4]. Biomimetics is a new field of interest in nanotechnology by replicating the nature to create artificial nanostructures. The biological structures of organisms are studied and applied in the fabrication of material. Skin scales of sharks [49.5, 6], cellulose nanowhiskers of the marine tunicate, *Ascidiella aspersa* [49.7], and microlens array from the marine echinoderm, *Ophiocoma wendtii* [49.8] are various marine sources used for successful biomimicking for engineering purposes.

The present chapter describes various sources and applications of marine-derived products and possible marine-based designs used for fabrications in the field of nanotechnology.

49.2 The Ocean as Source for Nanomaterials and Nanodevices

The ocean comprises many organic and inorganic matter either in living (marine organisms) or nonliving forms (their exudates). The organisms and their biomolecules are involved in the synthesis or processing of many nanoparticulate matters. This chapter discusses the utilization of various marine sources for fabricating nanomaterials and nanodevices.

49.2.1 Metal Nanoparticles

The physical properties of NPs vary significantly from the bulk material; in that the particle possesses a large surface area, surface energy, spatial confinement, and decreased imperfections. Metal NPs are advantageous over bulk material because of their surface plasmon resonance (SPR), enhanced Rayleigh scattering, surface

enhanced Raman scattering (SERS), the quantum size effect in semiconductors and super magnetism in magnetic materials. These metal NPs are useful in various applications of optoelectronics, electronics, and in various chemical and biochemical sensors. Metal NPs are synthesized by top-down and bottom-up approaches. The former one breaks down the bulk material and the latter works by constructive synthesis of atomic or molecular structures.

Biological synthesis of NPs is the recent trend because of its controlled synthesis at normal temperatures and it is nonhazardous to the environment. Since physical and chemical methods result in the release of toxic and hazardous chemicals to the environment, the biological route of synthesis is focused for safe and ecofriendly usage of nonhazardous, economically sustainable raw materials. The major concern in the biological synthesis of metal NPs is their polydisperse nature and slow rate of synthesis. Modifications of pH, temperature, substrate concentration, and other physiological conditions enhance the controlled size and increase the rate of NPs synthesis.

Exploitation of microbes in the fields of bioremediation, biomineralization, bioleaching, and biocorrosion are emerging trends in the research for reducing metal toxicity in the environment. There are two modes of detoxification, either extracellularly through biomineralization, biosorption, complexation, precipitation, or by intracellular accumulation. The presence of various detoxification mechanisms in the microbes is responsible for the resistance towards toxic heavy metals. The application of various methodologies aims to detoxify the metal complexes and synthesize metal NPs, which has more commercial applications in different fields. Metabolic redox reactions are the major mechanisms underlying the synthesis/conversion to metal NPs. Energy-dependent ion efflux from the cell by membrane proteins such as ATPase, chemiosmotic cation/proton antitransporters, detoxifies either by reduction or precipitation of the soluble inorganic particles to insoluble nontoxic metal particles.

Treatment by ultrasonication and various detergents release the intracellularly synthesized metal NPs. Metal NPs are produced inside region of the cell, depositing onto inside cell wall [49.9, 10], precipitating in periplasmic space [49.11–13] and accumulating on the cell surface [49.14, 15]. Extracellular production of metal NPs are due to the presence of cell wall reductive components or soluble secreted enzymes. Modes of synthesis of metal NPs, their growth, and controlled structural development have been addressed by various research groups. Mechanism of modes of action and the components involved should be unraveled to increase the efficiency of biological synthesis.

Amino acid moieties in proteins help in the nucleation reaction for the formation of silver NPs. The face centred cubic (FCC) form of silver crystals are synthesized by the AG3 (AYSSGAPPMPPF) and AG4 (NPSSLFRYLPSD) peptides using the aqueous solution of silver ions [49.16]. Peptides rich with proline and hydroxyl group are found to be effective for the formation of metallic particles from the solution. The outer membrane bound *c*-type cytochrome (*Mtrc*) of the dissimilatory metal-reducing bacterium, *Shewanella oneidensis* MR-1, is involved in the reduction of uranium(VI) forming the extracellular polymeric substance uranium dioxide (UO_2) NPs and the enzyme was also previously reported in the reduction of Mn(IV) and Fe(III) [49.17, 18]. Other than enzymes that fall in the major part of the reduction; membrane bound quinines, the derivatives of naphthoquinones and anthraquinones and cytosolic pH-dependent oxidoreductases were also involved in the catalytic reduction of soluble metal complexes [49.19].

Viral mediated conversion of soluble organic complexes to pure inorganic metal ions were also defined [49.20]. Biological synthesis of NPs are aided by both aquatic and terrestrial forms. Since aquatic forms have quiet different habitats, the synthesis of NPs is focused on them. A few marine microbes are described here with their respective applications. Among them, gold, silver, and lead NPs are well studied.

Gold (Aurum) Nanoparticles (AuNPs)

Monodisperse AuNPs ranging in size between 7–20 nm were synthesized for the first time using aqueous extracts of marine sponge, *Acanthella elongate* by reducing the aqueous solution of $AuCl_4$ [49.21]. Cells of the marine bacteria *Marinobacter pelagius* were able to synthesize monodisperse AuNPs of 10 nm size using $HAuCl_4$ solution [49.22]. Synthesis of stable monodisperse AuNPs ranging between 8–12 nm from $HAuCl_4$ was done with the marine algae *Sargassum wightii* [49.23].

Silver (Argentum) Nanoparticles (AgNPs)

Because of its usage in various applications, such as selective coatings for solar panels, polarizing filters, reaction catalysts, bio-labeling, and as an antimicrobial agent, the biological synthesis of AgNPs is of increas-

ing interest to make the synthesis simple, reliable, eco-friendly, and economical. For the last few years the trend in AgNPs synthesis has marched towards using marine organisms, especially bacteria, fungi, and yeast because of their ability to reduce metal ions to metallic silver [49.24]. The major difference observed among the AgNPs with respect to their terrestrial source and their counter aquatic source, is size. AgNPs synthesized by the *Stapylococcus aureus* of terrestrial origin range from 160–180 nm [49.25]. *Penicillium* sp. isolated from soil was shown to synthesize AgNPs with a size of 75 nm [49.26]. Marine isolates have showed AgNPs in the range of 1–100 nm. Extracellular synthesis of AgNPs ranging from 5 to 25 nm at faster rate was done using the culture filtrate of the marine fungus *Penicillium fellutanum*, isolated from coastal mangrove sediment. Silver ions started converting to AgNPs within 10 min of coming in contact with the culture filtrate [49.27].

The rapidity of NPs synthesis was previously reported by *Bhainsa* and *D'Souza* [49.24] by synthesizing NPs using *Aspergillus fumigatus*. Maximum production was observed using culture supernatant of *P. fellutanum* under the optimized conditions of pH (6.0), temperature (5 °C), NaCl (0.3%) and concentration of $AgNO_3$ as 1 mM for 24 h incubation and the formation of NPs was confirmed by observation of the wavelength peak at 430 nm, which is a typical characteristic for AgNPs [49.27]. Thermophilic *Bacillus* sp. obtained from the marine environment of the Ramanathapuram district (India) synthesized AgNPs with sizes ranging between 10–100 nm [49.28]. *Streptomyces albidoflavus* isolated from marine sediment of the Bay of Bengal; it synthesized AgNPs with sizes ranging 10–40 nm, with an average mean size of 14.5 nm [49.29]. AgNPs show potent antimicrobial action compared to bulk silver. The synergistic action of the AgNPs in combination with standard antibiotics increased the drug sensitivity of the pathogen [49.30]. AgNPs were synthesized intracellularly in the marine bacterium, *Idiomarina* sp. PR58-8 with a size of 26 nm, and x-ray diffraction calculated the crystal size to be 25 nm [49.31].

Lead (Plumbum) Nanoparticles (PbNPs)

Synthesis of stable lead sulfide NPs from lead nitrate was carried out using the lead resistant marine yeast *Rhodosporidium diobovatum*. Stable PbNPs are used as quantum dots applied in solar concentrators and as bioconjugates with antibodies for targeted near-infrared molecular imaging because of their semiconductor properties [49.32].

49.2.2 Nanoparticles in MRI

Biocompatible chitosan and dextran sulfate hybrid biopolymer were used as coating agents for magnetic NPs to develop potential magnetic resonance imaging (MRI), T2 contrasting agent [49.33]. For controlled size distribution and to prevent aggregation, polyelectrolyte complexes are made to form a colloidal solution with metal oxides, resulting in the even dispersal of NPs throughout the polymer. Chitosan is one of the most abundant polymers present in the exoskeleton of crustacean shells, and in many marine mollusks, squid, etc. The polymer of chitosan and dextran sulfate are preferred because of the unique properties of chitosan with its biological properties, biodegradability, biocompatibility, and with its degree of acetylation responsible for charge density; and dextran sulfate for its negatively charged sulfur residues. The iron chloride salts of ferrofluids are directly co-precipitated inside the above mentioned complex polymeric matrixes. Cellular toxicity was checked using BALB/c 3T3 fibroblast cells that were loaded with different concentrations of ferrofluid and detected using propidium iodide staining. No toxicity was observed in the range of 5–80 μg/ml.

49.2.3 Biosurfactants

Surfactants are amphiphilic molecules containing both hydrophobic tails and hydrophilic heads. These molecules are in interface between two immiscible liquids, such as oil and water, and help in miscibility, solubilizing the polar compounds in nonpolar solvents [49.34]. Besides solubility, surfactants are also of interest because of their stability, lower toxicity, biodegradability, higher foaming capacity, and optimum activity at extreme temperature, pH, and salinity, which has led to significant growth in industrial and research fields. Biologically/chemically synthesized surfactants possess a hydrophilic shell comprised of acids, peptide cations, anions, mono, di, and polysaccharides, and a hydrophobic shell comprised of unsaturated or saturated hydrocarbon chains or fatty acids. Surfactants are synthesized either by chemical/physical methods/biological processes. Many microbial fauna are characterized for synthesizing stable and diverse biosurfactants. The use of these biosurfactants increases the stability, shelf-life, and solubility of NPs. Stability of NPs is important for their sustained usage for many applications. Green synthesis of biosurfactants have increased in recent years because of the eco-friendly nature and availability of a variety of low-cost raw materials, such

as plant-derived oils, starchy materials, and distillery waste [49.35]. Many biological systems have been discovered for screening reliable and stable biosurfactants. Marine sponge-derived microbes were screened for the presence of surfactants. *Brevibacterium casei*, an actinobacterium associated with *Dendrilla nigra*, collected from the southwest coast of India was reported to produce a glycolipid biosurfactant [49.36]. The synthesis and stability of AgNPs using biosurfactants was monitored and was proved to be stable for 2 months, which elicits the usage of biologically-derived surfactants to stabilize NPs [49.36].

49.2.4 Nanocomposites

Nanocomposites are polymeric matrixes that enhance and alter the stability, then physical and chemical properties of the different kinds of NPs embedded within them. Their reliability in extreme environments, high mechanical strength, and electrical properties are applicable in various fields such as biological implant materials, electronic packages, and automotive or aircraft components. Various reinforcing materials used are mineral particles; exfoliated clay stack sheets, carbon nanotubes, and electrospun fibers. Addition of nanocomposite material enhances the optical, dielectric properties, heat resistance, mechanical properties and resistance to wear and tear. Mostly synthesized nanocomposites are categorized into ceramic-matrix nanocomposites, metal-matrix nanocomposites, and polymer-matrix nanocomposites. Ceramic-matrix nanocomposites are mixture of ceramic and titanium oxide (TiO_2)/copper (Cu) in which metal particles are comprised as secondary components. The inclusion of metal components improves the optical, electrical, and magnetic properties, and the resistance to corrosion [49.37].

However, the only constraint is the requirement of higher temperatures to form polydispersed immiscible ceramic nanocomposites. The use of high tensile strength and electrical conductive carbon nanotubes in metal matrix nanocomposites improves the electrical, thermal, corrosive resistance, and hydrogen storage properties [49.38]. NPs filling the matrices of polymer composites enhance performance, such as mechanical, optical, and crystallization behavior. For example, polymers of poly(propylene fumarate) nanocomposites, carbon nanotubes, single and multiwalled grapheme oxide nanoribbons, graphene oxide nanoplatelets, and dodecylated nanocomposites are used in medical implants as a scaffolds for bone tissue engineering increased with mechanical properties [49.39]. But chemically or physically synthesized nanocomposites release by-products that are toxic to humans and require extreme conditions for synthesis.

Increased utilization of biological sources for various applications is focused towards the synthesis of nanocomposite materials. Many marine organisms are involved in the synthesis of structures made out of composites throughout their lifetime. For example, mollusk shells, nacre in gastropods, eventhough not marine wings of vultures, the enamel of mammal teeth, sponge spicules, diatom shells, and so on are various fabrications made by diverse organisms. These naturally occurring nanocomposites inspire the synthesis of nanocomposites through biological route. Shells of diatoms and mollusks and silicatein molecules of sponges are the major sources for synthesizing nanocomposites [49.40]. *Gutu* and his co-workers incorporated germanium into the shell of the marine diatom, *Nitzschia frustulum*, by a two-stage photobioreactor cultivation process where organic materials were removed by specific hydrogen peroxide treatment to incorporate germanium [49.41].

Germanium embedding into dielectric silica at nanoscale is of significant interest for development of optoelectronic devices and nonvolatile memory devices. Fabrication of nanostructured materials by bio-inspired or biologically mediated synthesis is carried out at low temperatures and at ambient pressures. In a study done by *Jeffryes* et al. [49.3], the cell division cycle of a diatom was focused for incorporation of germanium oxide into silica matrix. In the first stage, diatom cells were grown until the starvation period for silicon uptake. In the second stage, the cells were co-fed with soluble silicon and germanium to promote one cell division for uptake of germanium oxide into the cell. The nanocomposite material of the silicon-germanium incorporated into the diatom frustules increased the mechanical and optoelectronic properties [49.3].

Marine sponges are very peculiar in synthesizing skeletal elements such as calcareous lamina, siliceous spicules, and other organic filaments. The most noticed siliceous element is attracting researchers to understand the molecular mechanism for its controlled nanofabrication, which could be used for many industrial applications [49.2]. *Schröder* et al. [49.2] synthesized recombinant silicatein with the replacement of cysteine residues by serine to enhance the rate of reaction. The various applications of silicatein in nanotechnology are for the preparation of surface coating of metal oxides, metals, biomaterials glasses, the synthesis of nanocon-

tainers, nanodevices, and the encapsulation of bioactive molecules – which allows controlled release of these substances – biosilicification in lithography and for the production of microelectronics [49.2].

Titanium oxide from water stable alkoxide-titanium was synthesized biocatalytically by purified silicatein protein from composites of *Tethya aurantia* collected from Santa Barbara, California (USA). Purified silicatein filaments were mixed with aqueous solution of titanium(IV) bis(ammonium lactato)-dihydroxide and incubated at 20 °C for 24 h at 50 rpm. The controlled fabrication of titanium oxide and nanocrystalline anatase molecules upon photoluminescence showed a broad peak between 450 and 550 nm, with a maximum absorbance at 475 nm (ca.) (2.76–2.26 eV with 2.61 eV ca.). In comparison with photoluminescence of standard anatase powders (2.92 eV), titanium/protein composites showed a lower value by 0.31 eV. Avoiding higher temperatures and extreme pH the enzymatic method leaves space for accessing potentially useful parameter space of structures and properties [49.42]. In another study, surface immobilized silicatein was shown to synthesize titanium oxide and zirconium oxide particles from precursor molecules of titanium bis(ammonium lactato)-dihydroxide and anionic hexafluorozirconate (ZrF_6^{2-}). Recombinant silicateins having a histidine tag was used for the experiment described below. A reactive polymer, poly(acetoxime methacrylate) bound to amine group of cysteamine coated on gold particles, can provide the groups for binding of nitrilo-triacetic acid (NTA) which complexes with metal ions to hold histidine tag of recombinant silicatein. The hydrolytic activity of surface immobilized silicatein helped in the formation of titania and zirconia NPs which was confirmed by scanning electron micrographs of 50–60 nm sized TiO_2 and ZrO_2 particles. The enzymatic activity of silicatein was not found good in the synthesis of titania and zirconia, since there is a vast difference in the charge density and co-ordination number of metal complexes [49.43].

49.2.5 Nanomachines

Nature's molecular mechanisms are inspirations for developing nanoscale machines that are applicable in various fields, such as aerospace, medical, environment and climate sensing, and military applications. Controlled assembly, sensing, signaling, information processing, intelligence and swarm behavior should be the in-built behavior for an ideal machine to work at the nanoscale level. The development of essential properties, dynamic laws, design, and computation are key factors for an active machine. The exploration of molecular components from a biological source and applying its conventional activity in an artificial manner at qualitative and quantitative levels are a challenging issue.

In the medical field such devices help to perform operations in diagnosis and treatment in situ in the patient body, and to deliver drugs right way to the infected site. In the following sections, wide ranges of applications of nanomachines are discussed, including applications of nanomachines in the fields of medicine, optics, sensors, photonics, purification, electronics, sonar, and so on.

Medicine

Technological advances in computation methods are taking another step towards diagnosis and treatment of disease. Qualitative and quantitative diagnostic tools have been developed in the form of microarrays; one-touch strips that require only a minute quantity of sample. However, diagnosing the disease in vivo requires much intellectuality and more defined programming devices that specifically target the mutilated tissue. Nanomachines have been developed to sense change in molecular levels in vitro and act as miniaturized detectives. Preprogrammed devices are injected into the biological system and targeted a particular site either by an external magnetic field or by using cell/tissue specific ligands [49.44, 45]. Two broad fields in medicine: diagnosis and therapeutics, targeted by molecular and computational biologists, are briefly discussed below.

Diagnosis. Replacing silica chips, biological molecules themselves are used for targeting the diseased condition. Autonomous biomolecular computer can be used in medical diagnosis and treatment. The *Benenson* [49.46] research group developed molecular devices made out of DNA that can analyze the messenger RNA levels and produce a molecule in response to affect the gene expression. Models of small-cell lung cancer and prostate cancer are identified for the levels of messenger ribonucleic acid (mRNA) in vitro using computer programmed devices [49.46, 47]. A nanochip is a microelectronic chip designed to diagnose samples at nano-quantities. The integration of electronics and molecular biology has resulted in the development of nanochips that are useful in diagnostics, biomedical research, genomics, and genetic testing. Fluorescence imaging probes such as quantum dot bioconjugates, quantum dot-fluorescent protein, fluorescent resonance energy transfer (FRET) probes, molecular beacons,

and different NP-based MRI and computer tomography (CT) contrast agents are diverse components of nanomachines towards diagnosis [49.48].

Therapy. An ideal drug delivery should target the infected area of cell/tissue, be 100% reliable in the biological system, be biocompatible, site specific, have controlled supervision, be manipulative, and excrete out from the body without any side effects [49.49]. The transport of drugs from the external environment to inside tissue can be done by bacterial systems that possess engineered flagellum and type III injectisome [49.50]. Nanodiscs made of apolipoproteins conjugated with hydrophobic molecules are used as drug delivery vehicles for hydrophobic drugs. In vitro studies have been carried out using amphotericin B and trans-retinoic acid against fungal and protozoal diseases, and against cancer, respectively [49.51]. The usage of NPs for drug delivery applications has implicated necessary choices in the reduction of hazardous chemical usage. Biological materials that are modified and conjugated with different NPs are made biocompatible in delivering the drug at the targeted site. Chitosan-bound carriers, microcapsule devices from cnidarians, are a few biological sources studied for drug delivery vehicles.

Chitosan and Carrageenan NPs. Chitosan and carrageenan are naturally occurring polymers from the exoskeleton of crustaceans and extract of seaweed respectively. Both have been well appraised for their low toxicity, biocompatibility, and biodegradability. These polymers are currently used to deliver sodium diclofenac and diltiazem hydrochloride in the form of beads and tablets. The use of these polymers for sustained release of NPs in the infected site would be a promising one [49.52]. A model protein, ovalbumin, was used for loading into NPs of chitosan and carrageenan as drug delivery vehicles. The ionic complexation of both polymers by their opposite charges, such as positively charged amino groups of chitosan, interact with negatively charged sulfate groups on the carrageenan formed the nanosized range of 640 nm. Ovalbumin was loaded into carrageenan NPs at concentrations of 10, 20, and 30% (w/w) of protein with respect to the chitosan material. In vitro assays were done to determine the protein release into supernatants using the Micro BCA Protein assay. The controlled release of the protein for more than 3 weeks suggested the application of chitosan/carrageenan NPs as a nanocarriers in the field of tissue engineering and regenerative medicine, where an adequate release profile of a specific growth factor for a prolonged time in the infected area is ensured [49.52].

Microcapsules as Drug Delivery Vehicles. A transdermal route of drug administration is efficient for local and systemic drug delivery. Cnidarians, distinguished by the presence of cnidocytes, comprise both sessile (sea anemones, corals, sea pens) and swimming (jellyfish) forms of life and possess specialized cells called sting cells that are equipped with submicron-diameter injection system with a condensed matrix of short chains of polyglutamate and cations. Upon activation by cellular sensors, water flows through the microcapsules, disassociating the matrix and resulting in an increase in osmotic pressure to 150 mbar, releasing the folded tubule at an acceleration of $5 \times 10^6 g$ at less than 3 ms. The physical phenomenon of the injection system could serve as a vehicle for hydrophilic drug delivery systems [49.53]. The marine sea anemone *Aiptasia diaphana* was used to isolate the acontia (microfilaments) consisting of microcapsules. Microcapsules were prepared in 2% hydroxypropyl cellulose in absolute ethanol; lidocaine HCl was used as a drug to demonstrate its delivery using microcapsules, and methylene blue dye was used to activate the hydrophilic dye. Eight week-old female nude mice skin was used for the drug delivery assay. The amount of drug encapsulated in 1×10^6 microcapsules was applied to per square centimeter of skin above this number of microcapsules the delivery was inefficient. The experimental results confirmed the delivery of the drug through the transdermal route with less than 1 μm diameter and 50 μm length with controlled and rapid release [49.53].

Optical Devices

Traditional microscope are restricted with diffraction limit and there is an urgent need to develop nano-optics and photonic devices. Near-field scanning optical microscopy (NSOM), photo-assisted scanning tunneling microscopy, and surface plasmon optics are technologies based on nano-optics [49.54]. Research is focused on optical devices at the nanolevel to explore in nanolithography, high-density optical data storage, and high-efficiency electronic devices for engineering applications. Nano-optics are found in natural biological systems such as beetles, butterflies, birds, moths, and marine diatoms. Marine-based nano-optics that assists in development of microlenses is discussed below.

Microlenses. Frustules of the marine diatom, *Coscinodiscus wailesii* have been well studied for their

lensless light-focusing nature – for designing microoptic devices [49.55]. Microlenses are made of peculiar cell wall composite, hydrated silica valves, that are interconnected in specific patterns giving a regular array of holes (areolae). Within the distance of 104 μm, the 100 μm spot size of a red laser beam was narrowed to less than 10 μm after its transmission through the diatom frustules. Numerical simulations of the hypothesis proved to be the same in experimental results. The surface of the diatom valve comprising numerous holes scattered at regular intervals that lead to the superimposition of waves resulting in a focusing effect. Its high reproduction rate and reproducible nanoscale patterns which are at cost effective, could be beneficial in fabricating optical microarrays, semiconductor devices, and organic light-emitting devices [49.55].

Nanophotonics. Nanophotonics is another contribution of nanotechnology. The eyes of marine shrimp has 12 different photo receptors and can visualize in polarized light, whereas humans can not and have only three photoreceptors. This capability could be used in compact disc (CDs) and digital video disc (DVDs). Special light-sensitive cells in mantis shrimp eyes act as quarter-wave plates – which can rotate the plane of the oscillations (the polarization) of a light wave as it travels through them. This capability makes it possible for mantis shrimps to convert linearly polarized light to circularly polarized light and vice versa. Man-made quarter-wave plates perform this essential function in CD and DVD players and in circular polarizing filters for cameras. However, these artificial devices only tend to work well for one color of light, while the natural mechanism in the mantis shrimp's eyes work visible spectrum and ultra violet to infra-red light [49.56]. Photonic devices are fabricated using biological models such as shells of diatoms and spicules of sponges. Biocatalytically formed silica can form ultrafast holographic patterning. For this a nanostructure of silica sphere was made using a polycationic peptide obtained from *Cylindrotheca fusiformis* into a polymer hologram created by two-photon-induced photopolymerisation. The polycationic peptide was obtained from silaffin-1 protein of the organism.

These materials could be used for fabricating photonic devices, and in diagnosing antigens by incorporating the respective antibodies in the holograms [49.57]. In natural environments of the glass sponge, *Hyalonema sieboldi*, silicon dioxide present in the water is extracted by the spongin protein giving unique optical and mechanical properties. The synthesis and assembly of silicon dioxide NPs on the macromolecules of proteins (collagen) and polysaccharides (chitin) which acts as a matrix form a natural organic-inorganic hybrid nanocomposite material that combines both the elastic nature of polymer matrixes and the flexibility and durability of silica [49.58]. These nanocomposites comprising hydrated quartz in the axial layers were studied for their optical characteristics [49.59]. A fluorescence intensity at longer wavelengths of 770 nm was observed as second-harmonic pulses from an Nd:YAG (neodymium-doped yttrium aluminum garnet) laser, which excited the basal spicules with longer durations compared to common multimode quartz optical fibers. The transmission spectra of ultra-short pulses (USP) of spicules showed comparatively stronger nonlinear optical properties than the quartz optical fibers. Because of the high heterogeneous distribution of organic complexes, these nonlinear energy conversions in the spicules are classified into a new type of self-organizing photonic crystals, which could be used for optoelectronic instruments such as lasers, fiber amplifiers, and compressors of ultrashort pulses [49.59].

Biosensors

Biosensors are coming up with more accuracy to detect the underlying changes in concentration, displacement, gravitational, electrical, magnetims, pressure, temperature, velocity, and volume of cells. Nanosensor research has made an immense development of sensory devices to measure the release of toxic gases/fumes in the atmospheric environment, coal mines, and monitoring pollutants. An ideal sensor converts the physiological/physical/chemical changes into electrical signals that can be amplified and detected. Nanosensors are made of inorganic/organic molecules such as cadmium telluride (CdTe) nanocrystals for oxygen sensors [49.60]; perovskite barium titanate ($BaTiO_3$) [49.61] and polyacrylic acid/poly(vinyl) alcohol for humidity sensors [49.62]; metal oxide nanotubes for nitrogen dioxide and methanol sensors [49.63]; gold-coated carbon nanotubes (Au-CNT) for glucose sensors, hydrogen peroxide sensors, DNA and other biomolecular sensors, arsenic and parathion sensors, tramadol sensors, ethanol sensors, hydrogen sulfide sensors [49.64–69]; carbon nanotube-copper sulfide NP hybrid material for infrared sensors [49.70]; palladium wires deposited on graphite surfaces for hydrogen sensors [49.71]; and boron doped silicon nanowires for chemical and biological sensors [49.72]. Marine-derived biological components are used in the preparations of biosensors for controlled

synthesis at normal temperatures for eco-friendly functioning.

Detection of Pathogens. With an insight for rapid and early detection of pathogenic bacteria in an infected site, nanotechnology stepped into another approach through biosensors. The rapid and efficient identification of bacteria using gold NP-poly(*para*-phenylene ethynlyene) (PPE) constructs was developed to diagnose the bacteria in environmental samples and in food samples. The negatively charged polymer loses its fluorescent nature on binding with AuNP [49.73]. On addition of the bacterial sample, due to the negative charges on the cell wall, the positively charged NPs bind to them and release the fluorescent PPE into the solution, which results in fluorescence. So, the live bacteria increases the fluorescence by releasing PPE from AuNP-PPE. Different responses can be generated by different types of bacteria, resulting in specific identification [49.73, 74].

Nitrogen Sensors. Advances in nanotechnology using biological processes for various applications have recently been involved in sensing chemicals from released gaseous molecules. Marine diatoms made of amorphous silica shells with a complex and intricate skeleton (frustule) have been reported as possessing gas sensitive optical properties. The marine diatom *Thalassiosira rotula Meunier* has been identified for its photoluminescence emission to quench or enhance in the presence of nitrogen dioxide gases and organic vapors because of its polarization ability [49.75].

Nanofilters

Removal of total dissolved salts, inorganic and organic matter, and the treatment of water co-produced from oil and gas manufacturing companies increases the quality and reusability of water in this water scarce era [49.76–79]. Nanotechnology improved the water of purification systems. The development of nanofilters/membranes and co-filtration of samples with reverse osmosis has radically increased the quality of filtration compared to what was obtained with conventional technologies. The pore sizes of nanofilters are measured by the molecular weight cut-off ranging less than 1000 Da. Nanofiltration technology is capable of the removal of soluble salts, organic/inorganic chemicals, taste, odor, parasites and their cysts, pathogenic microbes, and viral particles. The protozoans *Giardia* and *Cryptosporidium* are the main parasites targeted in surface water and drinking water systems in USA, which are qualitatively treated by nanofiltration [49.80]. Pharmaceutical drug contaminants such as hydrochlorothiazide, ketoprofen, diclofenac, propyphenazone, and carbamazepine in surface water were successfully removed using nanofiltration and a reverse osmosis membrane, and the retentates were analyzed by liquid chromatography-mass spectrometry (LC-MS) for confirmation [49.81]. In vitro studies have been carried out on the removal of 60–90% of arsenic content in waste water treatment using nanofiltration [49.82]. Naturally occurring nanofilters have been discovered in marine environments that possess nanofabricated structures comprised of both filtration and sensing properties. Marine diatoms such as *Glyphodiscus stellatus*, *Cyclotella meneghiniana*, *Roperia tesselata*, and *Isthmia nervosa* are involved in the synthesis of amorphous silica glass valves on micro and nanoscale with particular shapes and sizes that are useful in various applications of biomedicine, electronics, sensing, semiconductor, nanolithography, and as drug delivery vehicles [49.83, 84]. A thermodynamically favored reaction was executed by replacing the silicon atoms in the silicon oxide structures of diatom shells by keeping at the atmosphere of magnesium gas at 900 °C for 4 h. A replicate of a diatom shell of magnesium oxide that resembles silicon oxide with no change in its 3-D shape was formed, thus transforming to magnesium oxide from a silicon dioxide, which has limited applications in nanotechnology. The modified diatom shells could be useful in the preparation of microcapsules for medications, water filters, catalytic substrates, sensors, optical diffraction gratings, and actuators. In another case, the frustules of the diatoms were proposed for water purification by filtration. Diatomaceous earth consists of fossils of dead diatoms remaining in the earth. The different grades of the composition of silica and its sizes determine the porosity and its permeability [49.83].

Nanoelectronics

The next generation of electronics is becoming completely transformed into nanometer-scale electronics that utilize the quantum mechanics phenomenon instead of field-effect transistor technology, which currently used in the conventional transistors ranging between 100–250 nm [49.85]. The fundamental approaches in designing nanoelectronics are nanofabrication, nanomaterial electronics, molecular electronics, nanoionics, and nanophotonics. Various devices under production applying the principles of nanoelectronics are computer devices, memory storage, optoelectronics, displays, quantum computers, radios, energy produc-

tion (solar cells), and medical diagnostics. *Xu* and *Lavan* [49.86] discovered biological cells that synthesize a high voltage of current in the electric eel *Electrophorus electricus*. The miniature structure of the cells impressed researchers and led them to produce a quantum of voltage to be utilized for electronic devices. Electric eels process a special kind of cells (electrocytes) known to produce a high voltage of current of about 600 V, which could be sufficient to kill a human-being. Electrocytes consist of numerous ion channels and ion pumps, which could trigger the release of action potential by forming ion concentration gradients throughout the membrane. The physiological process is used for designing artificial cells that could be used as medical implants for supplying energy and in other tiny devices [49.86]. The artificially synthesized cells performed 31% more efficiently than biological cells. A synthetic biological battery has been developed by *Xu* and his colleagues [49.87], which comprises two droplets separated by a lipid bilayer stabilized with membrane proteins. It acts as a biological battery that converts transmembrane ionic gradients by membrane-protein regulated ion transport into electricity. An energy conversion efficiency of 10% is observed while using synthetic cells other than lead-acid batteries [49.87].

Bio-Sonar

Animals of either terrestrial or aquatic habitats possess a typical property called echolocation. Determining the presence of an object, prey, or hurdle by measuring the time the echo takes to return, is called echolocation. Echolocation can be seen in mammals such as dolphins, whales, and in bats. Artificial SONAR (sound navigation and ranging), which comprises a sound transmitter and a receiver have been developed for submarines, aircraft radar systems, and in industrial, robotic, and medical applications. Whales and dolphins communicate with the surrounding environment and other mammals by producing sounds and listening to the echoes returning to them. This method is used either to communicate, avoid obstacles, hunt prey, and localize objects. The detection of fish by echolocating bottlenose dolphins and harbor porpoises have been studied. In noise-limited environments, the bottlenose dolphin's echolocation of fish was calculated to be 70–93 mts and in quiet environments it was calculated to be 107–173 mts [49.88]. The anatomy of the inner ear, called cochlea, is used as a model for artificial sonar devices that could be used in the medical field, audio frequency range, and ultrasonic transducers. The polyvinylidene fluoride (PVDF) ultrasonic transducer resembles a basilar membrane in which acoustic pressure is amplified by a piezoelectric effect into electrical signals [49.89]. PVDF is a ferroelectric polymer, applicable in the fields of electroacoustics, electro-mechanics, audio frequency, ultrasonics, underwater, optics, and pyroelectric transducers. It is largely used in the medical field as an ultrasonic transducer [49.90, 91].

Tolerating Pressure

Deep-sea animals survive under extreme conditions of osmotic pressures quite easily because of the presence of piezolytes, which regulate the osmotic pressure of the cell. Piezolytes are osmolytes present in soluble form in cytoplasms composed of oligomers of β-hydroxy butyrate (β-HB) units found in the deep-sea bacterium *Photobacterium profundum* strain SS9 [49.92]. This strain was isolated from an amphipod-enriched material collected in the Sulu Sea at a depth of 2551 m [49.93]. In vitro experiments were performed on *P. profundum*, maintaining the cultures at 0.1 MPa and 28 MPa pressure in glucose fermentation conditions. Cells were harvested, extracted with ethanol, and analyzed by natural abundance using ^{13}C nuclear magnetic resonance (NMR) for intracellular osmolytes. At lower pressure conditions, betaine and glutamates were observed in major concentrations, with minor amounts of alanine and glycine. At higher pressure conditions, along with the constant concentrations of betaine, glutamate, and alanine, larger amounts of β-HB units and oligomers of β-HB were observed. This suggests that accumulated β-HB monomers and oligomers are involved in maintaining osmotic balance at higher pressures. The effect of temperature and sodium chloride on the levels of solutes were also assayed. Accumulation of β-HB units was pronounced to be higher in lower temperatures and at increased concentrations of sodium chloride. From these results, piezolytes that function to tolerate extreme pressures could also tolerate osmotic pressures [49.92].

49.3 Ocean in Climate Control

49.3.1 Cooling Effects of Ocean and Cloud Formation

Due to their alkalinity sea salt aerosols are involved in the production of sulfate particles from sulfur dioxide (SO_2) oxidation by ozone and thus act as a sink SO_2. The major protion of SO_2 are released from the combustion of fossil fuels, industrial processes, and volcanoes, and in the reduced form of organic sulfur, such as dimethyl sulfide which is released into the atmosphere by phytoplankton [49.94]. Once the aerosol particles become cloud condensation nuclei (CNN), a major part of the sulfate production occurs on the cloud particle. Because of its hygroscopic nature and optical properties, the sulfate particles scatter the radiation from sunlight, acting as main climate cooling aerosols and are thus effective CNN [49.95]. The mixture of aerosols and sulfate particles possesses different optical properties and hygroscopic nature depending on the size of the particles, which diminishes the cooling effect of sulfate particles. Thus an increase of sea salt aerosols and their interactions with the sulfate particles result in climatic control and formation of a cloud in the marine troposphere and marine boundary layer [49.94–96].

49.3.2 Effect of Environmental Parameters

Temperature and evaporation results in the precipitation of NPs in water bodies. Such an incident happened in 2001 at Lake Michigan in North America. The lake, which is based in a limestone basin, precipitated out calcium carbonate at the end of the summer. Calcium carbonate remained in soluble form throughout the winter and on an increase in temperature during the summer decreased the solubility, which resulted in precipitation in the form of nanometer-scale particles of clouds that could be viewed as bright swirls from satellite images. The precipitation was probably due to the warming up of the ocean, resulting in dissolved carbon dioxide leaving the water body, thus increasing the pH (making it basic) which decreases the solubility of calcium carbonates [49.97]. The salt aerosols were used to treat severe impairment of mucillary transport in patients affected with cystic fibrosis [49.98].

49.4 Detoxification of Nanomaterials

49.4.1 Biotransformation

The increased usage of different kinds of NPs of various sizes, applicable in diverse fields, has increased the accumulation of toxic NPs in the environment. Besides their advantages, the accumulation in aquatic and biological habitats has had an adverse affect over life. Ultrafine NPs can easily pass through the pores of the lipid layers and accumulate in tissues [49.99]. Along with natural aerosols (asbestos), prolonged exposure of man-made fibers are also associated with an increased risk of pulmonary fibrosis and cancer [49.99]. Mussels are well known as biological markers for polluted environments. The aquatic mussel *Corbicula fluminea* was studied for uptake and biological accumulation of bovine serum albumin-coated AuNPs [49.100]. Marine mussels are proficient in taking up the metal NPs by filtering them out from the water body [49.100, 101]. Filtration of engineered cerium oxide (CeO_2) and zinc oxide (ZnO) NPs were studied in an artificial set-up of a water body containing suspension feeding mussels, *Mytilus galloprovincialis*, supplemented with dissolved CeO_2 and ZnO NPs. Mussels are likely to accumulate higher amounts of ZnO than CeO NPs, which was confirmed from the quantification of pseudofeces. The solubility differences between the ZnO and CeO_2 are responsible for the difference in accumulation. Soluble ZnO is readily taken up by mussels, where insoluble CeO_2 is filtered out through the water column and packed in the pseudofeces. Besides the differences in the accumulation in engineering NPs, the experiment proves the biotransformation of NPs released into the environment [49.101].

49.5 Biomimetics

Living organisms are well known for their diverse biological activities, and their significant changes through the course of evolution have adapted to the changing environments. The study of the biological route and the components involved in the activities are trends in research for mimicking its usage. The natural activities that have inspired research groups are nacre synthesis from pearl oysters (for materials science and coatings in biomedical science) [49.102], skin scales of sharks [49.5, 6] (Fig. 49.2a), cellulose nanowhiskers of the marine tunicate *Ascidiella aspersa* [49.7] (Fig. 49.2b), and microlens arrays from the marine echinoderm *Ophiocoma wendtii* [49.8] (Fig. 49.2c). The marine originated species involved in the biomimetic approach for various applications are briefly discussed below.

49.5.1 Nanocoatings and Fabrication

Shark skin scales of a superoleophobic nature are used as a model for preparing low-drag surface materials in swim suits. The superoleophobic nature provides self-cleaning and anti-fouling properties and avoids oil contaminants in the air and water interface to adhere with the surface. Riblets (shark scales) have a lower shear stress than smooth surfaces. The physical phenomenon behind the decrease in shear stress is the presence of small ribs between the previous ribs, which results in sloshing the water particles away from the surface [49.104]. It is hoped that the model can be applied to paintings on airplanes, ships, and wind mills to decrease air drag and to resist the flow of air currents [49.103]. This attempt will reduce the costs of paint and result in more economical fuel savings every year and a decrease in carbon emissions. These superoleophobic surfaces can be used for reducing residual oil loss in oil tanks and pipes [49.105]. The models of shark skin denticles, which reduce water drag during swimming, are used in artificial shark skin-like surface materials in swimsuit preparations. These artificial shark skin preparations reduce the hydrodynamic drag and were proved through a comparison with the self-propelled swimming speed of a flapping foil robotic device on Speedo shark-line swim suit fabric, a silicone riblet-made material, and shark skin membranes. There was a 12.3% increase in swimming speed with the shark skin membranes, compared to a 7.2% increase in speed with the riblet material, and no change in speed with the Speedo shark-line fabric [49.106].

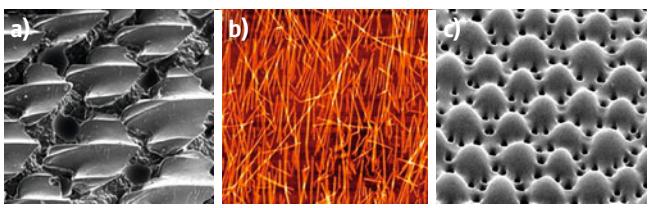

Fig. 49.2a–c Electromicrographs of marine-originated biological designs used for various biomimetic applications. **(a)** shark skin replica [49.103]; **(b)** cellulose nano whiskers [49.7]; **(c)** array of microlenses [49.8]

49.5.2 Nanowhiskers

Chitin Whiskers

Chitin, a naturally occurring biodegradable biopolymer found as the exoskeleton of shrimps, crustaceans (sea crabs, beetles), pens of squid, and cell walls of fungi and yeasts [49.107]. Since chitin is one of the most abundant biocompatible polymers, its usage in various applications is increasing. Suspensions of acid hydrolyzed chitin polymer are used as composite films in preparing complex materials involving various synthetic and nonsynthetic matrixes. Chitin whiskers are used as reinforcing fillers in natural polymeric matrixes such as chitosan to improve their water resistance and mechanical properties [49.108]. Along with their major application as reinforcing fillers, chitin whiskers are also applicable in preparing scaffolds, wound dressings, water purification, adsorbents in industry, transformation in bacteria by exogenous genes, protein immobilization, and preparation of hydrogels [49.107, 109].

Cellulose Whiskers

Besides its abundance as a natural polymer, its biodegradable, renewable, and nontoxic nature, a variety of applications in food, paper production, and pharmaceutics, and as biomaterial, cellulose material is a new area of research in nanotechnology. It is used in preparing cellulose nanofibrils or nanowhiskers for applications in drug delivery, nanocomposite films, protein immobilization, and metallic reaction templates [49.110]. Cellulose nanowhiskers are extracted from the native cellulose by removing lignin, extractives, inorganics, and hemicelluloses [49.111]. Acid hydrolyzed cellulose materials are the main source for the production of cellulose nanowhiskers. The marine tunicate *Ascidiella aspersa* was the first ani-

mal source used for extracting cellulose nanowhiskers. These nanowhiskers were used as nanoscale materials for engineering skeletal muscle tissues [49.7].

49.5.3 Microlens Array

Due to their acute sight capabilities, marine deep-sea organisms are used as a model in developing microlens arrays for highly tunable optical elements, which are used in various applications. The ophiuroid *O. wendtii*, which is called brittle star and belongs to echinoderms, forms a different class because of its strong responses to the light [49.8]. The physiological and phototactic behavior is noted as having a dark brown color during the day and being gray and black during night as an escape mechanism from predators. The dorsal arm plates (DAPs) composed of an array of calcite crystal structures that possess the typical double lens design involved in high photosensitivity. The optical properties of DAPs have the perfect features of being micron-scale, lightweight, mechanically strong, aberration-free, birefringence-free, and having individually addressed lenses that provide a unique focusing effect, signal enhancement, intensity adjustment, angular selectivity, and photochromic activity. A biomimetic model of optical microarray lenses was developed by *Aizenberg* and *Hendler* [49.8] based on numerical simulations of the existing natural model of ophiuroid photosensing calcite structures. A porous hexagonal microlens of a diameter of $1-8\,\mu m$ was created from photoresist materials using multi-beam interface lithography. The controlled size, shape, symmetry, connectivity, and polarization of the lens are adjusted by beam wave vectors, and pore sizes are controlled by laser intensity, exposure time, and concentration of the photosensitizer. The synthetically manufactured microarrays possess nearly perfect structures as naturally occurring lenses and gear up for potential use in optical devices [49.8]. In the same way, deep-sea dragon fishes (*Malacosteus niger, Aristostomias tittmanni*, and *Pachystomias microdon*) and a teleost (*Lestidiops affinis*) were characterized with specialized cells for increasing sensitivity for visualization [49.112, 113]. Most of them are bioluminescent and capable of converting luciferin by photophores present in specialized glandular cells [49.112, 113]. These species could also be studied well for exploiting the interactions of the eye lens with a wide range of radiations for designing new optical devices with a diverse range of applications of nano and bioimaging, optical lithography, optical memory storage, and nanosensing [49.114].

49.6 Conclusions

Nanotechnology plays an important role in the advances of modern technology. There is an important need for the synthesis of various biomaterials and bioparticles in the field of nanotechnology by the improvement of reliable and eco-friendly sources. This can only be accomplished in biological ways. The incorporation of biological sources and derivatives or the biology of the organism or organisms themselves for the synthesis are a better way to achieve a sustainable environment. The synthesis may be partially difficult if only biological sources are used and, moreover, changes in the pH, temperature, and to a lesser extent pressure may favor the synthesis.

The formation of naturally occurring biomaterials and the study of their biological mechanisms may improve the incorporation of diverse elements during their in vitro synthesis. Applications of nanotechnology in utilizing renewable sources in energy production will decrease the rate of extinction of nonrenewable sources. Marine sources are portrayed as being best for the various applications. The uptake of marine sources for the field of nanotechnology may increase the processes of various commercial applications. Thus, the knowledge of the biological mechanisms underlying the synthesis may be used in applications of medicine, optics, electronics, filtration, space, military, environmental monitoring, sensors, and detoxification.

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50. Algal Photosynthesis, Biosorption, Biotechnology, and Biofuels

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The issues relating to the global warming, air pollution, and energy security have been one of the most important public policy issues nowadays and with the increasing global population, food security has also become a major public policy issue. The development of algal biofuels as a third-generation biofuel has been considered as a major solution for these global problems. Although there have been many reviews on the use of the marine algae in energy, medicine, and health care and there been a number of scientometric studies on the algal biofuels, there has not been any study on the citation classics in the algal photosynthesis, biosorption, biotechnology, and biofuels as in other research fields. As North's New Institutional Theory suggests, it is important to have up-to-date information about the current public policy issues to develop a set of viable solutions to satisfy the needs of all the key stakeholders. Therefore, a selected set of citation classics in the algal photosynthesis, biosorption, biotechnology, and biofuels are presented in this chapter to inform the key stakeholders about the use of marine algae for the solution of these problems in the long run complementing a number of recent scientometric studies on the biofuels and global energy research.

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50.1 Overview

50.1.1 Issues

The global warming, air pollution, and energy security have been one of the most important public policy issues nowadays [50.1–3]. With the increasing global population, food security has also become a major public policy issue [50.4]. The development of biofuels generated from the biomass has been a long awaited solution to these global problems [50.5–7]. However, the development of the early biofuels produced from the agricultural plants such as sugar cane [50.7] and agricultural wastes such as corn stovers [50.8] have resulted in a series of substantial concerns about the food security [50.9]. Therefore, the development of algal biofuels as a third-generation biofuel has been considered as a major solution for the global problems of global warming, air pollution, energy security, and food security [50.5].

Although there have been many reviews on the use of the marine algae in energy, medicine, and health care [50.10–14], and there been a number of scientometric studies on the algal biofuels [50.15], there has not been any study on the citation classics in the algal photosynthesis, biosorption, biotechnology, and biofuels as in the other research fields [50.16–24].

As North's New Institutional Theory suggests, it is important to have up-to-date information about the current public policy issues to develop a set of viable solutions to satisfy the needs of all the key stakeholders [50.25–34].

Therefore, a selected set of citation classics in the algal photosynthesis, biosorption, biotechnology, and biofuels are presented in this chapter to inform the key stakeholders relating to the global problems of warming, air pollution, food security, and energy security about the use of marine algae for the solution of these problems in the long run complementing a number of recent scientometric studies on the biofuels and global energy research [50.15, 35–47].

50.1.2 Methodology

A search was carried out in the SCIE (science citation index expanded) and SSCI (social sciences citation index) databases (version 5.9) in February 2013 to locate the papers relating to the algal research using the keyword set of (*micro** *alga** or *macro** *alga** or algal or algae or microalga* or macroalga*) in the abstract pages of the papers. For this paper, it was sufficient to focus on the key algal search terms rather than for a collection of the search terms on the specific names such as diatoms, cyanobacteria, etc., for the algal plants.

It was found that there were 88 582 papers indexed by these indexes between 1956 and 2013. The key subject categories for the algal research were Marine Freshwater Biology (24 358 references, 29.7%), Plant Sciences (14 249 references, 17.4%), Environmental Sciences (9655 references, 11.8%), and Ecology (8951 references, 10.9%) comprising 79% of all the references on the algal research.

Next, the results were refined to Document Types = (Article or Review) and Web of Science Categories = (Biotechnology Applied Microbiology or Multidisciplinary Sciences or Energy Fuels or Engineering Chemical or Chemistry Medicinal or Agricultural Engineering or Agriculture Multidisciplinary or Materials Science Multidisciplinary) and 11 852 articles and reviews were located in both indices. The topical focus was necessary to locate the key papers on the applications of the algal technology in many areas from solar fuels to Medicine and Fuels. It was also necessary to focus on the key references by selecting articles and reviews.

The located papers were arranged in the order of the decreasing number of citations and a cut-off point of 100 citations was used to locate the citation classics in algal research. Then the abstract pages of the papers related to biotechnology, biofuels, photosynthesis, and metal biosorption were grouped under the headings of the topical areas. In order to check whether these collected abstracts correspond to the larger sample on these topical areas new searches were carried out for each topical area.

The summary information about the located citation classics are presented under the four headings of algal biofuels, algal photosynthesis, algal biotechnology, and algal biosorption in the order of the decreasing number of citations for each topical area.

50.2 Algal Photosynthesis

50.2.1 Introduction

The research on the algal photosynthesis has been one of the most dynamic research areas in recent years. Papers of 2011 were located as indexed in the SCIE and SSCI as of February 2013. The United States and Germany produced over 45% of these papers whilst University of California was the most prolific institution. The research output increased significantly in the last two decades with 155 papers in 2012. *Photosynthesis Research* was the most prolific journal publishing over 8% of the papers whilst nearly 41% of the papers were indexed under the subject category of *Plant Sciences*. The citation impact of the papers on the algal photosynthesis has been significant with 29.2 citations per paper and H-index of 97.

Ten citation classics in the field of algal photosynthesis with more than 100 citations were located and the key emerging issues from these papers were presented below in the decreasing order of the number of citations [50.48–57]. These papers give strong hints about the structural determinants of the algal photosynthesis,

informing the following sections. Photosystems I and II (PSI and PSII) which reaction centers that capture light energy in order to drive oxygenic photosynthesis play an important role in this field of research.

50.2.2 Research on the Algal Photosynthesis

Jordan et al. study three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution in a paper with 1156 citations [50.48]. They find that the photosynthetic conversion from light energy to chemical energy in algae is driven by the involvement of two large protein–cofactor complexes, namely, photosystems I and II, located in the thylakoid photosynthetic membranes of algae. The structural information on the proteins and cofactors and their interactions provides valuable information on photosynthetic efficiency of photosystem I in the stages of light capturing and electron transfer. For example:

> *the crystal structure of photosystem I from the thermophilic cyanobacterium Synechococcus elongatus provides a picture at atomic detail of* 12 *protein subunits and* 127 *cofactors comprising* 96 *chlorophylls,* 2 *phylloquinones,* 3 Fe_4S_4 *clusters,* 22 *carotenoids,* 4 *lipids, a putative* Ca^{2+} *ion, and* 201 *water molecules.*

Loll et al. study complete cofactor arrangement in the 3.0 angstrom resolution structure of cyanobacterial photosystem II in a paper with 977 citations [50.49]. They note that as in plants, oxygenic photosynthesis in algae is initiated at photosystem II, a homodimeric multisubunit protein–cofactor complex embedded in the thylakoid membrane. Photosystem II captures sunlight and powers the unique photo-induced oxidation of water to atmospheric oxygen. They find that:

> *Crystallographic investigations of cyanobacterial photosystem II provide several medium-resolution structures (*3.8–3.2 Å*) that explain the general arrangement of the protein matrix and cofactors. Assignment of* 11 *beta-carotenes yields insights into electron and energy transfer and photoprotection mechanisms in the reaction center and antenna subunits. The high number of* 14 *integrally bound lipids reflects the structural and functional importance of these molecules for flexibility within and assembly of photosystem II.*

They propose a lipophilic pathway for the diffusion of secondary plastoquinone that transfers redox equivalents from photosystem II to the photosynthetic chain. The structure provides information about the Mn_4Ca cluster, where oxidation of water takes place. They conclude that these near-atomic details are necessary to understand the processes that convert light to chemical energy.

Escoubas et al. investigate whether light-intensity regulation of cab gene-transcription is signaled by the redox state of the plastoquinone pool in eukaryotic green alga *Dunaliella tertiolecta* in a paper with 395 citations [50.50]. They note that the eukaryotic green alga *D. tertiolecta* acclimates to decreased growth irradiance by increasing cellular levels of light-harvesting chlorophyll protein complex apoproteins associated with photosystem II (LHCIIs), whereas increased growth irradiance elicits the opposite response:

> *Light intensity-dependent changes in LHCII are controlled at the level of transcription, cab gene transcription in high-intensity light is partially enhanced by reducing plastoquinone with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), whereas it is repressed in low-intensity light by partially inhibiting the oxidation of plastoquinol with 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB):*

They find that uncouplers of photosynthetic electron transport and inhibition of water splitting has no effect on LHCII levels. These results strongly implicate:

> *the redox state of the plastoquinone pool in the chloroplast as a photon-sensing system that is coupled to the light-intensity regulation of nuclear-encoded cab gene transcription.*

They conclude that:

> *the accumulation of cellular chlorophyll at low-intensity light can be blocked with cytoplasmically directed phosphatase inhibitors, such as okadaic acid, microcystin L–R, and tautomycin.*

Cells grown in high-intensity light contain proteins that bind to the promoter region of a cab gene carrying sequences homologous to higher plant light-responsive elements. In their model for a light intensity signaling system, cab gene expression is reversibly repressed by a phosphorylated factor coupled to the redox sta-

tus of plastoquinone through a chloroplast protein kinase.

Hoganson and *Babcock* propose a metalloradical mechanism for the generation of oxygen from water in photosynthesis in a paper with 384 citations [50.51]. They note that in algae, photosystem II uses light energy to oxidize water to oxygen at a metalloradical site that comprises a tetranuclear manganese cluster and a tyrosyl radical. They propose a model that, the tyrosyl radical functions by abstracting hydrogen atoms from substrate water bound as terminal ligands to two of the four manganese ions. They find that molecular oxygen is produced in the final step in which hydrogen atom transfer and oxygen–oxygen bond formation occur together in a concerted reaction. They conclude that this mechanism establishes clear analogies between photosynthetic water oxidation and amino acid radical function in other enzymatic reactions.

Stroebel et al. study an atypical haem in the cytochrome b_6f complex in alga *Chlamydomonas reinhardtii* in a paper with 346 citations [50.52]. They note that photosystems I and II (PSI and II) are reaction centers that capture light energy in order to drive oxygenic photosynthesis. However, they can only do so by interacting with the multisubunit cytochrome b_6f complex. This complex receives electrons from PSII and passes them to PSI, pumping protons across the membrane and powering the Q-cycle. They find that:

> *unlike the mitochondrial and bacterial homologue cytochrome* bc_1, *cytochrome* b_6f *can switch to a cyclic mode of electron transfer around PSI using an unknown pathway and the X-ray structure at* 3.1 Angstrom *of cytochrome* b_6f *from the alga* C. reinhardtii *provides information about this process.*

The structure bears similarities to cytochrome bc_1 but also exhibits some unique features, such as binding chlorophyll, beta-carotene and an unexpected haem sharing a quinone site. They conclude that *this haem is atypical as it is covalently bound by one thioether linkage and has no axial amino acid ligand* and this haem is perhaps the missing link in oxygenic photosynthesis.

Collini et al. investigate coherently wired light harvesting in photosynthetic marine algae at ambient temperature in a paper with 295 citations [50.53]. They note that photosynthesis makes use of sunlight to convert carbon dioxide into useful biomass and is vital for life on Earth. Crucial components for the photosynthetic process are antenna proteins, which absorb light and transmit the resultant excitation energy between molecules to a reaction center. The efficiency of these electronic energy transfers has inspired much work on antenna proteins isolated from photosynthetic organisms to uncover the basic mechanisms at play. Intriguingly, recent work has documented that light-absorbing molecules in some photosynthetic proteins capture and transfer energy according to quantum-mechanical probability laws instead of classical laws at temperatures up to 180 K. This contrasts with the long-held view that long-range quantum coherence between molecules cannot be sustained in complex biological systems, even at low temperatures. They present:

> *two-dimensional photon echo spectroscopy measurements on two evolutionarily related light-harvesting proteins isolated from marine cryptophyte algae, which reveal exceptionally long-lasting excitation oscillations with distinct correlations and anti-correlations even at ambient temperature.*

They conclude that:

> *these observations provide compelling evidence for quantum-coherent sharing of electronic excitation across the* 5 nm*-wide proteins under biologically relevant conditions, suggesting that distant molecules within the photosynthetic proteins are wired together by quantum coherence for more efficient light-harvesting in cryptophyte marine algae.*

Niyogi et al. study the roles of specific xanthophylls in photoprotection in green alga *Chlamydomonas reinhardtii* in a paper with 245 papers [50.54]. They note that xanthophyll pigments hare critical structural and functional roles in the photosynthetic light-harvesting complexes of algae and vascular plants. Genetic dissection of xanthophyll metabolism in the green alga *C. reinhardtii* reveal functions for specific xanthophylls in the nonradiative dissipation of excess absorbed light energy, measured as nonphotochemical quenching of chlorophyll fluorescence. They find that:

> *mutants with a defect in either the alpha- or beta-branch of carotenoid biosynthesis exhibited less nonphotochemical quenching but were still able to tolerate high light.*

In contrast:

> *1a double mutant that was defective in the synthesis of lutein, loroxanthin (alpha-carotene*

branch), zeaxanthin, and antheraxanthin (beta-carotene branch) had almost no nonphotochemical quenching and was extremely sensitive to high light.

They conclude that:

in addition to the xanthophyll cycle pigments (zeaxanthin and antheraxanthin), alpha-carotene-derived xanthophylls such as lutein, which are structural components of the subunit of the light-harvesting complexes, contribute to the dissipation of excess absorbed light energy and the protection of plants from photo-oxidative damage.

Amunts et al. investigate the structure of a plant photosystem I supercomplex at 3.4 Å resolution in a paper with 160 citations [50.55]. They note that all higher organisms on Earth receive energy directly or indirectly from oxygenic photosynthesis performed by plants, green algae and cyanobacteria. Photosystem I (PSI) is a supercomplex of a reaction center and light-harvesting complexes. It generates the most negative redox potential in nature, and thus largely determines the global amount of enthalpy in living systems. They report the structure of plant PSI at 3.4 Å resolution, revealing 17 protein subunits. PsaN is identified in the luminal side of the supercomplex, and most of the amino acids in the reaction center are traced. The crystal structure of PSI provides a picture at near atomic detail of 11 out of 12 protein subunits of the reaction center. They find at this level:

168 *chlorophylls (*65 *assigned with orientations for* Q_x *and* Q_y *transition dipole moments),* 2 *phylloquinones,* 3 Fe_4S_4 *clusters and* 5 *carotenoids.*

They conclude that this structural information extends the understanding of the most efficient nano-photochemical machine in nature.

Bonardi et al. investigate whether photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases in green alga *Chlamydomonas* and the plant *Arabidopsis* in a paper with 138 citations [50.56]. They note that illumination changes elicit modifications of thylakoid proteins and reorganization of the photosynthetic machinery. This involves, in the short term, phosphorylation of photosystem II (PSII) and light-harvesting (LHCII) proteins. PSII phosphorylation is thought to be relevant for PSII turnover, whereas LHCII phosphorylation is associated with the relocation of LHCII and the redistribution of excitation energy (state transitions) between photosystems. In the long term, imbalances in energy distribution between photosystems are counteracted by adjusting photosystem stoichiometry. They find that in the green alga *Chlamydomonas* and the plant *Arabidopsis*, *state transitions require the orthologous protein kinases STT7 and STN7, respectively*. They show that:

in Arabidopsis a second protein kinase, STN8, is required for the quantitative phosphorylation of PSII core proteins.

However:

PSII activity under high-intensity light is affected only slightly in stn8 mutants, and D1 turnover is indistinguishable from the wild type, implying that reversible protein phosphorylation is not essential for PSII repair.

They conclude that:

acclimation to changes in light quality is defective in stn7 but not in stn8 mutants, indicating that short-term and long-term photosynthetic adaptations are coupled.

Therefore, the phosphorylation of LHCII, or of an unknown substrate of STN7, is also crucial for the control of photosynthetic gene expression.

Zak et al. study the initial steps of biogenesis of cyanobacterial photosystems occur in plasma membranes in cyanobacteria in a paper with 101 citations [50.57]. They note that during oxygenic photosynthesis in cyanobacteria and chloroplasts of plants and eukaryotic algae, conversion of light energy to biologically useful chemical energy occurs in the specialized thylakoid membranes. Light-induced charge separation at the reaction centers of photosystems I and II, two multisubunit pigment–protein complexes in the thylakoid membranes, energetically drive sequential photosynthetic electron transfer reactions in this membrane system. In general, in the prokaryotic cyanobacterial cells, the thylakoid membrane is distinctly different from the plasma membrane. They have recently developed a two-dimensional separation procedure to purify thylakoid and plasma membranes from the genetically widely studied cyanobacterium *Synechocystis* sp. PCC 6803. Immunoblotting analysis demonstrates that the purified plasma membrane contained a number of protein components closely associated with the reaction centers of both photosystems. Moreover:

these proteins are assembled in the plasma membrane as chlorophyll-containing multiprotein com-

plexes, as evidenced from nondenaturing green gel and low-temperature fluorescence spectroscopy data.

Furthermore, electron paramagnetic resonance spectroscopic analysis shows that:

in the partially assembled photosystem I core complex in the plasma membrane, the P700 reaction center is capable of undergoing light-induced charge separation.

Based on these data, they propose that:

the plasma membrane, and not the thylakoid membrane, is the site for a number of the early steps of biogenesis of the photosynthetic reaction center complexes in these cyanobacterial cells.

50.2.3 Conclusion

The research on the algal photosynthesis has been one of the most dynamic research areas in recent years with 2011 papers and with H-index of 97. Ten citation classics in the field of algal photosynthesis with more than 100 citations were located and the key emerging issues from these papers were presented above in the decreasing order of the number of citations [50.48–57]. These papers give strong hints about the structural determinants of the algal photosynthesis, informing the following sections. Photosystems I and II (PSI and PSII) which reaction centers that capture light energy in order to drive oxygenic photosynthesis played an important role in this field of research.

50.3 Algal Biofuels

50.3.1 Introduction

The research on the algal biodiesel has been one of the most dynamic research areas in recent years with 909 papers and H-index of 54. Fifteen citation classics in the field of algal biodiesel with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.10, 11, 58–70]. These papers give strong hints about the determinants of the efficient algal biofuel production and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

The research on the algal biohydrogen has been one of the most dynamic research areas in recent years with 144 papers and H-index of 25. Seven citation classics in the field of algal biohydrogen with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.12, 71–76]. These papers give strong hints about the determinants of the efficient algal biohydrogen production and emphasize that marine algae are efficient biohydrogen feedstocks.

The research on the algal bio-oil has been one of the most dynamic research areas in recent years with 89 papers and H-index of 21. Three citation classics in the field of algal bio-oil with more than 100 citations are located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.77–79]. These papers give strong hints about the determinants of the efficient algal bio-oil production and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

50.3.2 Algal Biodiesel

Introduction

The research on the algal biodiesel has been one of the most dynamic research areas in recent years. Nine hundred and nine papers were located as indexed in the SCIE and SSCI as of February 2013. The United States and China produced over 45% of these papers whilst Chinese Academy of Sciences was the most prolific institution. The research output increased significantly in the last decade with 346 papers in 2012. In comparison with the research field of algal photosynthesis, it is a new research field as over 90% of the papers were published after 2008. *Bioresource Technology* was the most prolific journal publishing over 19% of the papers whilst nearly 50% and 46% of the papers were indexed under the subject category of *Biotechnology & Applied Microbiology* and *Energy & Fuels*, respectively. The citation impact of the papers on the algal biodiesel has been significant with 14.1 citations per paper and H-index of 54.

Fifteen citation classics in the field of algal biodiesel with more than 100 citations have been located and the

key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.10, 11, 58–70]. These papers give strong hints about the determinants of the efficient algal biofuel production and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

Research on the Algal Biodiesel

Chisti reviews the literature on the algal biodiesel in a paper with 1039 citations. He notes that renewable, carbon neutral, and transport fuels are necessary for environmental and economic sustainability as an alternative to the fossil-based fuels [50.10]. He argues that biodiesel derived from the biomass is a potential renewable and carbon neutral alternative to the fossil-based fuels and in comparison with oil crops, waste cooking oil, and animal fat, microalgae are the only source of renewable biodiesel as transport fuels. He notes that like plants, microalgae use sunlight to produce oils through the photosynthetic conversion in a more efficient way. He finds that oil productivity of many microalgae greatly exceeds the oil productivity of the best producing oil crops and there are a number of methodological approaches for making microalgal biodiesel economically competitive with petroleum-based diesel.

Chisti further reviews the literature on the algal biodiesel in a paper with 338 citations. He notes that renewable biofuels are needed to displace fossil-based transport fuels, which contribute to global warming and are scarce [50.11]. He points out that biodiesel and bioethanol are the two potential renewable fuels that have attracted the most attention. However, biodiesel and bioethanol produced from agricultural crops using existing methods cannot sustainably replace fossil-based transport fuels, but there is a viable alternative. He argues that algal biodiesel is perhaps the only renewable biofuel that has the potential to completely displace fossil-based transport fuels without adversely affecting supply of food and other crop product as a third-generation biofuel. Most productive oil crops, such as oil palm, do not come close to microalgae in being able to sustainably provide the necessary amounts of biodiesel and bioethanol from sugarcane is no match for microalgal biodiesel as the first- and second-generation biofuels.

Demirbas reviews the literature on the biomass resource facilities and biomass conversion processing for biofuels and biochemicals [50.58]. He notes that biomass resources include wood and wood wastes, agricultural crops and their waste byproducts, municipal solid waste, animal wastes, waste from food processing, and aquatic plants as well as algae. He further notes that biomass is used to meet a variety of energy needs, including generating electricity, heating homes, fueling vehicles, and providing process heat for industrial facilities. He separates the conversion technologies for utilizing biomass into four basic categories: direct combustion processes, thermochemical processes, biochemical processes, and agrochemical processes. Furthermore, he subdivides thermochemical conversion processes into gasification, pyrolysis, supercritical fluid extraction, and direct liquefaction. On the other hand, pyrolysis is:

> *the thermochemical process that converts biomass into liquid, charcoal and noncondensable gases, acetic acid, acetone, and methanol by heating the biomass to about* 750 K *in the absence of air.*

He argues that to maximize the yield of liquid products resulting from biomass pyrolysis, a low temperature, high heating rate, short gas residence time process are necessary. On the other hand, for high char production, a selection of a low temperature and low heating rate process are necessary. However, to maximize the yield of fuel gas resulting from pyrolysis, a high temperature, low heating rate, and long gas residence time process are required.

Mata et al. recently reviews the literature on the biodiesel production from microalgae and other applications in a paper with 285 citations [50.59]. They note that sustainable production of renewable energy is a recent public policy issue since the first-generation biofuels, primarily produced from food crops, and mostly oil seeds are limited in their ability to achieve targets for biofuel production, climate change mitigation, and economic growth. They argued that these concerns about the first- and second-generation biofuels have increased the interest in developing third-generation biofuels produced from nonfood feedstocks such as microalgae, which potentially offer greatest opportunities in the longer term. They review the current status of microalgae use for biodiesel production, including their cultivation, harvesting, and processing. They present the microalgae species most used for biodiesel production and describe their main advantages in comparison with other available biodiesel feedstocks. They further focus on the various aspects associated with the design of microalgae production units, giving an overview of the current state of development of algae cultivation systems such as photobioreactors and open ponds.

They also discuss the other potential applications and products from microalgae such as for biological sequestration of CO_2 wastewater treatment, in human health, as food additive, and for aquaculture.

Miao and *Wu* study biodiesel production from heterotrophic microalgal oil in a paper with 295 citations [50.60]. They introduce an integrated method for the production of biodiesel from microalgal oil. They find that heterotrophic growth of *Chlorella protothecoides* result in the accumulation of high lipid content (55%) in cells and they extract large amount of microalgal oil from these heterotrophic cells by using *n*-hexane. They obtain algal biodiesel comparable to traditional diesel from heterotrophic microalgal oil by acidic transesterification. They point out that the best process combination is

> 100% *catalyst quantity based on oil weight with* 56 : 1 *molar ratio of methanol to oil at temperature of* 30 °C*, which reduced product specific gravity from an initial value of* 0.912 *to a final value of* 0.8637 *in about* 4 h *of reaction time.*

They conclude that the new integrated process, which combined bioengineering and transesterification, is a feasible and effective method for the production of high quality biodiesel from microalgal oil.

Schenk et al. discuss second-generation biofuels: high-efficiency microalgae for biodiesel production from the third-generation biofuel feedstocks in a paper with 258 citations [50.61]. They note that the use of fossil fuels is unsustainable due to depleting resources and the accumulation of greenhouse gases in the environment that have already exceeded the *dangerously high* threshold of 450 ppm CO_2–*e*. They further note that to achieve environmental and economic sustainability, fuel production processes are required that are not only renewable, but also capable of sequestering atmospheric CO_2 and currently, nearly all renewable energy sources such as hydroelectric, solar, wind, tidal, and geothermal energy sources target the electricity market, while fuels make up a much larger share of the global energy demand. The market demand for biofuels resulted in the third-generation microalgal systems having the advantage that they can produce a wide range of feedstocks for the production of biodiesel, bioethanol, biomethane, and biohydrogen. They argue that producing biodiesel from algae is one of the most efficient ways of generating biofuels and represents the only current renewable source of oil that could meet the global market demand for transport fuels. They conclude that the main advantages of third-generation microalgal systems are that:

> *they have a higher photon conversion efficiency as evidenced by increased biomass yields per hectare; they can be harvested batch-wise nearly all-year-round, providing a reliable and continuous supply of oil; they can utilize salt and waste water streams, thereby greatly reducing freshwater use; they can couple* CO_2*-neutral fuel production with* CO_2 *sequestration; and they finally produce nontoxic and highly biodegradable biofuels.*

However, the harvesting process and in the supply of CO_2 for high efficiency production limit the efficient algal biofuel production.

Brennan and *Owende* recently review the literature on the algal biofuels with a focus on the technologies for production, processing, and extractions of algal biofuels and co-products in a paper with 209 citations [50.62]. They note that sustainability is a key principle in natural resource management, and it involves operational efficiency, minimization of environmental, impact and socioeconomic considerations where all of them are interdependent. The continued reliance on fossil fuel energy resources is unsustainable, owing to both depleting world reserves and the green house gas emissions associated with their use. Therefore, there are large-scale research initiatives to develop alternative renewable and potentially carbon neutral solid, liquid, and gaseous biofuels as alternative energy resources. However, alternate energy resources based on the first-generation biofuels derived from land crops such as sugarcane, sugar beet, maize, and rapeseed place an enormous strain on world food markets, contribute to water shortages and precipitate the destruction of the world's forests. Second-generation biofuels derived from lignocellulosic agriculture and forest residues and from nonfood crop feedstocks address some of the above problems although there is still concern over competing land use or required land use changes. Therefore, they argue that third-generation biofuels specifically derived from microalgae are a technically viable alternative energy resource that is devoid of the major drawbacks associated with first- and second-generation biofuels. They note that microalgae are photosynthetic microorganisms with simple growing requirements such as light, sugars, CO_2, N, P, and K that can produce lipids, proteins, and carbohydrates in large amounts over short periods of time and these prod-

ucts can be processed into both biofuels and valuable co-products. They review:

> *the technologies underpinning microalgae-to-biofuels systems, focusing on the biomass production, harvesting, conversion technologies, and the extraction of useful co-products.*

They also review the

> *synergistic coupling of microalgae propagation with carbon sequestration and wastewater treatment potential for mitigation of environmental impacts associated with energy conversion and utilization.*

They conclude that although there are outstanding issues related to photosynthetic efficiencies and biomass output, algal biofuels can progressively substitute a significant proportion of the fossil fuels required to meet the growing global energy demand.

Xu et al. investigate high quality biodiesel production from a microalga *C. protothecoides* by heterotrophic growth in fermenters in a paper with 182 citations [50.63]. They produce high quality biodiesel from a microalga *C. protothecoides* through the technology of transesterification. They apply the technique of metabolic controlling through heterotrophic growth of *C. protothecoides*, and obtain the heterotrophic *C. protothecoides* with the crude lipid content of 55.2%. They use corn powder hydrolysate instead of glucose as organic carbon source in heterotrophic culture medium in fermenters to increase the biomass and reduce the cost of alga. They find that cell density significantly increases under the heterotrophic condition, and the highest cell concentration reach $15.5\,g\,L^{-1}$. They extract large amount of microalgal oil from the heterotrophic cells by using *n*-hexane, and then transmute into biodiesel by acidic transesterification. The algal biodiesel has a high heating value of $41\,MJ\,kg^{-1}$, a density of $0.864\,kg\,L^{-1}$, and a viscosity of 5.2×10^{-4} Pa s at 40 °C. They conclude that the transesterification method has great potential in the industrial production of liquid fuel from microalga.

Gouveia and *Oliveira* investigate biofuels production from microalgae – *Chlorella vulgaris*, *Spirulina maxima*, *Nannochloropsis* sp., *Neochloris oleabundans*, *Scenedesmus obliquus* and *D. tertiolecta* – in a paper with 149 citations [50.64]. They note that market demand for biofuels is substantial in order to reduce gaseous emissions such as fossil CO_2, nitrogen, and sulfur oxides and their greenhouse, climatic changes and global warming effects, to reduce energy dependence, contributing to security of supply, promoting environmental sustainability, and meeting the EU target of at least of 10% biofuels in the transport sector by 2020. Although the biodiesel is usually produced from the first-generation biofuel feedstocks such as rapeseed, soybean, sunflower, and palm, microalgae is a suitable alternative feedstock as the third-generation biofuel feedstocks because certain species contain high amounts of oil, which could be extracted, processed, and refined into transportation fuels, using currently available technology. Microalgae have also:

> *fast growth rate, permit the use of nonarable land and nonpotable water, use far less water and do not displace food crops cultures*

and their production is not seasonal and they can be harvested daily. They screen microalgae – *C. vulgaris, S. maxima, Nannochloropsis* sp., *N. oleabundans*, *S. obliquus*, and *D. tertiolecta* – in order to choose the best one(s), in terms of quantity and quality as oil source for biofuel production. They find that:

> N. oleabundans – *fresh water microalga – and* Nannochloropsis *sp. – marine microalga – are suitable as raw materials for biofuel production, due to their high oil content*

at 29.0, and 28.7%, respectively. Both microalgae, when grown under nitrogen shortage, show a great increase by 50% in oil quantity. They conclude that:

> S. obliquus *have the most adequate fatty acid profile, namely in terms of linolenic and other polyunsaturated fatty acids*

when used alone. However, they argue that the microalgae *N. oleabundans*, *Nannochloropsis* sp., and *D. tertiolecta* can also be used if associated with other microalgal oils and/or vegetable oils.

Griffiths and *Harrison* investigate lipid productivity as a key characteristic for choosing algal species for biodiesel production in a paper with 123 citations [50.65]. They note that microalgae are a promising alternative source of lipid for biodiesel production and one of the most important decisions is the choice of species to use as the high lipid productivity is a key desirable characteristic of a species for biodiesel production. They review information available in the literature

on microalgal growth rates, lipid content, and lipid productivities for 55 species of microalgae, including 17 Chlorophyta, 11 Bacillariophyta, and 5 Cyanobacteria as well as others. They find that the data available in the literature are far from complete and rigorous comparison across experiments carried out under different conditions is not possible. However, they argue that:

> *the collated information provides a framework for decision-making and a starting point for further investigation of species selection.*

They highlight the shortcomings in the current dataset and show the importance of lipid productivity as a selection parameter over lipid content and growth rate individually.

Carvalho et al. provides a review of enclosed system designs and performances for microalgal photobioreactors in a paper with 122 citations [50.66]. They note that one major issue in:

> *industrial microalgal culturing is to devise and develop technical apparata, cultivation procedures and algal strains susceptible of undergoing substantial increases in efficiency of use of solar energy and carbon dioxide.*

They argue that there is no such thing as *the best reactor system* – defined, in an absolute fashion, as the one able to achieve maximum productivity with minimum operation costs, irrespective of the biological, and chemical system at stake. In fact, they argue that, choice of the most suitable system is situation dependent, as both the species of alga available and the final purpose intended play a role. As the need of accurate control impairs use of open-system configurations, current investigation has focused mostly on closed systems. They review several types of closed photobioreactors described in the technical literature to support production of microalgae using transport phenomenon and process engineering methodological approaches with a focus on the reactor design, which includes tubular reactors, flat plate reactors and fermenter-type reactors as well as the processing parameters, which include gaseous transfer, medium mixing, and light requirements.

Li and *Liu* review the literature on the perspectives of microbial oils for biodiesel production in a paper with 119 citations [50.67]. They note that biodiesel has become more attractive fuel recently because of its environmental benefits, and the fact that it is made from renewable resources and biodiesel is prepared through transesterification of first-generation biofuel feedstocks such as vegetable oils or animal fats with short chain alcohols. However, the lack of oil feedstocks limits the large-scale development of biodiesel to some extent. Recently, much attention has been paid to the development of microbial oils and many microorganisms, such as algae, yeast, bacteria, and fungi, have the ability to accumulate oils under some special cultivation conditions. They argue that:

> *compared to other plant oils, microbial oils have many advantages, such as short life cycle, less labor required, less affection by venue, season and climate, and easier to scale up.*

They predict that with the rapid expansion of biodiesel, microbial oils become one of potential oil feedstocks for biodiesel production in the future, though there are many limitations associated with microorganisms producing oils.

Sialve and *Bernard* review the literature on the anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable in a paper with 118 citations [50.68]. They note that the potential of microalgae as a source of biofuels and as a technological solution for CO_2 fixation has been studied immensely. They argue that in the perspective of setting up massive cultures, the management of large quantities of residual biomass and the high amounts of fertilizers must be considered and anaerobic digestion is a key process that can solve this immense issue as well as the economical and energetic balance of such a promising technology. Indeed, they find that the conversion of algal biomass after lipid extraction into methane is a process that can recover more energy than the energy from the cell lipids. They identify three limitations to digest microalgae. First, the biodegradability of microalgae can be low depending on both the biochemical composition and the nature of the cell wall. Second, the high cellular protein content results in ammonia release which can lead to potential toxicity. Finally, the presence of sodium for marine species can also affect the digester performance. They argue that:

> *physicochemical pretreatment, co-digestion, or control of gross composition are strategies that can significantly and efficiently increase the conversion yield of the algal organic matter into methane.*

They conclude that when the cell lipid content does not exceed 40%, anaerobic digestion of the whole biomass

is the optimal strategy on an energy balance basis, for the energetic recovery of cell biomass. Lastly, they discuss the ability of these CO_2 consuming microalgae to purify biogas and concentrate methane.

Dismukes et al. review the literature on the aquatic phototrophs as efficient alternatives to land-based crops for biofuels in a paper with 118 citations [50.69]. They propose the use of biofuels derived from aquatic microbial oxygenic photoautotrophs (AMOPs), more commonly known as cyanobacteria, algae, and diatoms to mitigate some of the potentially deleterious environmental and agricultural consequences associated with current land-based-biofuel feedstocks. They review their productivity in mass culturing and aspects of their physiology that are particularly attractive for integration into renewable biofuel applications. They argue that:

> *compared with terrestrial crops, AMOPs are inherently more efficient solar collectors, use less or no land, can be converted to liquid fuels using simpler technologies than cellulose, and offer secondary uses that fossil fuels do not provide.*

However, AMOPs pose a new set of technological challenges to be used as biofuel feedstocks.

Huang et al. discuss biodiesel production by microalgal biotechnology in a paper with 107 citations [50.70]. They note that although numerous reports are available on the production of biodiesel from the first generation biofuel feedstocks such as soybean, sunflower, and palm oils, the production of biodiesel from microalgae is a newly emerging field. They argue that microalgal biotechnology have high potential for biodiesel production because a significant increase in lipid content of microalgae is now possible through heterotrophic cultivation and genetic engineering approaches. They provide an:

> *overview of the technologies in the production of biodiesel from microalgae, including the various modes of cultivation for the production of oil-rich microalgal biomass, as well as the subsequent downstream processing for biodiesel production.*

Conclusion

The research on the algal biodiesel has been one of the most dynamic research areas in recent years with 909 papers and H-index of 54. Fifteen citation classics in the field of algal biodiesel with more than 100 citations were located and the key emerging issues from these papers were presented below in the decreasing order of the number of citations [50.10, 11, 58–70]. These papers give strong hints about the determinants of the efficient algal biofuel production and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

50.3.3 Algal Biohydrogen

Introduction

The research on the algal biohydrogen has been one of the most dynamic research areas in recent years. 144 papers were located as indexed in the SCIE and SSCI as of February 2013. The United States, India, and China produced over 48% of these papers whilst Chinese Academy of Sciences, Ruhr University, and University of California were the most prolific institutions. In comparison with the research field of algal photosynthesis, it is a new research field as over 80% of the papers were published after 2008 starting with only one paper in 1997 and ending up with 45 papers in 2011. *International Journal of Hydrogen Energy* was the most prolific journal publishing nearly 28% of the papers whilst nearly 54% and 42% of the papers were indexed under the subject category of *Energy & Fuels* and *Biotechnology & Applied Microbiology*, respectively. The citation impact of the papers on the algal biodiesel has been significant with 22.7 citations per paper and H-index of 25.

Seven citation classics in the field of algal biohydrogen with more than 100 citations were located and the key emerging issues from these papers were presented below in the decreasing order of the number of citations [50.12, 71–76]. These papers give strong hints about the determinants of the efficient algal biohydrogen production and emphasize that marine algae are efficient biohydrogen feedstocks.

Research on the Algal Biohydrogen

Das and *Veziroglu* provide one of the first surveys of the literature on the algal biohydrogen in a paper with 676 citations [50.71]. They note that hydrogen is a major alternative to fossil-based fuels due to its high conversion efficiency, recyclability, and nonpolluting nature. Furthermore, biological hydrogen production processes are more environment friendly and less energy intensive as compared to thermochemical and electrochemical processes. They are mostly controlled by either photosynthetic or fermentative organisms whilst nitrogenase and hydrogenase play very important role. They find

that genetic manipulation of cyanobacteria – hydrogenase negative gene – improves the hydrogen generation. They further find that the focus of the research is on the microorganisms and biochemical pathways involved in hydrogen-generation processes and they argue that immobilized system is suitable for the continuous hydrogen production. They conclude that as about 28% of energy can be recovered in the form of hydrogen using sucrose as substrate, fermentative hydrogen production processes have some edge over the other biological processes.

Kapdan and *Kargi* study biohydrogen production from waste materials in a paper with 357 citations [50.12]. They note that hydrogen is a valuable gas as a clean energy source and as feedstock for some industries resulting in an increased market demand for hydrogen production in recent years. They argue that although electrolysis of water, steam reforming of hydrocarbons and auto-thermal processes are the usual methods for hydrogen gas production, they are not cost-effective due to high energy requirements. On the other hand, they argue that biological production of hydrogen gas has significant advantages over chemical methods:

> *The major biological processes utilized for hydrogen gas production are biophotolysis of water by algae, dark and photofermentation of organic materials, usually carbohydrates by bacteria.*

However, *sequential dark and photofermentation process is a rather new approach for bio-hydrogen production* although it has raw material cost limitations. They argue that:

> *carbohydrate rich, nitrogen deficient solid wastes such as cellulose and starch containing agricultural and food industry wastes and some food industry wastewater such as cheese whey, olive mill, and baker's yeast industry wastewater can be used for hydrogen production by using suitable bioprocess technologies.*

Utilization of these wastes for hydrogen production provides inexpensive energy generation with simultaneous waste treatment. They conclude that the types of potential waste materials, bioprocessing strategies, microbial cultures to be used, and bioprocessing conditions are the important parameters in the production of hydrogen gas from algae and waste materials.

Ghirardi et al. review the literature on the microalgae as a green source of renewable hydrogen in a paper with 217 citations [50.72]. They summarize recent advances in the field of algal hydrogen production. They find that there are two fundamental approaches for the algal biohydrogen production. The former one involves the temporal separation of the usually incompatible reactions of O_2 and H_2 production in green algae, and the latter one involves the use of classical genetics to increase the O_2 tolerance of the reversible hydrogenase enzyme. They also discuss the economic and environmental impact of a renewable source of H_2.

Barbosa et al. investigate acetate as a carbon source for hydrogen production by photosynthetic bacteria in a paper with 171 citations [50.73]. They note that hydrogen is a clean energy alternative to fossil-based fuels and photosynthetic bacteria produce hydrogen from organic compounds by an anaerobic light-dependent electron transfer process. They look into the:

> *hydrogen production by three photosynthetic bacterial strains* – Rhodopseudomonas *sp.,* Rhodopseudomonas palustris *and a nonidentified strain–, from four different short-chain organic acids – lactate, malate, acetate, and butyrate.*

They also study the effect of light intensity on hydrogen production by supplying two different light intensities, using acetate as the electron donor and they compare hydrogen production rates and light efficiencies. They find that:

> Rhodopseudomonas *sp. produce the highest volume of* H_2 *where this strain reach a maximum* H_2 *production rate of* 25 ml H_2 L^{-1} h^{-1}, *under a light intensity of* 680 µmol photons m^{-2} s^{-1}, *and a maximum light efficiency of* 6.2% *under a light intensity of* 43 pmol photons m^{-2} s^{-1}.

Furthermore, they find that *a decrease in acetate concentration from* 22–11 mM *result in a decrease in the hydrogen evolved from* 214–27 ml H_2 *per vessel.*

Ghirardi et al. study the oxygen sensitivity of algal hydrogen production in an early paper with 108 citations [50.74]. They note that photoproduction of H_2 by green algae utilizes electrons originating from the photosynthetic oxidation of water and does not require metabolic intermediates. However, they find that algal hydrogenases are extremely sensitive to O_2, which limits their usefulness in future commercial H_2 production systems. They design:

> *an experimental technique for the selection of* O_2*-tolerant,* H_2*-producing variants of* C. reinhardtii *based on the ability of wild-type cells to survive*

a short (20 min) exposure to metronidazole in the presence of controlled concentrations of O_2.

They find that the number of survivors depends on the metronidazole concentration, light intensity, preinduction of the hydrogenase, and the presence or absence of O_2. Finally, they demonstrate that some of the selected survivors in fact exhibit H_2-production capacity that is less sensitive to O_2 than the original wild-type population.

Melis studies green alga hydrogen production with a focus on the progress, challenges and prospects in a paper with 107 citations [50.75]. He argues that:

the recently developed single-organism, two-stage photosynthesis and H_2*-production protocol with green algae is of fundamental importance because it reveals the occurrence of hitherto unknown metabolic, regulatory, and electron-transport pathways in the green alga* C. reinhardtii.

It is also of practical importance that the sustainable light-dependent production and accumulation of significant amounts of H_2 gas, generated from sunlight and water. He argues that this method may serve as a tool to probe and improve photobiological hydrogen production. He concludes that:

the long-term advantage of photobiological hydrogen production is that it does not entail the generation of any toxic or polluting byproducts and it may even offer the advantage of value-added products as a result of the mass cultivation of green algae.

These issues are critical for the long-term success of a renewable hydrogen production process.

Kosourov et al. investigate sustained hydrogen photoproduction by *C. reinhardtii* with a focus on the effects of culture parameters in a paper with 107 citations [50.76]. They note that the green alga, *C. reinhardtii*, is capable of sustained H_2 photoproduction when grown under sulfur-deprived conditions as a result of the partial deactivation of photosynthetic O_2-evolution activity in response to sulfur deprivation. At these reduced rates of water oxidation, oxidative respiration under continuous illumination can establish an anaerobic environment in the culture. They find that after 10–15 h of anaerobiosis, sulfur-deprived algal cells induce a reversible hydrogenase and start to evolve H_2 gas in the light. Using a computer-monitored photobioreactor system, they investigate the behavior of sulfur-deprived algae and find that:

(1) The cultures transition through five consecutive phases: an aerobic phase, an O_2*-consumption phase, an anaerobic phase, a* H_2*-production phase and a termination phase.*
(2) Synchronization of cell division during pregrowth with 14 : 10 h *light: dark cycles leads to earlier establishment of anaerobiosis in the cultures and to earlier onset of the* H_2*-production phase.*
(3) Re-addition of small quantities of sulfate (12.5–50 μM $MgSO_4$*, final concentration) to either synchronized or unsynchronized cell suspensions results in an initial increase in culture density, a higher initial specific rate of* H_2 *production, an increase in the length of the* H_2*-production phase, and an increase in the total amount of* H_2 *produced.*
(4) Increases in the culture optical density in the presence of 50 μM *sulfate result in a decrease in the initial specific rates of* H_2 *production and in an earlier start of the* H_2*-production phase with unsynchronized cells.*

They conclude that:

the effects of sulfur re-addition on H_2 *production, up to an optimal concentration, are due to an increase in the residual water-oxidation activity of the algal cells.*

They also conclude that, in principle:

cells synchronized by growth under light: dark cycles can be used in an outdoor H_2*-production system without loss of efficiency compared to cultures that up until now have been pregrown under continuous light conditions.*

Conclusion

The research on the algal biohydrogen has been one of the most dynamic research areas in recent years with 144 papers and H-index of 25. Seven citation classics in the field of algal biohydrogen with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.12, 71–76]. These papers give strong hints about the determinants of the efficient algal biohydrogen production and emphasize that marine algae are efficient biohydrogen feedstocks.

50.3.4 Algal Bio-oil

Introduction

The research on the algal bio-oil has been one of the most dynamic research areas in recent years. Eighty nine papers were located as indexed in the SCIE and SSCI as of February 2013. The United States and China produced over 49% of these papers whilst University of Michigan and Tsing Hua University were the most prolific institutions. In comparison with the research field of algal photosynthesis, it is a new research field like algal biohydrogen as over 86% of the papers were published after 2008. *Bioresource Technology* and *Energy Fuels* were the most prolific journals publishing over 29% and 11% of the papers, respectively, whilst nearly 68% and 37% of the papers were indexed under the subject category of *Energy & Fuels* and *Biotechnology & Applied Microbiology*, respectively. The citation impact of the papers on the algal biodiesel has been significant with 16.0 citations per paper and H-index of 21.

Three citation classics in the field of algal bio-oil with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.77–79]. These papers give strong hints about the determinants of the efficient algal bio-oil production and emphasize that algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

Research on the Algal Bio-oil

Rodolfi et al. investigate microalgae for bio-oil with a focus on the strain selection, induction of lipid synthesis, and outdoor mass cultivation in a low-cost photobioreactor in a paper with 256 citations [50.77]. They screen 30 microalgal strains in the laboratory for their biomass productivity and lipid content. They select four strains – two marine and two freshwater – since they are robust, highly productive, and with a relatively high lipid content and cultivate them under nitrogen deprivation in 0.6 L bubbled tubes. They find that only the two marine microalgae accumulate lipid under such conditions. One of them, the eustigmatophyte *Nannochloropsis* sp. F&M-M24, which attain 60% lipid content after nitrogen starvation, is grown in a 20 L Flat Alveolar Panel photobioreactor to study the influence of irradiance and nutrient – nitrogen or phosphorus – deprivation on fatty acid accumulation. They find that fatty acid content increases with high irradiances up to 32.5% of dry biomass and following both nitrogen and phosphorus deprivation up to about 50%. To evaluate its lipid production potential under natural sunlight, the strain is grown outdoors in 110 L Green Wall Panel photobioreactors under nutrient sufficient and deficient conditions. They find that:

> *lipid productivity increased from* 117 mg L^{-1} d^{-1} *in nutrient sufficient media with an average biomass productivity of* 0.36 g L^{-1} d^{-1} *and* 32% *lipid content to* 204 mg L^{-1} d^{-1} *with an average biomass productivity of* 0.30 g L^{-1} d^{-1} *and more than* 60% *final lipid content in nitrogen deprived media.*

They find further

> *in a two-phase cultivation process which is nutrient sufficient phase to produce the inoculum followed by a nitrogen deprived phase to boost lipid synthesis, the oil production potential can be projected to be more than* 90 kg ha^{-1} d^{-1}.

They provide the first report of an increase of both lipid content and areal lipid productivity attained through nutrient deprivation in an outdoor algal culture. They conclude that this

> *marine eustigmatophyte has the potential for an annual production of* 20 metric tons *of lipid per hectare in the Mediterranean climate and of more than* 30 tons lipid ha^{-1} *in sunny tropical areas.*

Miao and *Wu* study high-yield bio-oil production from fast pyrolysis by metabolic controlling of *C. protothecoides* in a paper with 105 citations [50.78]. They note that the use of renewable energy sources is increasingly necessary to mitigate global warming and recently much research has been focused on identifying suitable biomass species, which can provide high-energy outputs, to replace conventional fossil fuels. They report an approach for increasing the yield of bio-oil production from fast pyrolysis after manipulating the metabolic pathway in microalgae through heterotrophic growth. They find that the yield of bio-oil (57.9%) produced from heterotrophic *C. protothecoides* cells is 3.4 times higher than from autotrophic cells by fast pyrolysis and the bio-oil has a much lower oxygen content, with a higher heating value (41 MJ kg^{-1}), a lower density (0.92 kg L^{-1}), and lower viscosity (0.02 Pa s) compared to those of bio-oil from autotrophic cells and wood. They note that these properties of the bio-oil are comparable to fossil oil. They conclude that the research could contribute to the creation of a system to produce

bio-oil from microalgae, and microalgae also can have great commercial potential for bio-oil production.

Minowa et al. investigate bio-oil production from algal cells of *D. tertiolecta* by direct thermochemical liquefaction in a paper with 105 citations [50.79]. They convert *algal cells of D. tertiolecta with a moisture content of* 78.4 wt% *directly into bio-oil by thermochemical liquefaction at around* 300 °C *and* 10 MPa and they find that the oil yield is about 37% on an organic basis. They conclude that the bio-oil obtained at a reaction temperature of 340 °C and holding time of 60 min have a viscosity of 150–330 mPa s and a calorific value of 36 kJ g^{-1}, comparable to those of fuel oil.

Conclusion

The research on the algal bio-oil has been one of the most dynamic research areas in recent years with 89 papers and H-index of 21. Three citation classics in the field of algal bio-oil with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.77–79]. These papers give strong hints about the determinants of the efficient algal bio-oil production and emphasize that algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

50.3.5 Conclusion

The research on the algal biodiesel has been one of the most dynamic research areas in recent years with 909 papers and H-index of 54. Fifteen citation classics in the field of algal biodiesel with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.10, 11, 58–70]. These papers give strong hints about the determinants of the efficient algal biofuel production and emphasize that algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

The research on the algal biohydrogen has been one of the most dynamic research areas in recent years with 144 papers and H-index of 25. Seven citation classics in the field of algal biohydrogen with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.12, 71–76]. These papers give strong hints about the determinants of the efficient algal biohydrogen production and emphasize that algae are efficient biohydrogen feedstocks.

The research on the algal bio-oil has been one of the most dynamic research areas in recent years with 89 papers and H-index of 21. Three citation classics in the field of algal bio-oil with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.77–79]. These papers give strong hints about the determinants of the efficient algal bio-oil production and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

50.4 Algal Biotechnology

50.4.1 Introduction

The research on the algal biotechnology has been one of the most dynamic research areas in recent years. 455 papers were located as indexed in the SCIE and SSCI as of February 2013. The United States and Germany produced over 31% of these papers whilst Wageningen University, Ben Gurion University, and University of California were the most prolific institutions. In comparison with the research fields of algal biodiesel, algal biohydrogen, and algal bio-oil, it is a more established research field as only 53% of the papers were published after 2008 with papers starting back in 1984. *Journal of Applied Phycology* and *Journal of Bioscience and Bioengineering* were the most prolific journals publishing over 8% and 4% of the papers, respectively, whilst nearly 48% and 17% of the papers were indexed under the subject category of *Biotechnology and Applied Microbiology*, and *Marine and Freshwater Biology*, respectively. The citation impact of the papers on the algal biodiesel has been significant with 24.1 citations per paper and H-index of 55.

Eighteen citation classics in the field of algal biotechnology with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.13, 80–96]. These papers give strong hints about the determinants of the efficient algal biotechnology applications and emphasize that marine algae are more efficient biofuel feedstocks in com-

parison with the first- and second-generation biofuel feedstocks necessitating the efficient design of the algal photobioreactors.

50.4.2 Research on the Algal Biotechnology

Spolaore et al. reviews the literature on the commercial applications of microalgae in a paper with 382 citations [50.13]. They note that although the first consumer use of microalgae by humans dates back 2000 years to the Chinese, who used *Nostoe* to survive during famine, microalgal biotechnology only really began to develop in the middle of the last century and nowadays, there are numerous commercial applications of microalgae for consumers. For example, microalgae can be used to enhance the nutritional value of food and animal feed owing to their chemical composition; they play a crucial role in aquaculture; and they can be incorporated into cosmetics. Moreover, they are cultivated as a source of highly valuable molecules. For example, polyunsaturated fatty acid oils are added to infant formulas and nutritional supplements and pigments are important as natural dyes. Stable isotope biochemicals help in structural determination and metabolic studies. They conclude that microalgal products would in that way become even more diversified and economically competitive with a focused research on the improvement of production systems and the genetic modification of strains.

Borowitzka study the commercial production of microalgae with a focus on the ponds, tanks, tubes, and fermenters in a paper with 241 citations [50.80]. He notes that the commercial culture of microalgae is now over 50 years old with the main microalgal species grown being *Chlorella* and *Spirulina* for health food, *Dunaliella salina* for p-carotene, *Haematococcus pluvialis* for astaxanthin and several species for aquaculture. The culture systems used in the 1990s to grow these algae are generally fairly unsophisticated. For example, *D. salina* is cultured in large (up to approx. 250 ha) shallow open-air ponds with no artificial mixing. Similarly, *Chlorella* and *Spirulina* also are grown outdoors in either paddle-wheel mixed ponds or circular ponds with a rotating mixing arm of up to about 1 ha in area per pond. The production of microalgae for aquaculture is generally on a much smaller scale, and in many cases is carried out indoors in 20–40 L carboys or in large plastic bags of up to approximately 1000 L in volume. Furthermore, a helical tubular photobioreactor system has been developed which allows these algae to be grown reliably outdoors at high cell densities in semi-continuous culture and other closed photobioreactors such as flat panels are also developed. He argues that the main problem facing the commercialization of new microalgae and microalgal products is the need for closed culture systems and the fact that these are very capital intensive as the high cost of microalgal culture systems relates to the need for light and the relatively slow growth rate of the algae. He concludes that although this problem has been avoided in some instances by growing the algae heterotrophically, not all algae or algal products can be produced this way.

Lorenz and *Cysewski* discuss the commercial potential for *Haematococcus microalgae* as a natural source of astaxanthin in a paper with 237 citations [50.81]. They note that as a result of high production costs, commercial products from microalgae have high prices. Astaxanthin produced by *Haematococcus* is a product that has become a commercial reality through novel and advanced technology. They further note that cultivation methods have been developed to produce *Haematococcus* containing 1.5–3.0% astaxanthin by dry weight, with potential applications as a pigment source in aquaculture, poultry feeds and in the worldwide nutraceutical market.

Pulz and *Gross* review the literature on the commercial products from biotechnology of microalgae in a paper with 222 citations [50.82]. They note that the biotechnology of microalgae has gained considerable importance in recent decades as the applications range from simple biomass production for food and feed to valuable products for ecological applications. However, for most of these applications, the market is still developing and the biotechnological use of microalgae will extend into new areas. They argue that considering the enormous biodiversity of microalgae and recent developments in genetic engineering, microalgae represent one of the most promising sources for new products and applications. They conclude that with the development of sophisticated culture and screening techniques, microalgal biotechnology can already meet the high demands of both the food and pharmaceutical industries.

Grima et al. reviews the literature on the recovery of microalgal biomass and metabolites with a focus on the process options and economics in a paper with 213 citations [50.83]. They note that:

> *commercial production of intracellular microalgal metabolites requires large-scale monoseptic production of the appropriate microalgal biomass, recovery of the biomass from a relatively dilute broth,*

extraction of the metabolite from the biomass, and purification of the crude extract.

They examine the options available for recovery of the biomass and the intracellular metabolites from the biomass and they discuss the economics of monoseptic production of microalgae in photobioreactors and the downstream recovery of metabolites using eicosapentaenoic acid (EPA) recovery as a representative case study.

Pulz reviews the literature on the algal photobioreactors with a focus on the production systems for phototrophic microorganisms in a paper with 182 citations [50.84]. He notes that microalgae have a large biotechnological potential for producing valuable substances for the feed, food, cosmetics, and pharmacy industries as well as for biotechnological processes whilst the design of the technical and technological basis for algal photobioreactors is the most important issue for economic success in the field of phototrophic biotechnology. He argues that for future applications, open pond systems for large-scale production seem to have a lower innovative potential than closed systems. He concludes that for high-value products in particular, closed systems of algal photobioreactors are more promising fields for technical developments despite very different approaches in design.

Schwarz et al. investigate the production of the pharmaceuticals from cultured algae in a paper with 177 citations [50.85]. They undertake an algae screening program, including cultured macroalgae, cultured cyanobacteria, and cultured eukaryotic microalgae and develop methods for the isolation, purification, preservation, and cultivation of axenic cyanobacteria and eukaryotic cultures. They find that screening of these groups of algae for biologically active components has lead to the isolation of pachydictyol and caulerpenyne from cultured macroalgae, while they have isolated a series of hapalindoles and an antifungal depsipeptide from cyanobacteria

Borowitzka discusses microalgae as sources of pharmaceuticals and other biologically active compounds in a paper with 137 citations [50.86]. He notes that in the 1980s the screening of microalgae, especially the cyanobacteria (blue–green algae), for antibiotics and pharmacologically active compounds has received substantial interest. A large number of antibiotic compounds, many with novel structures, have been isolated and characterized. Similarly many cyanobacteria have been shown to produce antiviral and antineoplastic compounds and a range of pharmacological activities have also been observed with extracts of microalgae, although the active principles are as yet unknown in most cases. He predicts that several of the bioactive compounds may find application in human or veterinary medicine or in agriculture whilst others should find application as research tools or as structural models for the development of new drugs. He concludes that:

the microalgae are particularly attractive as natural sources of bioactive molecules since these algae have the potential to produce these compounds in culture which enables the production of structurally complex molecules which are difficult or impossible to produce by chemical synthesis.

Smit reviews the literature on the medicinal and pharmaceutical uses of seaweed natural products in a paper with 136 citations [50.87]. He notes that in the last four decades the discovery of metabolites with biological activities from macroalgae has increased significantly although very few products with real potential have been identified or developed. Based on MEDLINE and Aquatic Biology, Aquaculture & Fisheries Resources databases, he searches the literature for natural products from marine macroalgae in the Rhodophyta, Phaeophyta, and Chlorophyta with biological and pharmacological activity. He finds that substances that currently receive most attention from pharmaceutical companies for use in drug development, or from researchers in the field of medicine-related research include sulphated polysaccharides as antiviral substances, halogenated furanones from *Delisea pulchra* as antifouling compounds, and kahalalide F from a species of Bryopsis as a possible treatment of lung cancer, tumors, and AIDS (acquired immune deficiency syndrome). Furthermore, other substances such as macroalgal lectins, fucoidans, kainoids, and aplysiatoxins are routinely used in biomedical research and a multitude of other substances have known biological activities. He then discusses the potential pharmaceutical, medicinal, and research applications of these compounds.

Kobayashi et al. study astaxanthin production by a green-alga, *H. pluvialis* accompanied with morphological changes in acetate media in a paper with 121 citations [50.88]. They develop two acetate containing media for astaxanthin production by a green unicellular alga, *H. pluvialis*. They find that the basal medium, a vegetative growth medium facilitate the algal cell growth, whereas the modified medium induces morphological changes with the formation of

large cysts and bleached cells which seemed to consequently enhance the carotenoid biosynthesis. They further find that in the two-stage culture, the injection of ferrous ion with acetate into the basal medium on the fourth day, is greatly stimulative for both the algal cell growth and the astaxanthin formation at a high light intensity. In addition, carotenoid precursors, mevalonate and pyruvate are effective on the carotenoid formation in the modified medium. They conclude that pyruvate is an especially good carbon source both for the algal cell growth and the carotenoid synthesis.

Belay et al. assess the current knowledge on potential health benefits of algae with a focus on *Spirulina* in an early paper on the algal biotechnology with 113 citations [50.89]. They note that spirulina is a microscopic filamentous alga that is rich in proteins, vitamins, essential amino acids, minerals and essential fatty acids like γ-linolenic acid (GLA). It is produced commercially and sold as a food supplement in health food stores around the world. Up to very recently, the interest in *Spirulina* is mainly in its nutritive value. Currently, however, they argue, numerous the possible therapeutic effects of *Spirulina* are also considered as:

> *many preclinical studies and a few clinical studies suggest several therapeutic effects ranging from reduction of cholesterol and cancer to enhancing the immune system, increasing intestinal lactobacilli, reducing nephrotoxicity by heavy metals and drugs and radiation protection.*

They present a critical review of the literature on therapeutic effects of *Spirulina*.

Ugwu et al. review the literature on the photobioreactors for mass cultivation of algae in a paper with 109 citations [50.90]. They note that algae have attracted much interest for production of foods, bioactive compounds and also for their usefulness in procuring the clean environment. They argue that in order to grow and tap the potentials of algae, efficient photobioreactors are required as only a few of them can be practically used for mass production of algae. They point out that one of the major factors that limit their practical application of photobioreactors in algal mass cultures is mass transfer. Thus, they argue that a detailed understanding of mass transfer rates in photobioreactors is necessary for efficient operation of mass algal cultures. They discuss various photobioreactors with a substantial potential for mass production of algae.

Hayashi et al. investigate calcium spirulan, an inhibitor of enveloped virus replication, from a blue–green alga *Spirulina platensis* in an early paper with 109 citations [50.91]. They find that

> *bioactivity-directed fractionation of a hot* H_2O *extract from a blue–green alga* S. platensis *lead to the isolation of a novel sulfated polysaccharide named calcium spirulan (Ca-SP) as an antiviral principle.*

This polysaccharide is composed of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid, galacturonic acid, sulfate, and calcium. Ca-SP inhibits the replication of several enveloped viruses, including Herpes simplex virus type 1, human cytomegalovirus, measles virus, mumps virus, influenza A virus, and HIV-1 (human immunodeficiency virus). They show that Ca-SP selectively inhibits the penetration of virus into host cells and they conclude that retention of molecular conformation by chelation of calcium ion with sulfate groups is indispensable to its antiviral effect.

Rosenberg et al. study engineered algae to redirect metabolic engineering to fuel a biotechnology revolution in a recent paper with 104 citations [50.92]. They note that microalgae have the potential to revolutionize biotechnology in a number of areas including nutrition, aquaculture, pharmaceuticals, and biofuels. They argue that although algae have been commercially cultivated for over 50 years, metabolic engineering now seems necessary in order to achieve their full processing capabilities as recently, the development of a number of transgenic algal strains boasting recombinant protein expression, engineered photosynthesis, and enhanced metabolism encourage the prospects of designer microalgae. Given the vast contributions that these solar-powered, carbon dioxide-sequestering organisms can provide to current global markets and the environment, an intensified focus on microalgal biotechnology is necessary. They conclude that recent advances in cultivation techniques coupled with genetic manipulation of crucial metabolic networks will further promote microalgae as an attractive platform for the production of numerous high-value compounds.

Grima et al. look into algal photobioreactors with a focus on the light regime, mass transfer, and scale-up in an early paper with 104 citations [50.93]. They discuss design and scale-up of tubular photobioreactors for outdoor culture of microalgae as culture productivity is invariably controlled by availability of light, particularly as the scale of operation increases. Thus, they emphasize light regime analysis with details of a methodology for computation of the internal culture illumination levels in outdoor systems. They also dis-

cuss supply of carbon dioxide as another important feature of algal culture. Finally, they outline potential scale-up approaches including novel concepts based on fundamentals of the unavoidable light–dark cycling of the culture.

Chen looks into the high cell density culture of microalgae in heterotrophic growth in an early paper with 102 citations [50.94]. He notes that microalgae are a great source of many highly valuable products such as polyunsaturated fatty acids, astaxanthin, and bioactive compounds. He argues that large-scale production of these products, however, has been hindered by an inability to obtain high cell densities and productivities in conventional photoautotrophic systems as high cell density processes suitable for heterotrophic cultures of microalgae may provide an alternative means for the large-scale production of algal products of high value. He reviews recent studies on the formation of algal products in various cultivation systems, with emphasis on the use of heterotrophic techniques and discusses the potential employment of heterotrophic high cell density strategies for commercial production.

Olaizola discusses the commercial development of microalgal biotechnology from the test tube to the marketplace in a paper with 100 citations [50.95]. He notes that only recently that we have come to realize the potential of microalgal biotechnology as microalgal biotechnology has the potential to produce a vast array of products including foodstuffs, industrial chemicals, compounds with therapeutic applications, and bioremediation solutions from a virtually untapped source. He argues from a commercial perspective that, the goal of microalgal biotechnology is to make money by developing marketable products and for such a business to succeed:

> *identifying a desirable metabolite and a microalga that produces and accumulates the desired metabolite and establishing a large-scale production process for the desired metabolite, and market the desired metabolite*

are necessary. He finds that microalgae that produce dozens of desirable metabolites have been identified and he argues that aided by high throughput screening technology even more leads will become available. He discusses achievements and drawbacks from the market point of view with examples from industry with a focus on Mera Pharmaceuticals.

Lee looks into microalgal mass culture systems and methods with a focus on their limitation and potential in a paper with 100 citations [50.96]. He notes that cultivation of microalgae using natural and designed open ponds is technologically simple, but not necessarily cheap due to the high downstream processing cost. Furthermore, products of microalgae cultured in open ponds can only be marketed as value-added health food supplements, specialty feed, and reagents for research. The need to achieve higher productivity and to maintain monoculture of algae necessitates the development of enclosed tubular and flat plate algal photobioreactors. He finds that despite higher biomass concentration and better control of culture parameters, data show that the illuminated areal, volumetric productivity, and cost of production in these enclosed photobioreactors are not better than those achievable in open-pond cultures. The technical difficulty in sterilizing these algal photobioreactors hinders their application for the production of high value pharmaceutical products and the alternative of cultivating microalgae in heterotrophic mode in sterilizable fermentors has some commercial success. He finds that the:

> *maximum specific growth rates of heterotrophic algal cultures are in general slower than those measured in photosynthetic cultures.*

The biomass productivity of heterotrophic algal cultures is not at a level that is comparable to the industrial production of yeast and other heterotrophic microorganisms. He concludes that:

> *mixotrophic cultivation of microalgae takes advantage of their ability to utilize organic energy and carbon substrates and perform photosynthesis concurrently.*

Moreover, the production of some algal metabolites is light regulated. He concludes that design of sterilizable bioreactors for mixotrophic cultivation of microalgae needs to consider the organic substrate as the main source of energy and light as the supplemental source of energy as well as a change in mindset.

50.4.3 Conclusion

The research on the algal biotechnology has been one of the most dynamic research areas in recent years with 455 papers and H-index of 55. Eighteen citation classics in the field of algal biotechnology with more than 100 citations were located and the key emerging issues from these papers were presented

above in the decreasing order of the number of citations [50.13, 80, 82–96]. These papers give strong hints about the determinants of the efficient algal biotechnology applications and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks necessitating the efficient design of the algal photobioreactors.

50.5 Algal Biosorption

50.5.1 Introduction

The research on the algal biosorption has been one of the most dynamic research areas in recent years. Eleven thousand one hundred and forty eight papers were located as indexed in the SCIE and SSCI as of February 2013. *Volesky* was the most prolific author with 39 papers and India, Turkey, and China produced over 35% of these papers whilst McGill University and CSIR (Council of Scientific Industrial Research) of India were the most prolific institutions. In comparison with the research fields of algal biofuels, it is a more established research field as only 37% of the papers were published after 2008 with papers starting back in 1990. *Journal of Hazardous Materials* and *Bioresource Technology* were the most prolific journals publishing over 10% and 8% of the papers, respectively, whilst nearly 36% and 28% of the papers were indexed under the subject category of *Environmental Sciences* and *Biotechnology and Applied Microbiology*, respectively. The citation impact of the papers on the algal biodiesel has been significant with 26.1 citations per paper and H-index of 78.

Twenty two citation classics in the field of algal biosorption with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.14, 97–117]. These papers give strong hints about the determinants of the efficient algal biosorption applications and emphasize that algae are efficient organisms in comparison with other organisms necessitating the efficient design of the algal biosorption processes.

50.5.2 Research on the Algal Biosorption of Heavy Metals

Volesky and *Holan* review the literature on the biosorption of heavy metals from metal-bearing industrial effluents in an early paper with 877 citations [50.14]. They note that biomass materials have potential of metal biosorption as they serve as a basis for newly developed metal biosorption processes for the detoxification of metal-bearing industrial effluents. For example:

> *lead and cadmium have been effectively removed from very dilute solutions by the dried biomass of some ubiquitous species of brown marine algae such as* Ascophyllum *and* Sargassum*, which accumulate more than* 30% *of biomass dry weight in the metal.*

Additionally, Mycelia of the industrial steroid transforming fungi *Rhizopus* and *Absidia* are excellent biosorbents for lead, cadmium, copper, zinc, and uranium and also bind other heavy metals up to 25% of the biomass dry weight.

> *Biosorption isotherm curves, derived from equilibrium batch sorption experiments, are used in the evaluation of metal uptake by different biosorbents and new methodologies are developed for the mathematical modeling of biosorption systems and their effective optimization.*

They argue that elucidation of mechanisms active in metal biosorption is essential for successful exploitation of the phenomenon and for regeneration of biosorbent materials in multiple reuse cycles. They conclude that the complex nature of biosorbent materials makes this task particularly challenging with a focus on the composition of marine algae polysaccharide structures, which are instrumental in metal uptake and binding.

Kratochvil and *Volesky* review the advances in the biosorption of heavy metals in an early paper with 406 citations [50.97]. They find that the biosorption of heavy metals by certain types of biomass is a highly cost-effective new alternative for the decontamination of metal-containing effluents and argue that packed-bed sorption columns are perhaps the most efficient for this purpose. They conclude that regenerating the biosorbents increases the process economy by allowing their reuse in multiple sorption cycles whilst the process results in metal-free effluents and small vol-

umes of solutions containing concentrated metals to be recovered.

Juhasz and *Naidu* review the literature on the bioremediation of high molecular weight polycyclic aromatic hydrocarbons with a focus on the microbial degradation of benzo[*a*]pyrene (BaP) in a paper with 293 citations [50.98]. They note that in recent years, research on the microbial degradation of polycyclic aromatic hydrocarbons (PAHs) has resulted in the isolation of numerous genera of bacteria, fungi, and algae capable of degrading low molecular weight PAHs which are compounds containing three or less fused benzene rings. They find that high molecular weight PAHs which are compounds containing four or more fused benzene rings are generally recalcitrant to microbial attack, although some fungi and algae are capable of transforming these compounds. They point out that:

> *only a few genera of bacteria have been isolated with the ability to utilize four-ring PAHs as sole carbon and energy sources while cometabolism of five-ring compounds*

has been reported. They focus on the high molecular weight PAH (BaP) as

> *there has been concern about the presence of BaP in the environment because of its carcinogenicity, teratogenicity and toxicity. BaP accumulate in marine organisms and plants which could indirectly cause human exposure through food consumption.*

They finally provide the outline of the occurrence of BaP in the environment and the ability of bacteria, fungi and algae to degrade the compound, including pathways for BaP degradation by these organisms.

Holan et al. study the biosorption of cadmium by biomass of marine-algae in an early paper with 286 citations [50.99]. They note that biomass of nonliving, dried brown marine algae *Sargassum natans*, *Fucus vesiculosus*, and *Ascophyllum nodosum* exhibit high equilibrium uptake of cadmium from aqueous solutions. They evaluate quantitatively the metal uptake by these materials using sorption isotherms. They find that biomass of *A. nodosum* accumulate the highest amount of cadmium exceeding 100 mg Cd^{2+} g^{-1} at the residual concentration of 100 mg CdL^{-1} and pH 3.5, outperforming a commercial ion exchange resin DUOLITE GT-73. They obtain a new biosorbent material based on A. nodosum biomass by reinforcing the algal biomass by formaldehyde cross-linking. They find that the prepared sorbent possess good mechanical properties, chemical stability of the cell wall polysaccharides and low swelling volume and desorption of deposited cadmium with 0.1–0.5 M HCl result in no changes of the biosorbent metal uptake capacity through five subsequent adsorption/desorption cycles. They also find that there is no damage to the biosorbent which retain its macroscopic appearance and performance in repeated metal uptake/elution cycles.

Aksu studies the equilibrium and kinetic modeling of cadmium(II) biosorption by *C. vulgaris* in a batch system with a focus on the effect of temperature as a function of the temperature, initial pH and initial metal ion concentration in a paper with 246 citations [50.100]. She finds that:

> *the algal biomass exhibits the highest cadmium(II) uptake capacity at* 20 °C*, at the initial pH value of* 4.0 *and at the initial cadmium(II) ion concentration of* 200 mg L^{-1}.

Additionally, biosorption capacity decreased from 85.3–51.2 mg g^{-1} with an increase in temperature from 20–50 °C at this initial cadmium(II) concentration. She applies the Freundlich and Langmuir isotherm models to represent the equilibrium data of cadmium(II) biosorption depending on temperature and finds that:

> *equilibrium data fit very well to both the models in the studied concentration range of cadmium(II) ions at all the temperatures studied.*

She also applies the:

> *pseudo first- and pseudo second-order kinetic models to experimental data assuming that the external mass transfer limitations in the system can be neglected and biosorption is sorption controlled*

She concludes that:

> *cadmium(II) uptake process follows the second-order rate expression and adsorption rate constants decrease with increasing temperature.*

She finally evaluates the second-order kinetic constants and the activation energy of biosorption.

Ahluwalia and *Goyal* review the microbial and plant derived biomass for removal of heavy metals from wastewater in a recent paper with 282 citations [50.101]. They note that discharge of heavy metals

from metal processing industries have adverse effects on the environment as the conventional treatment technologies for removal of heavy metals from aqueous solution are not economical and generate huge quantity of toxic chemical sludge. On the contrary, they find that:

> *biosorption of heavy metals by metabolically inactive nonliving biomass of microbial or plant origin is an innovative and alternative technology for removal of these pollutants from aqueous solution*

and point out that:

> *due to unique chemical composition biomass sequesters metal ions by forming metal complexes from solution and obviates the necessity to maintain special growth-supporting conditions.*

They find that:

> *algal biomass of* Aspergillus niger, Penicillium chrysogenum, Rhizopus nigricans, A. nodosum, Sargassum natans, Chlorella fusca, Oscillatoria anguistissima, Bacillus firmus, *and* Streptomyces *sp. have the highest metal adsorption capacities ranging from* 5–641 mg g^{-1} *mainly for* Pb, Zn, Cd, Cr, Cu, *and* Ni.

They conclude that algal biomass generated as a by-product of fermentative processes offers great potential for adopting an economical metal-recovery system.

Holan and *Volesky* study the biosorption of lead and nickel by biomass of marine-algae in an early paper with 228 citations [50.102]. They find as a result of the screening tests of different marine algae biomass types *a high passive biosorptive uptake of lead up to* 270 mg Pb g^{-1} *of biomass in some brown marine algae* and *members of the order Fucales perform particularly well in this descending sequence:* Fucus > Ascophyllum > Sargassum. They argue that *although decreasing the swelling of wetted biomass particles, their reinforcement by cross linking may significantly affect the biosorption performance*. They also observe lead uptakes up to 370 mg Pbg^{-1} in cross-linked *F. vesiculosus* and *A. nodosum*. At low equilibrium residual concentrations of lead in solution, however, ion exchange resin Amberlite IR-120 has a higher lead uptake than the biosorbent materials.

Wilde and *Benemann* review the literature on the bioremoval of heavy metals by the use of microalgae in an early paper with 212 citations [50.103]. They note that bioremoval, the use of biological systems for the removal of metal ions from polluted water, has the potential to achieve greater performance at lower cost than conventional wastewater treatment technologies for metal removal. They find that:

> *although microalgae are not unique in their bioremoval capabilities, they offer advantages over other biological materials in some conceptual bioremoval process schemes*

as:

> *selected microalgae strains, purposefully cultivated and processed for specific bioremoval applications, have the potential to provide significant improvements in dealing with the global problems of metal pollution.*

In addition to strain selection:

> *significant advances in the technology are possible by improving biomass containment or immobilization techniques and by developing bioremoval process steps utilizing metabolically active microalgae cultures.*

They conclude that the latter approach is especially attractive in applications where extremely low levels of residual metal ions are required. They outline the current literature, highlighting the potential benefits and problems associated with the development of novel algal-based bioremoval processes for the abatement of heavy metal pollution.

Aksu looks into the equilibrium, kinetic, and thermodynamic parameters of the batch biosorption of nickel(II) ions onto *C. vulgaris* in a paper with 197 citations [50.104]. She notes that the kinetics of the metal uptake process and the description of the thermal properties of biosorption remain essentially unknown and she therefore studies the biosorption equilibrium, kinetics and thermodynamics of nickel(II) ions to *C. vulgaris* in a batch system as a function of temperature and initial metal ion concentration. She finds that:

> *algal biomass exhibits the highest nickel(II) uptake capacity at* 45 °C *at an initial nickel(II) ion concentration of* 250 mg L^{-1} *and an initial pH of* 4.5

and *biosorption capacity increased from* 48.1–60.2 mg g^{-1} *with an increase in temperature from* 15–45 °C *at this initial nickel(II) concentration*. She then applies Freundlich, Langmuir, and Redlich–Peterson isotherm models to experimental equilibrium data of nickel(II) biosorption depending on temperature and she finds that equilibrium data fit very well to all the equilibrium models in the

studied concentration range of nickel(II) ions at all the temperatures studied. She then applies the saturation type kinetic model to experimental data at different temperatures changing from 15–45 °C to describe the batch biosorption kinetics assuming that the external mass transfer limitations in the system can be neglected and biosorption is chemical sorption controlled. She also determines the activation energy of biosorption (EA) as 25.92 kJ mole^{-1} using the Arrhenius equation and evaluates the thermodynamic constants of biosorption (ΔG, ΔH, and $\Delta S°$) using the thermodynamic equilibrium coefficients obtained at different temperatures.

Donmez et al. carry out a comparative study on heavy metal biosorption characteristics of some algae in a paper with 199 citations [50.105]. They test the biosorption of copper(II), nickel(II), and chromium(VI) from aqueous solutions on dried (*C. vulgaris*, *S. obliquus* and *Synechocystis* sp.) algae under laboratory conditions as a function of pH, initial metal ion, and biomass concentrations and they determine optimum adsorption pH values of copper(II), nickel(II), and chromium(VI) as 5.0, 4.5, and 2.0, respectively, for all three algae. They find that at the optimal conditions, metal ion uptake increases with initial metal ion concentration up to 250 mg L^{-1}. They additionally find that alga concentration influences the metal uptake for all the species and they conclude that both the Freundlich and Langmuir adsorption models are suitable for describing the short-term biosorption of copper(IT), nickel(II), and chromium(VI) by all the algal species.

Leusch et al. investigate the biosorption of heavy-metals (Cd, Cu, Ni, Pb, Zn) by chemically reinforce biomass of marine-algae in a paper with 170 citations [50.106]. They crosslink particles of two different sizes (0.105–0.295 mm and 0.84–1.00 mm diameter) of two marine algae, *Sargassum fluitans* and *A. nodosum*, with formaldehyde (FA), glutaraldehyde (GA) or embed in polyethylene imine (PEI), followed by glutaraldehyde cross linking. They are used for equilibrium sorption uptake studies with cadmium, copper, nickel, lead and zinc. They find that the metal uptake by larger particles (0.84–1.00 mm) is higher than that by smaller particles (0.105–0.295 mm).

> *The order of adsorption for* S. fluitans *biomass particles is* Pb > Cd > Cu > Ni > Zn, *for* A. nodosum *copper and cadmium change places and uptakes of metals range from* $q(max) = 378$ mg Pb g^{-1} *for* S. fluitans *(FA, big particles), to* $q(max) = 89$ mg Zn g^{-1} *for S. fluitans (FA, small particles) as the best sorption performance for each metal.*

They conclude that generally, *S. fluitans* is a better sorbent material for a given metal, size, and modification, although there are several exceptions in which metal sorption by *A. nodosum* is higher. They also conclude that the metal uptake for different chemical modifications show the order GA > FA > PEI and the Langmuir sorption model fit the experimental data best.

Chong and *Volesky* investigate 2-metal biosorption equilibria by Langmuir-type models in a paper with 158 citations [50.107]. They examine a biosorbent prepared from *A. nodosum* seaweed biomass, FCAN2, for its sorption capacity and perform equilibrium batch sorption studies using two-metal systems containing either (Cu + Zn), (Cu + Cd), or (Zn + Cd). They further evaluate three Langmuir-type models in order to model the isotherm surfaces mathematically and they use the apparent one-parameter Langmuir constant (b) to quantify FCAN2 *affinity* for one metal in the presence of another one. They find that the uptake of Zn decrease drastically when Cu or Cd were present and the uptake of Cd is much more sensitive to the presence of Cu than to that of Zn. They conclude that the presence of Cd and Zn alter the *affinity* of FCAN2 for Cu the least at high Cu equilibrium concentrations and the mathematical model of the two-metal sorption system enable quantitative estimation of one-metal (bio)sorption inhibition due to the influence of a second metal.

Donmez and *Aksu* look into the removal of chromium(VI) from saline wastewater by *Dunaliella* species in a paper with 150 citations [50.108]. They note that some industrial wastewater contain higher quantities of salts besides chromium(VI) ions so they investigate effect of these salts on the biosorption of chromium(VI) and test the biosorption of chromium(VI) from saline solutions on two strains of living Dunaliella algae under laboratory conditions as a function of pH, initial metal ion and salt (NaCl) concentrations in a batch system. They find that the biosorption capacity of both Dunaliella strains strongly depends on solution pH and maximum. They then determine chromium(VI) sorption capacities of both sorbents at pH 2.0 in the absence and in the presence of increasing concentrations of salt and they perform chromium(VI)-salt biosorption studies at this pH value. They find that:

> *equilibrium uptakes of chromium(VI) increases with increasing chromium(VI) concentration up to*

> *250–300 mg L^{-1} and decreases sharply by the presence of increasing concentrations of salt for both the sorbents*

whilst *Dunaliella* 1 and *Dunaliella* 2 biosorb 58.3 and 45.5 mg g^{-1} of chromium(VI), respectively, in 72 It at 100 mg L^{-1} initial chromium(VI) concentration without salt medium. On the other hand, when salt concentration arises to 20% (w/v), these values drop to 20.7 and 12.2 mg g^{-1} of chromium(VI) at the same conditions. They point out that:

> *both the Freundlich and Langmuir adsorption models are suitable for modeling the biosorption of chromium(VI) individually and in salt containing medium by both algal species as they apply the pseudo second-order kinetic model successfully to single chromium(VI) and chromium(VI)–salt mixtures biosorption data.*

Matheickal and *Yu* study biosorption of lead(II) and copper(II) from aqueous solutions by pretreated biomass of Australian marine algae in a paper with 149 citations [50.109]. They note that chemically modified biomass of marine algae can effectively remove heavy metals from waste water. They develop and study Australian marine algae (*Durvillaea potatorum* and *Ecklonia radiata*)-based biosorbents (DP95Ca and ER95Ca) for their heavy metal removal properties from aqueous solutions. They find that a two stage modification process substantially improves the leaching characteristics of the biomass as batch equilibrium experiments show that the maximum adsorption capacities of DP95Ca for lead and copper are 1.6 and 1.3 mmol g^{-1}, respectively, and the corresponding values for ER95Ca are 1.3 and 1.1 mmol g^{-1}. They point out that these capacities are comparable with those of commercial ion exchange resins and are much higher than those of natural zeolites and powdered activated carbon. They then find that the:

> *heavy metal uptake process is rapid with* 90% *of the adsorption completed in about* 10 min *in batch conditions as heavy metal adsorption is observed at pH values as low as* 2.0 *and maximum adsorption is obtained approximately at a pH of* 4.5.

They conclude that both biosorbents are effective in removing lead and copper in the presence of chelating agents and other light metal ions in waste water.

Aksu and *Tezer* study biosorption of reactive dyes on the green alga *C. vulgaris* in a paper with 142 citations [50.110]. They examine biosorption of three vinyl sulphone type reactive dyes (Remazol Black B (RB), Remazol Red RR (RR) and Remazol Golden Yellow RNL (RGY)) onto dried *C. vulgaris*, a green alga in a batch system. They find that the:

> *algal biomass exhibit the highest dye uptake capacity at the initial pH value of* 2.0 *for all dyes whilst the effect of temperature on equilibrium sorption capacity indicates that maximum capacity is obtained at* 35 °C *for RB biosorption and at* 25 °C *for RR and RGY biosorptions.*

They further find that:

> *biosorption capacity of alga increases with increasing initial dye concentration up to* 800 mg L^{-1} *for RB and RR dyes, and up to* 200 mg L^{-1} *for RGY dye.*

Among the three dyes, RB is adsorbed most effectively by the biosorbent to a maximum of approximately 419.5 mg g^{-1}. They use the Freundlich, Langmuir, Redlich–Peterson, and Koble–Corrigan adsorption models for the mathematical modeling of the biosorption equilibrium and they evaluate isotherm constants at different temperatures. They find that equilibrium data of RB biosorption fit very well to all models except that the Langmuir model, while this model is most suitable for modeling the biosorption of RR and RGY dyes in the studied concentration and temperature ranges. They also apply the pseudo first- and second-order and saturation-type kinetic models to the experimental data assuming that the external mass transfer limitations in the system can be neglected. They conclude that the dye uptake process follows the pseudo second-order and saturation type rate expressions for each dye *C. vulgaris* system.

Mehta and *Gaur* discuss the use of algae for removing heavy metal ions from wastewater in a paper with 140 citations [50.111]. They note that many algae have immense capability to absorb metals and there is considerable potential for using them to treat wastewater. Metal sorption involves binding on the cell surface and to intracellular ligands where the adsorbed metal is several times greater than intracellular metal and carboxyl group is most important for metal binding. They find that concentration of metal and biomass in solution, pH, temperature, cations, anions, and metabolic stage of the organism affect metal sorption. Algae can effectively remove metals from multi-metal solutions

and dead cells absorb more metal than live cells. Various pretreatments enhance metal sorption capacity of algae and $CaCl_2$ pretreatment is the most suitable and economic method for activation of algal biomass. Algal periphyton has great potential for removing metals from wastewater whilst an immobilized or granulated biomass-filled column can be used for several sorption/desorption cycles with unaltered or slightly decreased metal removal. They argue that Langmuir and Freundlich models, commonly used for fitting sorption data, cannot precisely model metal sorption since they ignore the effect of pH, biomass concentration, etc. They conclude that for commercial application of algal technology for metal removal from wastewater:

> *emphasis should be given to selection of strains with high metal sorption capacity; adequate understanding of sorption mechanisms; development of low-cost methods for cell immobilization; development of better models for predicting metal sorption; genetic manipulation of algae for increased number of surface groups or over expression of metal binding proteins; and finally economic feasibility.*

Hashim and *Chu* look into the biosorption of cadmium by brown, green, and red seaweeds in a paper with 137 citations and examine seven species of these seaweeds for their abilities to sequester cadmium ions from aqueous solution [50.112]. They observe considerable variability in their biosorption performance although all the seaweed types investigated are capable of binding appreciable amounts of cadmium and they find that maximum cadmium uptake capacities at pH 5 range from the highest value of $0.74\,\mathrm{mmol\,g^{-1}}$ for the brown seaweed *Sargassum baccularia* to the lowest value of $0.16\,\mathrm{mmol\,g^{-1}}$ for the red seaweed *Gracilaria salicornia*, representing a 363% difference. In general, they find that brown seaweeds exhibit the best overall cadmium ion removal. They conduct additional experiments to evaluate the biosorption characteristics of the brown seaweed *S. baccularia* and they find that the equilibrium uptakes of cadmium are similar within the pH 3–5 range but decrease significantly when the solution pH is reduced to pH 2. Additionally, the presence of background cations such as sodium, potassium, and magnesium, and anions such as chloride, nitrate, sulphate, and acetate up to a concentration of $3.24\,\mathrm{mmol\,L^{-1}}$ have no significant effect on the equilibrium uptake of cadmium. However, the biosorbent uptake of cadmium inhibits substantially in the presence of calcium ions at $3.24\,\mathrm{mmol\,L^{-1}}$ and kinetic studies show that cadmium uptake is fast with 90% or more of the uptake occurring within 30–40 min of contact time.

Liu et al. investigate reduction kinetics of Fe(III), Co(III), U(VI) Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria in a paper with 125 citations and examine the reduction kinetics of Fe(III)citrate, Fe(III)NTA, Co(III)EDTA$^-$, U(VI)O_2^{2+}, Cr(VI)O_4^{2-}, and Tc(VII)O_4^- in cultures of dissimilatory metal reducing bacteria (DMRB) (*Shewanella alga* strain BrY, *Shewanella putrefaciens* strain CN32, *Shewanella oneidensis* strain MR-1, and *Geobacter metallireducens* strain GS-15) [50.113]. They find that reduction rates are metal specific with the following rate trend for the Fe(III)citrate: Fe(III)NTA > Co(III)EDTA$^-$ much greater than $UO_2^{2+} > CrO_4^{2-} > TcO_4^-$, except for CrO_4^{2-} when H_2 is used as electron donor. The metal reduction rates are also electron donor dependent with faster rates observed for H_2 than lactate$^-$ for all *Shewanella* species despite higher initial lactate (10 mM) than H-2 (0.48 mM). They also find that the bioreduction of CrO_4^{2-} is anomalously slower compared to the other metals with H_2 as an electron donor relative to lactate and reduction ceased before all the CrO_4^{2-} have been reduced. They perform transmission electron microscopic (TEM) and energy-dispersive spectroscopic (EDS) analyses on selected solids at experiment termination and find precipitates of reduced U and Tc in association with the outer cell membrane and in the periplasm of the bacteria. As the kinetic rates of metal reduction are correlated with the precipitation of reduced metal phases and they discuss their causal relationship. They model the experimental rate data by a Monod kinetic model with respect to the electron acceptor for all metals except CrO_4^{2-}, for which the Monod model is modified to account for incomplete reduction. However, they notice that the Monod models become statistically over-parametrized, resulting in large uncertainties of their parameters. A first-order approximation to the Monod model also effectively models the experimental results, but the rate coefficients exhibit far less uncertainty. They conclude that the more precise rate coefficients of the first-order model provide a better means than the Monod parameters, to quantitatively compare the reduction rates between metals, electron donors, and DMRB species.

Cruz et al. carry out kinetic modeling and equilibrium studies during cadmium biosorption by dead *Sargassum* sp. biomass in a paper with 117 citations and conduct an experiment on the removal of *cadmium*(II) ions from aqueous solutions by dead *Sargas-*

sum sp. in batch conditions where they focus on the effect of different experimental parameters such as initial pH, shaking rate, sorption time, temperature, and initial concentrations of cadmium ions on cadmium uptake [50.114]. They find that:

> *cadmium uptake can be modeled by the Langmuir adsorption model whilst being the monolayer capacity negatively affected with an increase in temperature.*

Analogously, the adsorption equilibrium constant decreases with increasing temperature. The kinetics of the adsorption process follows a second-order adsorption, with characteristic constants increasing with increasing temperature where the activation energy of biosorption is 10 kcal mol^{-1}. The biomass used is suitable for removal of cadmium from dilute solutions as its maximum uptake capacity is 120 mg g^{-1}. They conclude that *Sargassum* sp. has great potential for removing cadmium ions especially when concentration of this metal is low in samples such as wastewater streams.

Sakaguchi et al. study the accumulation of heavy-metal elements in biological systems with a focus on the accumulation of cadmium by green microalgae (*Chlorella regularis*) in an early paper with 108 citations [50.115]. They find that the amount of cadmium absorbed by *Chlorella* cells is rapid during the first 30 min following addition of cadmium and then continues to be absorbed more slowly and the uptake of cadmium by *Chlorella* is not markedly affected by temperature or metabolic inhibitors whilst most of the cadmium absorbed by *Chlorella* cells is easily released by EDTA. The amount of cadmium absorbed differ markedly with the pH value of the solution and is inhibited by the presence of other divalent cations whilst heat-killed *Chlorella* cells take up cadmium to a greater degree than living ones. They conclude that:

> *the uptake of cadmium into* Chlorella *cells is not directly mediated by metabolic processes, rather it is completely dependent upon physicochemical adsorption on the cell surface*

whilst

> *the ability to accumulate cadmium is species specific and is in decreasing order of* C. reinhardtii > C. regularis > Scenedesmus bijuga > S. obliquus > Chlamydomonas angulosa > Scenedesmus chlorelloides.

Volesky and *Prasetyo* investigate the cadmium removal in a biosorption column in a paper with 103 citations as they examine new biosorbent material derived from a ubiquitous brown marine alga *A. nodosum* in packed-bed flow-through sorption columns [50.116]. They find that algae effectively remove 10 mg L^{-1} of cadmium down to 1.5 ppb levels in the effluent representing 99.985% removal. The experimental methodology used is based on the early Bohart and Adams sorption model, resulting in quantitative determination of the characteristic process parameters which can be used for performance comparison and process design.

> *An average metal loading of the biosorbent (N_0) determined is* 30 mg Cd g^{-1}*, corresponding closely to that observed for the batch equilibrium metal concentration of* 10 mg Cd L^{-1}*. The critical bed depth (D_{min}) for the potable water effluent quality standard (*0.005 mg Cd L^{-1}*) varies with the column feed flow rate (*2.4 *to* 9.6 L $h^{-1}cm^{-2}$*) from* 20–50 cm.

They finally determine the sorption column mass transfer and dispersion coefficients, required for solving the sorption model equations.

Ying et al. investigate the uptake of cadmium and zinc by the alga *C. vulgaris* with a focus on the individual ion species in a paper with 102 citations [50.117]. They note that the ability of algae and bacteria to accumulate heavy metals from the surrounding environment has a number of important implications. They report on the development of a quantitative model that addresses the basic mechanisms inherent in many uptake processes. The model postulates two mechanisms where an initial rapid metal ion uptake due to attachment onto the cell wall followed by a relatively slow uptake due to membrane transport of the metal into the cell. They test this mathematical model using the alga *C. vulgaris* in the presence of cadmium and zinc in solution under various experimental conditions.

50.5.3 Conclusion

The research on the algal biosorption has been one of the most dynamic research areas in recent years with 1148 papers and H-index of 78. Twenty two citation classics in the field of algal biosorption with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.14, 97–117]. These papers give strong hints about the determinants of the efficient algal biosorption applications and emphasize that marine algae are efficient organisms in comparison with other organisms necessitating the efficient design of the algal biosorption processes.

50.6 Conclusion

The issues relating to the global warming, air pollution, and energy security have been one of the most important public policy issues nowadays [50.1–3]. With the increasing global population, food security has also become a major public policy issue [50.4]. The development of algal biofuels as a third-generation biofuel has been considered as a major solution for the global problems of global warming, air pollution, energy security, and food [50.5].

Although there have been many reviews on the use of the algae in energy, medicine, and health care [50.10–14], and there been a number of scientometric studies on the algal biofuels [50.15], there has not been any study on the citation classics on the algal photosynthesis, biosorption, biotechnology, and biofuels as in other research fields [50.16–24]. As *North*'s New Institutional Theory suggests, it is important to have up-to-date information about the current public policy issues to develop a set of viable solutions to satisfy the needs of all the key stakeholders [50.25–34].

Therefore, a selected set of citation classics in the algal photosynthesis, biosorption, biotechnology, and biofuels are presented in this paper to inform the key stakeholders relating to the global problems of global warming, air pollution, food security, and energy security about the use of marine algae for the solution of these problems in the long run complementing a number of recent scientometric studies on the biofuels and global energy research [50.15, 35–47].

The research on the algal photosynthesis has been one of the most dynamic research areas in recent years with 2011 papers and with H-index of 92. Ten citation classics in the field of algal photosynthesis with more than 100 citations were located and the key emerging issues from these papers were presented above in the decreasing order of the number of citations [50.48–57]. These papers give strong hints about the structural determinants of the algal photosynthesis, informing the following sections. Photosystems I and II (PSI and II) which reaction centers that capture light energy in order to drive oxygenic photosynthesis played an important role in this field of research.

The research on the algal biodiesel has been one of the most dynamic research areas in recent years with 909 papers and H-index of 54. Fifteen citation classics in the field of algal biodiesel with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.10, 11, 58–70]. These papers give strong hints about the determinants of the efficient algal biofuel production and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

The research on the algal biohydrogen has been one of the most dynamic research areas in recent years with 144 papers and H-index of 25. Seven citation classics in the field of algal biohydrogen with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.12, 71–76]. These papers give strong hints about the determinants of the efficient algal biohydrogen production and emphasize that marine algae are efficient biohydrogen feedstocks.

The research on the algal bio-oil has been one of the most dynamic research areas in recent years with 89 papers and H-index of 21. Three citation classics in the field of algal bio-oil with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.77–79]. These papers give strong hints about the determinants of the efficient algal bio-oil production and emphasize that marine algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks.

The research on the algal biotechnology has been one of the most dynamic research areas in recent years with 455 papers and H-index of 55. Eighteen citation classics in the field of algal biotechnology with more than 100 citations were located and the key emerging issues from these papers were presented above in the decreasing order of the number of citations [50.13, 80–96]. These papers give strong hints about the determinants of the efficient algal biotechnology applications and emphasize that algae are more efficient biofuel feedstocks in comparison with the first- and second-generation biofuel feedstocks necessitating the efficient design of the algal photobioreactors.

The research on the algal biosorption has been one of the most dynamic research areas in recent years with 1148 papers and H-index of 78. Twenty two citation classics in the field of algal biosorption with more than 100 citations were located and the key emerging issues from these papers are presented below in the decreasing order of the number of citations [50.14, 97–117]. These papers give strong hints about the determinants of the efficient algal biosorption applications and emphasize that algae are efficient organisms in comparison with

other organisms necessitating the efficient design of the algal biosorption processes.

The citation classics presented under the four main headings in this paper confirm the predictions that the marine algae has a significant potential to serve as a major solution for the global problems of warming, air pollution, energy security, and food security. These papers also provide evidence that marine algae has significant benefits for the biosorption of heavy metals, for the photosynthetic processes, and development of commercial products for a range of industries including healthcare, medicine, and pharmacy besides as a feedstock for biofuels.

Further research is recommended for the detailed studies in each topical area presented in this paper including scientometric studies and citation classic studies to inform the key stakeholders about the potential of the marine algae for the solution of the global problems of warming, air pollution, energy security, and food security.

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51. Biofuel Innovation by Microbial Diversity

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Biofuels represent a viable alternative to the use of fossil fuels. They contribute to the reduction of greenhouse gas emission and do not compete with agricultural land. However, biofuels are not yet capable of replacing the current energy matrix based on fossil fuels because they cannot compete with standard fuels such as diesel and gasoline. Therefore, innovation is necessary to promote technical, economical, and environmental viability of biofuels. For this purpose, the marine realm is a promising source of bioresources to promote innovation in biofuel production. The marine biomass can be converted into biofuels such as biodiesel, bioethanol, and biogases (i. e., methane and hydrogen) through microbial activity. Microbial diversity plays a fundamental role in marine ecosystems by recycling of organic matter, which is a reflection of the vast genetic diversity associated to the marine microbial species available for biotechnological exploitation. The present study provides an overview of the importance of microbial fuels and presents innovative findings which can be applied to the production of biofuels in the near future.

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51.1 Bioprospecting of Marine Microbial Diversity

Microorganisms are involved in biogeochemical cycles such as carbon, nitrogen, sulfur, and other key elements playing crucial role in the homeostasis of marine environments by recycling of nutrients [51.1, 2]. A major challenge currently is the sustainable use of marine bioresources. The ability to convert a wide range of organic molecules in the environment indicates that marine microbial communities represent an important source of enzymes that can be used in the conversion of biomass for biofuel production.

Biofuels are produced by converting of biomass of organisms or by using products of their metabolism. Microbes annually hydrolyze 10^{11} t of plant biomass, principally plant cell wall material, which contains the energetic equivalent of 640 billion barrels of crude oil [51.3]. Advancement in molecular techniques has enabled access to the genetic diversity of marine microorganisms that are able to convert biomass.

High-throughput sequencing is a powerful tool for evaluating the metabolic potential of marine microbial communities. Genomics and metagenomics approaches have shown to be very promising with regard to exploration of marine microbial diversity. This has allowed

a thorough analysis of their genetic content, expanding our understanding about metabolic potential of marine microorganisms. These advances have directed the search for microbes, clusters, and genes of biotechnological interest.

The development of relevant projects in recent years (i. e., Global Ocean Sampling and International Census of Marine Microbes) [51.4, 5] have been developed by the scientific community towards a better understanding and use of marine microbial diversity. The number of microorganisms in the oceanic subsurface is estimated to be up to 3.5×10^{30}, spread over thousands of species per milliliter [51.6, 7]. However, it is estimated that only 1% of microorganisms is detected on plates with culture medium due the selective conditions of the culture media. Isolation and characterization of 10^6 or 10^9 bacterial species are beyond the capabilities of current microbiologists. The difference between the known diversity (the 10^3 described bacterial species) and the estimated total diversity (10^6–10^9 bacterial taxa) is the unknown fraction.

The diversity and abundance of free-living marine microorganisms have been described using culture-independent methods by means of 16S ribosomal ribonucleic acid (rRNA) and metagenomes from a variety of marine environments worldwide [51.4, 5, 8–13]. These efforts represent further knowledge about a vast genetic diversity associated to the marine realm. Marine microbial communities present greater taxonomic diversity highlighting tropical marine regions [51.14–16]. This represents a potential source of new genes for biotechnological applications. However, the still limited number of studies pursued to date on bacterioplankton diversity has been unable to cover the whole marine microbial functional diversity [51.17–21]. Marine samples demonstrate a greater potential to mining enzymes involved with biomass conversion for biofuel production.

The aim of this chapter is to provide an overview about the main aspects of microbial fuels that make them an interesting alternative for fossil fuels. To find this goal, we present marine bioresources that can be explored by biotechnology for biofuel production and examples of strategies that are being developed to consolidate microbial fuels (bioethanol, biodiesel, and biogas such as hydrogen and methane) by using marine bioresources. These examples address the functional diversity of entire communities to specific taxonomic groups (i. e., Vibrios). The following sections will clarify the role played by marine microbial diversity towards to biofuel innovation.

51.1.1 Marine Biomass for Biofuel Production Through Marine Microbial Activity

Fuels are produced in large quantities and low unit value. Gasoline is the current standard fossil fuel consumed around the world and currently its price is about $1\,\text{US\$}\,\text{L}^{-1}$. Thus, raw material prices are crucial to the final cost due the high consumption required for its production. Hence, the ideal raw material for biofuel production be inexpensive, easy to obtain, and should not compete with land for food production [51.22]. Marine biomass meets these requirements and shows advantageous features relative to terrestrial biomass (i. e., sugarcane, corn, and soybeans).

Seaweed and phytoplankton, for example, produce more oxygen than used in breathing, being responsible for 90% of the world production of O_2, unlike forests which consume the same rates of O_2 produced during the breathing process. This means marine organisms are extremely efficient in the conversion of CO_2 into organic matter and consequently the production of biomass. Thus, marine biomass represents an abundant resource that can be obtained only with the input of solar energy, water, and CO_2 during the photosynthesis process, making it a lower cost production with high capability of producing energy by biomass conversion.

The marine biomass available in the oceans can be converted into different types of biofuels by microbial activities with the cooperative use of microbial enzymes. Biomass conversion into bioethanol can be accomplished by hydrolysis of the different sugars that comprise the marine organisms. Bioethanol produced by macroalgae biomass represents the third generation of ethanol. Seaweeds are rich in sugars such as alginate, mannitol, cellulose, laminarin, carrageenan, and starch, and has a low lignin content in its composition, which is an important advantage over terrestrial biomass [51.23]. First and second generations of ethanol are well established and are based on terrestrial biomass. The first generation of ethanol is based on fermentable sugars arising from sugarcane juice, while the second generation uses glucose from cellulose degradation, which constitutes the major part of the biomass present in the bagasse. Yet among liquid fuels, biodiesel production using microalgae shows great potential because they are able to duplicate their biomass several times per day and produce at least 15 times more oil per hectare than food crops. Some species can achieve an oil content

Table 51.1 Lipid (oil and/or fatty acids) content of marine microalgae commonly applied to biodiesel production

Marine microorganisms	Lipid content (% dry weight)
Schizochytrium sp.	50–77
Nitzschia sp.	45–47
Pavlova lutheri	35.5
Nannochloropsis sp.	31–68
Pavlova salina	30.9
Isochrysis sp.	25–33
Isochrysis galbana	22–38
Phaeodactylum tricornutum	20–30
Nannochloris sp.	20–35
Cylindrotheca closterium	17–20
Cylindrotheca sp.	16–37
Tetraselmis suecica	15–23
Dunaliella salina	14–20
Monallanthus salina	>20
Dunaliella tertiolecta	37
Dunaliella primolecta	23
Crypthecodinium cohnii	20
Dunaliella bioculata	8
Spirulina maxima	4–7

corresponding to about 80% of its dry weight (Table 51.1).

Beyond liquid biofuels, marine biomass allows biogas production as methane and hydrogen to produce energy through anaerobic fermentation. Methane may have been one of the early building blocks of life, and there are numerous areas of the ocean floor where methane produced by methanogenic microorganisms percolates up from the deep subsurface. Hydrogen production from microbial metabolism may be obtained from a photosynthetic process or by fermenting marine biomass [51.24, 25].

Microbial fuels represent a valuable alternative to promote diversification of the world energy production pattern. The main objective to introduce microbial biofuel is to innovate in service offerings, using technologies to reduce the carbon footprint, taking steps to preserve the environment. Currently, we are already aware about demand for biofuels aiming at reducing dependency on volatile petroleum products. For example, all cars in Brazil run on pure or mixed alcohol because the government has ruled that gasoline must include a 20–26% mixture of ethanol. The use of bioethanol of the second and third generations in the mixture might be stimulated by environmental and economical policies followed by the innovation of production processes. The same has applied for diesel since 2010, when Brazilian diesel started to require a 5% biodiesel blend. This has enabled a steady expansion of the Brazilian biodiesel market, with 43 plants operating today and production capacity currently at 3.6 billion L (950 million gallons) per year.

The increasing of use of blended fuel is a tendency around the world. Europe supports low biofuel blends (10% of ethanol in gasoline and 7% of biodiesel for diesel) as a first step. Higher biofuel blends seem attractive if they meet criteria agreed upon for greenhouse gas performance, quality, sustainability, and availability. Microbial biofuels have the potential to be applied as a mixture in fossil fuels, with the aim to replace them completely in the future. Reasons to support this idea are given in the following.

51.1.2 Critical Issues to Support Microbial Biofuels

Microorganisms have the widest genetic repertoire on Earth, and the biofuel sector has shown particular interest in microbial enzymes. This interest occurs primarily due to environmental, economic, and social issues [51.26], as discussed below.

The first aspect concerns the worn model of the energy matrix based on fossil fuels, which has been proved to be damaging to the environment. Currently, the fuel sector meets an annual demand of 13 TW, mainly by consumption of fossil fuels. Efforts to develop new alternatives for energy production based on the concept of sustainability encourage research on biofuels. The use of fossil fuels over the last 200 years has had serious consequences on the quality of environmental health. Among the main ones is the increased greenhouse effect, which has directly contributed to the acidification of the oceans, promoting significant changes in marine life, as well as an increase in global temperature. Therefore, many efforts are being made in an attempt to develop fuels that are environmentally friendly (or *greener*). The application of biocatalysts decreases environmental hazards related to industrial processes, as by-products, and generally are less harmful than those produced by physicochemical processes.

From an economic standpoint, microbial enzymes represent an important alternative for reducing the costs of procedures for processing biomass used as feedstock for biofuel production. Currently, biomass must be processed to remove the high lignin content. However,

the physical and chemical processes used represents much of the cost involved in biofuel production [51.23]. Microbial enzymes can be obtained by purification from natural sources such as environmental isolates and genetically improved microorganisms. The use of microbial enzymes is among the most viable alternatives in terms of technical, economic, and environmental optimization of biofuel production. Therefore, the study of the functional diversity of marine microbial communities allows us to discover and characterize a still underexploited enzymatic repertoire in the context of biotechnology.

From a social view, biotechnological innovation through the exploitation of marine resources for microbial biofuel production plays an important role in the development of industrial processes that require less use of agricultural land for biofuel production. It can reduce pressure on food prices. Also, production and commercialization of marine biomass can promote an economic and social insertion of people who have no farmland into the production chain of biofuels. Thus, exploitation of marine microbiological resources is promising for the development of alternatives to current patterns of fuel production. Much research is being conducted to enable sustainable ways to obtain microbial biofuels. Innovations of biofuel production are indispensable to become technically and economically viable.

51.2 Marine Microbial Diversity Applied to Biofuel Innovation

The increase of the global consumption pattern has increased the urgency to reduce greenhouse gas emission and the risk of increasing food prices. The demand for fuel can be met through biofuel innovation by exploration of marine microbial resources. This section provides some examples of how biodiversity has been exploited in the quest for sustainable energy in the near future.

Here we present different forms of the exploration of microbial diversity for the production of microbial fuel as an alternative to fossil fuels. The first topic gives an overview of the main advantages and challenges related to the use of cyanobacteria and microalgae for biodiesel production. In the following section, we show that the marine environment represents a vast untapped repertoire of microbial enzymes applied to bioethanol production. The third topic shows that in contrast to this entirely new fraction of functional diversity from microbial communities, studies of well-known marine bacteria such as Vibrios open doors to genetic manipulation to optimize the production of bioethanol and hydrogen using marine biomass. The same marine biomass also serves as a substrate for methane production by a fermentation process using methanogenic microbes associated to the marine sediments addressed in the last topic of this section. Thus, these four topics are connected, because they clearly show the potential of marine microbial diversity to promote innovations in biofuel production as an alternative to the use of terrestrial bioresources for bioenergy.

51.2.1 Biodiesel Production from Microbial Marine Resources

Biodiesel has been widely investigated as an alternative source to using fossil fuels. Such fuels are potentially carbon neutral because they do not result in a net increase in atmospheric greenhouse gases, resulting in negative carbon dioxide emission and net carbon dioxide removal from the atmosphere, and thus constitute a form of greenhouse gas remediation.

Biodiesel is a lipid-derived biofuel obtained by a transesterification reaction of triglycerides of vegetable oil or animal fats, or by esterification of free fatty acids with low chain alcohols, used to generate mechanical energy. The enzymes involved with this step are called lipases. Microbial lipases now occupy a special place among biocatalysts due to their ability to catalyze a variety of reactions in aqueous and non-aqueous media [51.27].

Biodiesel consists either of fatty acids-methyl-esters (FAME) from methanolysis reactions or fatty acids-ethyl-esters (FAEE) when using ethanolysis reactions catalyzed by strong alkali or acids. Considering the existing restrictions on greenhouse gas emissions, biodiesel provides significant advantages compared with petroleum-based fuel; the major problem in using and producing biodiesel is associated with feedstock resources, which nowadays is mainly edible seed crops, such as soybean, rapeseed, sunflower, etc., leading to a great controversy about food security, which is a great threat especially to the poor countries. Until 2007 about

14 million ha of farmland were used for the production of biofuels, which represent approximately 1% of the entire cultivated land in the world.

Characteristics such as performing oxygenic photosynthesis using water as an electron donor and growing to high densities, and having high per-acre productivity compared to typical terrestrial oil-seed crops are present in marine organisms and represents a requirement for sustainable biofuel production. Mass cultivation for commercial production can be performed efficiently, and, consequently, does not demand arable land. Indeed it utilizes a wide variety of water sources (fresh, brackish, seawater, and wastewater), and produces both biofuels and valuable co-products.

Prospecting of marine microbes for biodiesel production has been performed, and microalgae and cyanobacteria are the main focus for the development of technological strategies for biodiesel to become a competitive biofuel as an alternative to fossil fuels. Here, we discuss the main issues concerning the barriers and advantages related to the production and commercialization of microbial biodiesel.

Challenges in Biodiesel Production from Microbial Marine Resources

Although it may become a promising strategy, biodiesel production from microalgae and *Cyanobacteria* is not yet a viable process. There are still technical, economic, and environmental challenges that need to be addressed [51.28–30]. For example, the open pond cultivation system presents two difficulties in terms of water conditions: thermic and carbon dioxide regulation. Enclosed photobioreactors are still too expensive, as they need a material with high transparency to capture sunlight, or a special illumination system made of optical fiber cables.

The algae biofuel industry is pursuing pilot and demonstration-scale algae cultivation projects. Bioenergy industries are still developing numerous initiatives, but commercial algae biomass production is restricted to high-value nutritional products. Nevertheless, different algae cultivation systems, such as closed photobioreactors, raceway ponds, and closed ponds, in addition to different methods to produce biofuels, such as oil extraction and algal biorefinery, are being investigated worldwide [51.31]. Although biofuels from microalgae are considered technically viable, current barriers to the commercial implementation of microalgal-derived biofuels are expected to be overcome by technology developments. The limitations have a direct influence on oil production by microorganisms.

Microalgal oil contains a high degree of polyunsaturated fatty acids (with four or more double bonds) when compared to vegetable oils, which makes it susceptible to oxidation in storage and, therefore, reduces its acceptability for use in biodiesel [51.32]. The oil content (triacylglycerols – TAGs) is one of the positive aspects of using microalgae for biodiesel production, as TAGs are the main storage compound present in any algal species, especially in stress conditions such as nitrogen starvation. However in stress conditions the biomass of the microalgae is dramatically decreased and this is another point of research to make the industrial process viable. Nutrient limitation is one of the most efficient triggers to enhance lipid accumulation in a single microalgal cell when energy source (light) and carbon source (CO_2) are available [51.33]. Many aspects of lipid production are considered for the optimum cultivation of marine microalgae because lipid accumulation is a feature of an unbalanced metabolism. However, to achieve this performance it is pivotal to optimize strains and culture media to obtain biomass for the production of lipids in microalgae on a large scale [51.34]. The growth temperature, pH, micronutrient availability; salinity, and nutrient limitation (especially nitrogen and phosphorus supply) need to be under control to improve microalgae biomass and lipid production for industrial application.

Since algae production systems are a complex composite of several subsets of systems (i. e., production, harvesting, extraction, drying systems), reducing the number of steps in the production of algae biofuels is essential for providing easier, better, and lower cost systems [51.35]. Currently, the cost of biodiesel production by microalgae is still not competitive compared to that of diesel. The costs of biofuel production from algal biomass amounts to approximately $50\,€^{-1}$, that is, to high to be attractive for the commercial production of algal biofuels [51.32].

Moreover, it is necessary to pay attention to an obvious problem concerning environmental aspects related to biodiesel use. While promoting a significant reduction in CO_2 emissions, NO_x emission by biodiesel engines is a subject of considerable research. Biodiesel can be considered as an oxygenated fuel. A high oxygen content results in the improvement of its burning efficiency and reduction in the particulate matter, CO, and other gaseous pollutants. However, it also leads to higher production of NO_x (approximately 10%) com-

pared to fossil diesel, particularly in a high-temperature burning environment. Indeed, there are few studies on possible environmental impacts, since the toxicity of biodiesel has not yet been properly assessed. More ecotoxicity studies will be needed to develop the secure production and storage of biofuel. Strategies of containment and environmental decontamination in possible spill events need to be developed to allow the process of production and commercialization of biodiesel on an industrial scale. Nevertheless, efforts are being made because it is understood that there are positive aspects that make biodiesel a promising energy source.

Positive Aspects About Marine Microbial Biodiesel

There are several aspects of cyanobacterial and microalgal biodiesel production that have combined to capture the interest of researchers around the world. Cyanobacteria can convert up to 10% of the sun's energy into biomass by photosynthesis. It is highly efficient if compared to the 1% recorded by conventional energy crops (corn or sugarcane) or the 5% achieved by algae. Indeed, microalgae (phytoplanktons) and cyanobacteria (blue–green algae) are a reasonable choice [51.36] as an alternative energy source, because most are great lipid producers (7–23% of cell composition, 20–50% dry weight) (Table 51.1) and both display simple growth requirements using light, carbon dioxide, and other inorganic nutrients efficiently [51.37]. When microalgal cells grow actively, polar membrane lipids (phospholipids and glycolipids) generally predominate, but as the cell enters the stationary phase many species accumulate TAGs. The majority of microalgal TAGs include saturated or monosaturated C14–C18 fatty acids, but long-chain polyunsaturated fatty acids (PUFAs) are often present. Even so, compared to others sources used for biodiesel production, microalgae oil production is about 10–20-fold higher [51.32].

Another advantage of microalgae cultivation is the environmental benefits, as it does not require the application of herbicides or pesticides and can be used in wastewater bioremediation, because microalgae is capable of removing NH_4^+, NO_3^-, and HPO_3^- from a variety of wastewater sources (e.g., agricultural runoff, concentrated animal feed operations, and industrial and municipal wastewaters [51.28]. Moreover, by removing nitrogen and carbon from water, microalgae can help reduce eutrophication in the aquatic environment, leading to a successful sustainable process [51.38].

Many species of freshwater and marine microalgae have been isolated and characterized by their lipid content [51.32, 34, 38–42]. In general freshwater microalgae works better for this purpose, but marine microalgae such as the *Dunaliella*, *Nitzschia*, and *Schizochytrium* species present a higher lipid content, which can reach more than 35–50% of their dry weight (Table 51.1).

Recently, some efforts were made with respect to cost minimization, as done by the integration of the biodiesel production system with methane production via anaerobic digestion of microalgae, which can further be converted to generate electricity. The consumption of the energy required in microalgal cultivation, dewatering, extraction, and transesterification process could be surrogated by this approach and theoretically could reduce about 33% of the cost of biodiesel production [51.43]. Moreover, the main components of a typical algae feedstock are proteins, carbohydrates, lipids, and other valuable components, e.g., pigment, antioxidants, fatty acids, and vitamins; so as it occurs as another vegetable feedstock, microalgae oil is a by-product that can be sustained by other valuable higher priced products, such as carotenoids, lutein, and PUFAs [51.44]. This is the concept of an algal biorefinery, which can be a possible way to make microalgae biodiesel production a promising industrial process.

51.2.2 Marine Metagenomes as a Source of Novel Hydrolases for Bioethanol Production

The term metagenomics refers to a culture-independent approach based on the study of deoxyribonucleic acid (DNA) molecules in a mixture of microbial populations [51.45]. The application of metagenomics approaches to the study of microbial communities from environmental samples has allowed a fast increment in the number of new sequences in databases. This has been achieved by the impressive development of sequencing techniques and analysis tools to investigate the position of these sequences in the sequence space [51.46]. An unprecedented volume of data has been generated by high-throughput sequencing, and most of these sequences represent novel genes or uncharacterized sequences. This diversity represents a huge catalog of genes available to be chosen according to specific goals for a number of biotechnological applications, for instance, for biofuel production.

The search for high-performance enzymes such as hydrolases comes is meeting the interests of the bioenergy industries, with emphasis on the production of bioethanol from the second and third generation. The discovery of new glycoside hydrolases with improved biocatalytic properties for efficient conversion of cellulosic materials is a critical challenge for the development of economically viable routes to production of fuels and chemicals. Glycoside hydrolases (category EC 3.2.1) are a widespread group of enzymes that hydrolyze glycosidic bonds between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety from biomass.

Recalcitrance of organic matter can be overcome by the application of microbial enzymes, influencing the reduction of costs and the environmental impact generated by pretreatment of lignocellulosic material used for the production of second generation bioethanol. The search for marine microbial enzymes opens new avenues for the optimization of marine biomass conversion to produce third-generation bioethanol. The diversification of sources of organic molecules used as raw material for biofuel production also represents an important strategy for the development of a sustainable productive process. The exploration of microbial diversity by cultivation-independent methods has led to the discovery of an impressive number of new sequences and related genes of industrial interest for biofuel production.

Molecular Approaches Reveal the Functional Diversity of Marine Microbial Communities for Bioprospecting

The two main approaches to study microbial diversity include dependently and independently cultivated methods. Molecular studies using sequences of small-subunit rRNA genes have shown that the diversity of microbial communities is composed of two components: i) a set of abundant and actively growing taxa and, ii) a seed bank of many rare taxa [51.47–49]. Common organisms may not play a critical role in the dynamics of a given community despite their numbers, and organisms that only muster 0.1% prevalence can be of pivotal importance. This suggests the presence of a rare repertoire of enzymes related to specific metabolic pathways, which can be used for biotechnological applications. A deeper knowledge about marine microbial diversity allows us to access microbial genetic diversity for bioprospecting [51.50].

Metagenomics allows correlating members of microbial communities to functional gene sequences by whole metagenome sequencing. Quantitative analysis of gene content reveals habitat signatures that reflect the particular characteristics of an environmental sample. This allows us to compare the complexity of communities and distinguish them as depending on the metabolic repertoire represented by metagenome sequences [51.51–53].

In nature, organic matter is decomposed by complexes and efficient microbial processes by hydrolytic enzymes which act synergistically to decompose biomass. In recent years, there has been a tendency to search for hydrolases in typical plant biomass degrading environments [51.54–59]. The main targets are animal rumen microbiota, termite guts, and microbiota associated with decomposed biomass of forests. However, a low identification rate of specific hydrolases (0.01−0.5%) [51.57, 59–63] was recovered in those environments. On the other hands microbial communities of marine environments represent an underdeveloped alternative source to the discovery of new enzymes as glycoside hydrolases. Bioprospecting of glycosyl hydrolases has been performed for application to bioenergy production [51.64–66].

Bioprospecting Glycosyl Hydrolases in the Southwestern Atlantic Ocean

Functional screening based on whole metagenome sequencing is a strategy used to find new sequences of glycosyl hydrolases in the Abrolhos Bank, opposite to other metagenomics studies based on phenotypic screening from previous samples [51.54, 62, 63]. The strategy used in Abrolhos revealed a higher number of new sequences related to carbohydrate degradation. The advantages of screening based on sequences are that target genes can be isolated regardless of the gene expression.

Recently, metagenomic strategies have been applied for a better understanding of the genetic diversity associated with bacterioplankton along the Southwestern Atlantic Ocean, with particular interest in the Abrolhos Bank. It is the most important coral reef area of the South Atlantic Ocean [51.67]. The Abrolhos Bank represents an interesting resource for bioprospecting of carbohydrate-active enzymes encoded by genes which participate in many important steps of carbohydrate metabolism (i. e., glycoside hydrolases). The region presents high turnover rates of organic matter. With the metagenomic approach it was possible to identify a high contribution of genes involved in carbohydrate metabolism [51.21]. The search for those genes in the Abrolhos Bank metagenomes re-

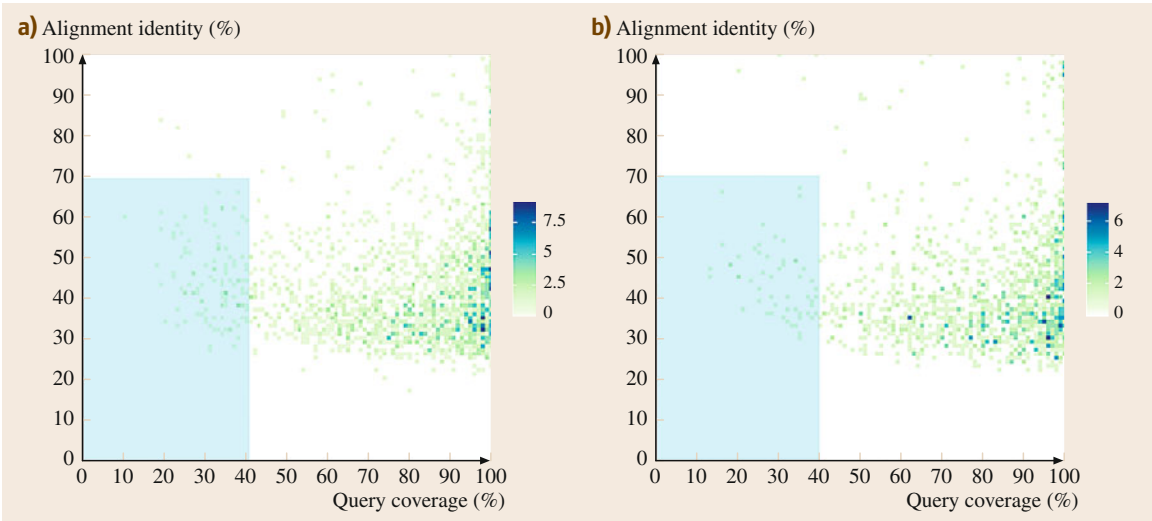

Fig. 51.1a,b Potential novel carbohydrate active enzymes from marine metagenomes. Sequences were submitted to BlastX against protein database (Swiss-Prot) and homology was evaluated by means of alignment identity and query cover. Sequences presenting identity and covering more than 40% and 70%, respectively, were assigned as homologs of known sequences from the database and those out of that cut-off were considered as potential novel proteins involved with carbohydrate metabolism. The *colored zone* highlights sequences assigned as potential novel carbohydrate active enzymes found in Southwestern Atlantic Ocean samples. **(a)** Coastal reefs surrounding water metagenome sequences from the Abrolhos Bank region and **(b)** metagenome sequences from the reef structure called Buraca (only found in the Abrolhos Bank region, Brazil) where an enrichment of sequences assigned as glycosyl hydrolases compared to coastal reef samples was found. The number gradients is the number of sequences for each dot

vealed a high diversity of glycoside hydrolases protein families and the presence of novel amino acid sequences.

Sequencing of five coastal reef regions in the Abrolhos Bank region (about 700 000 sequences) allowed the identification of thousands (about 2000 sequences) of glycoside hydrolase sequences. More than 50% of the total number of known proteins of the glycoside hydrolase family were identified (71 families). Those that do not have any close relatives form deeply branched lineages and even represent novel families. Among the sequences in the Abrolhos Bank, about 30% were identified as putative new glycoside hydrolases (Fig. 51.1). These sequences presented identity and covered less than 40 and 70%, respectively, compared to protein sequences deposited in curated databases (Swiss-Prot) [51.68]. Identification of glycoside hydrolase families was based in homology of conserved domains of amino acid sequences. Searches based on conserved domains have been shown to be more efficient than a simple search for similarity of nucleotide sequences for identification of new proteins [51.69]. That strategy is based on using domain signatures as probes to find isofunctional homolog proteins, increasing the chance of finding novel ones.

In addition to the coastal coral reefs of the Abrolhos Bank, a new reef structure that was recently described in this region has been addressed [51.70]. Within the large mesophotic reef, unusual sinkhole-like depressions (called *buracas*) are outstanding features with potentially important ecological roles in the middle and outer shelf [51.71]. These cup-shaped structures trap and accumulate organic matter, acting as productivity hotspots in the central portion of the Abrolhos Bank shelf. Buracas showed an increment in abundance of glycoside hydrolases gene sequences (2.0% of metagenome sequences) reaching a contribution five times higher than in a coral reef structure but with similar diversity of glycosyl hydrolase genes. This is clearly an interesting number if compared with other metagenomes that have been explored, where the contribution reaches up to 1.5% of (i. e., termite guts) glycosyl hydrolase gene sequences [51.72].

A recent search in the GOLD database (197 environmental studies) [51.73] for glycosyl hydrolase

putative homologs retrieved 7338 genes for different ecosystems (including terrestrial and microbiome ones, including only two for marine metagenomes). If this is compared with data obtained in the Abrolhos Bank, it is clear that there is an enormous potential of marine metagenomes to find hydrolase genes waiting to be discovered and applied to bioethanol production. So far, only 5% of metagenomes (a total of 2055 samples) available in GOLD are from marine environments. This reinforces the idea that marine metagenomes represent a still underexploited reservoir of new genes for hydrolytic enzymes.

The steps that will lead to the application of these gene sequences to bioprocesses need to be harnessed through several complex procedures that include gene synthesis and cloning, heterologous expression, and fermentation efficiency evaluation, which shows that there is a long way to their practical application.

51.2.3 Recent Advances in Biofuel Production by Marine Vibrios

In contrast with the last topic, which addressed microbial communities, here we explore a well-known cultivated marine microbial taxonomic group to access our biotechnological potential. Vibrios are huge groups that show facultatively anaerobic, widespread marine bacteria, but one of their major habitats is the gut of marine fish/shellfish. There are many kinds of seaweed-eating animals in which the unique gut bacteria may be harbored *Vibrio halioticoli*, and related species are isolated from *Haliotis* abalone (Gastropoda) as novel alginate degrading vibrios [51.74–76]. Intensive screening of marine microbes showing biofuel production from seaweed contributes to advances in innovative research for marine biofuel production because they are rich in seaweed carbohydrates and anaerobic environments which are likely to be suitable conditions of fermentation [51.77]. During the last 5 years, studies on evaluating the potentials of marine vibrios for biofuel production, namely, *V. halioticoli* have been undertaken, and a novel vibrio species has been raised as a candidate.

Degradation and utilization of marine resources are the key metabolic pathways to launching the idea of bioethanol and biohydrogen production using fast growing kelps as raw material. Kelp is not only cultured and/or harvested for food purposes but also for industrial purposes as polysaccharide production, especially in Asian countries, including Japan and Chile. Such traditional cultivation could easily be refined to be adapted to energy-aimed cultivation systems, the recently so-called *energy line* [51.78].

In the meantime, constructions of metabolically engineered *Escherichia coli* and *Sphingomonas* sp., implemented with homoethanologenic pathways, have been successful by Japanese groups [51.79, 80]. However, *Escherichia coli* and *Sphingomonas* did not originally inhabit marine environments and are incapable of showing the best fermentation performance in saline environments using kelp as feedstock. We must seek better microbes for excellent performance in biofuel production from seaweed feedstock under marine conditions.

Bioethanol Production

Generally, biomass consists of complex carbohydrates, seaweed as well. Carbohydrate compositions vary in the algae groups: brown, red, and green. The major carbohydrates in brown, red, and green algae are alginate-mannitol, carrageenan-agar, and starch-cellulose, respectively. Kelp is a common name for gigantic brown algae. Kelp has been used as a marine substrate in the fermentation process to obtain biofuel. It is an abundant algae in Hakodate, which is one of the biggest kelp mariculture points in Japan. Typical carbohydrate contents consist of mannitol (30 w/w % dry material), alginate (30%), and cellulose (< 10%). Mannitol, alginate, and glucose were selected as substrates for the optimization of ethanol production by vibrios. *Vibrio* sp. AM2, as well as *V. halioticoli* [51.76] were tested for ethanol production in a marine broth culture supplemented with mannitol, alginate, and glucose. Ethanol production was the highest in broth supplemented with mannitol, and lowest in alginate-supplemented broth (Fig. 51.2). Especially, when mannitol was used as the substrate, ethanol production reached one third of the total fermentative products. This production was ca. one and half times higher than that of glucose-supplemented broth in both marine vibrios.

We further optimized ethanol production in two kinds of marine vibrios: *Vibrio* sp. AM2 and *V. halioticoli*. Both strains required best mix conditions of glutathione (1.2 w/v %) and mannitol (5 w/v %) for maximum ethanol production (Fig. 51.3). The detailed mechanism of glutathione in ethanol production has not been clarified yet, but it might function as a biological reducing agent inside the vibrio cells. Further transcriptomic analysis is necessary. In addition to these two factors, pH control and hydrogen addition were also important physicochemical factors to improve ethanol production in vibrios (unpublished data). Under the best

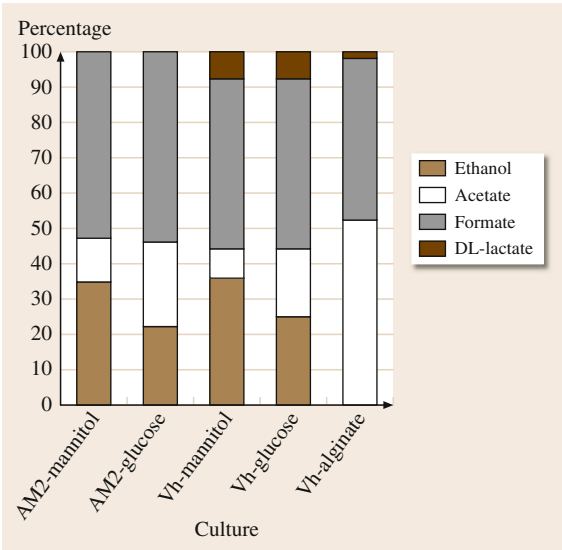

Fig. 51.2 Effect of carbohydrates on fermentation by marine vibrios. Ethanol (*light brown*), acetate (*white*), formate (*gray*), DL-lactate (*dark brown*). Glc: glucose, CMan-ol: mannitol, CAlg: alginate

culture condition, currently, 14.9 g L^{-1} ethanol production from mannitol under pH controlled batch culture (100 mL) has been achieved.

This shows an interesting potential of vibrios for biofuel production by consumption of marine algae biomass. Including this important finding, biogas production is another useful characteristic of vibrios that can help to establish this microbial group as a model for marine microbes for biomass conversion for biofuel production.

Biohydrogen Production

Hydrogen is one of the most reliable future clean energies in the world, nevertheless its infrastructure has not been devised yet. Biologically generated hydrogen by photosynthesis and dark fermentation is called as *biohydrogen* ($BioH_2$) [51.81]. The big advantages of dark fermentation are the applicability to practical use (especially on reactor-based production) and concerns about availability of a wide variety of feedstock, and faster production speed rather than H_2 production by photosynthesis [51.81, 82].

The recently isolated *Vibrio* sp. AM2 shows a unique gas production metabolism, which only small vibrio groups have [51.83–86]. As the gas was produced under culture supplemented by Glc and Man-ol, and the gas composition was determined as hydrogen and CO_2; the gases were estimated to be produced via a formate-hydrogen lyase pathway through formate. Intensive optimization of $BioH_2$ production in this strain from seaweed carbohydrates revealed that the strain possesses unexpectedly effective $BioH_2$ production machinery (unpublished data). Furthermore, based on the optimization experiments, a 3 L scale culture was achieved not only with mannitol-supplemented marine broth but also kelp powder-supplemented marine

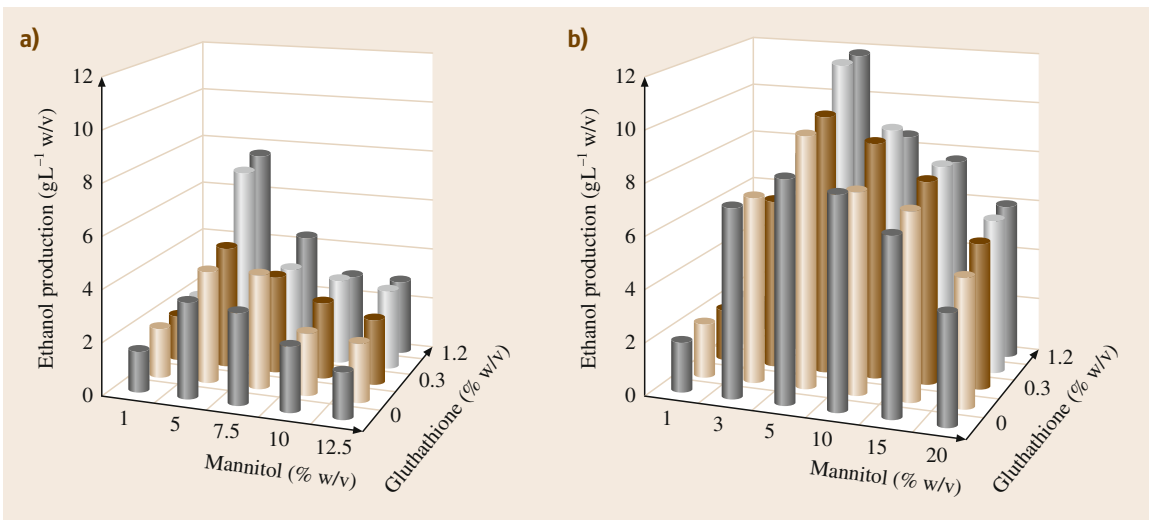

Fig. 51.3a,b Effect of mannitol and glutathione on ethanol production of marine vibrios. **(a)** *Vibrio halioticoli*, **(b)** *Vibrio* sp. AM2

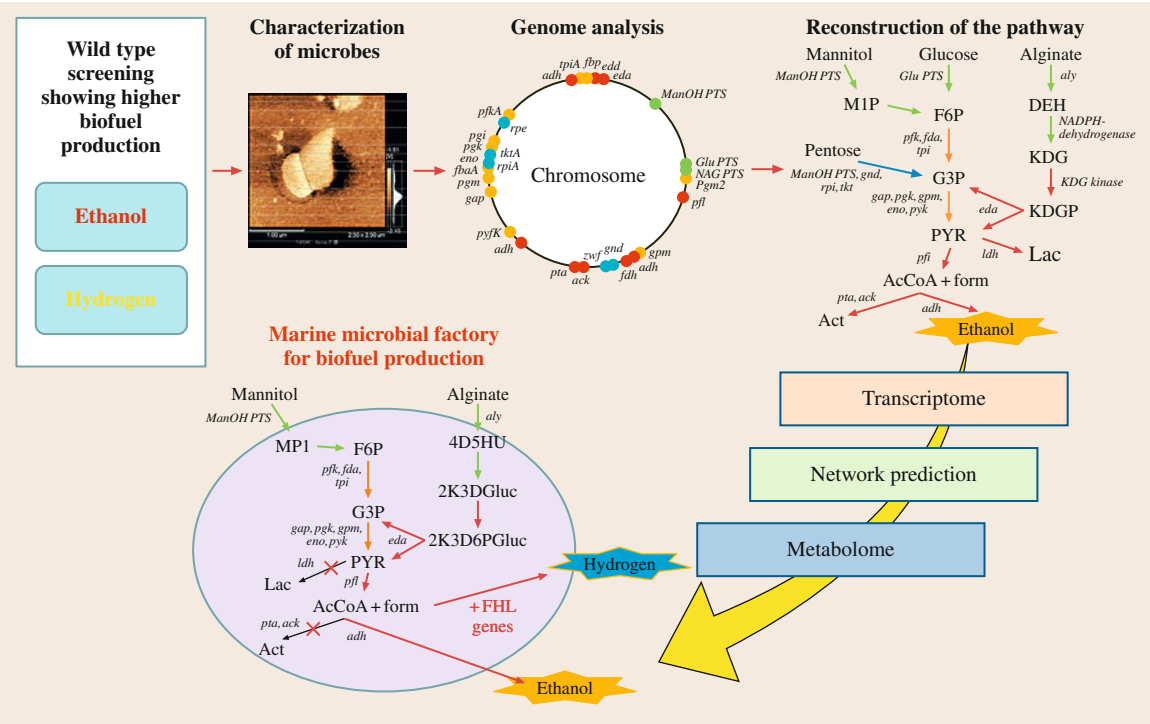

Fig. 51.4 Conceptual framework for the development of a marine microbial cell factory for biofuel innovation

broth with almost the same efficiency as a small-scale (100 mL) batch culture. Interspecific variation in $BioH_2$ production among AM2 related strains was also observed.

To power typical house life using 1 kW fuel cells, a total of 24 mol h^{-1} hydrogen is required. Reduction of the $BioH_2$ production system and the cost are major obstacles to achieving hydrogen economy and use in society. A variety of research concerning the understanding of the microbial hydrogen production machinery and metabolic engineering has been undertaken [51.82, 87].

Toward the Creation of a Marine Microbial Cell Factory

A conceptual framework for the development of a marine microbial cell factory based on marine vibrio cells has been established to obtain more efficient microorganisms for the fermentation of sugars of marine origins (seaweed) and bioenergy production. The workflow shown in Fig. 51.4 demonstrates how to obtain microorganisms with high capacity for fermentation. At the moment, we have two unique marine vibrios to push forward to construct a marine microbial cell factory in marine biofuel innovation. Using the vibrios and with genome analysis, transcriptomic analysis, and metabolic engineering, we shall elucidate these metabolic networks for seaweed carbohydrates. These results emphasize our technological advances.

Recently, whole genome sequences and draft genome sequences were obtained in *Vibrio* sp. AM2 and *Vibrio halioticoli* JCM 21271^T (IAM 14569^T), respectively (unpublished data). In addition to these two strains, draft genome sequences are available in five *Vibrio* species and ten are in progress. Based on the genome information, we recently succeeded in the reconstruction of three major central metabolic pathways for seaweed carbohydrates (Embden–Meyerhof pathway (EMP) Entner–Doudoroff pathway (EDP) and Pentose phosphate pathway (PPP)), and mining of the genes responsible for alginate degradation and utilization, diverse alcohol dehydrogenases, and hydrogen production machinery (unpublished data).

Nevertheless, ethanol production by *V. halioticoli* from alginate has not yet been achieved. However, the draft genome analysis also revealed many interesting aspects of the species for engineering the cell factory: the rather small genome size among Vibrionaceae, a

variety of alginate utilization gene clusters, and possible uptake hydrogenase (unpublished data). Based on the reconstructed metabolic network, we conducted mini-transcriptomic analysis using 44 genes focusing on seaweed carbohydrate metabolism, which revealed an unexpectedly higher expression on the genes responsible for mannitol uptake machinery. Using the *green fluorescent protein* (GFP) gene delivery vector for vibrios [51.74], engineered *Vibrio halioticoli* cells, which aim to biofuel production, were a little more successful; heterogeneous gene expression of *Zymomonas mobilis* genes in vibrio cells were recently successful.

The creation of a variety of engineered strains based on *V. halioticoli* or other species may open a new horizon in marine biofuel production. The development of techniques to convert seaweed to biofuels are quite challenging. Not only elucidation of the metabolic networks of seaweeds and metabolic engineering as shown in Fig. 51.4, but also breeding of energy-aimed seaweed or algae will be required in future.

51.2.4 Efficient Methane Production from Marine Biomass Resources

A variety of microorganisms are able to convert marine algae biomass beyond vibrios. Different biofuels can be produced using similar substrates from the marine realm by the action of different metabolic processes. Methane fermentation (anaerobic digestion) has been widely used to treat various organic matters such as food wastes, livestock wastes, or municipal wastes, and produce fuel gas simultaneously [51.88]. Methane fermentation is a multistep process, in which particular microorganisms participate in different steps such as hydrolysis, acidogenesis, (hydrogen forming) acetogenesis, and methanogenesis for methane formation from organic matter. In each step, many kinds of microorganisms with same metabolic function can be present simultaneously.

In particular, the recent development of high-rate methane fermentation processes has dramatically encouraged the broad use of the anaerobic treatment of high-strength wastewater [51.89]. This reinforces the needed to obtain microorganisms well adapted to marine conditions and execute methane production using marine biomass resources.

Since marine algae fall roughly into three categories; brown, red, and green algae as mentioned before. Their carbohydrate composition is complex, e.g. containing particular polysaccharides such as alginate and mannitol in brown algae or agar in red algae that are rarely contained in terrestrial biomass. Methane fermentation would be effective for bioconversion of macro algae because it can contain key player microorganisms which consume almost all of major carbohydrates in marine algae. However, it is still important to understand microbial diversity and characterize microorganisms that participate in each step for optimization of methane fermentation.

Alginate and Mannitol Anaerobic Degraders

Acclimation and characterization of microbial consortia that degrade alginate and mannitol are important because these are the main carbohydrates in brown algae, which are the most abundant macroalgae. A poor understanding of the microbial metabolism of such particular carbohydrates have hampered the efficient conversion of marine algae to biogas. A microbial consortium able to degrade alginate and mannitol has been developed and characterized.

Among the 70 microbial sources collected from sea environments in Japan and anaerobically cultured in a medium with alginate as the carbon source, 18 samples produced a detectable amount of end products such as ethanol and volatile fatty acids (VFA), mainly acetate. The microbial community obtained by continuous subcultivation (over 1 year) from sea sand collected from the seashore in Hiroshima Bay, Japan (named microbiota no. 11) showed a higher potential for alginate consumption.

Microbiota no. 11 continuously consumed alginate in the medium containing $5\,g\,L^{-1}$ of sodium alginate over 5 days (Fig. 51.5). From alginate, the major end product was acetate (22 mM) produced after 5 days. Additionally, 5mM ethanol was also produced. Since $4.2\,g\,L^{-1}$ of alginate was consumed during the culture, the carbon-based yield was 32 w/v % to acetate and 6% to ethanol. Phylogenetic assignment by means of rRNA 16S gene sequences identified sequences relative to *Clostridium saccharolyticum* (98% of 16s rRNA similarity), *Citrobacter freundii* (98%), and *Dysgonomonas capnocytophagoides* (95%).

Since stable microbial consortia consuming alginate and mannitol were established, a degradation of the brown algae *Sargassum fusiforme* was performed to evaluate VFA and alcohol production by a consortium composed of those taxonomic groups. Microbial growth and ethanol production were detected in the medium containing algae diluted preparations. This suggested that the dominant groups of the microbiota obtained could degrade actual macroalgae to produce VFAs and alcohols. Especially, with respect to the

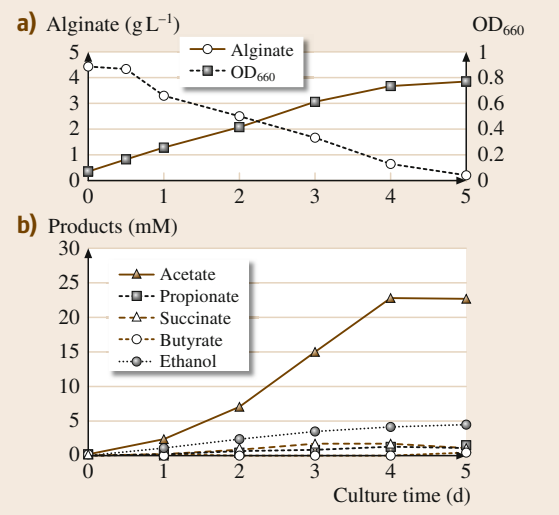

Fig. 51.5a,b Cell growth and alginate consumption **(a)**, and volatile fatty acids and ethanol production from consumed alginate **(b)** by microbiota no. 11 consisting of three kinds of microorganisms acclimated by continuous subcultivation for over 1 year from sea sand collected from the seashore in Hiroshima Bay

five times diluted medium, marine consortia yielded ethanol, lactate, succinate, and acetate as the main end products (Fig. 51.6a). The theoretical methane yield fermentation reached 0.08 L g^{-1}-VS (volatile solid).

This implies that microorganisms from marine samples can be efficiently used for the hydrolysis and acidogenesis steps in methane fermentation of brown algae.

Methane Production from Acetate Under Halophilic Conditions

To promote efficient marine biomass consumption by methane fermentation under halophilic conditions, it is desirable to use marine microbiota in the process. This limitation is significant for marine biomass resources such as macroalgae, microalgae, or other unused marine products because they are saline by nature. Thus, we investigated methane production by microbiota associated with marine sediments collected from Hiroshima Bay as a marine microbial source.

Marine sediment filled a UASB reactor kept at 37 °C for microbiota acclimation in the presence of substrates used to stimulate methane production as sodium acetate and sodium chloride (Fig. 51.6). A medium containing 5 g L^{-1} sodium acetate and 30 g L^{-1} NaCl was

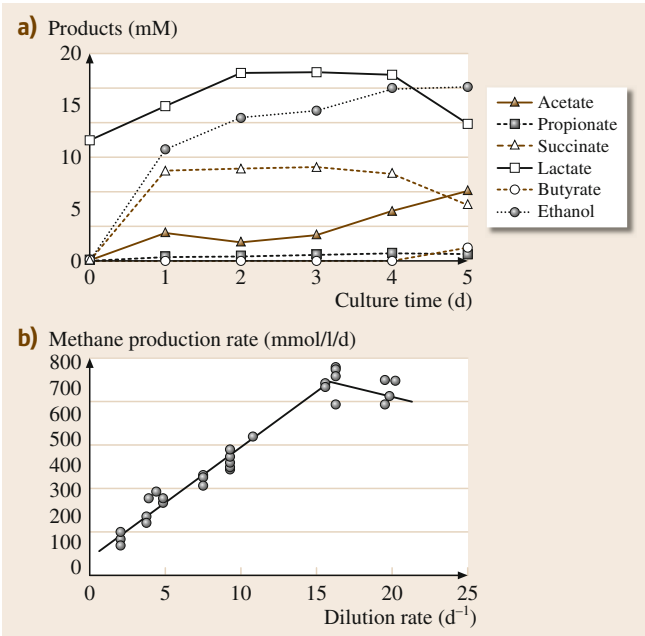

Fig. 51.6a,b Acidogenesis of brown algae and methanogenesis from acetate, a main product of acidogenesis of alginate, by marine microorganisms for methane production. **(a)** Acidogenesis of *Sargassum fusiforme* pretreated with a hydrothermal treatment by the microbiota no. 11; **(b)** continuous methane production from acetate under 3% NaCl with acetoclastic methanogens acclimated from marine mud sediments in the UASB (UASB) reactor

continuously fed from the bottom of the reactor and was withdrawn from the top of the reactor. The dilution rate of the medium was increased stepwise and methane production and acetate consumption were measured. From 2.0–4.9 d^{-1} acetate the removal efficiency was kept above 96%. A higher rate of methane production of 750 mmol L^{-1} d^{-1} (31 mmol L^{-1} h^{-1}) was achieved at 16.2 d^{-1} dilution rate (Fig. 51.6b), where the acetate removal efficiency was 77% and the ratio of the methane production rate and the acetate production rate was 1.

The effect of NaCl concentration on the specific methane production rate was investigated. Acclimated sludge could produce methane with a similar specific rate (0.15 h^{-1}) at 1.5 and 3.0% NaCl. This is much higher than the rates of the well-established acetoclastic methanogen *Methanosaeta soehngenii* (0.008 h^{-1}) [51.90]. Thereafter, although the specific methane production rate decreased, a significant amount of methane was produced even at 6.0% NaCl.

This investigation demonstrated that acetoclastic methanogens with tolerance to salt concentration contained in seawater could easily be acclimated. Moreover, acetate utilization under 3.0% NaCl was 3.2%. This is a significant rate because the specific acetate consumption of the known acetoclastic methanogen, *Methanosarcina barkeri* was only 0.7 mmol CH_4 g^{-1} MLVSS h^{-1} (MLVSS) [51.91].

These results suggest that the exploitation of acetoclastic methanogens in marine environments makes it possible to apply high-rate methane fermentation to marine biomass resources with high salinity. These findings imply that methane fermentation under high salinity may be a promising alternative energy source from marine macroalgae biomass.

51.3 Conclusions

It has been demonstrated that the use of fossil fuel has generated a high environmental impact during the last century. Its intensive use has promoted an increase of the greenhouse effect, in turn promoting significant changes in marine life. Biofuels represent a promising alternative to change the actual model of the energy production of the planet. Biofuels are alternative energy source that will lead to sustainable ways of production. This has stimulated research focused on innovation in biofuel production, because microbial diversity represents the largest genetic repertoire on Earth. Occupying about 70% of the planet, marine waters harbor a yet unexplored biodiversity, especially when it comes to the invisible-to-the eye but accounted for rich variety of metabolites that is still uncharacterized.

In this chapter we have addressed some challenging aspects of biodiesel production to highlight the main barriers that need to be overcome and positive features that make biodiesel one of the most important alternatives to fossil fuels. Indeed, positive initiatives in the exploration of marine microbial diversity can contribute to innovation with respect to different biofuels. The second and third bioethanol generations can benefit from microbial bioprospecting by mining new hydrolase genes from marine metagenomes.

Furthermore, vibrios have been presented as an interesting model to implement the microbial factory concept for ethanol and hydrogen production. Moreover, the potential application of marine microbial methane producers to obtain biogas by anaerobic fermentative processes has been discussed. These efforts show that there are good perspectives for innovation in biofuel production using marine bioresources in the near future. We can expect more utilization of enzymes and microbes prospected from marine microbial diversity, promoting an improvement in the bioprocesses applied to the bioenergy field.

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52. Maraine Biomaterials as Antifouling Agent

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Water is one of the most essential elements to good health – it is necessary for the digestion and absorption of food, helps maintain proper muscle tone, supplies oxygen and nutrients to the cells, rids the body of wastes, and serves as a natural air conditioning system. Polluted water is unsuitable for drinking, recreation, agriculture, and industry. The presence of highly toxic heavy metals and synthetic chemicals in ground water, surface water, drinking water, and aqueous effluent has impact on human and aquatic life. The methods used for the removal of heavy metals include filtration, precipitation, adsorption, ion exchange, reverse osmosis and electrolysis. These processes may be efficient but expensive. Biosorption is a feasible option because it is both efficient and cheap, compared with other conventional methods and having the advantage of low operating cost, minimization of volume of chemicals and biological sludge to be disposed off and high efficiency in detoxifying very dilute effluents. Chitin, chitosan, and alginate are polysaccharides from marine sources which are potential sorbents either in the native state or in the modified forms. Marine biofouling is the undesirable accretion of biological organisms on artificial surfaces immersed such as ship hulls, jetty pilings, navigational instruments, aquaculture net cages, and seawater intake pipes in sea-water. Membrane fouling is one of the most important challenges faced in membrane operations. Fouling results in flux decline, which increases the energy demand for filtration. Hence demands for antifouling membranes are high. This chapter describes the modification of chitin and chitosan marine products for effective antifouling membrane fabrication and the mechanism involved in the antifouling process.

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52.1 Pollution

52.1.1 Pollution of Water and Need for Treatment

Water is one of nature's most important gifts to mankind. Essential to life, a person's survival depends on drinking water. Water is one of the most essential elements to good health – it is necessary for the digestion and absorbtion of food, helps maintain proper muscle tone, supplies oxygen and nutrients to the cells, rids the body of wastes, and serves as a natural air conditioning system. Polluted water is unsuitable for drinking, recreation, agriculture, and industry. It diminishes the

aesthetic quality of lakes and rivers. More seriously, contaminated water destroys aquatic life and reduces its reproductive ability. Eventually, it is a hazard to human health. Nobody can escape the effects of water pollution.

The effects of water pollution strongly impact the balance of nature, which ultimately impacts all humans. Water is polluted in many ways like effluent of leather and chemical industries and dye industries [52.1]. The presence of highly toxic heavy metals and synthetic chemicals in ground water, surface water, drinking water, and aqueous effluent has impact on human and aquatic life [52.2]. The methods used for the removal of heavy metals include filtration, precipitation, adsorption, ion exchange, reverse osmosis, and electrolysis. These processes may be efficient but expensive [52.3]. As a result, biological methods such as biosorption may provide an attractive alternate to physiochemical methods [52.4, 5].

Biosorption is a feasible option because it is both efficient and cheap, compared with other conventional methods and having the advantage of low operating cost, minimization of volume of chemicals and biological sludge to be disposed off and high efficiency in detoxifying very dilute effluents [52.6]. However, the necessity of investigating more biomaterials is still important in order to obtain the best material for industrial application [52.7].

52.2 Use of Marine Biomaterials for Water Treatment

Biosorption is a process that utilizes biological materials as adsorbents, and several researchers have studied this method as an alternative technique to conventional methods for the heavy metal removal from wastewater.

Marine environment is a source of untold diversity of materials with specific biological and chemical features, some of which are not known in terrestrial organisms. For instance, macroalgae synthesize a great diversity of polysaccharides-bearing sulfate groups that find no equivalent in land plants [52.8]. Having normally a support function in those organisms, together with other properties, these biopolymers can be considered to be further used for environmental protection.

52.2.1 Chitin/Chitosan

Chitin is one of the most abundant marine biopolymer. Chitosan is the derivative of chitin. Chitin and chitosan are polysaccharides which at carbon-2 of their cellulose-like backbone have acetamido and amino groups, respectively. The seafood industry waste product chitosan has good physical and biological properties. Chitosan molecules contain a large number of reactive hydroxyl ($-OH$) and amine ($-NH_2$) groups. *Chui* et al. [52.9] confirms that the amino sugars of chitin and chitosan are the major effective binding sites for metal ions, forming stable complexes by coordination. The nitrogen electrons present in the amino and *N*-acetylamino groups establish dative bonds with transition metal ions and some of the deprotonated hydroxyl groups' functions as the donar [52.10] and involved in coordination. Chitin is the one of the most abundant marine biopolymer. Chitosan is the derivative of chitin.

52.2.2 Alginate

Alginate is an unbranched anionic polysaccharide composed of β-D-mannuronic acid (M) and α-l-guluronic acid (G) linked by 1−4 glycosidic bonds [52.11]. Alginate is the main component of the cell wall of brown algae, thus having not only a structural function but also an important participation in ionic exchange mechanisms. Up to 45% of dry weight of algae contains alginate, occurring together with other polysaccharides (cellulose and fucoidan) and proteins, with the amount, as well as molecular structure, being dependent on the algal species, environmental conditions, and life cycle.

The binding mechanisms of heavy metals by biosorption could be explained by the physical and chemical interactions between the cell wall ligands and adsorbates by ion-exchange, complexation, coordination, and microprecipitation. The diffusion of metal from bulk solution to active sites of biosorbents occurs predominantly by passive transport mechanism [52.12] and various functional groups such as carboxyl, hydroxyl, amino, and phosphate existing on the cell wall of the biosorbents can bind the heavy metals [52.13].

From *Krajewska* [52.14], it is observed that by considering the increase of public health and environmental awareness together with the increase of stricter environmental regulations on disposal, biopolymer from renewable resources had been focused as an alternative to synthetic polymer. In spite of potential applications

of chitin and chitosan, it is necessary to establish efficient and appropriate modifications to fully explore the high potential of these biomacromolecules. Chemical modifications of chitin are generally difficult owing to the lack of solubility, and the reactions under heterogeneous conditions are accompanied by various problems such as the poor extent of reaction, difficulty in selective substitutions, structural ambiguity of the products and partial degradation due to severe reaction conditions. Therefore, with regard to developing advanced functions, much attention has been paid to chitosan both in terms of physical and chemical modifications [52.1, 15].

52.2.3 Chitosan in Wastewater Treatment

The binding mechanism of metal ions to chitosan is not yet fully understood. Various processes such as adsorption, ion exchange, and chelation are discussed as the mechanism responsible for complex formation between chitosan and metal ions. The type of interaction depends on the metal ion, its chemistry, and the pH of the solution [52.16, 17]. Metal anions can be bound to chitosan by electrostatic attraction. It is likely that the chitosan-metal cation complex formation occurs primarily through the amine groups functioning as ligands [52.18]. It is well known that chitosan may complex with certain metal ions [52.19]. Possible applications of the metal-binding property are wastewater treatment for heavy metals and radioisotope removal with valuable metal recovery and potable water purification for reduction of unwanted metals [52.20]. Chitosan is a good scavenger for metal ions owing to the amine and hydroxyl functional groups in its structure [52.21–23].

Chitosan has a strong metal binding ability. It was found that their adsorption of uranium is much greater than of the other heavy metal ions [52.24]. Chitosan, a polymer of biological origin, has been reported to be an effective adsorbent for Crs(VI) removal from waste water [52.25]. *Lasko* and *Hurst* [52.26] studied silver sorption on chitosan under different experimental conditions, changing the pH in the presence of several ligands [52.27]. Molybdate anions are selectively bound to chitosan in the presence of excess nitrate (or) chloride ions, with selectivity to chitosan in the presence of Ni^{2+}, Zn^{2+}, Cd^{2+} ions, with selectivity coefficients in the range of 10–10 000 [52.28]. Nair and Madhavan used chitosan for the removal of mercury from solutions and the adsorption kinetics of mercuric ions by chitosan was reported. The result indicates that the efficiency of adsorption of Hg^{2+} by chitosan depends upon the period of treatment, and the particle size [52.29].

52.3 Modification of Marine Biomaterials

Modification of chitosan is to introduce special properties into these abundant biopolymer and enlarge its fields of potential applications [52.30]. Physical and chemical modifications have been performed for improving metal sorption selectivity by template formation (or) the imprinting method [52.31–33].

One of the most interesting advantages of chitosan is its versatility. The material can readily be modified physically, preparing different conditioned polymeric forms such as powder, nanoparticles [52.34], gel beads [52.35], membranes [52.36], sponge [52.37], honeycomb [52.38], fibers [52.39], and hollow fibers [52.40] for various fields of applications such as wastewater treatment, biomedical, and textiles.

52.3.1 Beads

Chitosan has a very low specific area ranging between 2 and 30 $m^2 g^{-1}$ [52.41]. Glutaraldehyde-crosslinked chitosan gel beads have a higher specific surface area around 180–250 $m^2 g^{-1}$ [52.42]. Gel bead conditioning significantly modifies the porous characteristics of the polymer, which may explain the differences in the sorption properties of the material [52.43].

The crosslinked chitosan beads have very high adsorption capacities to remove the anionic dyes whose maximum monolayer adsorption capacity ranges from 1911–2498 g kg^{-1} at 300 °C. The adsorption capacities of the crosslinked chitosan beads are much higher than those of chitin for anionic dyes. It shows that the major adsorption site of chitosan is an amine group – NH_2, which is easily protonated to form – NH_3^+ in acidic solutions. The strong electrostatic attraction between the $-NH_3^+$ of chitosan and anionic dye can be used to explain the high adsorption capacity [52.44]. Chitosan bead is a good adsorbent for the removal of Congo red from its aqueous solution and 1 g of chitosan

in the form of hydro gel beads can remove 93 mg of the dye at pH 6 [52.45].

Alginate–chitosan hybrid gel beads were prepared and shown to very rapidly adsorb heavy metal ions [52.46]. Modified chitosan gel beads with phenol derivatives were found to be effective in adsorption of cationic dye, such as crystal violet and Bismark brown [52.47].

52.3.2 Flakes

Maruca [52.48] used chitosan flakes of 0.4–4 mm for the removal of Cr(III) from wastewater. The sorption of arsenate on to chitosan flakes has been studied. The maximum adsorption capacity occurs at an initial pH 3.5 [52.49]. Chitosan was chemically modified by introducing xanthate group onto its backbone using CS_2 under alkaline conditions. The chemically modified chitosan flakes were used as an adsorbent for the removal of Cd ions from electroplating waste effluent under laboratory conditions. The maximum uptake of Cd was found to be 357.14 mg g^{-1} at an optimum pH of 8 whereas for plain chitosan flakes it was 85.47 mg g^{-1} [52.50]. The Cr(VI) removal ratio was optimized by surface response methodology. Accordingly a maximum of 92.9% Cr(VI) removal was attained at pH 3 with 13 g L^{-1} chitosan flakes from a solution initially concentrated as 30 mg L^{-1} [52.51].

52.3.3 Fibers

Chitosan in the fiber form is not used much for wastewater treatment. Chitosan fibers were tested with aqueous solutions of copper sulfate and zinc sulfate for different periods of time to prepare samples containing different levels of metal ion contents. On chelation of metal ions the chitosan fibers gained substantial increase in both dry and wet strength. The metal ions were readily removed from the chitosan fibers by treatment with an aqueous EDTA solution [52.52]. The recovery of direct dye by adsorption on cross linked fiber was developed and appeared technically feasible. The concentration of amino group fixed in the adsorbent phase was 3.30 mol kg^{-1} dry fibers. A typical direct dye, brilliant yellow was used. The breakthrough curves for adsorption of the dye were measured for different flow rates, bed heights, influent concentration of the dye and temperature [52.53]. Chitosan fibers have been studied for the recovery of dyes and amino acids [52.54]. But less attention has been paid to the use of this conditioning of the polymer for the recovery of metal ions.

52.3.4 Hollow Fibers

Hollow fibers have recently received attention with the objective of performing the simultaneous sorption and desorption of the target metal. Hollow chitosan fibers were prepared and the system was used for the recovery of chromate anions. The hollow fibers were immersed in the chromate solution while an extractant was flowed through the lumen of the fiber. Chromate anions adsorbed on the fiber were re-extracted by the solvent extractant. The hollow fiber acts simultaneously as a physical barrier that can make the extraction process more selective [52.39, 55].

52.3.5 Membrane

Membranes and membrane processes were first introduced as an analytical tool in chemical and biomedical laboratories; they developed very rapidly into industrial products and methods with significant technical and commercial impact. Today, membranes are used on a large scale to produce potable water from sea and brackish water, to clean industrial effluents and recover valuable constituents, purity, and to separate gases and vapor in petrochemical process [52.56, 57].

Muizzarelli reported a decrease in the metal ion sorption efficiency of chitosan membranes compared to chitosan flakes and attributed this effect to a decrease in contact surface, despite the thickness of the membrane [52.58]. *Krajewska* prepared chitosan gel membranes and extensively characterized their diffusion properties [52.59]. The permeability of metal ions through these membranes was measured, Cu < Ni < Zn < Mn < Pb < Co < Cd < Ag [52.60]. Crosslinked chitosan membranes with epichlorohydrin has been proposed to improve pore size, distribution, mechanical resistance, chemical stability, and adsorption properties [52.61–63]. The maximum Cr adsorption capacity occurred in epichlorohydrin-crosslinked chitosan at pH 6 [52.64]. The removal of divalent metal ions including Cu(II), Co(II), Ni(II), and Zn(II) from aqueous solutions by chitosan-enhanced membrane filtration was studied. At neutral condition, the removal of Cu(II) was more efficient compared to other metals [52.65].

Chitosan membranes as sorbents for the trace elements determination in surface water have been tried by *Mladenova* et al. [52.66] and the authors have successfully used their membranes as an efficient sorbents for the pre concentration and they have recommended their membranes for solid phase extraction of Cd(II), Eu(II), Ni(II), and Pd(II) from surface water.

52.4 Antifouling Marine Biomaterials for Water Treatment

52.4.1 Fouling

Fouling is a problem encountered with most filtration processes as it reduces productivity and impairs the capabilities of the membrane due to the decline in flux because of concentration polarization or fouling of the membrane [52.67]. If the fouling effect is not controlled, membrane processes can become nonviable. Permeate flux usually decreases during fouling because of retained molecules that accumulate on, or within the membrane pores.

Biofouling is a major challenge for the biomedical industry as well. Healthcare-associated infections are attributed to biofilms on surfaces such as countertops, doors, beds, surgical tools, or medical devices such as catheters. The Centers for Disease Control and Prevention have reported that these healthcare-associated infections account for an estimated 1.7 million infections and 99 000 deaths annually in the United States [52.68]. Furthermore, these infections accounted for nearly $45 billion of patient costs in 2007 [52.69]. The formation of an atherosclerotic plaque within the arterial wall can be broadly described as a biofouling process [52.70].

Marine Organisms in Fouling

Marine biofouling is the undesirable accretion of biological organisms on artificial surfaces immersed such as ship hulls, jetty pilings, navigational instruments, aquaculture net cages, and seawater intake pipes in sea-water. There are over 4000 species worldwide, and marine biofouling communities will begin to grow on substrates immediately upon submersion. The groups of organisms that contribute to marine biofouling such as bacteria, algae, plant and animal colonies include seaweeds, bivalves, crustaceans, and barnacles. Biofouling on ships reduces their speed and manoeuvrability, resulting in increased fuel and maintenance costs. On static structures, such as buoys, piers, and jetties, biofouling can accelerate corrosion as well as increase the risk of mechanical failure. Fouling is a serious problem causing the blockage of seawater intake pipes. Fouling organisms and organisms hitch-hiking in the ballast tanks of ships are the major vectors for invasive organisms causing environmental impacts in many countries.

Based on the size of fouling material the marine biofouling can be divided into two groups. They are microfouling and macrofouling. The micron/submicron-size organisms such as bacteria's, algae, and microbes and quickly colonize any substrate placed in seawater forms microfouling. The macrofouling includes many larger animals and plants that may attach as individuals or in large colonies, such as barnacles, mussels, polychaetes, and various species of bryozoans and hydroids. Surface chemistry is a significant factor in the formation, stability, and release of adhesion of fouling organisms to surfaces. The work by *Baier* in the late 1960s demonstrated a correlation between relative adhesion of fouling organisms and the energy of the surface [52.71].

Membrane fouling is one of the most important challenges faced in membrane operations [52.72, 73]. Fouling results in flux decline, which increases the energy demand for filtration [52.74]. To counteract this problem, membranes are cleaned, often with aggressive chemicals, to remove the foulants adsorbed on membrane surfaces. When cleaning becomes ineffective, the membranes must be replaced [52.75]. Fouling by proteins, other biomolecules, and organic matter is generally attributed to the hydrophobic nature of membrane materials, which leads to a high interfacial energy with water-rich media that is reduced upon biomolecules adsorption [52.76].

The fouling is usually described by the formation of a *cake* on membrane surface [52.77]. The protein adhesion is affected by interactions between molecules and membrane surface, solution chemistry, pH, ionic strength, and membrane morphology [52.76]. An adjustment of these parameters could be the key to solve the antifouling phenomena. In food and biotechnological industries, ultra and microfiltration processes besides proteins often deal with polysaccharides. In fouling investigations, polysaccharide solution can be of great interest, namely starch that is an important dietary energy source and frequently present in complex mixtures of biomacromolecules.

Biofouling is a very dynamic process, which spans numerous length and time scales. Fouling of a new surface in the marine environment is typically described as a four-phase process: formation of a conditioning layer of organic molecules, primary colonization by microorganisms such as bacteria and diatoms, unicellular colonization by algal spores, and attachment of multicellular macrofoulers [52.78].

Marine Biomaterials as Antifouling Agents in Water Treatment

In general, membrane fouling occurs more seriously on hydrophobic membranes than hydrophilic ones because of hydrophobic interactions between solutes, microbial cells, and the membrane surfaces [52.79]. As a result, much attention has been made to reduce membrane fouling by modifying hydrophobic membranes to relative hydrophilic [52.80, 81].

Polysulfone (PSF) membranes have been widely used in the fields of ultrafiltration, gas separation, pervaporation, and hemodialysis, playing a decisive role in plasma separators, membrane oxygenators, and artificial organs [52.82]. However, due to the hydrophobic nature of PSF material, conventional PSF membranes easily suffer serious membrane fouling and may cause life-threatening complications [52.83].

On the basis of the empirical criteria recently proposed by *Ostuni* et al. [52.84], nonfouling polymers should be hydrophilic, electrically neutral and possess hydrogen-bond acceptors. Accordingly, several polymer classes have been explored [52.85], including polyacrylates [52.86], polyzwitterions [52.87], poly(ethylene glycol) (PEG) derivates [52.88] and chitosan derivatives. In a facile and effective method, chitosan coating and glutaraldehyde crosslink combined with pretreatment by ozone was developed for endowing nonwoven membrane with an antifouling and antibacterial surface [52.89].

Hydrogels – crosslinked polymer networks that swell in the presence of water – have also been investigated for antifouling applications. *Rasmussen* et al. demonstrated that hydrogel surfaces of alginate, chitosan, and polyvinyl alcohol substituted with stilbazolium groups (PVA-SbQ) inhibited settlement of *Balanus amphitrite*. This group also showed that the PVA-SbQ surface inhibited adhesion of the marine bacterium *Pseudomonas* sp. [52.90].

Biocompatible and naturally occurring chitosan was used as an additive for the preparation of a polysulfone ultrafiltration membrane. Two different compositions of polysulfone in *N*-methylpyrrolidone (NMP) and chitosan in 1% acetic acid were blended to prepare PSf–CS ultrafiltration membranes by the diffusion induced phase separation (DIPS) method. The PSf–CS membrane showed an enhanced hydrophilicity compared to a PSf ultrafiltration membrane. An improved antifouling property was observed for PSf–CS blend membranes as compared to pristine PSf ultrafiltration membranes. Both the permeation and antifouling properties of PSf–CS membranes increased with an increase in chitosan composition [52.91].

Using the very promising technology in water purification industry, *Lin* et al. [52.92] coupled chitosan/UF process for dissolved organic matter and arsenic removal. In this investigation, they concluded that even chitosan has weak affinity for arsenic but adsorbs effectively the dissolved organic matter. Also chitosan retards membrane fouling effectively. Under the experimental condition arsenic rejection is only about 10% but the humic compounds about 22%.

Strategies that are used to reduce fouling on membranes can be divided into three groups, i. e.:

i) Changing operating conditions
ii) Modification of the membrane
iii) Modification of the feed [52.93].

Suitability of chitosan as an antifouling agent by the biocatalyst (yeast cells) for the fouling of membrane was studied by *Le Roux* et al. [52.94].

It is assumed that the reduced fouling in the presence of chitosan flakes is largely due to the physical restriction (entrapment) of the yeast cells by the chitosan preventing the yeast cells from forming a cake on the membrane surface. However, at pH 7.5 (buffer solution) 5% of the amino groups of chitosan are positively charged (assume $pKa = 6.2$) [52.95] which implies that chitosan at pH 7.5 is polycationic. In a recent study, it was shown that the yeast cells of *Rhodosporidium toruloides* have the ability to adhere both to polycationic and polyanionic surfaces due to distinct cationic and anionic regions on the cellwall [52.96]. This means that irrespective of the essential charge of the chitosan (due to possible Donnan effects) there will be attractive forces between chitosan and yeast cells. This implies that apart from the physical restrictions, electrostatic attractions will contribute to the retainment of the cells by the chitosan.

Protein fouling in membrane processes is a complicated mechanism due to many factors affecting fouling formation. It is known that the electrostatic force and the hydrophobic interaction between certain domains in protein molecules and the hydrophobic membrane surfaces as well as between protein molecules are the main factors affecting membrane fouling [52.97]. The chitosan/alginate multilayers with either alginate or chitosan showed that the outermost layer displayed very low interaction with albumin showing the use of Chi/Alg multilayers as antifouling coatings to avoid interaction with proteins and to decrease cell uptake [52.98].

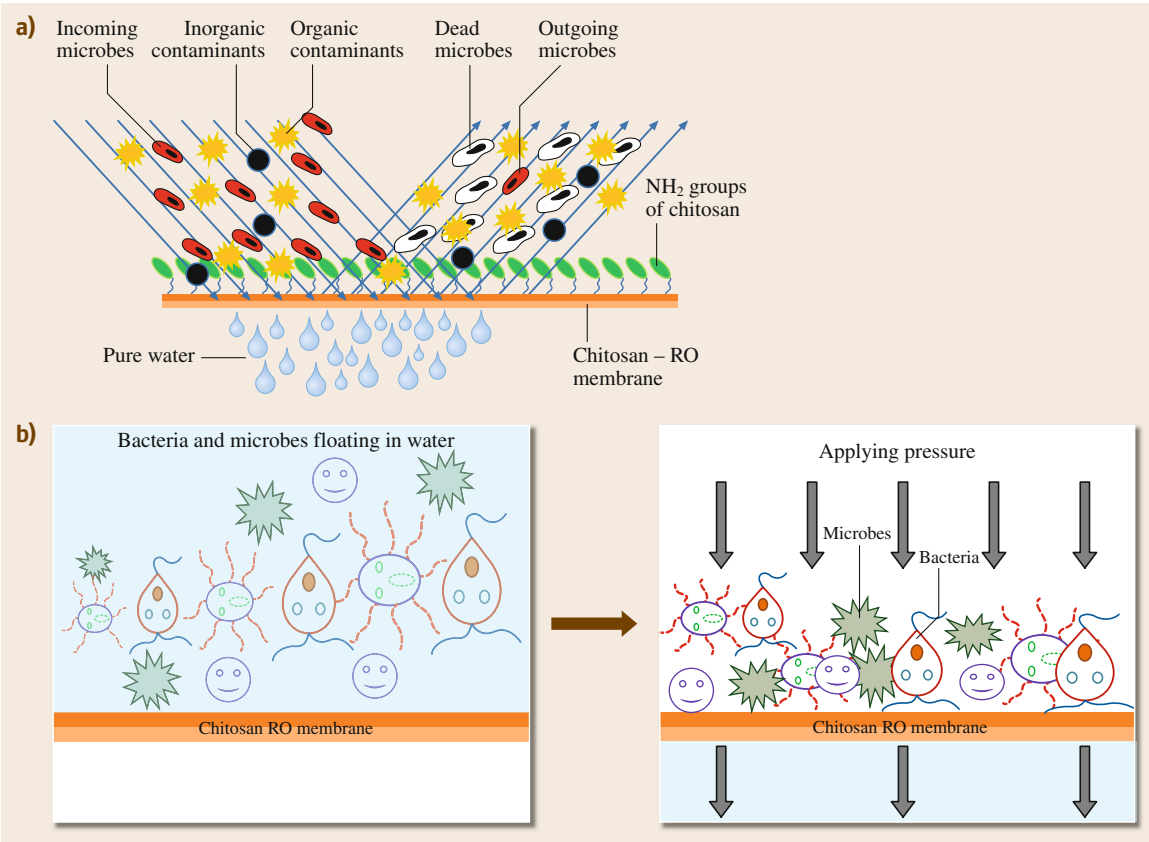

Fig. 52.1a,b Rejection of the microbes along with contaminants through RO membranes and removal of the same from membrane due to the pressure of feed. **(a)** Rejection of microbes through RO membrane. **(b)** Removal of microbes by applying pressure.

Possible mechanisms for the antifouling properties of chitosan-containing membranes are presented below:

1. Rejection of the microbes along with contaminants through RO membranes and removal of the same from membrane due to the pressure of feed (Fig. 52.1a,b).
2. Removal of the microbes due to the zwitterions complex formation with chitosan-based membranes (Fig. 52.2).
3. Synergistic effect of chitosan and nanosilver in killing the microbes (Fig. 52.3a,b).
4. Electrostatic repulsion of charged microbial cells by chitosan/alginate-based membranes (Fig. 52.4).

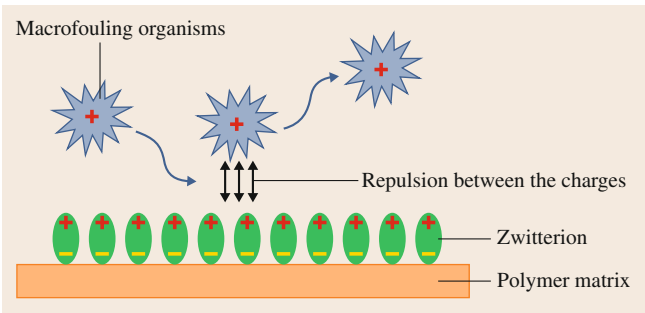

Fig. 52.2 Removal of the microbes due to the zwitterions complex formation with chitosan-based membranes

Huisman et al. [52.99] reported that membrane–protein interactions influenced the fouling behavior in the initial stage of filtration and in the later stage of filtration, protein–protein interactions dictated the overall performance. The interactions also depend on other parameters such as membrane materials, solution type, and operating conditions. Solution pH is an impor-

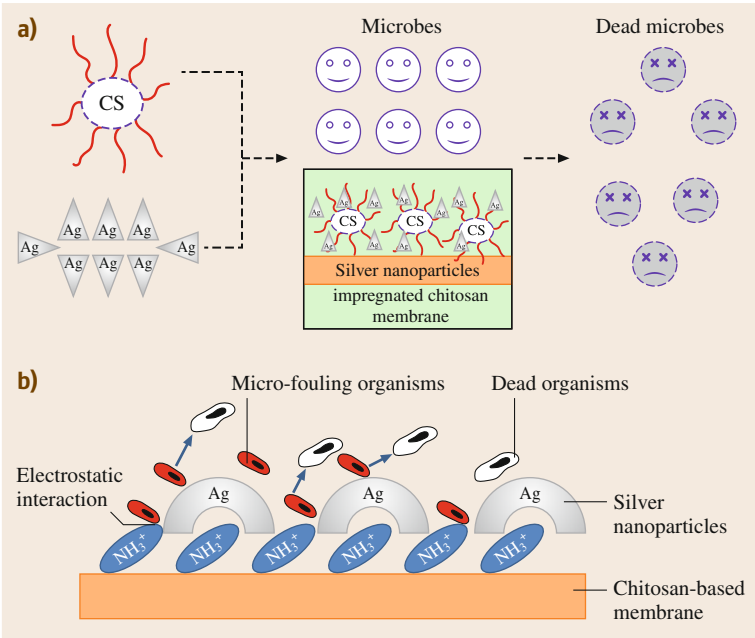

Fig. 52.3a,b (a) Synergistic effect of chitosan and nanosilver in killing the microbes. (b) Electrostatic interaction between the membrane and microbes

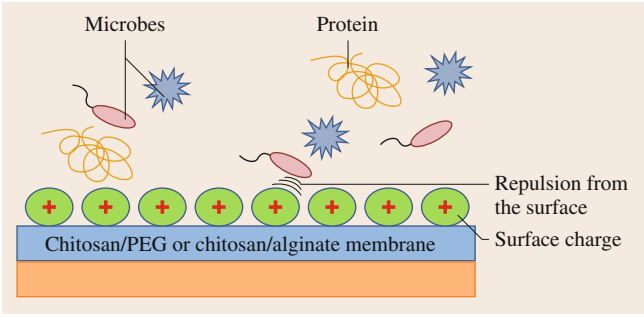

Fig. 52.4 Electrostatic repulsion of charged microbial cells by chitosan/alginate-based membranes

tant factor which can strongly affect membrane fouling and filtration performance. It was reported that change in pH could cause fouling in protein filtration. *Zhao* et al. [52.100] found that bovine serum albumin adsorption on chitosan/PES (polyethersulfone) composite microfiltration (MF) membrane was highest at the IEP (isoelectric point) and at low pH (3.0–4.7), the MF composite membranes had higher adsorption capacities of BSA than that at higher pH range (6.0–8.0). *Mo* et al. [52.101] emphasized the effect of pH on BSA fouling in reverse osmosis (RO) process.

To date, effective prevention strategies have primarily relied upon antimicrobial agents that usually show toxicity against nontarget organisms. While a variety of nontoxic surface modification technologies have been employed, their efficacy has been very limited in in-situ environments. A number of extracellular polysaccharide (EPS) formulations purified from marine bacteria were evaluated as potential antifouling agents. EPS from *Alteromonas, Pseudomonas*, and *Vibrio* spp. were dip-coated onto cleaned glass slides, placed into a flow cell apparatus with real-time imaging, and then exposed to natural flowing seawater under laboratory conditions over a 5 d timeframe. All six of the purified EPS formulations inhibited biofouling (primarily, bacteria) over the test period, with HYD 657, HE 800, HYD 1644, and HYD 1545 showing the most significant reductions in fouling. Surface area fouling was reduced by 90% relative to a cleaned glass control substrate [52.102]. EPS, proteins, and DNA are highly hydrated hydrophilic molecules, but some EPS have hydrophobic properties. There are also clear advantages to using natural polymers and especially bacterial exopolysaccharides for antifouling purposes. Exopolysaccharides do not contain toxic heavy metals or other molecules that adversely affect the local ecology. In addition, such polymers can easily be produced using relatively simple bacterial cultivation protocols and commercially available fermentation equipment.

Nanosilver is regarded as a new generation of antibacterial agents and has great potential to be utilized in antibacterial surface coatings for medical devices,

food package, and industrial pipes. However, disadvantages such as easy aggregation, uncontrollable release of silver ions, and potential cytotoxicity greatly hinder its uses. Recently, polymers possessing unique functions have been employed to fabricate nanocomposite coatings with nanosilver for better biocompatibility and enhanced antibacterial activity [52.103]. Regarding antimicrobial activity, chitosan film forming solutions showed antimicrobial behavior against *Escherichia coli* and *Lactobacillus plantarum* [52.104].

52.5 Conclusion

It is clear that protein, polysaccharide adsorption, and subsequent biofouling are strongly influenced by surface chemistry. Correlations have been observed between protein adsorption and biofouling in both the marine and biomedical environments. Resistance to protein adsorption could be used as an inexpensive way to screen new materials for antifouling properties. A single chemistry has not yet emerged as a universal antifouling strategy. However, a variety of surface chemistries have shown promise as fouling-release coatings. A combination of chemical and physical antifouling strategies is therefore necessary to produce an optimal coating.

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Part I

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53. Marine Biomaterials

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In recent years, significant development has been achieved in marine biomaterials for various biological and biomedical applications. In the present chapter, we discuss isolation techniques and the application of marine-derived polymers and ceramics in detail. The main marine polysaccharides are alginate, chitin, chitosan, and fucoidan. The marine-derived polymers show substantial biological properties such as those of anti-inflammation, antimicrobial, anticancer, and osteoporosis. In addition, marine-derived ceramics play an important role in bone related treatment. Isolation procedures and the application of hydroxyapatite (HA) and biosilica are also discussed. HA that has been isolated or developed from different sources such as fish bone, fish scales, and coral is discussed along with its biomedical applications. Properly implemented marine-derived biomaterials will be promising materials for mankind.

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53.1 Examples of Marine Biomaterials

Approximately 72% of the earth is covered by water that is divided by oceans, lakes, rivers, etc. The oceans contain 97% of the earth's water. They do not only consist of salty water but are an abundant resource for food, medicine, and various raw materials. Marine species are economically important to humans in various ways, including food fish. In recent years, marine-derived biomolecules (proteins, natural compounds, etc.) have been given much importance in medicine and engineering. Marine environments are the household to many exotic biological materials that may inspire biomimetic materials.

Biomaterial science is concerned with the interaction of substances with biological metabolism. Biomaterials can be derived from synthetic sources and natural sources. Synthetic materials are usually metallic, polymeric, and ceramic, or in the form of composite materials. These materials are often used for biomedical applications, including surgery, tissue engineering, and drug delivery. If the substance comes from a natural source (marine), it can be called marine biomaterial. The important sources of marine biomaterials are fish, invertebrates, mammals, reptiles, fungi, and corals. Fish skin is a rich source of collagen and bone for hydroxyapatite. Algae are a rich source for several polysaccharides. Marine-derived biomaterials have been checked to solve the bone related defects; they include materials from polymers, ceramics, and biomimetic materials. The main polymers derived include alginate, chitin, chitosan, fucoidan, etc. (Fig. 53.1).

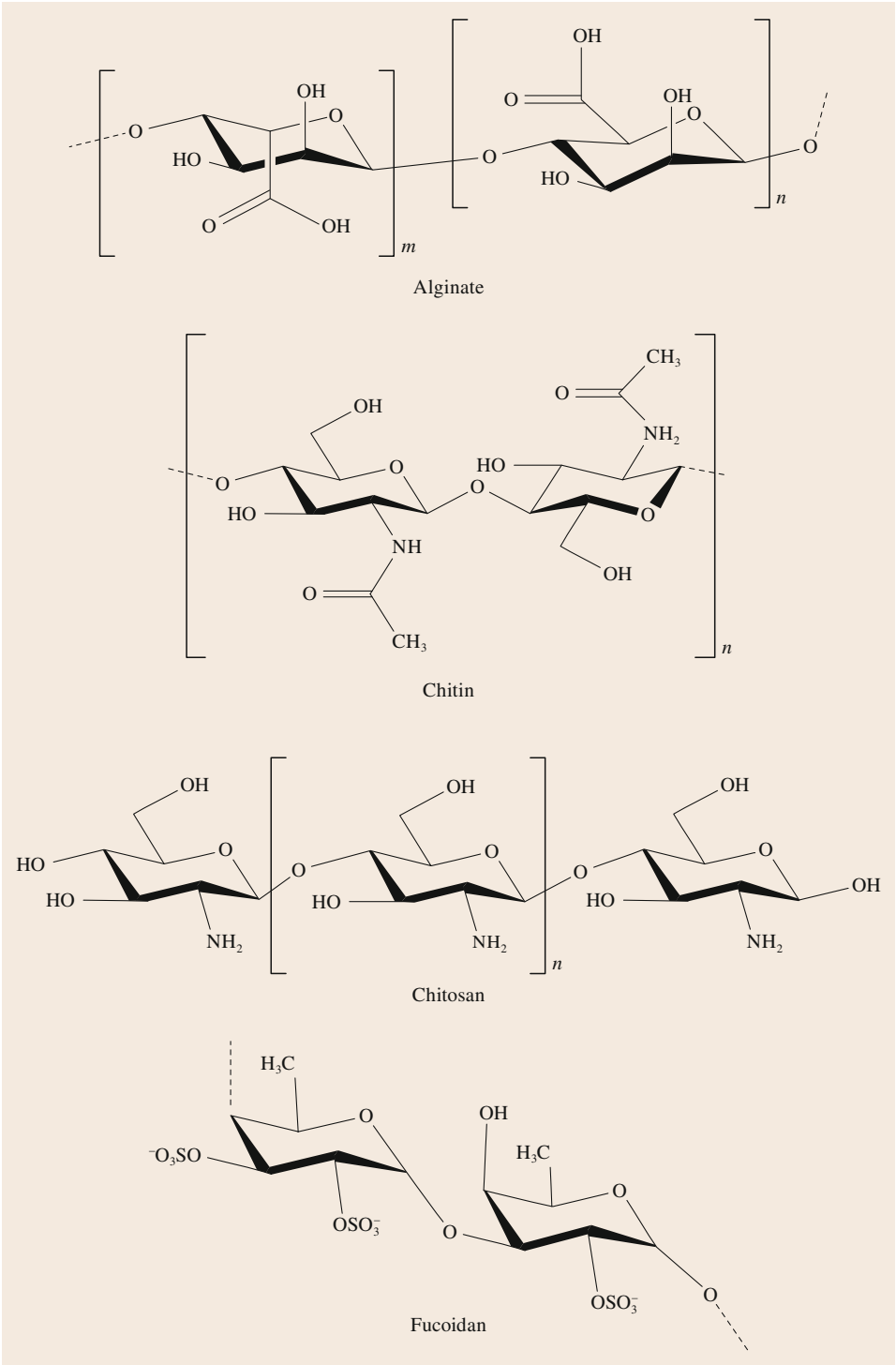

Fig. 53.1
Structure of important biomedical marine polysaccharides

53.2 Marine Polysaccharides

53.2.1 Alginate

Alginate is a biopolymer and found in seaweed and typically extracted from brown algae (*Phaeophyceae*) including *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis prrifere* by treatment with aqueous alkali solutions, typically with NaOH. The extract is filtered with calcium chloride and added to the filtrate in order to precipitate alginate. This alginate salt can be transformed into alginic acid by treatment with diluted HCl. Further purification produces water-soluble sodium alginate [53.1–3] (Fig. 53.2).

Bacterial alginate can be produced from *Azotobacter* and *Pseudomonas* [53.4]. The pathway of alginate biosynthesis is generally divided into four steps:

- Synthesis of precursor substrate
- Polymerization and cytoplasmic membrane transfer
- Periplasmic transfer and modification
- Export through the outer membrane.

Alginate is composed of guluronic acid (G) and mannuronic acid (M), which is considered to be biocompatible, nontoxic, nonimmunogenic, and biodegradable. Alginate extracted from different sources differs in mannuronate and gluluronate content, as well as in the length block. The alginate is known to form a hydrogel by hydrogen bonding at low pH. The alginate and alginate chemical modification can bring about new biomaterials, which are useful in cell immobilization, tissue engineering, and drug delivery. Alginate hydrogels have been proven to improve the neocartilage and neo-bone formation [53.5–8]. Alginate hydrogels are particulary used for several biomedical applications such as tissue engineering, drug delivery, and wound healing, due to their structural similarity with extracellular tissues [53.1]. Hydrogels are three-dimensionally cross-linked networks composed of hydrophilic polymers with high water contents. Chemical or physical cross-linking of hydrophilic polymers are typical approaches to form hydrogels and their physicochemical properties are highly dependent on the cross-linking type and amount of cross-linking agents [53.9]. The most widely used method to make the alginate gel is with calcium chloride, calcium carbonate, calcium sulfate, and sodium hexametaphosphate (ion cross-linking agents). Covalent cross-linking of alginate with poly (ethylene glycol)-diamines of various molecular weights was first investigated in order to prepare gels with a wide range of medicinal properties. Thermal gelation and cell cross-linking are some other processing methods to make the alginate hydrogels [53.1].

Biomedical Applications

Drug Delivery. The conventional application of alginate in pharmaceutics is to serve as thickening, gel forming, and stabilizing agents. Multiple drugs have been incorporated with alginate hydrogels with different materials such as poly (caprolactone) [53.10], carbon nanotubes [53.11], and chitosan [53.12]. Alginate is an excellent biomaterial for the delivery of drugs. Alginate microspheres are used to encapsulate or load the desired amount of protein for protecting the protein functions, transporting it to the targeted sites, and controlling the kinetics of the protein release. Alginate microspheres were prepared small scale with a water in oil emulsion technique and loaded with fluorescently labeled immunoglobulin G (IgG) [53.13]. Alginate microspheres have been coated with Bombyx mori silk fibroin using layer-by-layer deposition techniques, which provided mechanically stable shells as well as a diffusion barrier to the encapsulated proteins [53.14]. Calcium-cross-linked alginate microspheres and microspheres modified with CpG oligonucleotides are mixed with soluble *matrix* alginate in phosphate buffer saline (PBS) containing soluble IL-2. Diffusion of calcium ions in the microspheres into the surrounding solution induces cross-linking of the soluble alginate and gel formation. The inset figure outlines the process of calcium reservoir alginate microsphere synthesis via water-in-oil emulsion of alginate in isooctane in the presence of surfactants (Fig. 53.3) [53.15].

Wound Dressing. Alginate dressing materials are typically produced by ionic cross-linking of an alginate solution with calcium ions to form a gel, which is subsequently free-dried to obtain a porous sheet. Alginate dressing can retain a physiologically moist environment by absorption and desorption of the water from the gels. Several bioactive alginate wound dressing materials have been studied to date [53.16–19].

Tissue Engineering. Bone is a complex tissue with a hierarchical structure consisting of hydroxyapatite (HA) and collagen as a major portion. Bone defects can occur in several ways such as through trauma, neoplasm, congenital defects, motor accidents, osteoporo-

Raw material of alginate, a brown seaweed growing abundantly in the Ocean

Crushed seaweed, after it has been dried and pulverised for the production of alginate

By reaction with divarent metal cations from seawater (such as calcium), the alginic acid in seaweed forms a water insoluble alginategel. This alginate is partially cross-linked and retains its shape. After beeing rinsed in water, the seaweed swells in acidic water

To isolate alginic acid from thin sodium alginate solution, an acid is added. In an acidic system, insoluble alginic acid is precipitated and isolated. This is the so-called "acid precipitation method"

Precipitated alginic acid is dehydrated and the fibrous wet body (gel) is produced. The fibrous wet body is dried and pulverized to make alginic acid powder

Fig. 53.2 Isolation procedure of alginate from seaweed

sis, arthritis, etc. Various techniques have been used to solve bone defects, for example, autografting, allografting, and xenografting. However, all these techniques have advantages and disadvantages, for example, insufficient donor sites and transmittable disease [53.20]. Thus, considerable attention has been given by researchers and orthopedists to synthetic and natural materials that can solve the bone defect problem. Experiments are currently taking place at the laboratory and clinical levels. Synthetic materials are often made of hydroxyapatite or other naturally occurring biocompatible materials.

Alginate is the best-known material to form a scaffold-forming property, which can be useful to treat loss or failure of organs. Alginate gels have advantages for bone and cartilage regeneration due to their ability to be introduced into the body in a minimally invasive manner, their ability to fill irregularly shaped defects, the ease of chemical modification with adhesion ligands (e.g., arginylglycylaspartic acid (RGD)), and controlled release of tissue induction factors (e.g., BMP (bone morphogenetic protein), TGF-β (transforming growth factor beta)) [53.21–23] (Fig. 53.4).

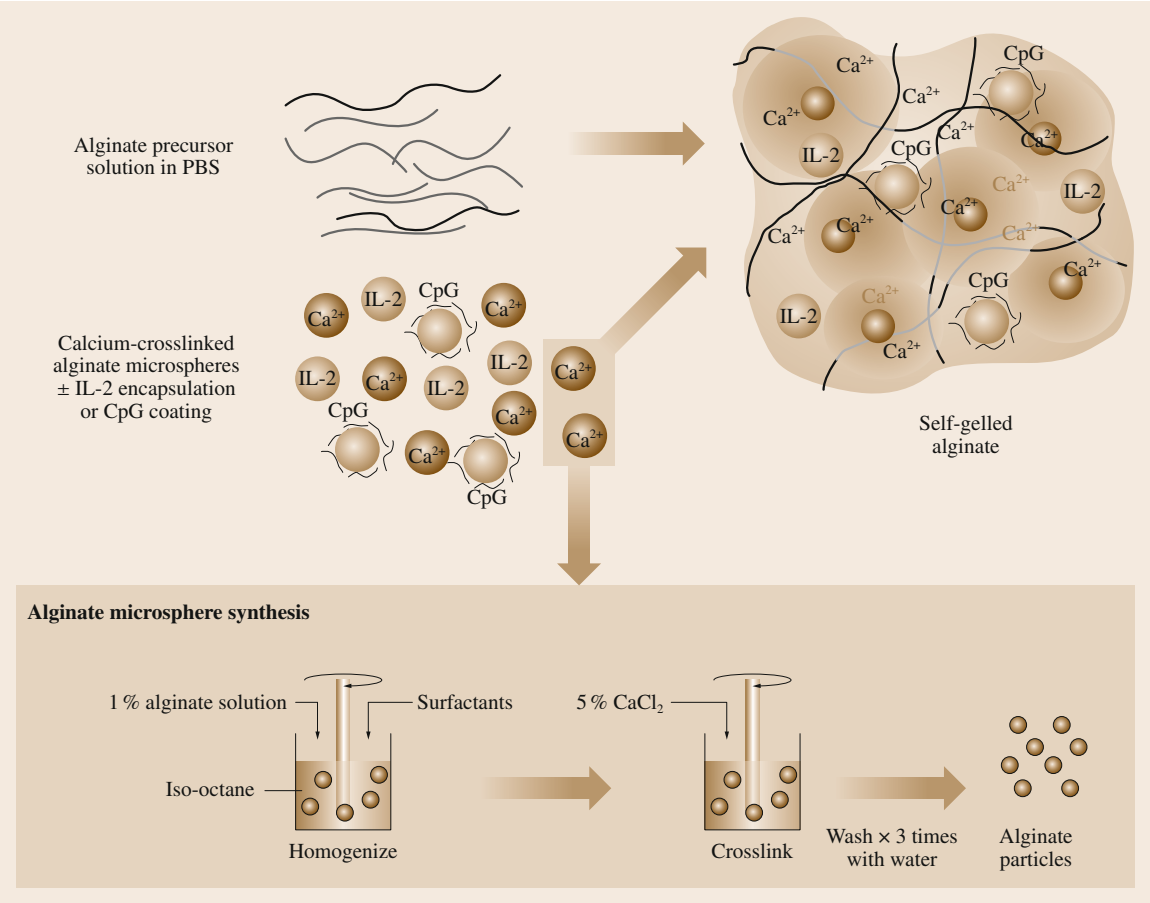

Fig. 53.3 Schematic of self-gelling alginate formulations based on calcium reservoir alginate microspheres (after [53.15])

The transplantation of stem cells using alginate hydrogels has been widely explored in bone tissue engineering [53.24]. The most widely examined application of alginate gels to promote blood vessel formation has exploited their ability to provide sustained and localized release of heparin binding growth factors such as VEGF [53.25, 26]. Due to a lack of mechanical strength of the alginate scaffold to mimic the natural function of bone, it is combined with inorganic materials to enhance strength as well as bone tissue formation. Alginate with hydroxyapatite is the better combination of the porous scaffold. This has been prepared with the phase separation method, which enhances the cell adhesion of osteosarcoma cells [53.27, 28]. Human mesenchymal stem cells (MSCs) encapsulated in the alginate gel bead have been cultured in a serum-free medium with the addition of a transforming growth factor, dexamethasone, and ascorbate, and have been found to form cartilage in large osteochondral defects [53.29–31]. Alginate scaffolds are being actively investigated for their ability to mediate the regeneration of other tissues and organs, including skeletal muscles, nerves, pancreas, and liver. Current strategies for skeletal muscle regeneration include cell transplantation, growth factor delivery, or a combination of both approaches, and alginate was found to be a good candidate in these strategies [53.32–37].

53.2.2 Chitin and Chitosan

Chitin are naturally occurring mucopolysaccharides, usually found in fungi, diatoms, nematodes, arthropids, shrimps, crabs, lobsters, krill, and squid [53.38–47].

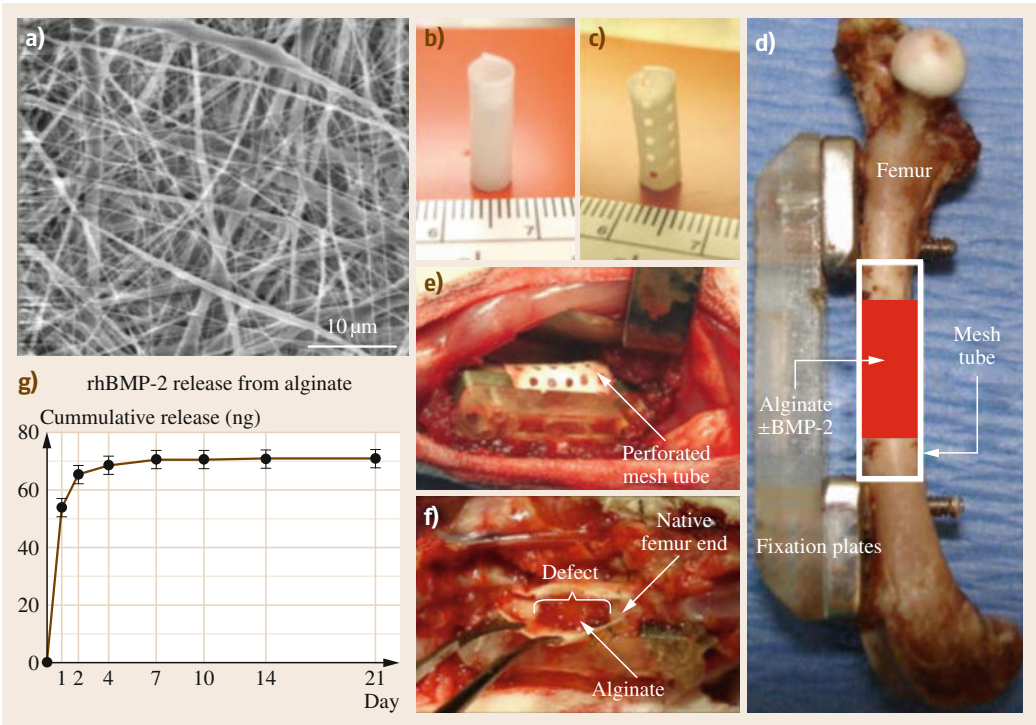

Fig. 53.4 **(a)** Nanofiber mesh tubes and alginate hydrogel for surgery. SEM (scanning electron microscope) image of an electrospun nanofiber mesh illustrating the smooth and bead-free nano-scaled fibers. **(b)** Hollow tubular implant without perforations made from nanofiber meshes. **(c)** Tubular implant with perforations. **(d)** Implants in a segmental bone defect. Modular fixation plates are used to stabilize the femur. A nanofiber mesh tube is placed around the 8 mm defect. In some groups, alginate hydrogel, with or without rhBMP-2, is injected inside the hollow tube. **(e)** Defect after placement of a perforated mesh tube. The alginate inside the tube can be seen through the perforations. **(f)** A specimen was taken after 1 week and the mesh tube was cut open. The alginate was still present inside the defect, with hematoma present at the bone ends, after [53.21]. **(g)** Alginate release kinetics over 21 d in vitro. Sustained release of the rhBMP-2 was observed during the first week

The chemical formula of chitin is 2-acetamido-2-deoxy-β-D-glucose through β (1–4) linkages (Fig. 53.1). This linkage can be easily degraded by the chitinase enzyme [53.48, 49]. Chitosan is a linear polysaccharide composed of randomly distributed β-(1–4)-linked glucosamine and *N*-acetyl-D-glucosamine. Chitosan can be obtained from chitin by a chemical method or an enzymatic production method. Chitosan can be isolated directly from the cell wall of certain fungi, but commercially available chitins are usually prepared from chitin. Chitin and chitosan are white, hard, inelastic nitrogenous polysaccharides and the major source of surface pollution in coastal areas. Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of the crustaceans crab and shrimp, and the cell walls of fungi. The degree of deacetylation can be determined by nuclear magnetic resonance (NMR) spectroscopy; it ranges from 60 to 100%, and the molecular weight is between 3800 and 20 000 Da.

Several methods have been introduced to isolate chitin and chitosan from shellfish waste, the most traditional and well-developed method is a chemical, enzymatic, fermentative method for industrial production, which is simple and convenient for large production [53.51–53].

Production of Chitosan by Chemical Methods

In the chemical hydrolysis method, four main steps are involved in the production from marine crustacean shells, as depicted in Fig. 53.5 [53.54–56]. They are:

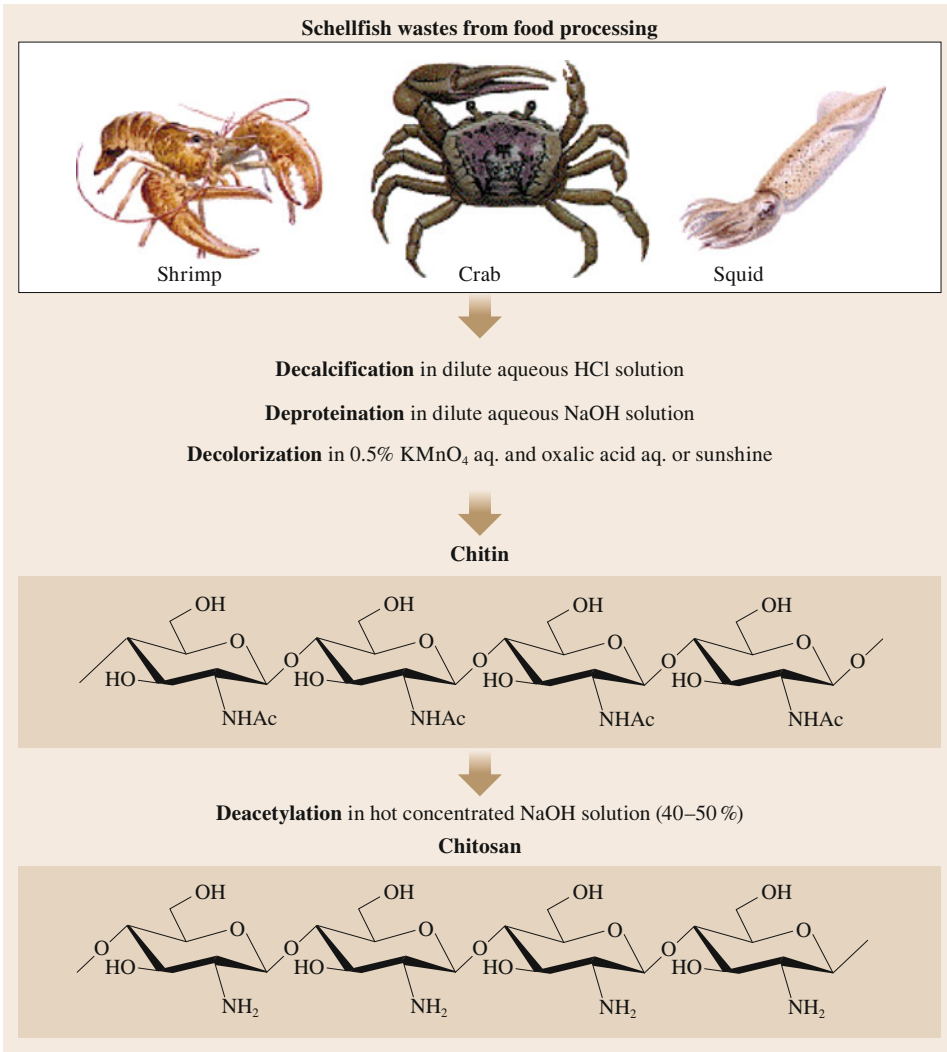

Fig. 53.5 Chemical method for the production of chitosan

- Decalcification in dilute aqueous HCl solution
- Deproteinization in dilute aqueous NaOH solution
- Discoloration in 0.5% aqueous $KMnO_4$ and aqueous oxalic acid or sunshine
- Deacetylation in hot concentrated NaOH solution (40–50%).

In general, proteins are first removed from ground shells by treatment with mild sodium hydroxide or potassium hydroxide solution at elevated temperature. Alkali concentration is usually between 1 to 10% with temperatures ranging from 30 to 100 °C, independent of the starting materials. These are the most common, and reaction times usually varies from 30 min to 12 h. Higher temperature reduces the molecular weight of the resultant chitosans. The removal of calcium carbonate, calcium phosphate, and other mineral salts found in shell waste is accomplished by extraction with dilute acids. To produce 1 kg of 70% deacetylated chitosan from shrimp shells, 6.3 kg of HCl and 1.8 kg of NaOH are required.

Enzymatic Methods

Enzymatic methods are an alternative to the chemical method for chitin and chitosan production. In addition, the protein often remains high and reaction times are significantly increased compared to chemical methods. Enzymatic methods are limited in industrial production

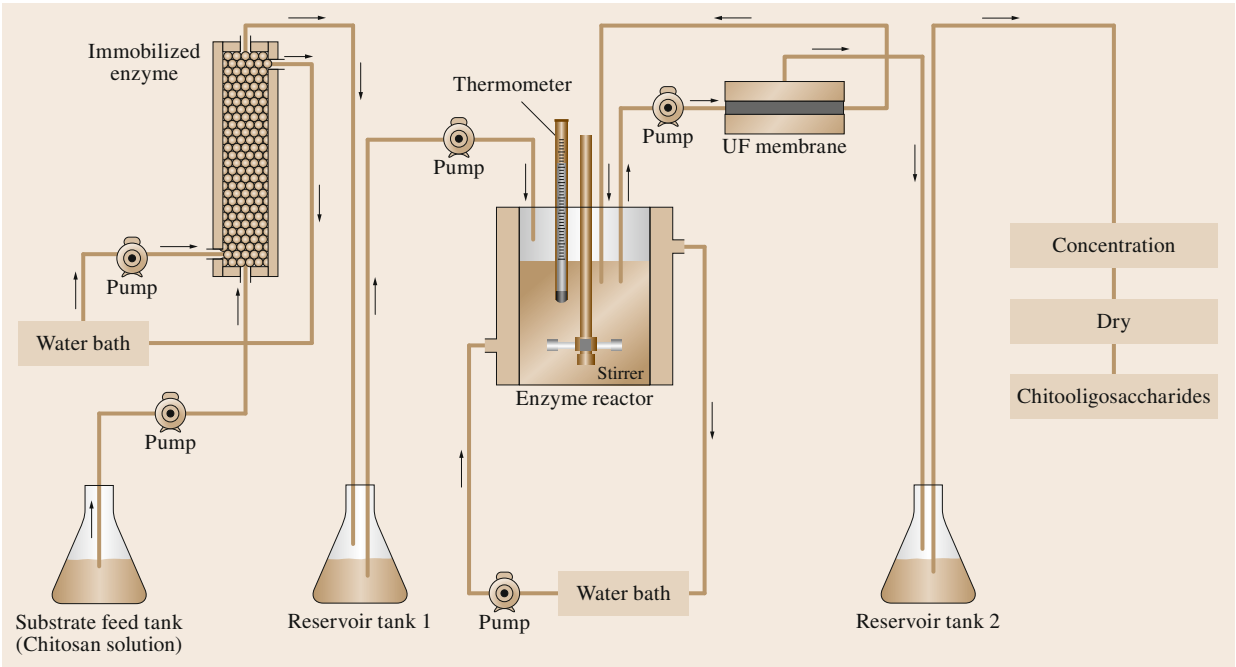

Fig. 53.6 Schematic diagram of the dual reactor system used for continuous production of chitooligosaccharide (after [53.50])

of chitosan, due to higher cost of enzymes [53.53]. Several commercially available enzymes such as alcalase, chymotrypsin, and papain are also used for the production of chitosan [53.57].

Fermentation Methods

Fermentation with bacteria producing proteolytic and chitinolytic enzymes has been researched as an alternative method [53.58]. Organic acid and protease produces a soil isolate of *Pseudomonas aeruginosa* F722 with crab shells. With an optimal fermentation temperature of 30 °C and a 10% glucose supplementation, the degree of demineralization was 92% and the degree of deproteinization was 63% after 7 days incubation [53.59].

Production of Chitooligosaccharides

Lower molecular and water soluble chitosan obtained by continuous hydrolysis of chitosan by chitinolytic enzymes, such as chitinase, chitosanase, papain, and lysozyme are widely used for the production of chitooligosaccharide (COS). Several research groups developed a method for the production of COS with a higher yield and a higher degree of polymerization [53.50, 60–63]. For the continuous production of COS by an enzymatic method, ultrafiltration reactors have been employed. The advantages of continuous production of COS is higher efficiency and greater enzyme productivity; it was found that high the viscosity of chitosan restricted continuous operation due to membrane fouling [53.50].

The continuous production of COS from chitosan has been attained with a dual reactor system with an ultrafiltration membrane reactor and a column reactor packed with an immobilized enzyme. The production of the COS was performed in two steps (Fig. 53.6):

1. Preparation of the partially hydrolyzed chitosan from viscose chitosan in the column reactor packed with an enzyme
2. Production of the oligosaccharides from partially hydrolyzed chitosan in the ultrafiltration membrane reactor.

Three kinds of partially hydrolyzed chitosan were obtained from three different outflow rates (3, 5, and 9 ml/min) in the column reactor and were supplied to a substrate feed tank of the following ultrafiltration (UF) reactor in order to identify the influence of the feed on membrane fouling. The partially hydrolyzed chitosan obtained with a 5 ml/min overflow rate was the most suitable substrate for alleviation of membrane

Table 53.1 Properties of chitosan and their applications

	Properties	Applications
Cationic	Linear polyelectrolyte with high charge density	Water purification
	Chelates toxic metal ions	Excellent flocculent
Chemical	High molecular weight	High viscosity, film forming
	Reactive amino and hydroxyl groups	Chemical modification for specific end uses
Biological	Biocompatible and biodegradable	Non-toxic, packaging films, coating
	Bioactivity	Antimicrobial, antitumor, anticancer, immuno potentiator
Pharmaceutical	Biocompatible, biodegradable	Wound, healing, drug delivery, tissue engineering, contact lens
General cosmetics	Moisture retention, excellent feed, protective film-coating, excellent tactile properties	Skin care products, hair lotions, natural ingredient
Food and agriculture	Binds anions (bile acids or free fatty acids)	Hypocholesterolemic agents, anticarcinogenic agents, dietary fibre, antiulcer
	Fungistatic	Increased crop production
Biotechnological	Extrapment and adsorption	Immobilization matrix, chitosan beads/gels

fouling and efficient hydrolysis under the operating conditions of the dual reactor system [53.50].

Some of other methods are also used for the production of chitin, chitosan and COS such as, gamma irradiation [53.64]. The method of preparation as follows, heads and shells from prawns found on the Algerian coast were collected, dried at 60 °C, and cut into small pieces that were then irradiated at a dose of 75 Gy/min to a dose of 25 kGy. Irradiation reduced the time needed for deproteinization from 3 to 1 h using 1 N sodium hydroxide and a reaction temperature of 85 °C.

Applications

Because it is a natural resource and is thus biologically reproducible, biodegradable and environmentally nonpolluting, biocompatible, nontoxic, and biologically functional, chitosan is a versatile material for various biological and biomedical applications [53.55] (Table 53.1).

Antimicrobial Activity

The antimicrobial activity of any substance is always directed toward its applicability. The film forming ability of any polymers with antimicrobial property can be used for food packaging. Antimicrobial packaging is one of the most promising active packaging systems that have been found to be highly effective in killing or inhibiting spoilage of pathogenic microorganisms that contaminate food [53.65]. Chitosan is best known for its antimicrobial property from the literature [53.66–69].

Variations in chitosan's bactericidal efficacy arise from various factors. According to the roles played, these can be classified into four categories as follows:

1. Microbial factors, related to species and cell age
2. Intrinsic factors of chitosan including positive charge density, molecular weight, concentration, hydrophilic/hydrophobic characteristic, and chelating capacity
3. Physical state, namely water solubility and solidity of chitosan
4. Environmental factors, involving the ionic strength in the medium, pH, temperature, and reaction time.

Chitosan is an ideal biopolymer for developing such antimicrobial films due to its nontoxicity. The inherent antibacterial/antifungal properties and the film-forming ability of chitosan make it ideal for use as a biodegradable, antimicrobial packaging material. The antimicrobial properties of chitosan can be enhanced

by irradiation, ultraviolet radiation treatment, partial hydrolyzation, chemical modifications, synergistic enhancement with preservatives, synergistic enhancement with antimicrobial agents, or in combination with other hurdle technologies.

Anti-Inflammatory Activity

Anti-inflammatory refers to the property of a substance or treatment that reduces inflammation. Chitin is rarely checked for anti-inflammatory property due to its lack of solubility.

Yang et al. reported that COS have shown considerable anti-inflammatory activity with different molecular weights [53.70]. The effects of COS on nitric oxide (NO) production and the cytokine expression TNF-α (TNF: tumor necrosis factor), and interleukin-6 (IL-6) have been checked as lipopolysaccharide (LPS) stimuli from RAW264.7. Stimulation with increasing concentrations of COS, the LPS-stimulated TNF-α, and IL-6 secretion recovered significantly within the incubation media of RAW264.7 cells [53.71].

Anticancer Activity

Chitosan shows several biological activities, as discussed above. Moreover, chitosan significantly inhibits the tumor growth [53.72]. The intra tumoral administration of chitosan compounds alone has been shown to promote antitumoral effects in a metastatic breast cancer model [53.73]. Chitosan was also found to activate macrophages into cytotoxic macrophages and suppressed Meth-A tumor growth in Balb/c mice [53.74]. COS is also shown to inhibit the growth of Meth-A solid tumors transplanted in mice [53.75].

Biomedical Application of Chitin, Chitosan, and COS

The wide array of tissue engineering applications exacerbates the need for biodegradable materials with broad potential. Chitosan is an excellent biodegradable and biocompatibility biomaterial. Natural polymer composite materials are promising scaffolds for bone tissue engineering [53.76]. Next generation biomaterials should combine bioactive and bioresorbable materials, which mimic the natural function of bone and activate in vivo mechanisms of tissue regeneration. Composite materials based on combinations of biodegradable polymers and bioactive ceramics are highly suitable for bone regeneration [53.77]. The important biomedical applications of chitin and chitosan are tissue engineering and drug delivery [53.54, 78–83].

Tissue Engineering

Tissue engineering has been a fascinating area of research in recent years to develop the artificial organs [53.84]. Several materials have been widely used to develop artificial organs; these are synthetic and natural derived materials. Chitosan is a promising biomaterial used for various biomedical applications. Chitosan can be modified to any form such as film, fibers, beads, and scaffolds (Fig. 53.7).

Dimensional structures of scaffolds are used to simulate the extracellular matrices naturally found in the body. The design consists of a large surface area so that cells can be seeded and are able to penetrate the pores. The pores are interconnected so that wastes and nutrients can be exchanged between the scaffold and the surrounding environment, thereby promoting cellular development. The properties of the scaffold are influenced by the method used in the creation of the scaffold. There are several methods used to create highly porous scaffolds, such as supercritical fluid technology [53.85] and the freeze drying method [53.86]. While chitosan has many desirable properties, its mechanical strength is poor, and to enhance the mechanical strength, it is often blended with other polymers and ceramics. Ceramics such as hydroxyapatite are biomaterials that are widely used with chitosan to make the scaffolds [53.76, 87–90].

Drug Delivery

Considerable research efforts have been directed towards developing safe and efficient chitosan-based particulate drug delivery systems [53.91–97]. Chitosan has been used as excipient in oral formulations and vehicles for parenteral drug delivery devices. Chitosan has further been used to manufacture sustained release systems deliverable by other routes (nasal, ophthalmic, transdermal, and implantable devices) [53.98]. Chitosan forms colloidal particles and entraps bioactive molecules through chemical cross-linking, ionic cross-linking, and ionic complex formation for the association of bioactive molecules to polymers and to control drug release [53.99, 100].

53.2.3 Fucoidan

Fucoidan is sulfated polysaccharides, mainly in found brown algae such as mozuku, komby, limu moui, bladderwrack, wakame, hijiki, and sea cucumber. Other common fucoidans are sourced from edible species such as *Fucus vesiculosis*, *Cladosiphon okamuranus*,

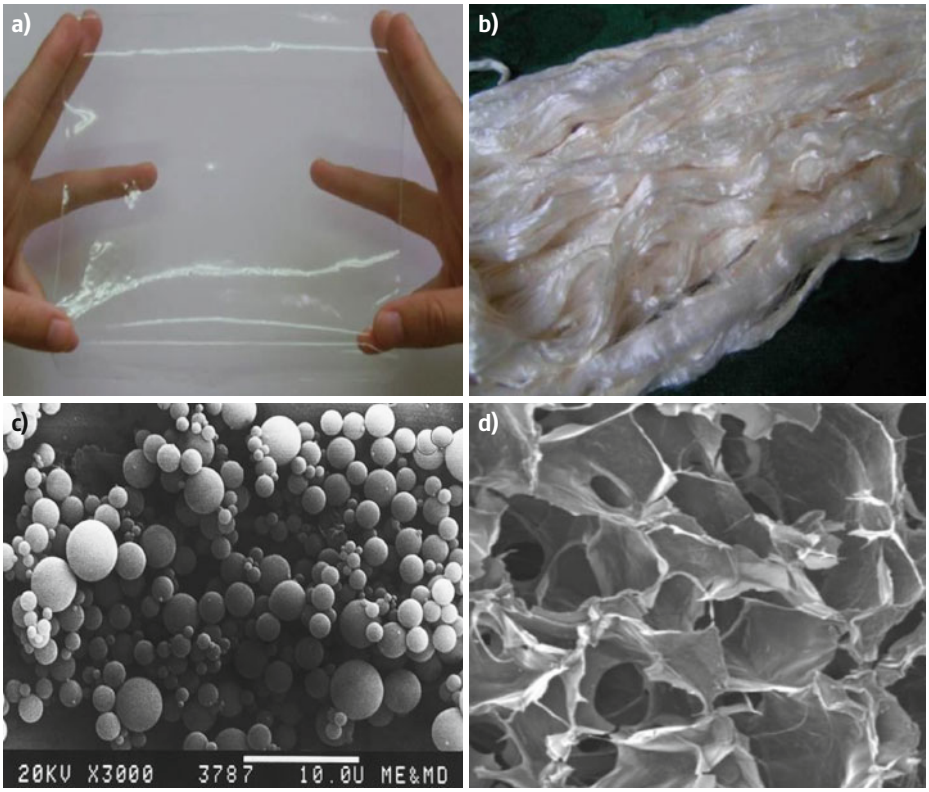

Fig. 53.7a–d Chitosan in different forms. **(a)** Film, **(b)** fiber, **(c)** beads, and **(d)** scaffolds

Laminaria japonica, and *Undaria pinnatifida*. The main skeleton of fucoidans consists of α-1,3-linked sulphated l-fucose; a repeating sequence of alternating $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 4)$ glycosidic bonds is also possible. Published research articles on fucoidans increased threefold between 2000 and 2010. Fucoidan plays a significant role in the control of acute and chronic inflammation via enzyme inhibition and inhibition of the complement cascade [53.101]. Fucoidans contain a high proportion of fucose in the sugar backbone of the polymer. They are sulfated, may be acetylated, and may also contain uronic acids. The yield of a crude first fraction of fucoidan is generally 2–10% by weight. Fucoidans derived from seaweed are all highly branched. A second source of linear rather than branched fucoidans is echinoderms, in particular sea cucumbers [53.102–104].

The chemical composition and structure of fucoidans are very diverse and vary significantly depending on the algae source, place of cultivation and habitat, harvesting time, etc. The biological activity and medicinal impact of fucoidans depends strongly on their structural properties. The extraction and purification procedures of fucoidan are shown in the Fig. 53.8. Fucoidan was isolated from the raw material by dilute acid extraction, ballast alginates were removed by $CaCl_2$ precipitation, and crude extract was purified by chromatography on DEAE-cellulose [53.105] (DEAE: diethylaminoethyl).

Biological Applications of Fucoidan

Anticancer Activity. Cancer is known to be one of the worst diseases that threaten human's lives. Unfortunately, drugs used for cancer therapy are toxic and affect not only cancer cells but also normal cells and tissues. It has been reported that fucoidans effectively inhibit proliferation and colony formation of cancer cells in vitro [53.107, 108] and also inhibitory activity in tumors growing in vivo [53.106]. Studies suggest that sulfate content, molecular weight, monosaccharide composition, and the structure of the main polymer chain of fucoidan have a great influence on their biological activities. A higher amount of sulfated content in fucoidan shows a higher antitumor activity with low degree substitution [53.109–113].

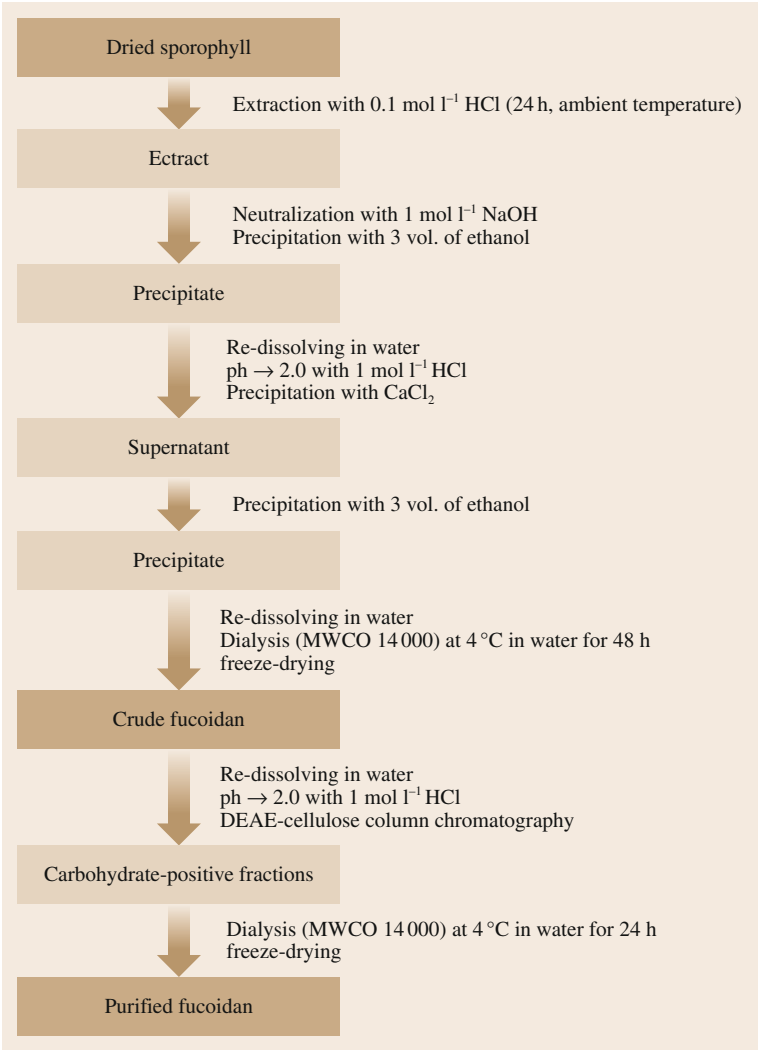

Fig. 53.8 Isolation and purification of the Miyeokgui fucoidan (after [53.106])

Anti-inflammatory Activity. *Park* et al. checked the inhibitory effects of fucoidan on production of lipopolysaccharide (LPS)-induced pro-inflammatory mediators in BV2 microglia. Results indicated that fucoidan treatment significantly inhibited excessive production of nitric oxide (NO) and prostaglandin E_2 (PGE2) in LPS-stimulated BV_2 microglia [53.114, 115].

Osteoporosis. Fucoidan has several biological activities and recent studies indicate that fucoidan can be useful in treating osteoarthritis. *Park* et al. used an animal model of collagen-induced arthritis and showed that orally administered *Undaria pinnatifida* fucoidan successfully inhibited pain [53.116]. Osteoarthritis symptoms were significantly inhibited by oral administration of fucoidan-rich seaweed extract; the symptoms were reduced by 52% [53.117].

Biomedical Applications of Fucoidan. *Changotade* et al. [53.118] reported that low molecular weight fucoidan explored to bone extracellular matrix to support human osteoblastic behaviors in 3-D culture. Fucoidan promotes cell proliferation, collagen type I expression, alkaline phosphatase activity, and mineralization [53.118, 119].

53.3 Marine Ceramics

Ceramics of marine origin such as corals, nacres, fish bone, and sponges provide significant amounts of ceramic materials for biomedical applications.

53.3.1 Hydroxyapatite

Hydroxyapatite (HA), is a naturally occurring mineral form of calcium apatite with the formula $Ca_{10}(PO_4)_6(OH)_2$. Several sources have been identified and used for isolation of HA, such as fish bone [53.120–123]. Considerable interest has been given to fish bone for the production of HA, which has several advantages such reduction of environmental pollution and results in value-added products. Several synthetic methods have been reported in the literature such as hydrothermal [53.124], liquid membrane [53.125], precipitation [53.126], radio frequency thermal plasma [53.127], ultrasonic precipitation [53.128], reverse microemulsion [53.129], sol-gel [53.130] and polymer-assisted methods [53.131], for example.

Hydroxyapatite from Fish Bone and Scales

The simplest method for the production of HA from fish is thermal treatment. HA is the main component of fish and the other is collagenous and noncollagenous protein. The amount HAp at different temperatures 600 °C, 900 °C, and 1200 °C were 62.12, 59.33, and 57.64% (Table 53.2 and Fig. 53.9). HA was isolated from *Thunnus obesus* bone using alkaline hydrolysis and thermal calcination methods. The results indicate

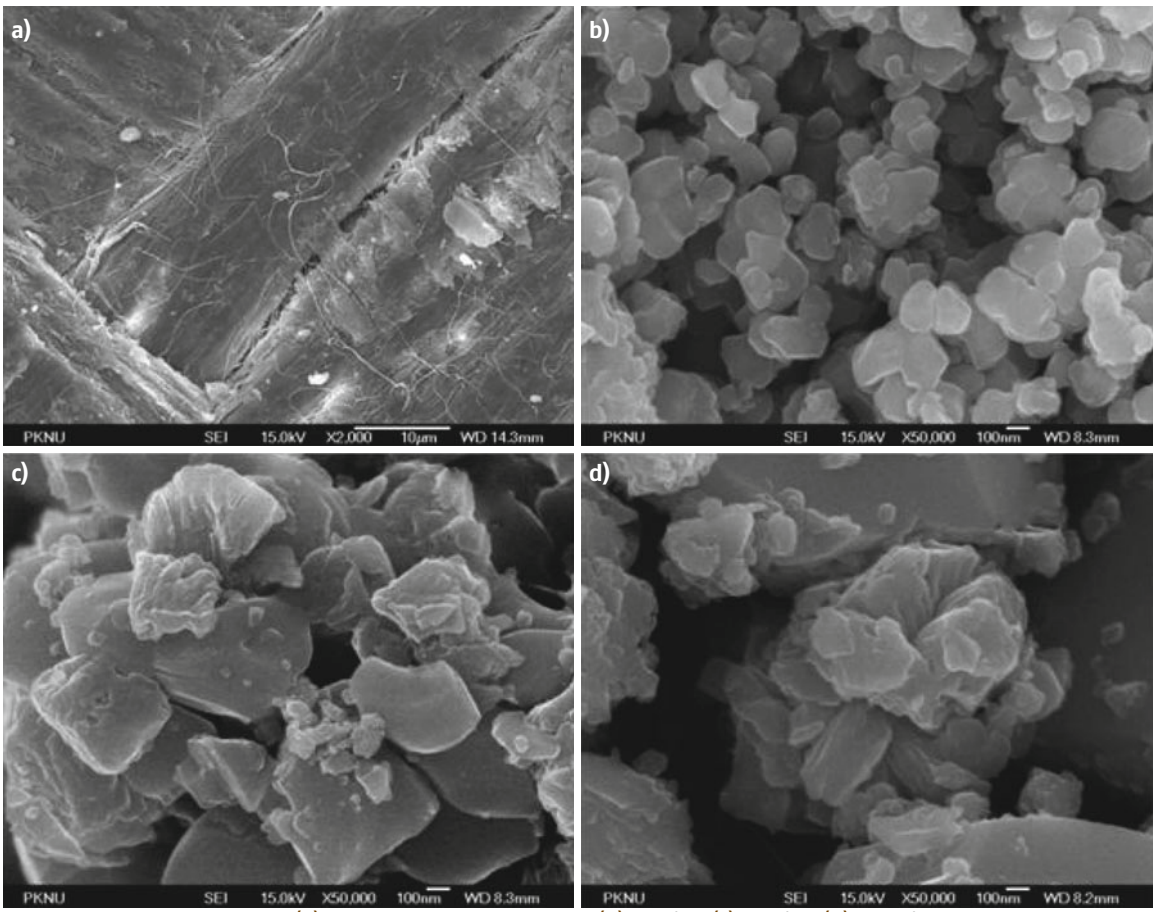

Fig. 53.9a–d SEM results of **(a)** raw fish bone and treated at **(b)** 600 °C, **(c)** 900 °C, **(d)** 1200 °C (after [53.120])

Table 53.2 Residues and color of calcined *Thunnus Obesus* bone (after [53.120])

Sample No.	Calcination temperature (°C)	Calcination period in (h)	Initial weight (g)	Weight after calcination (g)	Residue (%)	Description
1	1200	5	2.0000	1.1527	57.6350	White
2	1100	5	2.0020	1.1529	57.5874	White
3	1000	5	2.0024	1.1771	58.7845	White
4	900	5	2.0011	1.1872	59.3274	White
5	800	5	2.0030	1.1936	59.5906	White
6	700	5	2.0032	1.2129	60.5481	Off-white
7	600	5	2.0017	1.2434	62.1172	Off-white
8	500	5	2.0052	1.2688	63.2755	Tan
9	400	5	2.0031	1.3402	66.9063	Tan
10	300	5	2.0061	1.5162	75.5795	Black
11	200	5	2.0000	1.7360	86.8000	Black
12	Raw fish bone	–	–	–	–	Yellow

that there are significant differences between the ceramics and *Thunnus obesus* bone, the thermal calcination method produces good crystallinity with dimensions 0.3–1.0 μm, whereas the alkaline hydrolysis method produces nanostructured HAp crystals with 17–71 nm length and 5–10 nm width [53.120–123].

HA ceramics isolated from natural sources like cuttlefish bone [53.132], bovine bone [53.133–138], and fish bone [53.120, 139–142] have the advantage of providing inexpensive raw materials from bone and teeth. *Ooi* et al. developed HAp from a bovine source with the thermal calcination method at a temperature range 600–1000 °C, which exhibited HA in a pure form [53.135]. The most important parameters that can affect the properties of HA are the temperature and the duration of the heat treatment [53.134]. While synthetic materials have been widely used in the biomedical field with great success, natural structural materials are now providing an abundant source for novel biomedical applications. Carbonate groups present in carbonated HA are eliminated by heating, which affects the biological properties of the apatite extracted by thermal calcination method [53.143]. *Ozawa* and *Suzuki* reported that HA was isolated from Japanese sea bream by the thermal calcination method up to 1300 °C. Weight loss was observed at the three temperature ranges 30–250 °C, 250–380 °C, and 380–520 °C. The first one corresponds to water content and other two are removal of organic substances [53.144]. Some researchers use fish scale for the production of HA [53.145]. Some researchers have used cuttlefish for the production of HA [53.146–148].

Hydroxyapatite from Corals

Coral is widely used for the production of HA [53.149, 150]. The main component of coral is calcium carbonate, normally in the form calcite. Some chemical reaction is required for conversion of coral to HA. Usually phosphate-containing substances are used.

$$10CaCO_3 + 6(NH_4)_2HPO_4 + 2H_2O \Rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6NH_4CO_3 + 4H_2CO_3 \,.$$

Several commercially available HA based products are available based on the above reaction; they are ProOsten and Interpore200. These materials are widely used in several biomedical applications [53.151]. The above reaction condition can be varied according to the coral substance [53.152, 153].

Biomedical Applications

The natural trabecular structure of coralline HA is similar to that of bone by the hydrothermal conversion of the calcium carbonate skeleton of coral to HA. The benefits of carbonated hydroxyapatite (CHA) for bone grafts are predominantly its safety, biocompatibility, and osteoconductivity; it is used as a bone graft substitute and bone void filler [53.154–158].

53.3.2 Biosilica

Biogenic silica, commonly known as biosilica, consist of glassy amorphous silica and are formed in many aquatic organisms (and in terrestrials as well), such

as sponges, diatoms, radiolarians, and choanoflagellates [53.159]. In addition to being inspiring and a valuable source of marine collagen, as mentioned above, some sponge species are also an important source of biosilica.

There are two classes of sponge that have a silica skeleton: demospongiae and hexactinellida. The third class, calcarea, has a calcium carbonate skeleton. The process of biosilica formation in sponges is enzyme-mediated. The axial filament consists predominantly of the silicatein, which mediates the silicification process around it through the formation of the said concentric layers [53.160].

53.4 Current Understanding and Future Needs

Recent screening techniques have revealed the vast chemical diversity of oceans, which is much higher than what can be achieved by synthesis and standard chemical approaches; this opens new and exciting research scenarios. In fact, the real value of marine-derived materials and compounds can only be roughly checked; it is still to be discovered. Thus, the sustainable exploitation of ocean diversity for industrial and medical purposes is of enormous interest and promises a huge impact not only on research, but particularly on the progress of society, which is reflected on the emergence of marine biotechnology, also known as blue biotechnology, as a fast-growing sector [53.161].

53.5 Conclusions

Oceans not only consist of water, but it has been proven that they are an abundant source of various materials. There is a need to develop several bioactive biomaterials from the marine source. Isolation procedures need to be developed in order to obtain medical grade materials for human purposes. In recent years, several biotechnological processes have improved to help us obtain medical grade biomaterials from the marine source. Chemical derivatization is one promising approach to modify marine-derived polymers for use in tissue engineering, tissue delivery, or biosensors.

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54. Marine Materials: Gene Delivery

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The hurdle in the gene therapy is a lack of suitable gene carrier although animal virus vectors have been mostly used in vivo and in clinical applications due to their high transfection efficiency. However, there are several problems such as potential infectivity, inflammation, immunogenicity, and complicated production. Therefore, nonviral vectors have been tried as alternatives. Among nonviral vectors, natural cationic polymers have been used as the gene carriers because they are biocompatible, less immunogenic, no-limitation of gene size to deliver, and easily produced with low cost. This review chapter covers the recent development of chitosan, alginate, and pullulan among marine polymeric materials as gene carriers.

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54.1 Nonviral Vectors for Gene Delivery

Gene therapy, for curing inherited and acquired diseases by substituting missing genes, replacing defective genes or silencing gene expression, is one of a novel form of molecular medicine because it has a strong impact on human health in the next generation [54.1]. However, one of the big hurdles in the gene therapy is lack of a suitable gene carrier although recombinant animal viral vectors have been mostly used because they have high efficiency of gene transfer in vivo and in clinical applications. However, the use of animal viral vectors for the clinical trials often leads to several problems such as potential infectivity, inflammation, immunogenicity, and complicated production [54.2]. Therefore, nonviral vectors using liposomes and polymers have been promising alternatives because they have several advantages such as low immune response, biocompatibility, flexibility of delivered gene size, and easy production [54.3].

Among nonviral vectors, cationic polymers such as polyethylenimine (PEI) [54.4], poly(amino ester) [54.5], reducible poly(amido amine) (PAMAM) [54.6], PAMAM dendrimer [54.7], and chitosan [54.8] have been recently attracted as gene delivery carriers than cationic lipids because rapid clearance of deoxyribonucleic acid (DNA) from the bloodstream, acute of inflammation, tissue infiltration, and injury of liver and spleen by the lipids have been found [54.9]. Therefore, the objective of this chapter is to discuss the possibility of chitosan, alginate, and pullulan among marine polymeric materials as gene delivery carriers. These polysaccharides as natural polymers will be expected as promising nonviral vectors due to biocompatibility, low immunogenicity, and low toxicity. Moreover, these polymers can be easily manipulated for the proposed applications with desirable physicochemical and physiological properties.

54.2 Chitosan

Among marine polymeric materials, chitosan and chitosan derivatives have been mostly tried as gene delivery carriers because they have biodegradable, biocompatible, low toxic, and less immunogenic properties with positive charges as shown in Fig. 54.1. There are several parameters such as pH, molecular weight (MW), and degree of deacetylation of chitosan, N/P ratio, serum and cell type which affect transfection efficiency of chitosan/DNA complexes or gene silencing of chitosan/siRNA complexes [54.8]. Also, there are several steps for gene transfection of DNA or gene silencing of siRNA, including DNA or siRNA complexation and cellular uptake of the polyplexes at extracellular environment, release of DNA or siRNA from endosomes in cytoplasm, release of DNA or siRNA from the chitosan carrier, and DNA transfer into the nucleus at intracellular environment [54.10]. Particularly, one of the important issues of low transfection efficiency of DNA or gene silencing of siRNA by chitosan is slow release of DNA of chitosan/DNA complexes from endosomes into the cytoplasm [54.11]. Therefore, in this section, only pH-sensitive chemical modification of chitosan as the gene delivery carriers will be explained. The pH-sensitive moieties such as PEI, spermine, and amino acids will be included.

54.2.1 PEI

PEI has been used as a golden standard among nonviral vectors in vitro and in vivo because of high transfection efficiency and due to its excellent buffering ability [54.4] although high MW PEI has high cytotoxicity and nondegradability. On the other hand, low MW PEI has low cytotoxicity but it has low transfection efficiency. Therefore, much attention has been given to couple low MW PEI to the chitosan through degradable linkages for enhancing transfection efficiency of chitosan.

PEI-graft-chitosan (PEI-g-C) was first prepared by *Wong* et al. through cationic polymerization of aziridine by low MW chitosan [54.12]. Gel electrophoresis showed that the migration of DNA was completely retarded at the N/P ratio of 2.5. The PEI-g-C showed higher transfection efficiency in HepG2, HeLa, and hepatocyte cells as well as in bile duct of rat liver than PEI 25 K with a lower cytotoxicity.

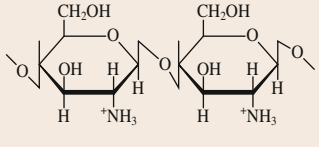

Fig. 54.1 Schematic diagram of chitosan

Chitosan-graft-PEI (C-g-PEI) was differently obtained by *Jiang* et al. [54.13] through an imine reaction between periodate-oxidized chitosan and low MW PEI (1800 Da) as shown in Fig. 54.2. The C-g-PEI showed good DNA binding capacity and protection of DNA from enzyme. Also, the C-g-PEI showed higher transfection efficiency in HeLa, 293T, and HepG2 cells than PEI 25 K with a lower cytotoxicity owing to the synergistic effect of proton sponge ability by PEI and low cytotoxicity by chitosan.

N-maleated chitosan-graft-PEI (NMC-g-PEI) was prepared by reaction of low MW PEI (800 Da) with *N*-maleated chitosan obtained by reaction of maleic anhydride with chitosan [54.14]. The NMC-g-PEI showed a good binding ability with DNA. Also, transfection efficiency of NMC-g-PEI in 293T and HeLa cells was comparable to PEI 25 K with a low cytotoxicity. However, the transfection efficiency depended on MW of NMC-g-PEI. They also prepared *N*-succinyl chitosan-graft-PEI (NSC-g-PEI) by reaction of low MW PEI (800) with *N*-succinyl chitosan obtained by introducing succinyl groups into the chitosan [54.15]. The NSC-g-PEI had good binding ability with DNA. Also, the transfection efficiency of NSC-g-PEI in 293T, HeLa, and CHO cells was higher than that of PEI 25K with a lower cytotoxicity. While the transfection efficiency of NSC-g-PEI increased with an increase in grafting degree of PEI, it was not affected in the presence of serum.

Low MW PEI (600 Da) was introduced into chitosan through a low MW PEG having biterminal epoxide rings as a spacer to increase water solubility and transfection efficiency of chitosan [54.16]. The PEI-PEG-chitosan showed higher transfection efficiency in 293T cells than chitosan with a higher cell viability although they did not compare with PEI 25 K.

Similarly, *Gao* et al. used 1,1′-carbonyl-diimidazole as another spacer to prepare chitosan-linked-PEI (CLP) for improving the transfection efficiency of chitosan [54.17]. The CLP showed higher and long term transfection efficiency in HepG2, A549, and HeLa cells with a lower cytotoxicity. Also, the tumor growth rate was significantly decreased by delivering CCL22 gene with CLP into mice due to the buffering capacity of introduced PEI.

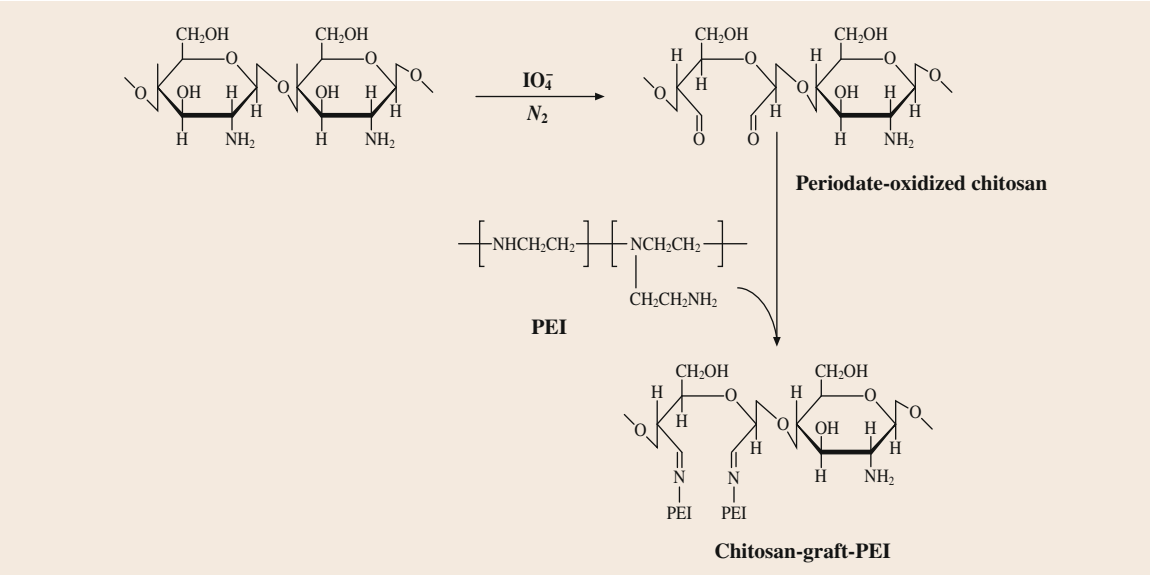

Fig. 54.2 Proposed reaction scheme for synthesis of chitosan-graft-PEI

PEG-graft-chitosan-graft-PEI (PCP) was prepared through polymerization of aziridine by PEG-g-chitosan to improve water solubility and transfection efficiency of chitosan [54.18]. The PCP showed higher tranfection efficiency in vitro than PEI 25 K with a lower cytotoxicity, and the transfection was markedly facilitated by serum owing to the PEG in the PCP.

PEI-conjugated stearic acid-graft chitosan (PEI-g-SAC) had good buffering capability and DNA binding capacity [54.19]. The transfection efficiency of PEI-g-SAC in HeLa and MCF-7 cells was comparable with Lipofectamine 2000 with a lower cytotoxicity and it was increased in the presence of serum. Furthermore, the PEI-g-SAC/pigment epithelium-derived factor DNA complexes effectively suppressed tumor growth without systematic toxicity after intravenous injection in mice.

Gene silencing experiment was also performed by using C-g-PEI to deliver Akt1 siRNA in A549 cells [54.20]. The C-g-PEI silenced more Akt1 protein, increased apoptosis, and decreased lung cancer proliferation malignancy than PEI 25 K.

Chitosan-graft-PEI-β-cyclodextrin (CPCD) was prepared through reductive amination between peroxidized chitosan and β-CD-PEI obtained by reaction of low MW PEI (600 Da) and tosylated β-CD to deliver siRNA in HEK293 and L929 cells [54.21]. The CPCD showed higher gene silencing in both cells than PEI 25 K with a lower cytotoxicity. The β-CD moieties in the pendent provided supramolecular architecture through self-assembly of adamantly modified PEG with the β-CD moieties and resulted in improved stability of the polyplexes under physiological conditions.

PEI-graft-chitosan was prepared by reaction of low MW linear PEI (2.5 K) with epichlorohydrin-modified chitosan containing three types of amines (1, 2, and 3°) for good buffering, proper binding and release of siRNA [54.22]. The PEI-g-chitosan showed higher green fluorescent protein (GFP) gene silencing than the Fugene system and 60% knockdown of JNKII, comparable to Lipofectamine, in HEK 293 cells.

54.2.2 Spermine

Spermine, which contains two primary and two secondary amine groups, is safe and naturally present in the body tissues [54.23]. It has a high buffering effect due to the secondary amine groups.

Chitosan-graft-spermine (CGS) was prepared through a reductive amination between periodate-oxidized chitosan and spermine to improve transfection efficiency of chitosan as shown in Fig. 54.3 [54.24]. The CGS showed good DNA binding ability and high protection of DNA from nuclease. The CGS also showed higher transfection efficiency in A549, WI-38, and HepG2 cells than chitosan itself and higher GFP expression in mouse lung compared with chitosan

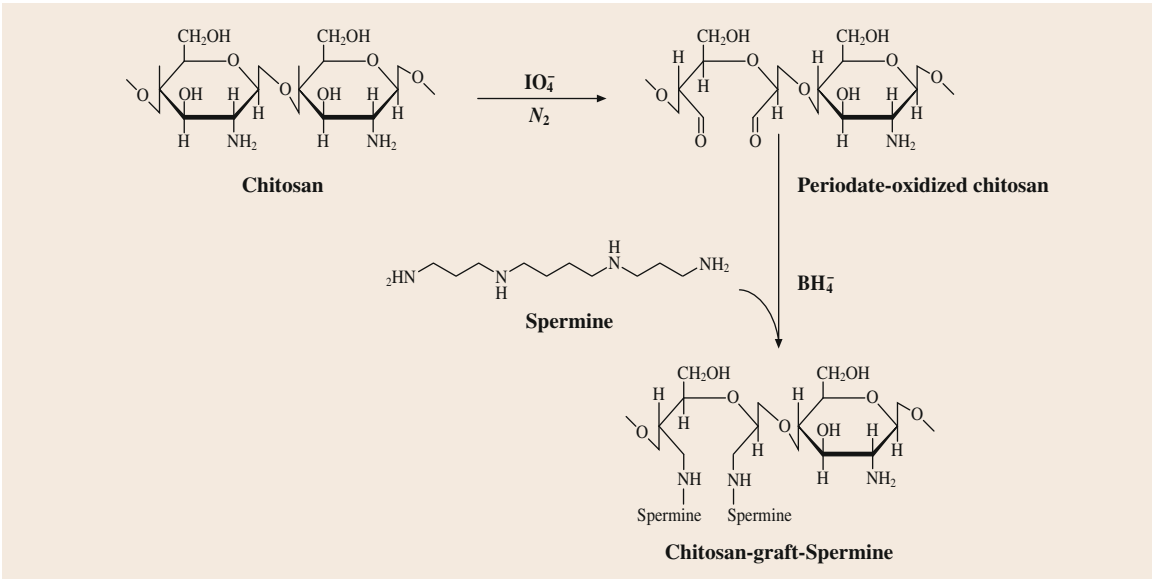

Fig. 54.3 Proposed reaction scheme for synthesis of chitosan-graft-spermine

after aerosol delivery due to high buffering ability of spermine.

Alex et al. [54.25] coupled spermine with galactosylated chitosan (GC) to improve transfection efficiency of chitosan and to target liver cells because asialoglycoprotein receptors on the hepatocytes can be recognized by galactose ligand [54.26]. The GC-g-spermine (GCS) showed higher transfection efficiency in HepG2 cells and the transfection efficiency was dramatically decreased by pretreatment of asialofetuin suggesting the receptor-mediated internalization of polyplexes. Similarly, GCS, prepared by different method, specifically delivered gene into HepG2 in vitro and liver cells in vivo due to the galactose ligand in the carrier [54.27]. Recently, they also prepared galactosylated PEG-chitosan-graft-spermine (GPCS) to deliver programmed cell death 4 (PCD4) as a therapeutic gene for suppression of tumor growth [54.28]. The GPCS had lower cytotoxicity in HepG2, HeLa, and A549 cells than PEI 25 K and showed good hepatocyte-targeting ability. Also, the GPCS showed higher GFP expression in the liver of mice after intravenous injection (IV) than PEG-chitosan-graft-spermine without remarkable inflammation. Furthermore, the GPCS/PCD4 complexes significantly suppressed tumor growth, proliferation and angiogenesis in liver tumor-bearing H-ras 12 V mice after intravenous injection, indicating the GPCS as a potential hepatocyte-targeting gene carrier.

54.2.3 Amino Acids

The introduction of amino acid moieties to the chitosan has been shown to increase the solubility and transfection efficiency of the chitosan. Among them, histidine, arginine, lysine, cysteine, or phenylalanine have been reported [54.29]. In this section, only pH-sensitive amino acid moieties will be covered.

Histidine

Histidine is mostly used as a modifying amino acid to improve transfection efficiency of chitosan due to its ability of endosomal escape as the imidazole ring present in the side chain of histidine has a pK_a of approximately 6.0 [54.30].

Urocanic acid (UA) containing imidazole group was first introduced into chitosan to increase transfection efficiency of chitosan [54.31]. The UA-coupled chitosan (UAC) showed about 1000-fold higher transfection efficiency in 293T cells than chitosan itself. Also, PCD4 gene was delivered to the K-ras null lung cancer model mouse via inhalation by UAC [54.32]. The UAC suppressed tumor angiogenesis and facilitated apoptosis in comparison with UAC. Furthermore, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor gene, were delivered into a K-ras lung cancer model mouse via aerosol route using the UAC [54.33]. The UAC suppressed lung tumors through Akt-related signal pathway and cell cycle ar-

rest mechanism. The UAC was also used to deliver p53 into BALB/c nude mouse via intratumoral injection where the UAC similarly suppressed tumor growth in mouse [54.34].

Another type of imidazole acetic acid (IAA) was introduced to chitosan to improve transfection efficiency of chitosan by the buffering capacity of imidazole ring [54.35]. The IAA-coupled chitosan (IAAC) showed about 100-fold higher transfection efficiency than chitosan. Also, the IAAC was used to deliver β-galactosidase gene in 293T and HepG2 cells [54.36]. Although the transfection efficiency was increased by introduction of IAA into chitosan, the transfection efficiency was dependent on the substitution degree of IAA into chitosan. Furthermore, the IAAC was used to deliver glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA to the mouse through intravenous route after PEGylation of IAAC [54.37]. The carrier significantly knocked down GAPDH enzyme in lung and liver of the mouse at 1 mg kg^{-1} siRNA dose after intravenous administration.

4-Imidazole carboxaldehyde was introduced to the carboxymethyl chitosan obtained by reaction of monochloroacetic acid with chitosan to improve water solubility and transfection efficiency of chitosan [54.38]. The *N*-imidazolyl-*O*-carboxymethyl chitosan was soluble in a wide pH range (4−10) and showed higher transfection efficiency in HEK293T cells than chitosan without remarkable cytotoxicity. However, the transfection efficiency was dependent on the degree of imidazole substitution.

Arginine

Arginine-rich peptides as the cell-penetrating peptides (CPPs) have attracted much attention in gene delivery owing to promotion of cell internalization by the cationic property of the arginine [54.39]. Therefore, arginine-graft-chitosan (AGC) was prepared in order to deliver DNA into HEK293 and COS-7 cells [54.40]. The AGC showed higher transfection efficiency in both cells with pH-dependency than chitosan itself but the transfection efficiency was still lower compared to Lipofectamine 2000.

Noh et al. [54.41] prepared PEG-graft-chitosan-graft-polyarginine (PCPA) to deliver siRNA such as a murine survivin-specific siRNA in vitro and in vivo. The PCPA showed higher gene silencing than chitosan due to enhanced cellular delivery of siRNA by polyarginine, reduced hemolysis of erythrocytes and increased stability of the polyplexes in the biological conditions by PEGylation. Also, intratumoral administration of PCPA/red fluorescent protein (RFP) siRNA complexes silenced the expression of RFP in RFP-expressing tumor tissues in vivo.

Zhang et al. [54.42] studied endocytic pathways of AGC after conjugation of Alexa Flour 488 in A10 cells in the presence of several inhibitors of endocytosis pathways. Interestingly, they reported that the increase of cellular uptake and transfection efficiency by the AGC was probably due to preferentially caveolin-mediated endocytosis pathway in comparison with the chitosan because the caveolin-mediated endocytosis can avoid the endosomal/lysosomal degradation of the gene.

54.3 Alginate

Alginate has been produced from the primary structural component of brown seaweed. The alginate as the monovalent form of alginic acid is a linear polysaccharide consisting of β(1–4)-linked D-mannuronic acid and (1–4)-linked L-guluronic acid as shown in Fig. 54.4. Nonetheless, the ratio of mannuronic acid and guluronic acid in the alginate is dependent on the source of brown seaweed. The alginate has been widely applied for the delivery of cells and drugs because cells and drugs are easily loaded in the alginate due to rapid gelation property in the presence of divalent cations. In this section, we only explain alginate nanoparticles to deliver genes due to page limitation although alginate microspheres and alginate microcapsules have been used to deliver genes.

54.3.1 Alginate/Chitosan Nanoparticles

Alginate/chitosan nanoparticles were prepared by controlling the ratio of alginate to chitosan MW of both polymers and pH to apply for the gene delivery system [54.43]. The smallest nanoparticles around 323 nm were formed using low MW polymers with pH between 5 and 5.6 and having an alginate/chitosan weight ratio of 1 : 1.5. But, they did not perform gene transfection. On the other hand, alginate/chitosan nanoparticles were used to deliver smad3 antisense oligonu-

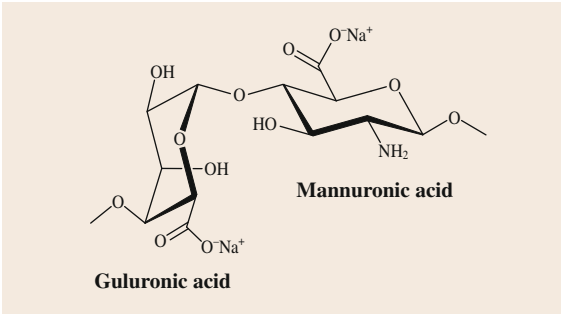

Fig. 54.4 Schematic diagram of alginate

cleotides for wound healing of excisional wounded mouse because the smad3 plays an important role in the elaboration of matrix pivotal to cutaneous wound healing [54.44]. The results indicated that wound closure and histological healing process were faster than other groups such as nanoparticles without gene, gene, and gauze dressing. Furthermore, *Azizi* et al. delivered epidermal growth factor receptor (EGFR) antisense by alginate/chitosan nanoparticles for checking release of the antisense and stability of nanoparticles in aqueous medium and after freeze drying [54.45]. The results showed that the antisense was released from antisense-loaded nanoparticles for 50 h and sizes of the nanoparticles were not much changed after freeze drying, but they did not perform the gene knock-down property.

54.3.2 Alginate/PEI Nanoparticles

Due to high MW PEI has high cytotoxicity in cells, alginate was used to shield partially positive charges of PEI [54.46]. Although the transfection efficiency of the PEI in COS-1 cells was improved by about 2 to 16 fold by hybrid with alginate, the optimum weight ratio of alginate to PEI is very critical in transfection efficiency. Also, alginate/PEI nanoparticles delivered siRNA into mammalian cells and resulted in 80% suppression of GFP expression. They also compared effect of MW of used PEIs on gene silencing of siRNA in COS-1 cells [54.47]. The results showed that 20 times lower concentration of GFP siRNA by the PEI 25 K was needed for gene silencing than PEI 750 K.

Recently, low MW PEI (2000 Da) was grafted onto alginate in order to achieve low cytotoxicity and high transfection efficiency of the polymer [54.48]. The alginate-g-PEI retained the buffering capacity of native PEI and mediated higher vascular endothelial growth factor (VEGF) expression in BEL7402, MSC, and RVMSC cells with a lower cytotoxicity than PEI25K.

54.3.3 Alginate/Calcium Carbonate Nanoparticles

Calcium carbonate ($CaCO_3$) has been used to deliver DNA because of its good biocompatibility and biodegradability. However, nanoparticles of $CaCO_3$/DNA complexes are uncontrollable, resulting in large sizes which affects cellular internalization and transfection efficiency of gene. Therefore, alginate was used to control the growth of gene-loaded $CaCO_3$ nanoparticles [54.49]. The results indicated that alginate retarded the growth of $CaCO_3$-based DNA co-precipitates and enhanced stability of $CaCO_3$/DNA nanoparticles. Also, gene transfection efficiency of alginate-modified nanoparticles was significantly enhanced as compared with the nanoparticles without alginate modification. They also loaded doxorubicin (DOX) as an antitumor drug into alginate/$CaCO_3$/p53 DNA nanoparticles for synergistic effect of antitumor activity by antitumor gene and drug [54.50]. The alginate/$CaCO_3$/DNA/DOX nanoparticles exhibited higher cancer cell inhibition rate of about 80% than $CaCO_3$/DNA/DOX, alginate/$CaCO_3$/DOX and alginate/$CaCO_3$/DNA nanoparticles.

54.3.4 Pullulan

Pullulan is a nonionic linear polysaccharide produced by the *Aureobasidium pullulans* isolated from marine environments [54.51]. It consists of maltotriose type units, i. e., α-(1–6)-linked (1–4)-α-D-triglucosides as shown in Fig. 54.5 [54.52]. The pullulan has been widely applied for the food, pharmaceutical and paper production and as a enzyme immobilization support due to nontoxic, biocompatible, nonimmunogenic, and biodegradable properties. In this section, pullulan derivatives will be explained as gene carriers.

54.3.5 PEI

PEI 25 K was grafted into pullulan-succinimidyl carbonate for liver cell gene delivery [54.53] because the pullulan has inherent liver affinity. The transfection efficiency of pullulan-graft-PEI in HepG2 cells was similar to PEI 25 K, with hemocompatibility and low cytotoxicity, and increased even in the presence of serum.

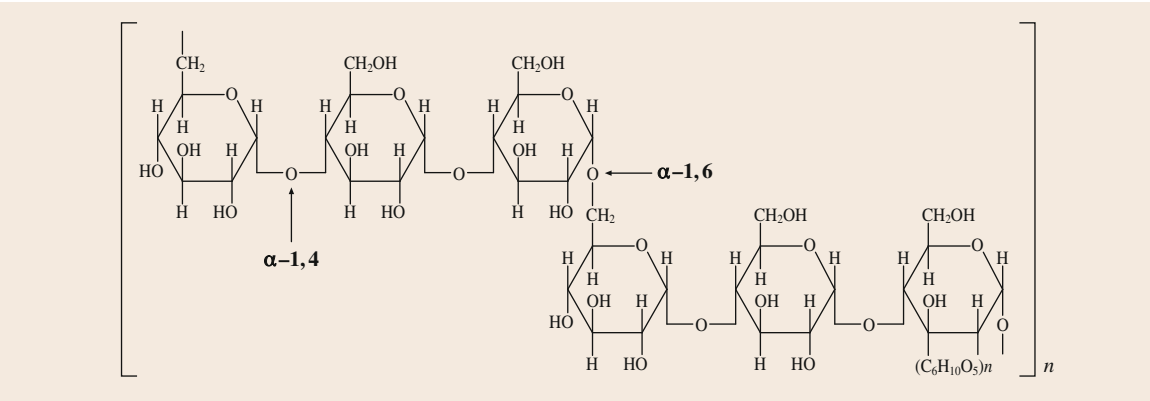

Fig. 54.5 Schematic diagram of pullulan

54.3.6 Spermine

Spermine was firstly introduced into the hydroxyl groups of pullulan using a *N*,*N*′-carbonylidiimidazole activation agent for liver targeting of gene [54.54]. The gene expression in the liver by pullulan-graft-spermine (PU-g-SP) was dependent on the degree of introduced spermine in the pullulan. Also, the tumor-bearing mice survived for a longer time and the number of tumor cells grown in the liver were lower than free DNA after intravenously injection of NK4 DNA by PU-g-SP. The efficient gene expression by the PU-g-SP was confirmed by the clathrin- and raft/caveolae-dependent endocytosis pathways [54.55]. The transfection efficiency of the PU-g-SP depended on the degree of introduced spermine [54.56] and MW of pullulan [54.57] in the PU-g-SP. They also found the highest transfection in rat sensory neuron cells by negatively charged PU-g-SP/DNA complexes obtained at the N/P ratio of 1 due to the glycoprotein-specific interactions and claveolae-dependent endocytosis cellular uptake [54.58]. However, they did not explain why negative charge zeta potential was obtained by polyplexes of N/P ratio of 1. Furthermore, the reverse transfection method was used to deliver mouse Notch intracellular domain (NICD) gene into bone marrow stomal cells (BMSCs) for induction of dopamine-producing neuronal cells by the PU-g-SP [54.59]. The introduced exogenous genes were expressed in the cytoplasm of BMSCs and the differentiation of induced neuronal cells into dopamine-producing cells was promoted by treatment with trophic factors.

Additionally, gene was delivered into rat mesenchymal stromal cells (MSCs) seeded into the biodegradable gelatin scaffold by the PU-g-SP [54.60]. The transfection efficiency was dependent on the method of complex during scaffold preparation. When hepatocyte growth factor (HGF) gene as the therapeutic gene for the treatment of liver antifibrosis was delivered in a rat model of liver fibrosis through intravenous injection by the PU-g-SP, the fibrosis area in the rat was significantly decreased compared with that of original MSCs [54.61].

Recently, human growth hormone 1 (hGH1) gene was delivered to rat brain endothelial (RBE) cells and human brain microvascular endothelial (HBME) cells by the PU-g-SP for allowing hGH1 protein to enter the brain [54.62]. It was found that transfection of HBME cells by the PU-g-SP led to secretion of hGH1 protein into the growth medium but they did not perform in vivo.

54.3.7 Diethylaminoethyl (DAE)

2-chloro-*N*,*N*-diethylethylamine hydrochloride was reacted with pullulan to deliver secreted form of alkaline phosphatase (SEAP) DNA in smooth muscle cells [54.63]. The pullulan-graft-DAE showed 150-fold higher SEAP activity than SEAP gene. Also, the pullulan-graft-DAE was crosslinked by phosphorus oxychloride to make 3-D matrices for loading DNA. The 3D matrices protected SEAP gene from enzyme and showed significant gene transfer without cell toxicity. Also, the tubular pullulan-graft-DAE hydrogel was used to deliver DNA in vascular smooth muscle cells [54.64] where the transfection efficiency was dependent on the degree of DAE in the pullulan-graft-DAE. Furthermore, the pullulan-graft-DAE hydrogel was used to coat stent for delivery of siRNA targeted to matrix metalloproteinase 2 (MMP2) in vascular

cells [54.65]. The siRNA-loaded pullulan-graft-DAE hydrogel in rabbit balloon-injured carotid arteries provided release of siRNA from the stent into the arterial wall and a decrease of pro-MMP2 activity.

54.3.8 Glycidyl Trimethyl Ammonium (GTA)

GTA was introduced into pullulan to deliver gene to hepatocytes in vitro and liver in vivo [54.66]. The pullulan-graft-GTA showed higher transfection efficiency in HepG2 cells and higher liver binding affinity in mice than PEI 2 K with a lower cytotoxicity and blood compatibility owing to specific interaction of pullulan with liver cells.

54.3.9 Chelate Residues with Zinc Ion Coordination

Triethylenetetraamine (TTE), diethylenetriamine pentaacetic acid (DTP) and spermine as the chelate residues with Zn^{2+} coordination were conjugated with pullulan to deliver gene into liver cells [54.67]. The level of gene expression in the pullulan-graft-DTP with Zn^{2+} coordination was significantly higher than that in the pullulan-graft-DTP after injection to mice through intraveneous route. Although the gene expression was dependent on the degree of chelate residues introduced, the function of Zn^{2+} coordination was not clear.

54.3.10 Summary

In this chapter, chitosan, alginate, and pullulan among polymeric materials have been explained as nonvirus vectors for gene delivery carriers. Chitosan and chitosan derivatives have been widely used in vitro and in vivo studies than alginate and pullulan due to the positive charge property of chitosan itself. Further research is needed to find the relationship between structure of marine materials and their gene delivery function. A number of scientific researches are still required to support the evidence of potential use of marine materials before preclinical trials.

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55. Marine Organisms in Nanoparticle Synthesis

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The interdisciplinary science of nanotechnology requires input from physical, chemical, biological, and material scientists. The drawbacks associated with the conventional physical or chemical methods of synthesizing nanoparticles are often circumvented when biological systems are employed. The role of terrestrial organisms in nanoparticle synthetic procedures have been discussed on earlier occasions, and a summary on biological forms associated with marine ecosystems mediating such metal-reductive processes is given here. Organisms in the marine environments need to rapidly adjust to the dynamic conditions that they encounter. In order to resist metal fluxes, marine organisms synthesize novel biomolecules that can reduce metal ions and form nanostructures. In this chapter, the role of various marine organisms (bacteria, fungi, algae, plants, and sponges) in synthesizing metal nanoparticles, their structural characterization, applications in different fields, and the mechanisms involved in the synthetic procedures have been discussed. In the concluding section, the need to investigate the hitherto unexplored wide plethora of organisms in forming nanoparticles of different metals, means of increasing productivity, extending applications, and deciphering mechanisms is highlighted.

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55.1 Basic Concepts in Nanotechnology

55.1.1 Brief History of Nanotechnology

Nanotechnology is an interdisciplinary science encompassing the fields of physics, chemistry, biology, materials science and medicine. The word *nanotechnology* is derived from the Greek word *nanos* meaning *dwarf*. Unlike the parent bulk material, nanoparticles display different physical, chemical, mechanical, and optical features [55.1]. These unusual properties are due to the quantum confinement of electrons within particles

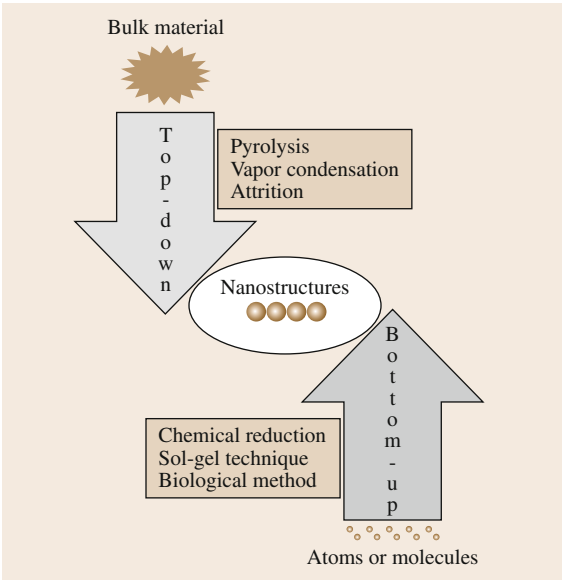

Fig. 55.1 General scheme for the synthesis of nanoparticles with representative methods

of dimensions smaller than the bulk electron delocalization length [55.2]. The concept of nanotechnology dates back to the ninth century. Nanoparticles of gold and silver were used by artisans to add glittering effects to pots and were also included in glass panes in cathedrals. The first scientific description of nanoparticles was given by Michael Faraday in an article entitled *Experimental relations of gold (and other metals) to light* [55.3]. In 1959, almost a century later, Richard Feynman explained the concept of nanotechnology in the lecture *There's plenty of room at the bottom* at the American Institute of Technology. The pioneering research article by *Drexler* entitled *An approach to the development of general capabilities for molecular manipulation* implicated the role of molecular engineering in the advancement of technology to different areas [55.4]. Thereafter, the concept of nanotechnology was revolutionized with advances in techniques such as the transmission electron microscope (TEM), the scanning electron microscope (SEM), the atomic force microscope (AFM), Fourier transform infra red spectroscopy (FTIR), and dynamic light scattering (DLS), to mention a few. At present, this field is at a juncture where it could provide solutions to a variety of challenges.

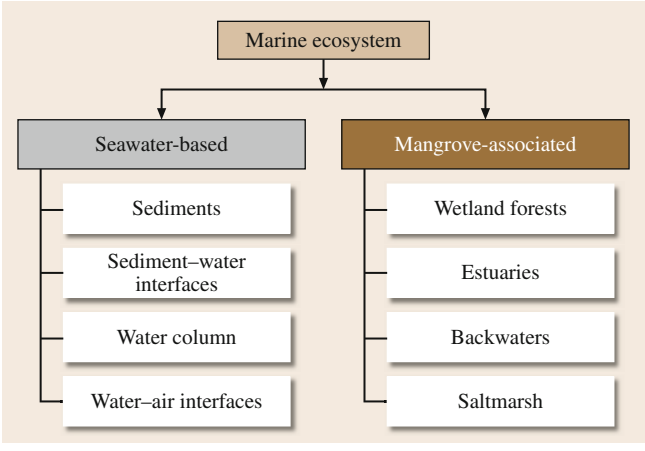

Fig. 55.2 Summary of marine ecosystems

55.1.2 Synthesis of Nanoparticles by Different Approaches

Nanoparticles can be synthesized by two approaches. The *top-down* method involves the gradual de-sizing of bulk material to nanostructures. Examples of such methods include pyrolysis, vapor condensation, and attrition. *Bottom-up* processes build nanostructures beginning at the atomic or molecular levels. The sol-gel technique and chemical reduction (with sodium borohydride or sodium citrate) are two representative examples involving this approach. Chemical and physical methods are often cost intensive, cumbersome, and may involve the use of toxic chemicals. These approaches have been summarized with representative examples in Fig. 55.1.

On account of these shortcomings, there was a need to develop some inexpensive, eco-friendly alternative methods. With pioneering studies on the use of biological systems in mediating nanoparticle synthesis, several of the aforementioned limitations were overcome. Nanoparticle synthesis by biological systems is a *bottom-up* approach based on oxidation-reduction potentials of different biomolecules. Various terrestrial and aquatic organisms such as plants, algae, filamentous fungi, yeasts, bacteria, and viruses are capable of synthesizing nanoparticles. Compared to the extensive reports on the synthesis of nanostructures by terrestrial or freshwater organisms, their counterparts in the marine environment have received less attention and are discussed in the following sections.

55.2 Marine Ecosystems

Oceans and seas account for about 71% of the total water on Earth. Ecosystems associated with these water bodies help in maintaining the balance of marine as well as terrestrial environments and contribute largely towards biological productivity. These systems can be broadly divided into two categories (i) seawater-based systems (sediments, sediment–water interfaces, water column, and water–air interfaces), and (ii) mangrove (wetland forests found along the coasts, in estuaries, backwaters, and marshes) associated ecosystems, as summarized in Fig. 55.2.

These are regarded as hotspots of biodiversity and varied biological forms with nutritional versatility are encountered here. Physical parameters such as light, temperature, pressure, and availability of organic substrates influence the biodiversity under these conditions.

55.3 Nanostructures Inherently Produced by Marine Organisms

Several marine biological forms inherently produce nanomaterials as a part of their physiology. The synthesis of such nanostructures involves the biomineralization and assimilation of inorganic compounds in definite sizes, morphologies, and patterns. Siliceous material in diatoms and magnetosomes in magnetotactic bacteria are classical examples of naturally occurring nanostructures in unicellular forms. In diatoms, specific proteins referred to as silaffins and long-chain polyamines mediate the conversion of silicic acid into silica particles [55.5]. In magnetotactic bacteria, biomineralization of iron occurs in specialized membrane-enveloped structures termed magnetosomes. Iron in the form of magnetite (Fe_3O_4) or greigite (Fe_3S_4) is accumulated in them. These structures are usually arranged in chains and enable the cells to align themselves along geomagnetic lines and move to microoxic zones favoring their growth [55.6, 7]. In both these cases, a degree of control over size, shape and assembly is observed. Higher forms such as seashells and fish bones also display natural nanostructures [55.8]. The pearly internal layer (nacre) of mollusc shells consists of aragonite (a form of calcium carbonate). These particles are layered in a brick-like arrangement and confer strength to the shells.

55.4 Metal Interactions in Marine Biological Forms

Conditions in the sea are dynamic, and organisms encountered here have developed the ability to adapt rapidly to these changes. Factors such as influxes of industrial effluents, transport, and oil-spills contribute

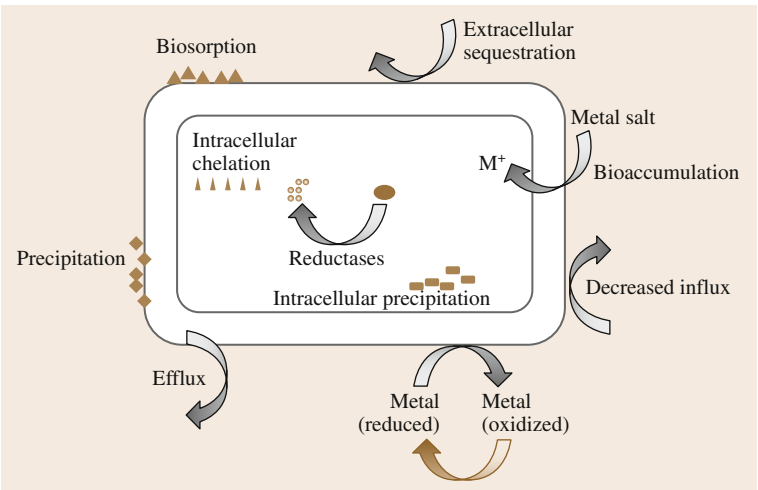

Fig. 55.3 Summary of biometal interactions

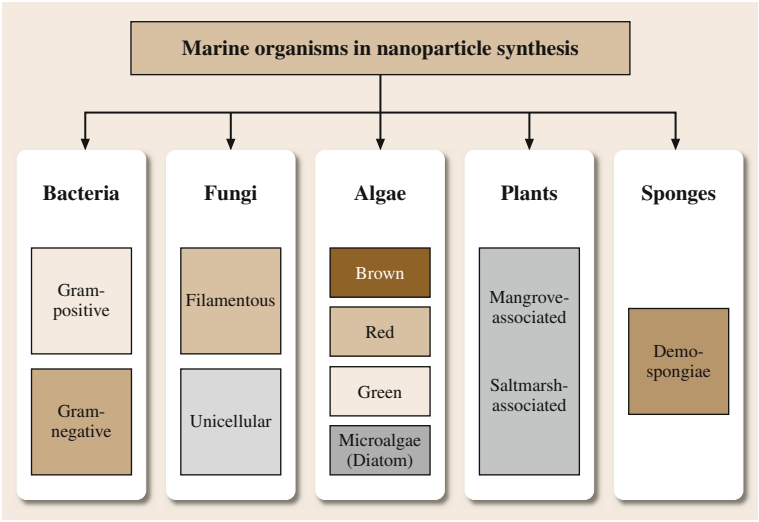

Fig. 55.4 Marine biosystems synthesizing metal nanomaterials

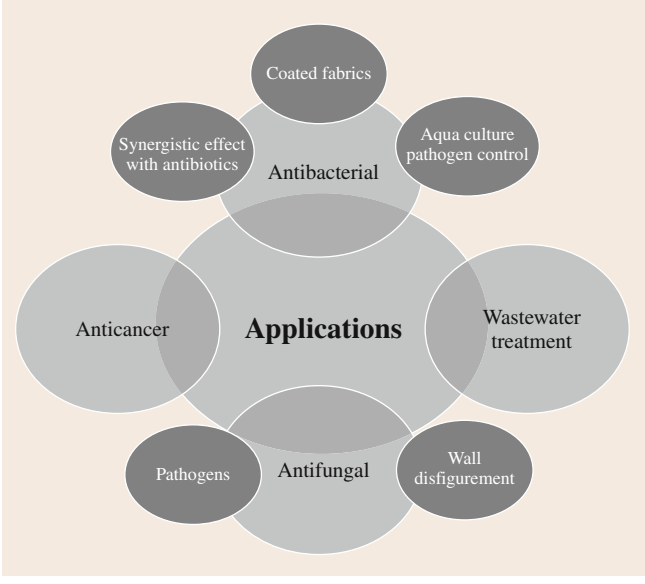

Fig. 55.5 Applications of nanoparticles derived from marine biosystems

towards shock loads of organic and inorganic constituents in the marine environment [55.9]. Organisms associated with such systems often synthesize a variety of biomolecules to combat the effect of different pollutants, including metal species. Most living forms adapt to the presence of metals via processes such as biosorption, bioprecipitation, extracellular sequestration, and/or chelation [55.10], as depicted in Fig. 55.3. An outcome of these processes may be the synthesis of metal nanoparticles either in a cell-associated form or in the extracellular manner [55.11].

Included here is an overview on the role of the different marine biosystems including bacteria, fungi, algae, plants, and sponges in synthesizing metal nanomaterials (Fig. 55.4).

The methodology adopted, characteristics, and mechanistic aspects of different types of nanostructures mediated by these marine systems are discussed. Most of the applications reported for these nanomaterials are in the field of medicine, particularly as antibacterial, antifungal, and anticancer agents as shown in Fig. 55.5. These applications are also described briefly in the appropriate sections.

55.5 Bacteria in Nanoparticle Synthesis

A variety of bacteria are associated with the marine environments. Most of them adapt to the physical and chemical conditions that they encounter, and diverse species have evolved. In general, bacteria have been extensively exploited for the synthesis of nanoparticles. One of the reasons for their preference is the ease with which they can be cultivated and manipulated in the laboratory. Since bacteria are exposed to different met-

als, they have developed mechanisms to resist them. They build up efflux systems, change the redox states of metal ions, mediate extracellular complexation or precipitation, and exclude metals via specific transport systems [55.12–14].

Marine species of *Escherichia*, *Pseudomonas*, *Serratia*, *Marinobacter*, *Shewanella*, *Halomonas*, *Brevibacterium*, *Bacillus*, and *Streptomyces* isolated from different sources are reported to mediate nanoparticle synthesis under laboratory conditions when challenged with metal salts. Most of these bacteria belong to the Gram-negative group. The low peptidoglycan content in the cell wall and the presence of a lipopolysaccharide layer with phosphate and carboxyl groups are said to be responsible for interaction with metal ions [55.15, 16].

Moreover, most of the reports are related to the synthesis of silver nanoparticles and the bacteria have mainly been isolated from mangroves or sponges. The following sections summarize the nanoparticle synthetic capabilities of these bacteria.

55.5.1 Gram-Negative Bacteria

Escherichia coli, a model system used for a variety of practical purposes, has been isolated from different environments. A strain of *E. coli* AUCAS 112 was obtained from a coastal mangrove sediment sample (Vellar estuary, India). This mediated the synthesis of silver nanoparticles in a short duration of time (6 h). When these nanoparticles were stabilized with polyvinyl alcohol, they displayed effective antimicrobial activity against a variety of clinical fungal and bacterial species as evaluated by a log reduction test. FTIR analysis suggested the role of proteins as capping and stabilizing agent [55.17].

Silver nanoparticles have also been synthesized by the cell-free extracts (CFE) of *Pseudomonas aeruginosa* M6 isolated from a mangrove forest in Pitchavaram, Tamil Nadu, India. The CFE was prepared by sonicating culture pellets and the cell debris was removed by centrifugation. Nanoparticle synthesis by two thermal methods was evaluated at pH 9 (i) by boiling, referred to as conventional thermal treatment (CTT), and (ii) via microwave treatment (MWT). The silver nanoparticles synthesized by the former method were smaller 10–12 nm and those by the latter technique were larger and were composed of silver oxide. The bioaccumulation of silver ions led to the synthesis of silver nanoparticles in the periplasmic space of the bacterium. The enzyme nitrate reductase was speculated to mediate the reductive process. Nanoparticles synthesized by both CTT and MWT displayed efficient antibacterial and anticandidal activities [55.18]. Another marine species of *Pseudomonas* (ram bt-1) has been effective in intracellular synthesis of silver nanoparticles. The nanoparticles ranged in size between 20–100 nm and they were stable even after 4 months. The particles were predominantly spherical although some nanotriangles were also observed [55.19].

A variety of culture collection strains and some marine bacteria were screened for their silver nanoparticle synthetic abilities. The marine bacteria screened were *Pseudomonas fluorescens*, *Vibrio natriegens*, and *Bacillus flexus*. While the marine cultures mediated rapid synthesis of silver nanoparticles (6–15 h), the terrestrial strains required longer incubation periods (24–48 h). Among the marine species, *P. fluorescens* mediated the extracellular synthesis of spherical silver nanoparticles (1–10 nm) in the shortest time duration (6 h). These nanoparticles could be coated on polycaprolactam (a biomaterial used extensively in medicine and the food industry) to form biocompatible composites. Moreover, the antibiofilm activities of the composites and the uncoated silver nanoparticles against *E. coli, Staphylococcus aureus* and *Candida albicans* were also evaluated. The nanoparticles caused extensive bacterial cell damage when assessed by the LIVE/DEAD kit [55.20].

Serratia marcescens associated with insect gut is known to mediate nanoparticle synthesis [55.21]. Marine isolates of *S. marcescens* have also effectively formed nanostructures of bismuth, antimony sulfide, and cadmium. A strain of *S. marcescens* was isolated from the Caspian Sea, Northern Iran and used for the biosynthesis of bismuth nanoparticles. The cell-associated nanoparticles were extracted by crushing with liquid nitrogen and purified by extraction with *n*-octanol : water (1 : 2). The biologically synthesized bis-

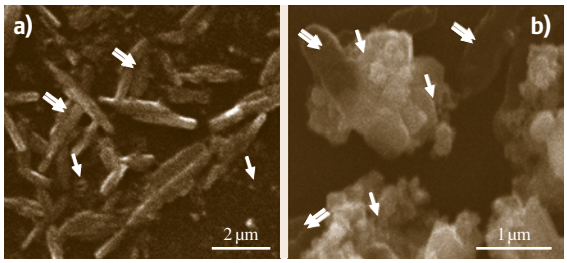

Fig. 55.6a,b Cell-associated synthesis of Cd nanostructures and aggregates by **(a)** *Serratia marcescens*, **(b)** *Bacillus pumilus*. The *white arrow* points to the nanostructures or aggregates and *double arrows* point to cells

muth nanoparticles formed amorphous aggregates. The strong magnetite properties displayed by bismuth at the nanoscale level caused the aggregation [55.22]. This bacterium also formed intracellular antimony sulfide (Sb_2S_5) nanoparticles (confirmed on the basis of energy dispersive spectra (EDS) profiles) that could be extracted by the above-mentioned protocol [55.23]. These nanoparticles were 10–35 nm in size and displayed a characteristic spectral peak at 258 nm. The role of a tropical marine strain of *Serratia marcescens* in synthesizing nanostructures of semiconducting materials was also reported recently. Cadmium oxide (CdO) and cadmium sulfide (CdS) nanostructures were effectively synthesized by this strain in a cell-associated as well as an extracellular manner (Fig. 55.6a). The CdO and CdS nanostructures were 40–45 nm and 15–20 nm, in size, respectively, and displayed fluorescence properties. These bio-inspired nanosized cadmium structures could be applied in biomedical imaging and in different devices [55.11]. The same strain also formed cadmium telluride quantum dots with strong photoluminescence properties. These non-toxic nanostructures were effectively used for imaging yeast and animal cells [55.24].

Different marine strains of bacteria (*Marinobacter pelagius* RS8, *M. pelagius* RS11, *Halomonas ventosae* RS15, *Pseudoalteromonas mariniglutinosa* LD4, *P. mariniglutinosa* LD14, *Alteromonas* sp., *A. macleodii*, *Marinobacter vinifermus*, *H. ventosae* K4, *M. salsuginis* K20, *Photobacterium ganghwense*) were isolated from Lakswadeep, Kakinada and West Bengal, India. Among these only *M. pelagius* RS11 mediated the synthesis of gold nanoparticles. This result suggests that not all marine bacteria have the ability to reduce metal salts to their nanostructures. The particles were 2–10 nm and a time-dependent change in particle size was observed. FTIR analysis indicated the possible involvement of proteins in the synthetic process [55.25].

The Gram-negative bacterium *Shewanella algae* is known to reduce metals such as iron, uranium, and plutonium under anaerobic conditions by using them as terminal electron acceptors. The resting cells of *S. algae* ATCC 51181 collected from the Great Bay estuary, USA, reduced platinum chloride to platinum nanoparticles within 60 min at pH 7.0 and 25 °C in the presence of lactate. A color change from yellow to black was observed. TEM images of thin sections of cells showed that the nanoparticles were 5 nm in size and were located in the periplasmic space. X-ray adsorption near edge spectroscopy (XANES) confirmed the presence of platinum in the elemental form. In this marine bacterium, the deposition of platinum was seen to occur in two steps: (i) $PtCl_6^{2-}$ ions were initially taken up from the aqueous solution into the periplasmic space, and (ii) the $PtCl_6^{2-}$ ions were reduced to platinum with lactate as the electron donor [55.26].

The halophilic archaeon, *Halomonas salina* has been exploited for the extracellular synthesis of gold nanoparticles. With 100 ppm gold salt concentration, optimum synthesis was observed at 30 °C, pH 9.0 in 24 h. These nanoparticles were found to be salt-stable (5 M NaCl). Under acidic conditions (pH 2.0, 3.0, and 4.0) the broad peaks and humps observed were indicative of the possible aggregation of the nanoparticles. Under alkaline conditions (pH 9.0 and 10.0), sharp peaks at 528 and 558 nm, respectively, typical of gold nanoparticles were obtained. The gold nanoparticles were anisotropic (spherical or triangular) between 30–100 nm at low pH. However, under alkaline conditions the formation of spherical nanoparticles was favored. It was hypothesized that some alkali stable components reduced and capped the nanostructures at specific facets, and the role of reductases was also suggested [55.27].

55.5.2 Gram-Positive Bacteria

A few Gram-positive marine bacteria are also reported to mediate the synthesis of nanoparticles under laboratory conditions. The role of cell wall-associated carboxyl groups and teichoic acids in metal deposition is well established in *Bacillus* sp. [55.28]. A strain of *Bacillus subtilis* MSBN17 associated with a marine sponge (*Collyspongia diffusa)* from the south-east coast of India was isolated. The production of a polysaccharide bioflocculant by this bacterium was optimized using response surface methodology. The ethanol precipitated bioflocculant mediated the synthesis of spherical silver nanoparticles (60 nm) at pH 7.0 after 12 h. The role of sugar derivatives in the formation and stabilization of nanoparticles was indicated by FTIR analysis. The synergistic antibacterial effect of silver nanoparticles with different antibiotics was demonstrated and these could also be applied in wastewater treatment procedures [55.29]. Another species, *Bacillus pumilus*, has been isolated from titanium coupons immersed in seawater near Kalpakkam, India [55.30]. This isolate mediated the synthesis of cell-associated and extracellular CdO and CdS nanostructures. The cell-associated nanostructures are depicted in Fig. 55.6b. These photoluminescent nanostructures displayed an absorption

maximum at 328 nm and could be used for a variety of applications [55.11]. The same marine culture of *Bacillus pumilus* also formed CdTe quantum dots that were photoluminescent. These were used for fluorescence staining of yeast and animal cells [55.24].

There are a few reports on marine actinomycetes involved in nanoparticle synthesis. An example of this is a glycolipid biosurfactant producing strain of *Brevibacterium casei* (MSA19). This has been isolated from the marine sponge *Dendrilla nigra* found along the south-west coast of India. Solid state fermentation on agro-industrial and industrial waste resulted in the increased production of this surfactant. Since there have been reports on biosurfactant mediated synthesis and stabilization of nanoparticles [55.31] further studies on the *Bacillus casei* surfactant were undertaken. This biosurfactant formed water-in-oil-microemulsions and reduced silver salts to nanoparticles. These nanoparticles were stable at room temperature for 2 months. The biosurfactant acted as a stabilizing agent and prevented aggregation of the nanostructures [55.32].

A few species of *Streptomyces* of marine origin are also implicated in the formation of nanostructures. Extracellular and intracellular synthesis of silver nanoparticles by *Streptomyces albidoflavus* has been reported. The nanoparticles were spherical and monodispersive in nature with a size ranging between 10–40 nm. The nanoparticles were capped with proteins and displayed antimicrobial activity [55.33]. Another species of *Streptomyces* (*S. hygroscopicus*) was isolated from the Pacific Ocean, near the Philippines. The extracellular synthesis of silver nanoparticles by using this strain was carried out at pH 6.5 for 24 h at 30 °C. The silver nanoparticles synthesized were spherical and 20–30 nm in size. On extended incubation, anisotropic structures were formed. The antimicrobial activity of silver nanoparticles was checked against Gram-positive and Gram-negative bacteria, and yeasts [55.34]. Zinc and copper oxide nanoparticles were synthesized by another *Streptomyces* sp. isolated from the Pichavaram mangrove, India. The supernatant and pellet mediated the formation of metal oxide nanoparticles that were 100–150 nm in size. These bio-inspired nanoparticles were coated on cotton fabric. The antibacterial activity of these coated fabrics was checked against pathogenic bacterial and fungal cultures. Copper oxide nanoparti-

Table 55.1 Summary of nanoparticles synthesized by different types of marine bacteria

Bacteria	Type of nanoparticle	Size (nm)	Location	References
Escherichia coli	Ag	5–20	Extracellular	[55.17]
Pseudomonas aeruginosa M6	Ag	12–42	Extracellular	[55.18]
Pseudomonas sp. *ram bt-1*	Ag	20–100	Intracellular	[55.19]
Pseudomonas fluorescens	Ag	1–10	Extracellular	[55.20]
Serratia marcescens	Bi	< 150	Extracellular	[55.22]
Serratia marcescens	Sb	10–35	Intracellular	[55.23]
Serratia marcescens	CdS, CdO, CdTe	40–45, 15–20, 10	Intracellular, extracellular	[55.11, 24]
Marinobacter pelagius	Au	< 20	Intracellular	[55.25]
Shewanella algae	Pt	5	Intracellular	[55.26]
Halomonas salina	Au	30–100	Extracellular	[55.27]
Bacillus subtilis MSBN17	Ag	60	Extracellular	[55.29]
Bacillus pumilus	CdS, CdO, CdTe	40–45, 15–20, 10	Intracellular, extracellular	[55.11, 24]
Brevibacterium casei	Ag	–	Extracellular	[55.32]
Streptomyces albidoflavus	Ag	10–40	Extracellular, intracellular	[55.33]
Streptomyces hygroscopicus	Ag	20–30	Extracellular	[55.34]
Streptomyces sp.	ZnO, CuO	100–150	Extracellular	[55.35]
Oscillatoria willei NTDM01	Ag	100–200	Extracellular	[55.36]
Phormidium cyanobacterium	Cu	10–40	Extracellular	[55.37]
Phormidium tenue NTDM05	CdS	5	–	[55.38]

cle coating was more effective and these fabrics could be used in medical settings to prevent or minimize infections [55.35].

55.5.3 Blue–Green Bacteria

A few species of blue–green bacteria are also reported to synthesize nanostructures. Washed cells of *Oscillatoria willei* (NTDM01) obtained from Kurusadai Island, India, were incubated with silver nitrate at 2 °C for 28 days and kept in the dark. Silver nanoparticles were synthesized in an extracellular manner. They were spherical nanoparticles and 100–200 nm in diameter. FTIR analysis indicated the role of proteins in the synthesis of the nanoparticles. The reduction of the silver ions was thought to be mediated by nitrate reductase and phytochelatin. The silver nitrate killed the cells and the biomolecules released outside mediated the synthesis of silver nanoparticles [55.36].

Biomass of another cyanobacterium *Phormidium cyanobacterium* was used for synthesizing copper oxide nanoparticles. After 36 h of incubation with $CuSO_4$ and centrifugation, the UV-visible spectra of the supernatants showed a blue shift, indicating the synthesis of nanoparticles. TEM images after 42 h of incubation showed that particles that were quasi-spherical with a size of about 10–40 nm. Some irregular aggregates were also seen. It was suggested that the uniform distribution of the nanoparticles was due to the presence of a protein moiety which formed a matrix-like structure in which they were embedded. Furthermore, three additional proteins were found to be induced when the cells were exposed to the gold salt. These possibly acted as reducing and capping agents [55.37]. Another species of *Phormidium*, (*P. tenue* NTDM05) was used to synthesize cadmium sulfide nanostructures. TEM analysis revealed the size of the CdS nanoparticles to be about 5 nm. The pigment-stabilized (C-phycoerthrin) nanoparticles could be applied in biolabeling studies [55.38]. A summary of nanoparticle synthesis by different types of marine bacteria is presented in Table 55.1.

55.6 Fungi in the Synthesis of Nanomaterials

Fungal species isolated from different marine environments represent approximately about 0.6% of the total fungi studied [55.39]. They are found as free-living forms or maybe associated with other marine organisms such as sponges [55.40]. It is known that some fungi play an important role in the bioremediation of toxic metals and possess the necessary enzymatic setup for their detoxification [55.41]. Filamentous fungi and yeasts isolated from marine habitats can mediate the synthesis of a variety of nanoparticles. A general observation is that most of the marine fungi mediating nanoparticle synthesis are ascomycetous in nature. Fungi offer certain advantages over bacteria when bulk production of nanoparticles is desired. They often secrete large quantities of enzymes, proteins and biomolecules in an extracellular manner and can grow rapidly on relatively simple media.

55.6.1 Filamentous Fungi

Compared to their terrestrial counterparts, there are relatively few reports on the synthesis of nanoparticles by fungi of marine origin. Most of the filamentous fungi reported to synthesize nanoparticles have been isolated from mangrove ecosystems. The growth of such saprophytic fungi is favored by the plant debris present in this habitat [55.42]. The conditions in these ecological niches fluctuate with respect to the presence of salt and other organic compounds depending on the tidal conditions [55.43]. In order to adjust to these changes, fungi associated with this ecosystem often produce novel biological compounds that may be involved in the synthesis of nanoparticles.

Aspergillus species are found in almost all climatic conditions worldwide. This fungus is often associated with environments that are rich in starchy material. Since mangrove ecosystems offer such conditions (due to the accumulation of debris from leaves, inflorescence, and stems), *Aspergillus* sp. have been isolated from these habitats [55.44]. A strain of *A. niger* (AUCAS 237) isolated from a mangrove sediment (Vellar estuary, India) synthesized silver nanoparticles. The nanoparticles were 5–35 nm in diameter and spherical in shape. They displayed effective antimicrobial activity towards clinical pathogens (especially Gram-negative bacteria and some fungi). The activity was enhanced when the nanoparticles were stabilized with polyvinyl alcohol. FTIR analysis revealed the possibility of proteins as possible capping and stabilizing agents [55.17]. Silver nanoparticles were also synthe-

sized by using another strain of *A. niger* isolated from the Gulf of Cambay, India. The nanoparticles were spherical, 5–26 nm in size, and displayed laser optical speckles, which could have several applications in the future [55.45]. Microorganisms are known to be symbiotically associated with different marine forms such as sponges. A strain of *A. terreus* (MP1) was isolated from a marine sponge. The mycelial extract of this fungus synthesized silver nanoparticles. SEM and TEM analysis showed the presence of spherical nanoparticles 15–20 nm in size. These particles effectively inhibited the growth of pathogenic bacterial strains of *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhi* [55.40].

Another fungus, *Penicilium fellutanum* associated with the rhizosphere of an Indian endemic mangrove plant (*Rhizophora annamala*) was isolated and evaluated for the synthesis of silver nanoparticles. The nanoparticles were synthesized optimally at 5 °C, with 1 mM silver nitrate concentration, 0.3% sodium chloride, and at pH 6.0 after 24 h. The extracellularly synthesized nanoparticles were spherical and had a size of 5–25 nm. A protein of about 70 kDa from the cell-free supernatant was proposed to be responsible for converting the metal ions to their zero valence state [55.46].

Apart from the above-mentioned *Aspergillus* and *Penicilium* species, *Hypocrea lixii* (MV1) associated with mangrove sediment soil also mediated the synthesis of silver nanoparticles. The cell-free supernatants of this fungus on incubation with silver nitrate at 28 °C produced nanoparticles that displayed antimicrobial activity against *S. aureus* (ATCC 29213) and *E. coli* (ATCC 25922) [55.47].

55.6.2 Yeast

Yeasts are unicellular fungi that predominantly reproduce by budding. Marine yeasts are important producers of enzymes such as amylases, lipases, proteases, and phytases [55.48]. On account of their inherent capabilities to synthesize a diverse range of products, they have been used as a source of riboflavin, siderophores, single-cell oil SCO, and as probiotics. A few species of marine yeasts are also reported to mediate the synthesis of nanoparticles. Twelve mangrove-associated yeast species, namely, *Candida tropicalis, C. albicans, Cryptococcus dimennae, Debaryomyces hansenii, Geotrichum* sp., *Pichia capsulata, P. fermentans, P. salicaria, Saccharomyces cerevisiae, Rhodotorula minuta, Trichosporon* sp., and *Yarrowia lipolytica* were screened for the synthesis of silver nanoparticles. After a preliminary screening procedure, it was concluded that *P. capsulata* had better synthetic abilities. This was followed by *S. cerevisiae*, *R. minuta*, *P. salicaria* and *D. hansenii*. Other species did not show significant nanoparticle synthetic activity. A rapid method for the synthesis of silver nanoparticles from *P. capsulata* was demonstrated. The optimum conditions were pH 6.0, 0.3% NaCl concentration, and a temperature of 5 °C. The nanoparticles were mostly spherical with a size of 5–25 nm. An NADH-dependent (NADH: nicotiamide adenine dinucleotide) protein similar to nitrate reductase was partially purified and was suggested to mediate the reduction process [55.49].

Y. lipolytica is biotechnologically important in the bioremediation of hydrophobic substrate contaminated environments, in the treatment or up-gradation of wastes, biotransformation of organic compounds, production of novel enzymes, and in the cloning and expression of heterologous proteins. This yeast has been isolated from polluted areas containing toxic and hazardous metals. Metal tolerance in this yeast is attributed to the presence of superoxide dismutase (a copper tolerating protein), reductases, CRF1, metallothioneins, efflux mechanisms, and melanin [55.50]. A tropical marine isolate of *Y. lipolytica* (NCIM 3589), obtained from oil-polluted seawater near Mumbai, India, mediated the synthesis of gold nanoparticles. The synthesis took place at 30 °C within 72 h. The cell wall-associated synthesis of nanoparticles was confirmed by TEM analysis. The yeast, as well as the mycelial forms of this dimorphic fungus, synthesized the gold nanostructures. The size of the nanoparticles was found to be pH-dependent. At pH 2.0, gold nucleation was observed within 15 min. Over a period of time, these developed into large triangular and hexagonal plates. The size of the nanostructures at pH 7.0 and at 9.0 was 15 nm [55.51]. In a further study, this system was used in the custom designing of gold nanoparticles with specific sizes. With increasing cell numbers and the same concentration of gold salt, the particle size was found to decrease. On the other hand, with increasing concentration of the gold salt and the same cell numbers, there was an increase in the size of the particles. The cell-associated gold nanoparticles could be released into the medium by incubation at 20 °C [55.52]. Melanin, a dark-colored pigment from this yeast was found to be one of the factors responsible for nanoparticle synthesis. Cell-extracted and induced melanin (obtained by incubating resting cells with L-3,4-dihydroxyphenylalanine (L-DOPA) mediated the

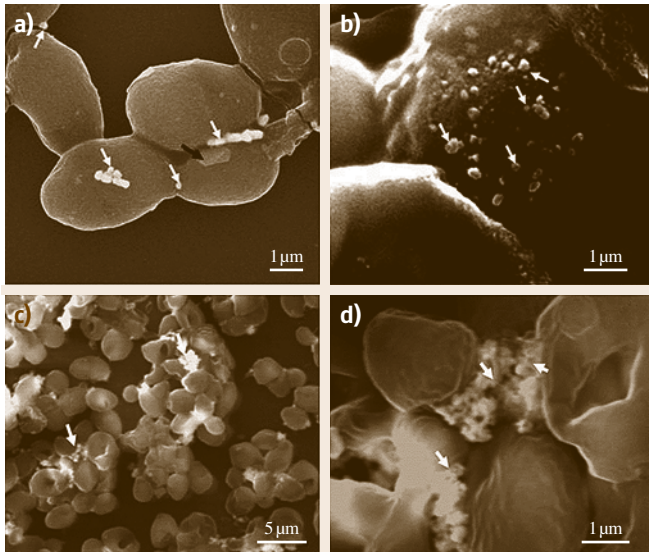

Fig. 55.7a–d Nanostructures associated with marine yeasts. **(a)** Gold nanoparticles and microplates synthesized by *Yarrowia lipolytica* NCIM 3590; **(b)** CdO nanoparticles mediated by *Y. lipolytica* NCIM 3589. *White arrows* point to nanostructures and the *black arrow* to a microplate

synthesis of gold nanostructures [55.53]. Another cold-adapted marine strain of *Y. lipolytica* (NCIM 3590) also synthesized gold nanostructures (Fig. 55.7a,b).

The synthetic ability in this case was also associated with the dark-colored pigment, melanin. These nanoparticles displayed antibiofilm activity against pathogenic bacteria [55.54]. Since the inherent content of melanin in this organism was low, the yeast was induced to overproduce melanin by incubation with a precursor, L-DOPA. This melanin also mediated the rapid formation of silver and gold nanoparticles. The former displayed effective antifungal properties against a wall-disfigurement causing fungus [55.55]. In addition, the tropical marine strain (*Y. lipolytica* NCIM 3589) described above was able to synthesize CdO and CdS nanostructures in a cell-associated and extracellular manner. The SEM images of cell-associated nanostructures in this yeast are depicted in Fig. 55.7c,d. Certain functional groups on the cell surface were thought to play a role in the reductive and stabilization processes [55.11].

A variety of the *Candida* species are also associated with the marine environment [55.8]. Three species of *Candida* were isolated from a coastal soil sample (Nicobar Islands, India) and screened for silver nanoparticle synthesis. Of the three isolates, the *Candida* sp. designated as VITDKGB was selected further. The nanoparticles were 87 nm and displayed antibacterial activity against the multidrug resistant pathogens *Staphylococcus aureus* and *Klebsiella pneumoniae* [55.56].

Rhodosporidium diobovatum, a marine yeast resistant to lead synthesized lead sulfide (PbS) nanostructures. These were characterized and found to be 2–5 nm in diameter. A sulfur-rich peptide was suggested to be the capping agent. In the presence of lead, this yeast produced increasing contents of non-protein thiols during the stationary phase. These were possibly involved in forming the nanoparticles [55.57]. A brief overview of nanoparticles synthesized by filamentous fungi and yeasts is depicted in Table 55.2.

Table 55.2 Marine fungi mediating the synthesis of nanoparticles

Fungi	Type of nanoparticle	Size (nm)	Location	References
Aspergillus niger AUCAS 237	Ag	5–35	Extracellular	[55.17]
Aspergillus niger	Ag	5–26	Extracellular	[55.45]
Aspergillus terreus MP1	Ag	10–30	Extracellular	[55.40]
Penicillium fellutanum	Ag	5–25	Extracellular	[55.46]
Hypocrea lixii MV1	Ag	–	Extracellular	[55.47]
Pichia capsulata	Ag	5–25	Extracellular	[55.49]
Yarrowia lipolytica NCIM 3589	Au CdO CdS	15 15–20 40–45	Intracellular Extracellular Intracellular	[55.11, 51–53]
Yarrowia lipolytica NCIM 3590	Au, Ag	20, 7	Extracellular	[55.54, 55]
Candida sp. VITDKGB	Ag	1–5	Extracellular	[55.56]
Rhodosporidium diobovatum	PbS	2–5	–	[55.57]

55.7 Algae in the Synthesis of Nanoparticles

Algae inhabit natural as well as metal-contaminated freshwater and marine environments. Various species of algae are also known to interact with heavy metal ions. Some of these species are involved in the detoxification and bioremediation of metal wastes from water [55.81, 82].

The first alga reported to synthesize gold nanoparticles was the brown alga *Sargassum wightii* [55.63]. Following this, there have been several other reports on the application of algal systems for the synthesis of metal nanoparticles. Eleven species of brown algae, four species of red algae, ten species of green algae, and three microalgae have been implicated in the synthesis of nanoparticles. The largest number of reports is on brown algae. This group has adapted to a wide variety of marine ecological niches, including tidal splash zones, rock pools, intertidal zones, and relatively deep near-shore waters. A summary of the nanoparticle biosynthetic capabilities of different algal species is listed in Table 55.3.

Table 55.3 Algal species involved in metal nanoparticle synthesis

Algae	Taxonomic features	Type of nanoparticle	Size (nm)	References
Padina tetrastromatica	Brown, Phaeophyceae	Silver	20	[55.58]
Padina pavonica	Brown, Phaeophyceae	Silver	47	[55.59]
Padina gymnospora	Brown, Phaeophyceae	Gold	53–67	[55.60]
Sargassum plaviophyllum	Brown, Phaeophyceae	Silver	20	[55.61]
Sargassum tenerrimum	Brown, Phaeophyceae	Silver	45	[55.62]
Sargassum wightii	Brown, Phaeophyceae	Silver, Gold	8–12	[55.63, 64]
Cystophora moniliformis	Brown, Phaeophyceae	Silver	81	[55.65]
Laminaria japonica	Brown, Phaeophyceae	Gold	15–20	[55.66]
Kjellamaniella crassifolia	Brown, Phaeophyceae	Gold	8–10	[55.67]
Cladosiphon okamuranus	Brown, Phaeophyceae	Gold	8–10	[55.67]
Fucus vesiculosus	Brown, Phaeophyceae	Gold	–	[55.68]
Gracilaria corticata	Red, Florideophyceae	Silver	18–46	[55.69]
Gelidiella acerosa	Red, Florideophyceae	Silver	22	[55.70]
Kappaphycus alvarezii	Red, Solieriaceae	Gold	10–40	[55.71]
Hypnea sp.	Red, Hypneaceae	Silver	10–20	[55.72]
Chaetomorpha linum	Green, Ulvophyceae	Silver	30	[55.73]
Enteromorpha compressa	Green, Ulvophyceae	Silver	40–50	[55.61]
Ulva reticulata	Green, Ulvophyceae	Silver	40–50	[55.61]
Chlorella salina	Green, Chlorophyceae	Silver	–	[55.74]
Tetraselmis gracilis	Green, Prasinophyceae	Silver	–	[55.74]
Isochrysis galbana	Green, Isochrysidaceae	Silver	–	[55.74]
Chlorella vulgaris	Green, Chlorophyceae	Gold	40–60	[55.75]
Tetraselmis suecica	Green, Prasinophyceae	Gold	79	[55.76]
Tetraselmis kochinensis	Green, Prasinophyceae	Gold	5–35	[55.77]
Ulva fasciata	Green, Ulvophyceae	Silver	33–40	[55.78]
Cylindrotheca fusiformis	Diatom, Bacillariaceae	Titanium dioxide	20–50	[55.79]
Navicula atomus CCALA 383	Diatom, Naviculaceae	Gold	9	[55.80]
Diadesmis gallica CCALA 766–DG	Diatom Diadesmidaceae	Gold	22	[55.80]

55.8 Marine Plants in Nanoparticle Synthesis

Various terrestrial and aquatic plants have been used for the detoxification of heavy metals via phytoremediation procedures. Polyphenols, metallothioneins, and glutathiones are a few examples of phytochemicals that interact with metals. While metallothioneins and glutathiones are metal binding entities, the antioxidant property of phenolic compounds is attributed to the presence of hydroxyl groups that alternate between the hydroxyl and quinone forms [55.83]. Mangrove plants often sequester metal ions and prevent their entry into aquatic ecosystems. In a representative mangrove species, namely, *Rhizophora*, the presence of metal ions does not affect plant growth significantly [55.84]. One of the strategies employed by such mangrove plants to overcome the toxic effect of metal ions is the synthesis of respective nanostructures. All reports on the synthesis of nanoparticles by marine plants are related to silver. The water soluble heterocyclic components such as polyols present in these plant systems are said to be responsible for the reduction and stabilization of silver ions [55.83].

Aqueous leaf extracts of *Rhizophora apiculata* (located in Pichavaram, India) mediated the synthesis of silver nanoparticles. For comparison purposes, silver nanoparticles synthesized by using 1% glucose as the reductant were evaluated. TEM observations showed that the nanoparticles were spherical and varied in size from 19–42 nm. Biologically synthesized silver nanoparticles showed higher antibacterial activity than the glucose-mediated ones [55.85]. Leaf bud extract of another *Rhizophora* species namely, *R. mucronata*, also formed silver nanoparticles when incubated with aqueous silver nitrate solution at 15 psi pressure and 121 °C for 5 min. High-resolution transmission electron microscope (HRTEM) observations revealed the diameter of these to be between 4–26 nm. These nanoparticles exhibited antimicrobial activity against the bacterial pathogens *Proteus* spp., *Proteus fluorescens*, and *Flavobacterium* spp., isolated from infected marine ornamental fish, *Dascyllus trimaculatus* [55.86].

Three plants from Parangipettai, south-east coast of India, were evaluated for the synthesis of silver nanoparticles. The leaf and callus extracts of the saltmarsh plant *Sesuvium portulacastrum* mediated the synthesis of spherical silver nanoparticles (5–20 nm) within 24 h. In comparison to the leaf extracts, the callus extracts were more effective in mediating the synthesis of monodisperse silver nanoparticles. Typically, callus cultures contain a mass of young cells with different ploidy states. Such cells were thought to be metabolically more active in producing the phytochemicals responsible for the reduction of silver ions. When stabilized with polyvinyl alcohol, these nanoparticles displayed prominent antibacterial activity [55.87]. The callus and leaf extracts of *Citrullus colocynthis* displayed moderate antibacterial activity. Spherical silver nanoparticles, 31 nm in diameter, were observed when callus extracts and silver salt were incubated at 35 °C for 24 h. The anticancer potential of the silver nanoparticles on the human epidermoid larynx carcinoma (Hep-2) cell line was evaluated. The toxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), caspase-3, lactate dehydrogenase leakage, and deoxyribonucleic acid (DNA) fragmentation assays [55.88]. Silver nanoparticles were also synthesized by another coastal plant *Prosopis chilensis* isolated from the same location. The nanoparticles were synthesized by leaf extracts after 24 h incubation with silver salts. TEM analysis showed that the nanoparticles were mostly spherical and ranged between 5–25 nm. The silver nanoparticles were applied in controlling vibriosis in the shrimp *Penaeus monodon* [55.89].

Avicennia marina plant was collected from Karangadu mangroves, Tamil Nadu, India. Among the different mangrove plants, this species is important because it displays antibacterial, antiplasmodial, and antiviral activities [55.93]. It is also rich in secondary metabolites such as polyphenols, flavonoids, alkaloids, and tannins [55.94]. On account of these characteristics, extracts of different plant parts (leaves, barks, and roots) were evaluated for the synthesis of silver nanoparticles. Of the three plant parts, the leaf extracts were most effective in nanostructure formation after 8 h of incubation. AFM analysis showed the particle size

Table 55.4 Marine plants mediating nanoparticle synthesis

Plants	Size (nm)	Reference
Rhizophora apiculata	19–42	[55.85]
Rhizophora mucronata	4–26	[55.86]
Sesuvium portulacastrum (L.)	5–20	[55.87]
Citrullus colocynthis (L.)	≈31	[55.88]
Prosopis chilensis (L.)	5–25	[55.89]
Avicennia marina	71–110	[55.90]
Mayaca fluviatilis	–	[55.91]
Suaeda monoica	≈31	[55.92]

to be between 71–110 nm. The silver nanoparticles synthesized using leaf aqueous extracts were effective against Gram-negative bacteria [55.90]. *Mayaca fluviatilis* another marine plant was effective in synthesizing silver nanoparticles at low temperatures (4 °C). The polydisperse particles were reported to be stable even after 6 weeks of incubation [55.91].

The leaves of *Suaeda monoica* were collected from the Kollidam coast, Tamil Nadu, India. The leaf extract was incubated with silver nitrate at 35 °C for 5 h to synthesize the silver nanoparticles. AFM analysis showed that the nanoparticles were spherical and 31 nm in size. The in vitro cytotoxicity effects of these nanoparticles were studied using human epidermoid larynx carcinoma cell lines (Hep-2) [55.92]. The role of marine plants in mediating nanoparticle synthesis is summarized in Table 55.4.

55.9 Nanoparticle Synthesis by Sponges

As described earlier, certain bacteria and fungi associated with sponges mediate nanoparticle formation. For example, *Bacillus subtilis* MSBN17 (associated with the sponge *Collyspongia diffusa*), *B. casei* MSA19 (isolated from *Dendrilla nigra*), and *Aspergillus terreus* (MP1) were able to form silver nanostructures [55.29, 32]. It is suggested that structurally unique natural products accumulated by invertebrates such as sponges and molluscs may bring about the conversion of metals to their respective nanostructures. There are two such reports on the use of sponges for the synthesis of nanoparticles.

The extract of the marine sponge *Acanthella elongata* (collected from the Gulf of Mannar, Tamil Nadu, India) synthesized stable gold nanoparticles within 4 h at 45 °C. TEM analysis showed that polydisperse spherical nanoparticles with variable sizes from 7–20 nm were present [55.95]. Another marine sponge (*Haliclona implexiformis*) was collected from the same region. The sponge extract was used for the synthesis of silver nanoparticles. Nanoparticles were formed after 12 h and their size was found to be 12 nm. The antimicrobial activity of silver nanoparticles was studied against *Pasteurella multocida*, *E. coli*, *Klebsiella pneumoniae*, *B. subtilis*, *Enterococcus fecalis*, and *Pseudomonas* sp. In vivo toxicity testing in chick embryos revealed their non-toxic nature [55.96].

55.10 Mechanistic Aspects

Reduction reactions involving the conversion of metal ions to their respective nanostructures are thought to be mediated by different biomolecules. The synthesis of silver nanoparticles is attributed to the presence of peptides, amino acids, reducing sugars, and enzymes such as reductases and proteases.

55.10.1 Proteins in Nanoparticle Synthesis

The role of some proteins in the biosynthesis of nanoparticles has been proposed by several investigators. For example, in bacteria such as *E. coli* AUCAS 112 and *Marinobacter pelagius* RS11, as well as in the fungus *Aspergillus niger* AUCAS 237, the role of proteins as capping and stabilizing agents has been suggested indirectly on the basis of FTIR profiles [55.17, 25]. In *Phormidium cyanobacterium* three proteins were actually induced in response to the presence of gold salt. Two of them demonstrated hydrolytic activity and the third probably acted as a capping

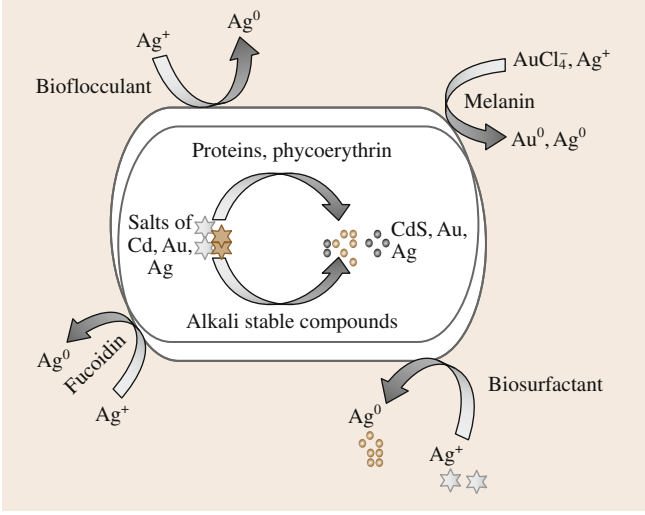

Fig. 55.8 Mechanisms involved in nanoparticle synthesis by marine systems

agent [55.37]. In the fungus *Penicillium fellutanum*, a protein of about 70 kDa was reported to be involved in nanoparticle synthesis [55.46]. In addition, certain non-protein thiols are proposed to form lead nanoparticles in the yeast *Rhodosporidium diobovatum* [55.57].

The importance of nitrate reductase in nanoparticle synthesis has been implied in the bacterium *Pseudomona aeruginosa* M6 and the yeast *Pichia capsulata* [55.18, 49]. In the cyanobacterium *Oscillatoria willei* NTDM01 this enzyme along with phytochelatin were thought to be involved [55.36]. In the Archaeon *Haloterrigena salina*, this enzyme in conjunction with alkali stable components mediated nanoparticle synthesis [55.27].

55.10.2 Role of Biosurfactants, Bioflocculants, and Polysaccharides

Biosurfactants, bioflocculants, and polysaccharides are also proposed to have a possible mechanism in nanostructure formation. For example, in *Bacillus subtilis* MSBN17 and *B. casei* MSA19, a bioflocculant and biosurfactant respectively, reduced silver salts to nanoparticles [55.29, 32]. Fucoidans (sulfated polysaccharides) from the two marine algae *Cladosiphon okamuranus* and *Kjellmaniella crassifolia* reduced gold salts and stabilized the nanoparticles [55.66].

55.10.3 Pigments as Mediators

The role of pigments in nanoparticle formation has been suggested in two marine systems. Melanin, a complex polymer of phenolic compounds has been described in the yeast *Yarrowia lipolytica*. The cell-associated and precursor-induced pigment plays a role in the formation of gold and silver nanostructures [55.53–55]. Another pigment, C-phycoerythrin (C-PE) extracted from the marine cyanobacterium *Phormidium tenue* NTDM05 was involved in the synthesis of CdS nanoparticles [55.38]. Some of the proposed mechanisms involved in nanoparticle synthesis in marine biological forms are summarized in the following section and in Fig. 55.8.

55.11 Current Understanding and Future Needs

A variety of prokaryotic and eukaryotic marine organisms have been investigated with respect to their nanoparticle synthetic abilities. Most of these reports deal with eukaryotic forms such as fungi, algae, and plants. The vast biodiversity encountered in the prokaryotic world has been relatively less explored. Furthermore, most of the studies are related to the synthesis of silver nanoparticles, followed by those of gold. One reason for this could be the relative ease with which the noble metal ions of gold and silver are reduced. Nanoparticles of platinum, bismuth, cadmium (CdO, CdS, CdTe), antimony sulfide, copper oxide, zinc oxide, and titanium oxide have been described on fewer occasions. It is observed that a majority of the reports deal with application of novel biological systems in mediating the synthesis of nanoparticles, their characterization, and applications in the biomedical field, particularly as antimicrobial agents. However, there is a need to understand the mechanisms involved in the synthetic process. Another limitation of the studies is that the experiments have been conducted at laboratory scale and there are hardly any efforts for the scale-up of these processes. In the future, these shortcomings need to be addressed in an effective manner to harness the actual nanoparticle synthetic potential of the marine resources to their full extent.

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56. Marine Biomaterials in Therapeutics and Diagnostics

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Marine organisms are constituted of compounds with several properties and characteristics. In the last decade lot of biomaterials like various types of polymers and bioactive ingredients have been identified, isolated, and characterized. These biomaterials have a vast range of applications in the medical sector including controlled drug delivery, tissue engineering, and diagnostic device etc. In this chapter, biomaterials have been defined and classified in general, followed by an account of mainly various polymers like collagen, chitin, chitosan, chondroitin sulfate, hyaluronic acid, alginate, biosilica, calcium carbonate and phosphate. Their structure, source, and application in the medical field have been discussed. A brief account of bioactive compounds as therapeutic agents has also been given. Marine biomaterials are also emerging as promising materials for the development of biosensors for medical diagnostics. This chapter also discusses biosensors and the role of biomaterials from marine origin in the development of biosensors for medical diagnostics. The chapter concludes with the scopes of the biomaterials in near future.

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56.1 Biomaterials

Biomaterials are materials with unique properties which make them suitable for intimate contact with living tissues. Biomaterials are produced through process that mostly employ or mimic biological phenomena.

As per the definition for biomaterials [56.1]:

A biomaterials can be defined as any material used to make devices to replace a part or a function of the body in a safe, reliable, economic and physiologically acceptable manner

Biomaterials are not necessarily of biological origin they may be of nonbiological origin as well. Most important criteria for the biological material are that they will be in intimate contact with living tissues. They must be biocompatible and either bioresorbable or biodurable.

The biomaterials should have four properties. There should be biocompatibility, sterlizability, functionability, and manufacturability [56.2]. Biomaterials are used for various purposes (Table 56.1).

56.2 Classification of Biomaterials

Various authors classified biomaterials in different ways. Broadly biomaterials can be classified in four groups:

a) Polymers
b) Metals
c) Composite materials and
d) Ceramics.

56.2.1 Polymers

There are a large number of polymers which have been used in the human body as implants or part of the implant system. These polymers include acrylics, polyamides, polyesters, polyethylene, polyurethane, polysiloxanes, and many others reprocess biological materials.

These polymers can be used in tissue engineering, implantation of medical devices, and artificial organs due to its inert nature, prostheses, dentistry, bone repair, drug delivery, and targeting into sites of inflammation or tumors, plastic tubing for intravenous infusion, and catheter etc. Advantages with the polymers are that they are resilient and easy to fabricate but disadvantages are many like they are not strong enough, they deforms with time and may degrade. Most common biodegradable polymers are the polylactic and glucolic acid polymers and copolymers, trimethylene carbonate copolymers, and polydioxanone.

56.2.2 Metals

Metals are used as biomaterial due to their excellent electrical and thermal conductivity and mechanical properties. Some metals are used as passive substitute for hard tissue replacement such as total hip, knee joints, for fracture healing aids as bone plates and screws, spinal fixation devices, dental implants, vascular stents, and catheter guide wires.

Following metallic systems are most frequently used in the body:

a) Stainless steel – (e.g., in fracture plates, screws, and hip nails)
b) Titanium and titanium-base alloys (e.g., in knee joint replacement, dental implants, etc.)
c) Cobalt-base alloys (e.g., in dentistry, in making artificial joints, for making the stems of prostheses for heavily loaded joints such as knee and hips).

Titanium and its alloys are getting great attention in both medical and dental fields because of excellent biocompatibility, light weight, excellent balance of mechanical properties, and corrosion resistance.

Table 56.1 Use of biomaterials in biomedical science

Use of Biomaterials	
1.	Replacement of diseased and damaged part (e.g., artificial hip joint)
2.	Assist in healing (e.g., sutyres, bone plates, and screws)
3.	Aid to treatment (e.g., catheters, drains)
4.	Aid to diagnosis (e.g., probes and catheters)
5.	Improve function (e.g., cardic pacemaker)
6.	Plastic surgery etc.

56.2.3 Composite Materials

Composite can be generally defined as those materials having two or more distinct material phase [56.2]. For example, combination of silane-coated inorganic filter particles with dimethacrylate resin which is used in dentistry. The filter particles used are either barium silicate glass, quartz, or zirconium silicate and are usually combined with 5–10% weight of 0.04 μm particles of colloids silica. A hydroxyapatite–polyethylene is also an example of composite which is used in orthopedic implants.

Many biomineralized tissues of many invertebrate origins are composite materials, containing a biologically produced organic matrix and nano or microscale amorphous or crystalline minerals. Composite materials have lot of advantages in comparison with homogenous materials. Those include the ability to have control over material properties. This is the potential for stiff, strong, light-weight materials, as well as for highly resilient and compliant materials.

Composite biomaterials can be used in various ways. Few of them are as follows:

(a) Dental filing
(b) Reinforced methyl methacrylate bone cement and ultrahigh molecular weight polyethylene
(c) Orthopedic implants with porous surfaces.

The properties of composite materials depend very much upon the structure.

56.2.4 Ceramics

Ceramics are used for the repair and restoration of diseased or damaged parts of the musculoskeletal systems. Four types of bioceramics are mostly used in human system:

Type 1 – Bioinert like aluminum oxide (Al_2O_3) and zirconium oxide (ZrO_2).
Type 2 – Micro-porus bioceramics like hydroxyapatite-coated metals, alumina.
Type 3 – Bioactive materials like hydroxyapatite, bioactive glasses, and glass–ceramics.
Type 4 – Resorbable like tri-calcium phosphate (TCP).

Application of the bioceramics includes: Replacement for hips, knees, teeth, tendons and ligaments, and repair for periodontal disease, maxillofacial reconstruction, augumentation and stabilization, spinal fusion, and bone fillers after tumor surgery.

56.3 Marine Biodiversity

Ocean is rich in biodiversity. The world's ocean covers more than 70% of our planet's surface with over five lakh species belonging to 32 animal phyla. Microbes constitute 90% of oceanic biomass. Marine organisms produce novel compounds to survive in extreme variation in pressure, salinity, and temperature, prevailing in the marine environment. With 3.5 billion years of existence on earth and experience in biosynthesis, the marine organisms especially microbes are the nature's best source of chemicals. Because of the immense biological diversity in the ocean, it is well established that a large number of natural products and novel chemical entities exist in the oceans, with biological activities that may be useful in finding drugs with greater efficacy and specificity for the treatment of many human diseases [56.3]. Some marine organisms are proved to be the potent sources of biomaterials for therapeutic and medical diagnostic purpose. These are mostly invertebrates that include sponges, soft corals, seafans, seahares, nudibranchs, bryozoans, tunicates, etc. It is believed that microorganisms associated with the invertebrates are responsible for the production of bioactive compounds. In addition to the marine invertebrates, microbes (bacteria, fungi, and actinomycetes), microalgae, seaweeds, and mangroves also provide compounds of therapeutic value [56.4]. The vast marine biodiversity offers great scope for new therapeutic biomaterials.

56.4 Biomaterials from Marine Origin

Marine organisms are constituted by materials with a vast range of properties and characteristics that explains their potential applications within the biomedical field. Some of the biomaterials from marine origin have been disused below.

56.4.1 Collagen

Collagen is the most widely found protein in mammals. Collagen is found in connective tissues. It is derived from the soluble precursor procollagen. Collagen is a left-handed-α helix and has three amino acid residue per turn (Gly-X-Pro or Gly-X-Hyp; X = any amino acid). Collagen is also a coiled coil, three separate polypeptide are super twisted in right-handed manner. Typically they contain about 35% Gly, 11% Ala, and 21% Pro-4-Hyp.

There are many types of collagen, differing in their structure, function, location, and other characteristics. The predominant form used in biomedical applications, however is type I collagen. Type I collagen is a *rope forming* collagen and can be found in skin and bones. Collagen may also be processed into a variety of formats, including porous sponges, gels and sheets, and can be cross-linked with chemicals to make stronger or to change its degradation rate.

The primary sources of industrial collagens are calf skin and bone. However, these carry a high risk of bovine spongiform encephalopathy or transmissible spongiform encephalopathy. Collagen has been used in

various types of surgery, cosmetics drug delivery, and also in bio-prosthetic implants and tissue engineering of multiple organs as well. Cells grown in collagen often come close to behaving as they do within the body, which is why collagen is so promising when one is trying to duplicate natural tissue function and healing.

Nagai et al. reported the isolation and characterization of collagen from rhizostomous jellyfish (*Rhopilema asamushi*) [56.5]. In a research report by *Song* et al., jellyfish collagen exhibited higher cell viability than other naturally derived biomaterials [56.6]. Jellyfish collagen scaffolds also had a highly porous and interconnected pore structure, which is useful for a high-density cell seeding, an efficient nutrient and an oxygen supply to the cells cultured in the three-dimensional matrices. *Song* et al. prepared the porous scaffolds composed of jellyfish collagen by freeze-drying and cross-linking with 1-ethyl-(3,3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide to be used in tissue engineering applications.

Collagen sponge is also one of the biomedical materials frequently used in clinical medicine, especially in dermatology and plastic reconstructive surgery [56.7]. Collagen sponges have been reported for the treatment of burn [56.8–10], trauma [56.11], chronic skin ulcer [56.12, 13], excised skin tumor [56.14–16], etc. Collagen sponge is also used as scaffold for tissue engineering because of its high ability of cell adherence and biological affinity. It has been used to regenerate various tissues such as skin [56.17, 18], cartilage [56.19, 20], bone [56.21, 22], cornea [56.23, 24], teeth [56.25, 26], etc., by being employed as a scaffold. *Matsumoto* et al. reported the usefulness of the collagen and elastin sponge (CES) derived from salmon as an artificial dermis and scaffold for tissue engineering. Report suggested that the CES has the same potential of forming the granulation tissue as the traditional artificial dermis and could be used as a scaffold for tissue engineering [56.7].

Sirbu et al. reported that collagenic gels containing marine algae extracts and chlorhexidine salt could be used in dental medicine as pharmaceutical formulae with optimum efficiency, having well-profiled organoleptic properties [56.27]. They tested the extract from marine algae *Ulvae lactuca, Cystoseira barbata*, and *Ceramium rubrum* from the Black sea. The marine algae extract and chlorhexidine salt were incorporated in type I nondenatured fibrillar collagen matrixes for the experiment.

Depending on the processing, collagen can cause alteration of cell behavior (e.g., change in growth or movement), have inappropriate mechanical properties, or undergo contraction. Because cells interact so easily with collagen, cells can actually pull and reorganize collagen fibers, causing scaffolds to lose their shape if they are not properly stabilized by cross-linking or mixing with another suitable material. Collagen can be easily combined with other biological or synthetic materials, to improve its mechanical properties.

56.4.2 Chitin and Chitosan

Chitin was first isolated from mushrooms by Braconnot in the year 1811. It remained unused biomass resource for a long time but in the last decade, lot of demand has been reported for the chitin and chitosan in different markets, like cosmetics, medicine, biotechnology, food, and textiles.

Chitin (polymer of β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose) are found in shells of marine crustaceans such as crabs and shrimps. These shells are available as waste from the seafood processing industry and used for commercial production of chitin. The shells contain approximately 15–40% chitin (Table 56.2).

Chitin is generally classified into two forms, β-chitin and α-chitin, depending on the crystalline structure. The most abundant and easily accessible form is α-chitin with maximum intermolecular hydrogen bonding [56.29]. Dissolution or swelling converts β-chitin to α-chitin, but not α-chitin to β-chitin. Chitosan is deacylated form of chitin.

Chitin and chitosan are attracting a great deal of attention because of their biological and physiochemical characteristics. They are nontoxic, biocompatible, and biodegradable. Chitosan may be easily combined with other materials like polyvinyl alcohol, polyethylene glycol, or collagen, in order to increase its strength and cell attachment potential.

Table 56.2 Contents of chitin from different marine resources [56.28]

Source	Chitin (%)
Crab cuticle	15–30
Shrimp cuticle	30–40
Krill cuticle	20–30
Squid pen	20–40
Clam/oyster shell	3–6

Chitin and chitosan have been reported for various bioactivities including promotion of wound healing [56.30, 31], hemostatic activity, immune enhancement, hypolipidemic activity [56.32], mucoadhession, eliciting biological responses, and antimicrobial activity [56.33, 34]. Chitosan is also used as supporting polymer for gene delivery, cell culture, and tissue engineering [56.28]. Nonwoven chitin fabrics and chitin threads are used as artificial skin and sutures with the advantages of wound healing, biocompatibility, and biodegradation. Glucosamine, the monomeric unit of chitosan, has proved clinically effective against osteoarthritis. Chitosan has already been investigated for use in the engineering of cartilage, nerve and liver tissues.

56.4.3 Chondroitin Sulfate (CS)

Chondroitin sulfate is the most valued glycosaminoglycan because of its abundance in mammalian tissues, physiological functions and high activity. CS contains repeating units of glucuronic acid and *N*-acetylgalactosamine linked by β-(1→3) glycosidic bonds and sulfated in different carbon positions. Classification and types of CS depend on sulfate group placing: carbon 4 (CS-A; present in dog fish and ray), 6 (CS-C; present in ray), both 4 and 6 (CS-E; present in squid, salmon, crocodile), position 6 of *N*-acetylgalactosamine and 2 of glucuronic acid (CS-D-present in shark and dog fish), and 4 of *N*-acetylgalactosamine/2 of glucuronic acid (CS-B) [56.35].

Chondroitin sulfate has been isolated from whale, shark, skate, squid, salmon, king crab, and sea cucumber. It is also present in marine invertebrates like Cnidaria, Polychaeta, and Mollusca [56.36]. Shark cartilage has been the most commonly used as a commercial source of nonmammalian CS. But price of raw material and ecological aspects are really concern today. Due to its high biocompatibility, CS demand in the industry is increasing day by day. It is used in engineering of biological tissues associated with the process of bone repair, cartilage, and cutaneous wound. Marine CS is also reported for its potential as biomedical gel forming polymer, anticoagulant activity, and as a supplement in arthritis-related diseases [56.36]. Moreover, it can also be combined with other polymers to formulate scaffolds with slow and controlled biodegradability. Recent studies also indicate that CS-E is a potent antiviral whereas CS-proteoglycan is a potential target for the development of vaccines against malaria. Sulfation pattern of CS can be used as potential biomarker to early detection of diverse types of cancer [56.35]. Fucosylated CS (CS-F) obtained from sea cucumber inhibit adenocarcinoma growth in lungs using mouse model [56.37].

56.4.4 Hyaluronic Acid (HA)

Hyauronic acid (HA) or hyaluronan is a naturally occurring nonsulfated glycosaminoglycan consisting of alternate disaccharide units of α-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine, linked by β (1→3) bonds. It is present in intracellular matrix of most connective tissues such as cartilage, vitreous of the human eye, umbilical cord, and rooster comb. HA is also present in cartilaginous fishes and in vitreous humor of different fish species [56.38]. However, the most important alternative in recent years has been development of microbial HA production by *Streptococcus* bacteria it also avoid the risk of animal derived pathogens [56.39, 40].

The activity of HA is dependent on its size [56.41, 42]. HA holds a large number of water molecules in its molecular domain. This *swelling* property and its chemical structure gives it a wide ranging of physiochemical and biological function such as lubricity, viscoelasticity, biocompatibility, angiogenic, and immunostimulatory. High concentration of high molecular mass HA in synovial fluid provides lubrication and serves as shock absorber. In osteoarthritis or rheumatoid arthritis, high molecular mass HA is degraded by reactive oxygen species, which reduces its viscosity and impairs its lubricant and shock absorbing properties leading to deteriorated joint movement and pain [56.36, 43]. In the skin, HA acts as scavenger of free radicals generated by the UV rays from sunlight, which otherwise would cause oxidative stress on cells [56.44]. This polymer has great economic value with numerous applications in biotechnology, regenerative medicine, major burns, and intraocular surgery [56.35]. HA is used as diagnostic marker for cancer, rheumatoid arthritis, and live pathologies [56.45], and also in drug delivery system [56.46].

56.4.5 Alginate

Alginate is a polysaccharide derived from brown seaweed. It is an unbranched anionic copolymer composed of two monomers, (1→4) linked β-D-mannuronic acid and α-L-guluronic acid. Commercial extraction of the alginate is from species of *Laminaria, Macrocystis*,

Ascophyllum, Eclonia, Lessonia, Durvillea, and *Sargassum* [56.47]. It is nontoxic and noninflammatory in nature due to which it has been approved in some countries for wound dressing and for use in food products.

Alginate is biodegradable and has controlled porosity. Alginate has been explored for being used in liver, nerve, heart, and cartilage tissue engineering. Drawbacks of the alginate include mechanical weakness and poor cell adhesion which can be overcome by mixing with other materials. The ability of alginate to cross-link with calcium ions may be used to produce scaffolds.

56.4.6 Biosilica

Biosilica or biogenic silica is formed in sponges, diatoms, radiolarians, and choanoflagellates [56.48]. There are two classes of sponges that have silica skeleton: Desmospongiae and Hexaactinellida. The silica skeleton is made up of siliceous spicules which are rod-like glassy spikes consisting of an axial filament surrounded by several hundreads concentric layers of hydrated silica. The process of biosilica formation catalyzed by the enzyme silicatein present in axial filament [56.36]. The silicateins were first identified in the axial filament of demosponge *Tethya aurantium*. Three isoforms have been identified, silicatein-α, silicatein-β, and silicatein-γ [56.49]. Sponge spicules have mechanical, optical, and electric properties. These natural biocomposites have been characterized as highly flexible and tough.

Another important source of biosilica is diatoms. Diatoms are a major group of microalgae. They are unicellular organisms, although some form chains or simple colonies. The living part of the diatoms is within a box, which is made up of silicon dioxide [56.50]. These exoskeleton-named frustules are made of silica nanoparticles assembled in a highly organized structure with porous network at different scales.

The biocompatibility of the biosilica is excellent and has no toxicity. Due to these properties, biosilica is being considered for biomedical approaches, namely, for bone replacement and regeneration strategies. It has been observed that human osteogenic sarcoma cells (SaOS-2) show increased mineralization when cultivated on biosilica surfaces in the presence of β-glcerophosphate. Moreover, concurrent coating of the substrate with biosilica and type1 collagen not only increased the cellular Ca phosphate deposition but also stimulated cell proliferation [56.36]. Biosilica has been identified for promoting morphogenesis on both osteoblast and osteoclasts. This polymer elicits cytokines that affect bone mineralization (hydroxyapatite formation) [56.49].

56.4.7 Calcium Carbonates and Phosphates

Calcium carbonate ($CaCO_3$; aragonic or calcite forms) can be found in many marine organisms like corals (*Coralline officinallis, Lithothamnion glaciale, and Phymatholithon calcareum*), sponges (calcareous sponge spicules from triactines of *Pericharax heteroraphis*) mollusc shells (nacre from *Haliotis* (abalone), *Mytilus galloprovincialis* and *Ostrea edulis* and *Pinctada maxima*) and fish bones (*Prionace glauca* (Blue shark)) [56.36]. Although there are many sources of calcium carbonate. Coral skeletal carbonate is a promising substitute material for orthopedics and dentistry. Coral-derived calcium carbonate has been in use as bone filler. Calcium carbonate can also be used as precursor material for bioceramics coatings. $CaCO_3$ can be the precursor material for obtaining different calcium phosphates.

Calcium phosphorous compounds such as hydroxyapatite (HAp) – $Ca_{10}(PO_4)_6(OH)_2$, have a special importance in the biomedical field due to its similarities with the mineral constituents of bones. Besides using $CaCO_3$ as precursors, hydroxyapatite can be found and obtained directly from marine resources like fish bones. Calcium phosphate has been used in drug delivery system as bioceramics, tissue engineering scaffolding, bone graft substitute, and facial fillers [56.36].

56.5 Status of Marine Natural Product as Therapeutic Agents

Marine flora and fauna continue to provide a prolific source of novel lead compounds for the pharmaceutical industry. The effort to extract bioactive compounds from the ocean started in the late 1960s. The systematic investigation began in the mid-1970s [56.4]. Approximately 200 patents were issued between 1969 and 1995 [56.51]. One hundred and thirty five patents were issued between 1996 and July 2003 [56.52]. Some of the recently patents compounds are given in Table 56.3.

Table 56.3 Some of the recently patented compounds (compiled from Ocean Drug Alert, Lucknow, India and [56.52])

Organism	Compound	Activity	Assignee	Year
1. Marine bacteria and fungi				
Marine bacteria *Myxobacterales* AJ 13395	Polyene SMP-2 and its isomers	Antifungal against *C. albicans, Aspergillus fumigates, A. niger* and *Mucor hiemalis*	Ajinomoto Co. Inc.	2000
Marine fungus *Aspergillus* CNC358	Avrainvillamide	Anticancer	University of California	2000
Ascomycete isolated from marine algae *Porphyra yezoensis*	M-3-A	Fungicide	Marine Biotechnology Institute of Japan	2001
Marine actinomycete strain ES20-008	Bafilomycin derivative	Anti-invasive and antitumour	Instituto Biomar SA	2001
Actinomycete from marine sponge	Indolocarbazole alkaloids	Anticancer	Instituto Biomar SA	2001
Marine *Pseudomonas* sp.	Acidic sulfated polysaccharide	Apoptosis in U-937 human leukaemia cells	Okuya Koichi	2002
Marine actinomycetes	Certain fermentation products	Antineoplastic agents	Regents of the University California, US	2005
Marine actinomycetes	Indole, carbozole, alkaloids	Anticancer	Institute of Biomarine SA, Spain	2005
2. Marine algae				
Bryothamnion seaforthii and *B. triquetrum*	Lectins	Analgesic	Braz. Pedido PI	2001
Brown seaweed *Cladosighon okamuranus*	Fucoidans	Antiulcer	University of South Coralina	2001
Green algae, *Chlorella* sp.	Glutamic Acid	Antagonist growth inhibitor	Yakult Honsha Co. Ltd.	2001
Laminaria sp.	Crude extract	Antioxidant, osmo protectors	Mekideche N	2001
Brown seaweed, *Padina pavonnica*	Crude extract	Calciotropic activity	Texinfine SA	2001
Brown seaweeds, *Laminaria japonica, Undaria pinnatifida, Kjellmaniella crassifolia*	Sulfated fucose containing polysaccharide	Anticancer and anticarcinostatic	Takaro Shuzo Co. Ltd. Japan	2001
Brown algae *Sargassum micracanthum*	Macrocyclic ether	Antioxidant and antiviral	Toyama-Ken and Lead Chemical Co. Ltd.	2002
Ascophyllum nodusum	Crude extract	Antiobesity	Riken Vitamin Co. Ltd., Japan	2002
Cynobacteria *Symploca hydnoides*	Peptid	Antitumor activity	Instituto Biomar SA	2003
Red algae, *Pophyra* sp.	Crude extract	Stress protein inducing	Larena 13, Avenue De-Segur F-75007, France	2003
Seaweed	Mixed extracts of brown, red and green algae	Antidiabetic	Endomatrix Inc USA	2004

Several bioactive ingredients have been isolated and characterized from the marine resources in the last decade. These active ingredients are against various types of diseases. Recently *de Jesus Raposo* et al. [56.53] reviewed the bioactivity and applications of sulfated polysaccharides from marine algae.

Aquired Immuno Deficiency Snydrom (AIDS) is a major concern around the world these days. It is a major viral disease caused by Human immunodefi-

Table 56.3 (continued)

Organism	Compound	Activity	Assignee	Year
3. Sponges				
Raspailia sp.	Asmarine A and B – novel diterpene alkaloids	Cytotoxic	Instituto Biomar SA	1999
Sponogosorites sp.	Toposentin D	Antineurogenic inflammatory property	Harbour Branch Oceanographic Inst. Inc.	1999
Sponge	Manzamine A	Inhibit growth of *Plasmodium falciparum* in mice	National Institute of Singapur et al.	1999
Sponge from South China Sea	Rhabdasterol acetate	Blood pressure reducing and arrhythmia-resisting utility	Guangzhou Inst. of Chem.	1999
Crambe crambe	Crambescidin 816, 844, and 800	Inhibit herpes simplex virus	Pharma Mar SA	1999
Sponge	Manzamine A–F	Antineurogenic inflammatory property	Harbor Branch Oceanographic Inst. Inc., Midwestern Univ.	2000
Marine sponge *Batzella* sp.	Aminoiminoquinone and Aminoquininie alkaloid compounds	Capsase inhibitor	Harbor Branch Oceanographic Inst. Inc.	2000
Haliclona tulearensis	Halitulin	Cytotoxic	Pharma Mar SA	2000
Stylotella aurantium	Sesquiterpenes	Antitumor	Pharma Mar SA	2001
Plakortis lita	3,6-dihydro-1,2-dioxins	Antitumor	Sagami Chem. Res. Center	2001
Marine sponge *Discodermia*	Discalamides A and B	Antitumor	Harbor Branch Oceanographic Inst. Inc.,	2001
Xestospongia exigua	Motuporamine C	Inhibition of angiogenesis	University of British Columbia	2002
Blue marine sponge *Cribrochalina*sp.	Cribrostatins 3-5	Anticancer, antibacterial, and antifungal	The Arizona Board of Regents	2002
Stelletta hiwasaensis	Orostanal	Anticancer	Kyushu TLO Co. Ltd.	2002
Sponge	Polymeric-1,3-alkylpyridinium	Toxins	Aberdeen University, UK	2004
Aplysina cavernicola, A. fulwa, Oeanapia sp.	A spiro hetrocyclic unit connected through a linker of certain length to another spiro cycle, an imidezole ring or an amide group	Against connitive and neuro-degenerative disorders	Neuropharma SA	2005
4. Cnidaria				
Soft coral *Sinularia dissecta*	Rameswaralide and its derivative	For the treatment of arthritis, psoriasis, and inflammatory bowel disease.	The regents of University of California	2000
Erythropodium caribaerum	Eleutherobin, desmethyleleutherobin, and isoeleutherobin	Antimitotic	University of British Col	2001
Jelly fish *Carybdea alata*	CaTX-A and CaTX-B	Hemolytic proteins	Suntory Ltd.	2001

ciency virus (HIV). Due to the emergence of resistance in virus, present anti-AIDS treatment is not successful. Therefore, the search for potential drug candidate with higher inhibitory activity against various HIV strains is the need of hour. Recently *Vo* and *Kim* [56.54] gave an overview for potential anti-HIV agents from marine resources.

Oxidative stress and inflammation are common pathophysiological features of artherosclerotic cardiovascular disease. Astaxanthin, a xanthophylls

Table 56.3 (continued)

Organism	Compound	Activity	Assignee	Year
5. Echinoderms				
Sea cucumber *Cucumaria echinata*	Proteins	Cytotoxic against leukaemia	National Institute of Advanced Industrial Sci. and Tech. et al.	2001
Sea urchin *Echinocardium cordatum*	Alkanesulfonic acids and derivatives	Antitumor activity and antibacterial activity against *Rhodospirillum salexigens* SCRS-113, a marine adhesive bacteria	Nagoya Industrial Sci. Res. Inst.	2002
Sea cucumber	Carotenoid lipid	Anti-inflammatory auto immune	Neptune technologies and bioress; Sampalis Tina,	2002
Holothuria scabra	Dye	Fluorescent dye	Council of Scientific and Industrial Research (CSIR-India)	2003
6. Molluscs				
Clam *Spisula polynyma*	Spisulosines 285	Antitumor activity	The Board of Trustees of the University of Illinois	1999
Bivalve mollusc shellfish *Mytilus galloprovincialis*	Myticin peptide	Antimicrobial activity	Center national De La Recherche Scientifique et al.	2001
Nudibanch *Jorunna funebris*	Jorumycin	Antitumor	Instituto Biomar	2001
7. Tunicates				
Cystodytes violatinctus	*n*-alkaloid-Shermilamine D	Antitumor	Instituto Biomar	1999
Ascidian *Lissoclinum* sp.	Tridecanolids	Antitumor	Sagami Chem. Res. Center	2000
Ascidian	Coproverdine – A carbazole alkaloid	Cytotoxicity against P-388, A-549, HT-29, MEL-28 and DU-145 cell lines	Pharma Mar.	2002

carotenoid present in various marine organisms like microalgae, seafood etc., is an antioxidant with anti-inflammatory properties. It may have a potential therapeutic role in cardiovascular disease [56.55]. *Fassett* and *Coombes* summarized various animal studies investigating the cardiovascular effects of astaxanthin [56.55]. Some studies also reported the role of astaxanthin in the prevention of diabetic nephropathy [56.56].

Anti-inflammatory ingredients obtained from marine algae can be used in regenerative therapy for tissues affected by the periodonthal disease [56.27]. *Sirbu* et al. presented the possibility of application of collagen gels with active compound from marine algae (*Ulva letuca, Cystoseira barbata*, and *Ceramium rubrum*) [56.27]. Report indicate that collagenic gels containing marine algae extracts and chlorhexidine salt could be used as pharmaceutical formulae with optimum efficiency, having well-profiled organoleptic properties. Some of the noble active pharmaceutical ingredients are listed in Table 56.4.

56.6 Marine Resources for Medical Diagnostic Devices

Since the past two decades, in vitro and in vivo implantable medical devices such as biosensor has been developed for the point of case medical diagnostics. The major advantage of the biomaterial directly obtained from marine source or biomaterial of marine origin is the biocompatibility and stability. This section will deal briefly with the biosensors types, and certain important examples of biosensor fabrication utilizing biomaterials of marine origin.

56.6.1 Biosensors: An Introduction

Biosensors are analytical devices that are based on the coupling of an immobilized biologically active com-

Table 56.4 Some of the bioactive compounds isolated from marine resources

Species/source	Active ingredient	Property	References
Algae			
Sargassum graminifolium (Turn.)	Polysaccharide (SGP)	Inhibit calcium oxalate crystallization and antioxidant. Could be a candidate for treating *urinary stones*	[56.57]
Caulerpa sp.	Caulerpin	Analgesic and anti-inflammatory activity	[56.4]
Laminaria	Laminine	Reduce hypertension	[56.4]
Eisenia bicyclis	Phloroglucinol – Eckol and dieckol	Inhibit glycation and α-amlyase, so many have an effect on complications of diabetes	[56.58]
Arthrospira	Extract	To lower hyperlipidaemia, hyperglycaemia and hypertension	[56.59–61]
Sponge			
Luffariella variabilis	Manoalide	Antibiotic	[56.62]
Batzella sp.	Batzelladine A&B	Potent inhibition to the binding of HIV glycoprotein on CD4 receptors of T cells	[56.63]
Corals			
Sarcophyton glaucum	Sarcophytol A, hydroperoxyl cembranoids	Anticancer	[56.64–66]
Lobophytum crassum	Ceramides	Antibacterial	[56.67]
Mollusc			
Dicathais orbita	Tyrindoleninone and 6-bromoisatin	Potential for development of treatment against female reproductive cancers	[56.68]
Dolabella auricularia	Dolastatin-10 and 15	Antitumor activity against breast and liver cancer	[56.69, 70]
Elysia rufescens	Kahalalide F	Antitumor activity against breast, hepatoma, melanoma, and pancreatic carcinoma	[56.71, 72]
Marine Tunicate			
Ecteinascidia turbinata	Trabectedin	For the platimum sensitive ovarian cancer and tumor soft tissue sarcoma	[56.73]
Eudistoma sp.	Eudistomins	Antiviral activity	[56.74]
Marine Fish			
Syngnathus acus	Protein syngnathusin	Antitumor	[56.75]
Shark	Protein	Antitumor	[56.76]
Manta birostris	Protein	Antitumor	[56.77]
Arca subcrenata Lischke	Protein	Antitumor	[56.78]

pound called a receptor with a signal transducer and an electronic amplifier. Physiochemical change produced by specific interactions between a target and the biorecognition element is measured by a transducer. The transducer then converts the biochemical response into quantifiable electronic signal that is processed into an analog or digital format. The concentration of the analyte is directly proportional to the amount of signal generated, allowing for both quantitative and qualitative measurements. A general schematic of a biosensor is shown in Fig. 56.1. Biosensors have improved the performance of the analytical detection over the conventional analytical methods due to certain advantages, such as specific, rapid, and simple to operate, can be easily fabricated with minimal sample pretreatment involved. The apparently alien marriage of two contrasting disciplines combines the specificity and sensitivity of biological systems with the computing power of microprocessor. The potential applications of biosensors have continued to lie in the clinical diagnosis, bioprocess, food analysis, environmental monitoring, and etc. [56.79–81].

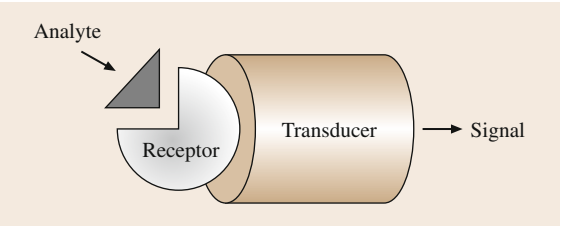

Fig. 56.1 Schematics diagram of the principle of biosensors

56.6.2 Components of Biosensors

Biosensors are highly selective technique due to the high substrate specificity of the enzyme and the interference free indication of the reaction product. Biosensors have offered the possibility of real-time analysis which is important for the rapid measurement of biological analysis. Various components have been received considerable interest in the fabrication of biosensors which included receptors, matrix for immobilization, methods of immobilization, and transducers as shown in the schematic diagram (Fig. 56.2).

Receptors

The biological elements that have been used in the creation of biosensors vary widely in type and include aptamers proteins, enzymes, nucleic acids, lipids, and living cells. The first step involved in a biosensor fabrication is the selection of biomolecule that forms a specific complex between an immobilized biologically active compound and a desired analyte.

The biological structure required for the biocatalytic receptor is usually derived from subcellular components and include enzymes, proteins, cells, and tissue [56.82]. These devices are based on immobilizing a biocatalyst into a transducer, transient, or steady-state responses are monitored by integrated detector that is finally displayed on a panel after processing and choice of the biological material and the adjusted transducer depends on the properties of each sample of interest and the type of physical magnitude to be measured. The type of a biocomponent determines the degree of selectivity or specificity of the biosensor. Thus, biosensors are classified, according to the type of involved active biological component in the mechanism, into biocatalytic and bioaffinity receptors (Fig. 56.3) [56.82–84].

56.6.3 Biosensor Fabrication Utilizing Biomaterials

For the immobilization of receptors or preparation of composites, several biomaterials such as collagen, chitosan, chitin, and alginate have been utilized in recent times for the fabrication of biocompatible, stable, and sensitive biosensors. For instance, a three-dimensional porous and biostable collagen scaffold has been developed to improve the biocompatibility of implantable glucose sensors by minimizing tissue reactions while stimulating angiogenesis around the sensors [56.85].

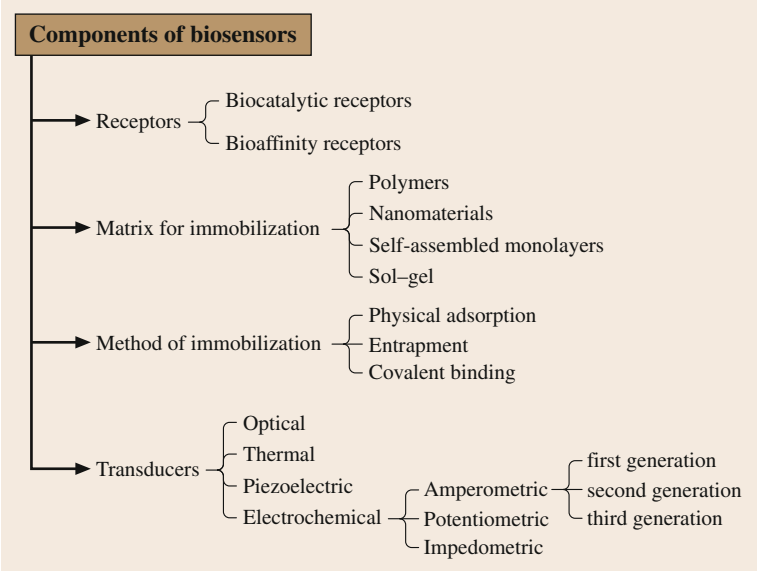

Fig. 56.2 A general component of biosensors

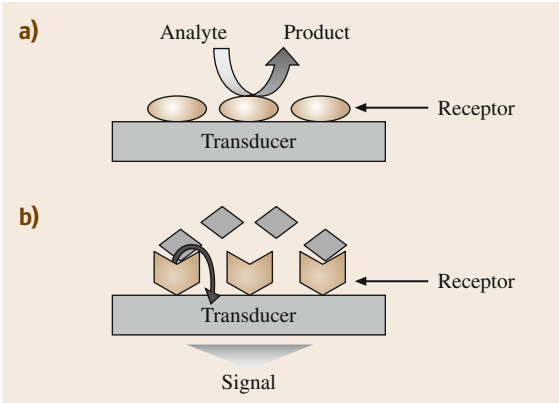

Fig. 56.3a,b Schematics illustration of the types of receptors: **(a)** biocatalytic and **(b)** bioaffinity receptors

The biostability of the collagen scaffold was enhanced through the cross-linking of nordihydroguaiaretic acid (NDGA) which was stable without any physical twist in the tissue of rats for 4 weeks. The response (i. e. sensitivity) of the developed sensors with porous scaffolds was not significantly changed when compared with sensors without, while the response time (T(95%)) was slightly delayed after a glucose concentration increase from 5 to 15 mM. These results indicate that the developed NDGA-cross-linked collagen scaffold biosensor was stable.

In another example, a multiwell plate-based biosensor containing B-cell hybridoma, Ped-2E9, encapsulated in type I collagen matrix, was developed for quick detection of living cells of *Listeria*, the toxin listeriolysin O, and the enterotoxin from *Bacillus* species [56.86]. This sensor measures the alkaline phosphatase release from infected Ped-2E9 cells colorimetrically. Pathogenic *L. monocytogenes* cells and toxin preparations from *L. monocytogenes* or *B. cereus* showed cytotoxicity ranging from 24 to 98% at 3–6 h postinfection. In contrast, nonpathogenic *L. innocua* (F4247) and *B. subtilis* induced minimal cytotoxicity, ranging only 0.4–7.6%. Laser scanning cytometry and cryo-nano scanning electron microscopy confirmed the live or dead status of the infected Ped-2E9 cells in gel matrix. The results shown by the author presents the first example of a cell-based sensing system using collagen-encapsulated mammalian cells for rapid point of the care detection system.

In recent times, chitosan has also been used extensively for the development of biocompatible biosensors. In some reports, chitosan is found to decrease the electron transfer process of the biosensors. Hence, a chitosan–nanomaterials composite are developed. In one example, direct electrochemistry of a glucose oxidase (GOD) – graphene–chitosan nanocomposite was studied [56.87]. The immobilized enzyme retains its bioactivity, exhibits a surface confined, reversible two-proton and two electron transfer reaction, and has good stability, activity. A much higher enzyme loading is obtained as compared to the bare surface without the nanobiocomposite. This nanobiocomposite film was used for sensitive detection of glucose from 0.08 to 12 mM glucose with a detection limit of 0.02 mM and much higher sensitivity as compared with other nanostructured supports. The excellent performance of the biosensor is attributed to a large surface-to-volume ratio and high conductivity of graphene and good biocompatibility of chitosan, which enhances the enzyme absorption and promotes direct electron transfer between redox enzymes and the surface of electrodes. Chitosan has also been widely used as electrochemical biosensor component for electrochemical nucleic acid biosensors, immunosensors, enzyme biosensors, and chitosan-modified voltammetric electrodes for trace analysis in real samples [56.88].

Chitin, the next most abundant biopolymer and imminent into its natural synthesis, enzymatic degradation, and chemical interactions with other biopolymers is vital in bioengineering and medical-device development such as implantable devices and detectors. Biosensors based on the ionic liquid (IL) and corn peroxidase immobilized on chemically cross-linked chitin have also been developed for the determination of adrenaline. The IL 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIPF_6$) were constructed in a homogenate as a source of peroxidase, which was immobilized in chitin chemically cross-linked with carbodiimide and glyoxal. In the presence of hydrogen peroxide, the peroxidase catalyzes the oxidation of adrenaline to the corresponding *o*-quinone, which is electrochemically reduced at a potential of −0.23 V *vs.* Ag/AgCl. The developed biosensor demonstrated long-term stability and reproducibility and it was also applied for the determination of adrenaline in pharmaceutical samples indicating the applicability of chitin as a good substrate for enzyme immobilization and biosensor fabrication. This strategy can easily be adopted for the immobilization of other biomolecules. Very recently, chitin films were formed by spin coating trimethylsilyl chitin onto gold or silica substrates, followed by regeneration to a chitin film for the development of biosensors [56.89]. Infrared and X-ray photoelectron spec-

troscopy, X-ray diffraction, ellipsometry, and atomic force microscopy were used to confirm the formation of smooth, homogeneous, and amorphous chitin thin films. Quartz crystal microbalance with dissipation monitoring (QCM-D) solvent exchange experiments showed these films swelled with 49% water by mass. The utility of these chitin films as biosensors was evident from QCM-D and surface plasmon resonance studies revealed the adsorption of a bovine serum albumin monolayer. This system in future can be extended for in vitro bioimaging and cell proliferation platform generation.

Another biomaterial alginate has also been well utilized in the biosensor fabrication. One of the important applications of alginate in the sensor development is toward cholesterol determination in the human body which is important in diagnosis of diseases like coronary heart disease, arteriosclerosis, diabetes, and obstructive jaundice. In this direction, fluorimetric cholesterol biosensor based on self-assembled mesoporous alginate-silica (algilica) microspheres has been reported. For the fabrication of the biosensor, Pt(II)-octaethylporphine (PtOEP; oxygen sensitive metalloporphyrin) dye was loaded in the algilica microspheres using the solvent-mediated precipitation process. Cholesterol oxidase (ChOx) was then covalently immobilized to PtOEP/algilica microspheres using the carbodiimide coupling reaction. The analytical response of the biosensor to standard cholesterol displayed a linear dynamic range from 1.25 to 10 mM of cholesterol with regression coefficient of 0.996 (1.25–3.75 mM), 0.976 (1.25–6 mM), and 0.959 (1.25–10 mM) with the response time of 10 min. Thus, the developed cholesterol biosensor based on the alginate shows great potential in the diagnosis of hypercholesterolemia. This kind of marix can also be extended toward the development other enzyme-based biosensors. In another example, two biosensor configurations based on the entrapment of algal cells of *Chlorella vulgaris* into either a regular alginate gel or pyrrole-alginate matrix have been reported [56.90].

These developed biosensors were compared in terms of their amperometric current measurements to *p*-nitrophenyl phosphate when used as substrate for the detection of an algal alkaline phosphatase activity. The high stability of the pyrrole–alginate gel when compared to that of the alginate coating is herein demonstrated.

56.6.4 Scope for Biomaterials

According to a market research report reported by Markets & Markets (www.marketsandmarkets.com), the total global biomaterials market is expected to be worth USD 88.4 billion by 2017. The biomaterials are broadly used in orthopedic, cardiovascular, plastic surgery, wound healing, neurological, dental, ophthalmology, and tissue engineering. Of these, cardiovascular biomaterial market dominated the biomaterial market in 2012, followed closely by orthopedic. Stents, pacemakers, and implantable cardiac defibrillators are the largest segments marking the growth of the cardiovascular biomaterial market. The plastic surgery and wound healing applications are expected to spur the demand for biomaterials in the coming years (www.marketsandmarkets.com). Metals and polymers dominate the biomaterials market.

Ocean is a huge source of materials, even though the available knowledge of marine materials and mechanism is still in its beginning stage. The diversity of the biomaterials from marine resources and its potential can be increased further by chemical and physical modifications. These modifications in marine polysaccharides or other compounds are continuing challenge to polymer and biomedical scientists but output from these kinds of researches will widen up perspective and potential applications in the future. It is envisaged that marine-derived biomaterials have role of major relevance in biomedical field, due to its use on tissue repair and regeneration. Lot of research is going on in this field.

56.6.5 Conclusion

Biomaterials are the focus of major research efforts around the world as they have huge potential for all. Progress in this field requires a multisidisciplinary approach. An increasing number of compounds are currently being identified and isolated from marine organisms ranging from bioactive ingredients to medical devices. However, strong efforts are still needed to obtain medical-grade biomaterials from marine resources. Till now most of the biomaterial-based biosensors are developed are synthetic biomaterials. It is interesting to obtain the marine biomaterials such as sponge collagen, chitin, etc., to obtain more biocompatible biosensors. It is also be interesting to compare the sensitivity of the sensor systems based on the various types of biomaterials.

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57. Enzymatically Synthesized Biosilica

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Structural biomaterials are hierarchically organized and biofabricated. Biosilica represents the main mineral component of the sponge skeletal elements, the spicules. We summarize recent data on the different levels of molecular, biological, and structural hierarchies controlling the synthesis of the picturesquely and intricately architectured spicules/skeletons.

Biosilica is a promising material/substance for the amelioration and/or treatment of human bone diseases and dysfunctions. It has been demonstrated that biosilica causes in vitro, a differential effect on the expression of the genes OPG (osteoprotegerin) and RANKL (ligand of the receptor activator of NF-κB), as well as induces the expression of the key mediator BMP-2 (bone morphogenetic protein 2); they are promising candidates for treatment of osteoporosis.

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57.1 The Sponges: The Earliest Ancestor of the Metazoa

Sponges (phylum Porifera) are aquatic, sessile, and multicellular organisms with a Bauplan that appears simple at a first glance and lacks similarities to any other living organism. Therefore, during early studies, it was difficult to determine morphological characters that would conclusively allow us to group sponges into either one of two kingdoms of multicellular life: Metazoa or Plantae. After comprehensive isolation, cloning, and phylogenetic analyses of many poriferan genes by our group, it became obvious that the phylum Porifera comprises of three classes – Hexactinellida, Demospongiae, and Calcarea – and forms the basis of the metazoan kingdom [57.1]. A few years later, it could be clarified that Hexactinellida (glass sponges), Demospongiae (silicate/sponging sponges), and Calcarea (calcareous sponges) are monophyletic and closely related to the common ancestor of all metazoans, the Urmetazoa [57.2].

Sponges appeared during the Neoproterozoic, the geologic period from 1000 to 542 Ma; [57.3]. Two major reasons contributed to the evolutionary success of the poriferan taxon: (a) symbiosis with microorganisms and (b) presence of hard skeletons [57.3]. The maintenance of symbiotic relationships with unicellular organisms allowed sponges to survive adverse environmental conditions because the autotrophic microbial symbionts represented rich organic carbon sources for them. On the other hand, the development of skeletal elements facilitated an increase in size, a common metazoan phyletic trend also known as Cope's rule [57.4]. Since changes in the body size affect almost every aspect of life, two strategies have been developed in animals to circumvent any constraints [57.5], first by acquisition of a hydrostatic skeleton, as it is known from the *worm*-like phyla of the Ediacaran and pre-Ediacaran Eon [57.6], or second

by acquisition of rigid solid skeletal elements [57.7, 8], as they were realized in Neoproterozoic siliceous sponges [57.3].

Skeletal elements (spicules) of siliceous sponges, Hexactinellida and Demospongiae, are composed of amorphous opal ($SiO_2 \cdot nH_2O$). They already existed in pre-Ediacaran sponges and represent a general and basic morphological character until today [57.9]. It is easily conceivable that why the animals integrated silicon instead of calcium as the fundamental element for their inorganic skeleton, since the Neoproterozoic oceans were rich in silicic acid and continuously replenished by products of the silicate weathering-carbonate precipitation cycle [57.10]. Sponges are provided with a sophisticated aquiferous canal system and hence, their body can be stabilized to some extent by internal hydrostatic pressure, even though a vasculomuscular system does not exist. The presence of a siliceous skeleton, however, allowed them to obtain sizes larger than 20 mm, even up to 2.5 m [57.11].

57.2 Silicatein-Based Siliceous Spicule Formation

The biogenic basis of spicule formation and the turnover of silica in spicules have already been depicted by *Duncan* [57.12]. He formulated *The spicule which has lived, has to decay, and may live again in another form*. However, it took up until 1999 until *Cha* et al. discovered that the main constituent of the proteinaceous filament within the axial canal of spicules is an enzyme [57.13], subsequently termed silicatein, which might be involved in biosilica formation. Soon after having identified this anabolic enzyme, also the corresponding catabolic enzyme (silicase) was discovered [57.14]. The identification of a biosilica degrading enzyme supported the view that the siliceous components in spicules are under metabolic turnover [57.15]. Studies on the metabolism of spicules on the cellular level became possible after the introduction of a poriferan cell culture system, primmorphs [57.16, 17]. Already the first contribution on that topic resolved that the spicule formation starts intracellularly in *J* sclerocytes, by the formation of an initial organic axial filament, around which the inorganic silica mantel is deposited. This result had later been corroborated by the application of more advanced immunochemical and electron microscopy techniques [57.18].

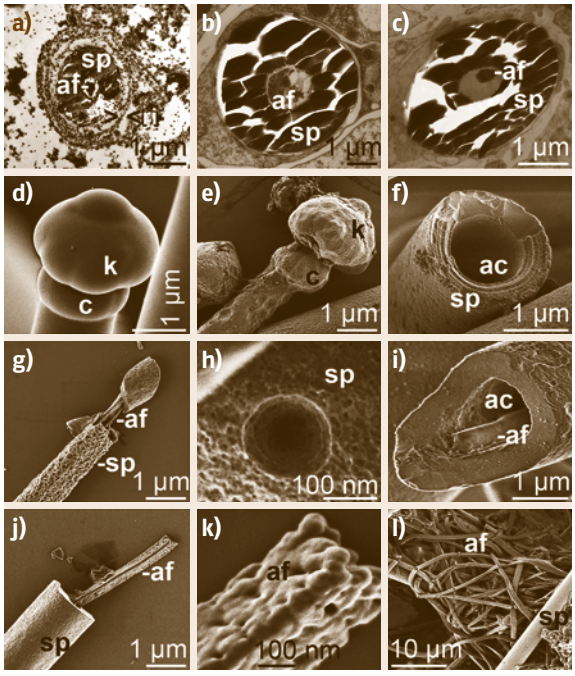

Fig. 57.1 Localization of the silicatein, packed within the axial filament, in the spicules from *S. domuncula*. **(a)** After raising of antibodies to silicatein it became possible to demonstrate, by electron immunogold labeling technique that concentric rings (ri) surround the growing spicules (sp) and react also with the axial filament (af). This approach also showed that the silicatein molecules are attached to the string- and net-like structures. **(b)** Immature spicule harboring a space-filling axial filament (af) within the axial canal. **(c)** In a more mature spicule (sp) the axial filament (af) had been shrunk. **(d)** A tylostyle head, the knob (k), that is based on a collar (c) at one end of the monaxonal pin. High-resolution scanning electron microscopy (HR-SEM): **(e)** after exposure of the tylostyle to HF vapor, the silica is progressively dissolved,exposing granular structures. **(f)** A broken *S. domuncula* spicule (sp), a tylostyle, displaying the axial canal (ac). **(j)** Progressive dissolution of the siliceous shell of the spicules (sp) releasing the axial filament (af). **(h)** At a higher magnification the plait cable-like organization of the axial filament (af) becomes obvious. **(i)** Bundles of axial filaments (af) released after HF treatment

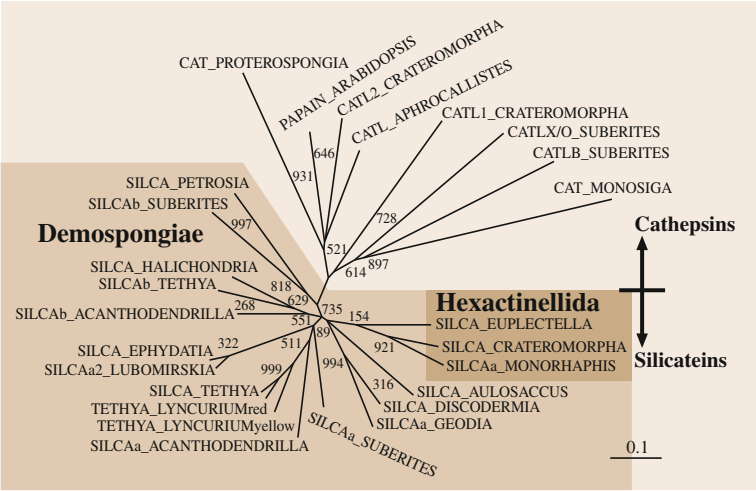

Fig. 57.2 Phylogenetic analysis of silicatein within the cathepsin family. The deduced proteins were aligned and the phylogenetic tree was constructed. The hitherto-known four hexactinellid sequences were included; silicatein from *Euplectella aspergillum* (SILCA_EUPLECTELLA; FR748156), *Crateromorpha meyeri* (SILCA_CRATEROMORPHA; AM920776), *Monorhaphis chuni* (SILCAa_MONORHAPHIS; FN394978) and the silicatein-like protein *Aulosaccus* sp. (SILCA_AULOSACCUS; ACU86976.1). The bulk of silicatein sequences have been identified in demosponges. Firstly, the silicateins-α sequences from *Suberites domuncula* (SILCAa_SUBERITES; CAC03737.1), *Tethya aurantium* (SILCAa_TETHYA; AAC23951.1), *Tethya lycurium* red variation (TETHYA_LYCURIUMred; FR748154) and *T. lycurium* yellow variation (TETHYA_LYCURIUMyellow; FR748155), *Discodermia japonica* (SILCA_DISCODERMiA; FR748157), *Geodia cydonium* (SILCAa_GEODIA; CAM57981.1) and *Acanthodendrilla* sp. Vietnam (SILCAa_ACANTHODENDRILLA; ACH92669.1), as well as from *Lubomirskia baicalensis* (SILCAa2_LUBOMIRSKIA; AJ968945) and *Ephydatia fluviatilis* (SILCA_EPHYDATIA; BAE54434.1). Secondly, the silicatein-β sequences from *S. domuncula* (SILCAb_SUBERITES; CAH04635.1), *T. aurantium* (SILCAb_TETHYA; AF098670_1) and *Acanthodendrilla* sp. Vietnam (SILCAb_ACANTHODENDRILLA; FJ013043.1). Thirdly, the silicateins that had been identified in marine sponges from which only one isoform had been obtained; silicatein from *Petrosia ficiformis* (SILCA_PETROSIA; AAO23671.1) and *Halichondria okadai* (SILCA_HALICHONDRIA; BAB86343.1). As reflected in the rooted tree, these silicateins derived from the cathepsins among which in this tree the following sequences have been included; cathepsin-like protein 2 *C. meyeri* (CATL2_CRATEROMORPHA; CAP17585.1), cathepsin-like protein 1 *C. meyeri* (CATL1_CRATEROMORPHA; CAP17584.1), mRNA for cathepsin L (catl gene) *Aphrocallistes vastus* (CATL_APHROCALLISTES; AJ968951); cathepsin B *S. domuncula* (CATLB_SUBERITES; CAH04630.1), cathepsin X/O *S. domuncula* (CATLX/O_SUBERITES; CAH04633.1). The resulting tree was rooted with the sequence from the papain-like cysteine peptidase XBCP3 *Arabidopsis thaliana* (PAPAIN_ARABIDOPSIS; AF388175_1). In addition, the cathepsins from choanoflagellates had been included to show that they derived, according to this tree, from the sponge cathepsins; the cysteine protease from *Proterospongia* sp. (PRL00000040) and the cathepsin from *Monosiga ovata* MNL00000103

57.2.1 Silicatein

Thorough dissolution of the inorganic silica from spicules (Fig. 57.1d; *Suberites domuncula* (demosponge) spicules) with hydrofluoric acid (HF) vapor revealed in addition to the presence of the axial filament a spicule-enfolding proteinaceous coat (Fig. 57.1e). The siliceous shell of the spicules can be dissolved by exposure to HF. During this process the polymerized silicic acid undergoes dissolution into monomeric silicic acid under the release of the organic component(s) of the spicules. Focusing on the tylostyles from the demosponge *S. domuncula*, both the knobs of the spicules (Fig. 57.1d,e) and the axial rod (Fig. 57.1f) undergo dissolution under release of the organic axial filament, located within the axial canal and additional proteins that are obviously initially localized within the silica coat of the spicule (Fig. 57.1d–f). While in the imma-

ture spicules the axial filament fills the complete axial canal, it shrinks at later stages (Fig. 57.1a–c). A pile of isolated axial filaments is shown in Fig. 57.1i.

After the discovery of the cathepsin L (cysteine protease)-related silicatein [57.13, 19] in spicules of the demosponge *Tethya aurantium*, several related genes were elucidated in both marine and freshwater demosponges [57.18]. The corresponding deduced polypeptides comprise of about 325 amino acids (aa) with a molecular weight of ca. 35 kDa. During maturation, this primary translation product (proenzyme) is processed by cleaving off a signal peptide (aa_1 to aa_{17}; *S. domuncula* (demosponge)silicatein-α) and the adjacent propeptide (aa_{18} to aa_{112}), resulting in the mature enzyme that has a size of 24–25 kDa. Similar to cathepsins, the catalytic center of silicatein contains His and Asn. However, the Cys of the cathepsins' catalytic triad is exchanged by Ser in silicatein. In addition to about 10 putative protein kinase phosphorylation sites, silicateins display a cluster of serine residues that is found close to the central aa residue of the catalytic triad, but is otherwise missing in cathepsins.

Subsequent phylogenetic analyses revealed that silicateins form a separate branch from cathepsins [57.18]; Fig. 57.2. The difficult accessibility of hexactinellids, which live primarily in depths of more than 300 m, generally results in a very poor sampling. Accordingly, only recently the first hexactinellid silicatein (*Crateromorpha meyeri*) could be identified and characterized [57.20]. This molecule shares high similarity to the demosponge sequences (expect value of 8×10^{-58}) and contains the same catalytic triad amino acids. However, striking in the *C. meyeri* sequence is a second Ser-rich cluster, which is located between the second and the third aa of the catalytic triad. Strong binding of the protein to the spicule silica surface has been attributed to this cluster [57.21]. The post-translational modifications of silicatein have been found to be essential for the enzyme activity with respect to (a) association with other structural and functional molecules within the tissue and (b) self-association/self-assembly. For those studies, silicatein had been isolated from spicules in the absence of HF, but in the presence of a glycerol-based buffer. Following this rationale, it could be demonstrated that silicatein exists not only in the axial canal but also in the extraspicular and extracellular space [57.22, 23]. The enzymatic reaction mechanism of silicatein had been proposed by *Cha* et al. [57.13]; the detailed properties of the reaction kinetics have been specified experimentally [57.24].

57.3 Spiculogenesis

With the primmorph cell culture system, the first solid evidence was provided that the synthesis of the spicules starts intracellularly [57.22]. The process of spicule formation can be divided into an initial intracellular phase and a subsequent extracellular shaping phase. Silicic acid, the substrate for silicatein, is actively taken up by cells (sclerocytes) via the $Na^+/HCO_3^-[Si(OH)_4]$ cotransporter. In parallel, silicatein is synthesized, processed, and stored with silicic acid in special organelles of the sclerocytes, the silicasomes. Within these organelles, the axial filaments are formed, around which silica is deposited enzymatically.

After formation of a first biosilica layer, immature spicules are released into the extracellular space, where they grow in length (axial/longitudinal direction) and in diameter (radial direction), by appositional layering of silica lamellae [57.23]. During growth in the axial direction, biosilica is formed through the enzymatic function of the axial filament silicateins. The growth of the spicule is driven by the elongating biosilica core cylinder, which is synthesized by the 23 kDa processed form of silicatein. The radial thickening of the spicules, their appositional growth (radial direction), occurs by deposition of silica on the surface of the growing spicule, and is mediated by the extraspicular 34.7 kDa immature silicatein. There is no evidence at all that, in demosponges, either in the axial filament or on the surface of the spicules, there is collagen that is causatively involved in biosilica formation.

57.3.1 Radial Growth

The radial, appositional thickening of the spicules and then the axial elongation process [57.23] were first understood (Fig. 57.3). In the extracellular space, the silicatein molecules are organized into larger entities, by concentric rings/cylinders around the spicule surface. These structures become stabilized by the protein galectin and Ca^{2+}; within these cylinders, the silicatein-mediated biosilica formation occurs. In hexactinellid spicules, these appositionally layered silica lamellae remain separated, and can reach 1000 in number [57.11].

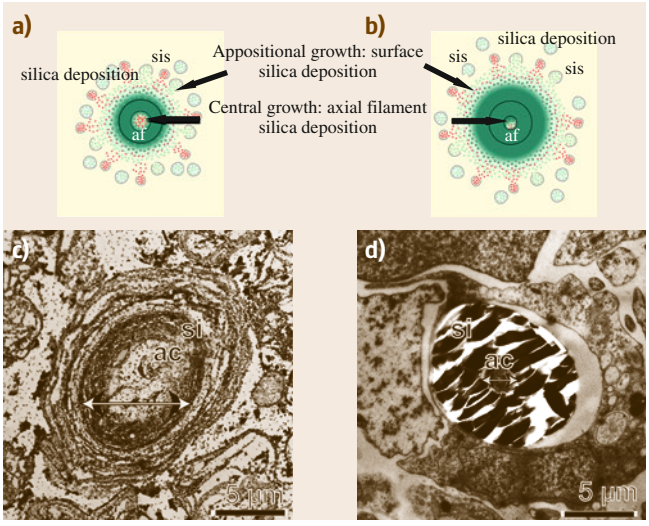

Fig. 57.3a–d The growth of the spicules occurs in two directions. This scheme shows the process of radial thickening of the spicule. **(a,b)** In the radial direction the spicules increase in thickness by appositional layering of the growing spicule (scheme). Both within the axial canal (ac), where the axial filament (formed of silicatein) is located (central growth), and on the surface of the spicules (appositional growth), the silica shell (si) increases in dimension through enzymatic synthesis of biosilica via silicatein. In the extra-spicular space, both silicatein and silica is released from silicasomes (sis); those silicasomes are set free from the surrounding cells. **(c,d)** Shrinkage of biosilica during biosilicification. **(c)** Growing siliceous spicules comprise a telescoped cylindrical arrangement. Approximately seven cylinders, formed from galectin, surround the axial canal (ac). Silicification, reflected by the densely packed grana, proceeds progressively from the central cylinders towards the surface of the spicule. Transmission electron microscopy (TEM): immunogold labeling with antisilicatein antibodies. **(d)** Section through a mature spicule, showing the axial canal in the center. The shrinking both of the spicule during formation/maturation, and of the axial canal is obvious

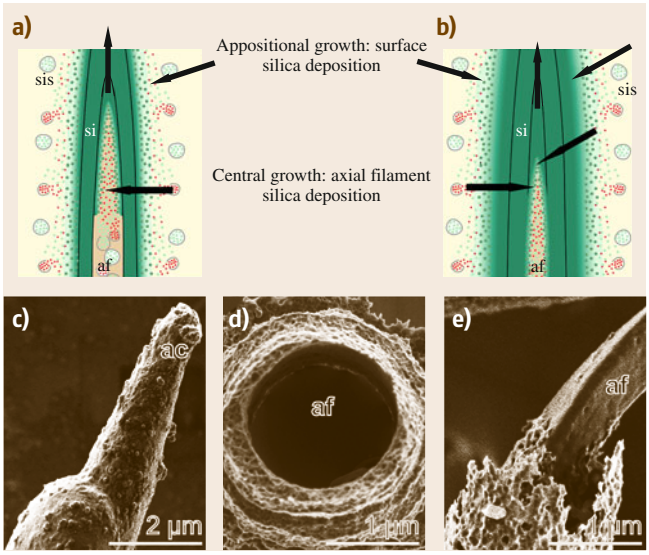

Fig. 57.4a–d Longitudinal growth of the spicules. **(a,b)** After the release of the immature spicule into the extracellular space via evagination of a cell protrusion the growing spicule is pushed away from the cell (sclerocyte) that initially has formed the immature spicule (scheme). This process is driven by an elongation of the cell protrusion and in turn an elongation of the axial filament (af) within the axial canal. Again it is highlighted that silica deposition occurs within the axial canal and onto the surface of the spicules. Around the spicules silicasomes (sis) exist that release silicatein and ortho-silicate to allow biosilica formation to occur. **(c)** A protruding axial canal (ac) is shown that harbors an axial filament. **(d)** Silica shell around the axial canal/axial filament (af). **(e)** A liberated axial filament (af) projecting from a spicule

In contrast, in demosponges, the individual lamellae fuse/bio-sinter together, and form a *solid* siliceous shell that surrounds the centrally located axial filament.

57.3.2 Longitudinal Growth

We provided, for the first time, experimental evidence for the involvement of cellular processes in the control of the axial growth of spicules (Fig. 57.4). Using the primmorph system, we demonstrated that these cell processes originate from evaginations of the spicule-forming cells (sclerocytes) into the growing and elongating axial canal. The experiments showed that, around a cell extension that protrudes from a sclerocyte into the axial canal of a given spicule, silicatein molecules are released into the extracellular space of the axial canal (the space between the cell membrane and the inner surface of the siliceous mantel) to catalyze biosilica deposition from the inner surface. This causes the axial canal to narrow from > 1 to $< 0.5\,\mu$m. Intracellularly, both the enzyme and its substrate (silica precursor) are stored in vesicles that have been termed as silicasomes [57.25]. These vesicles are released into the axial canal to allow the enzymatic polycondensation reaction.

Therefore, spicule formation requires two mechanistically independent biosilica condensation/deposition processes: first, biosilica formation, which gives rise to the inner core of the growing spicule, mediated by silicatein present in the axial filament; and second, lamellar, appositional growth (thickening), which proceeds on the elongating siliceous core via the formation of organic cylinders, enabling the layer-by-layer deposition of individual silica lamellae, which bio-sinter together in demosponges or remain separated, to a large extent, in hexactinellids.

57.4 Bio-Silica: The Enzymatically Formed Scaffold of Siliceous Sponge Spicules

Initially two different mechanisms had been proposed for silicatein reaction that might describe the condensation reaction of ortho-silicic acid or silicon alkoxide (TEOS) substrates [57.13, 26]. These models explain the hydrolytic activity of silicatein toward its synthetic substrate, TEOS, or the initial formation of disilicic acid from ortho-silicic acid as the natural substrate, but none of them is able to describe the biocatalytic formation of the polymeric silica from its monomeric precursors(s).

Recently, we proposed a new model which includes the two necessary assumptions for silicatein reaction: implication of the crucial Ser moiety and using ortho-silicic acid as substrate [57.27]. Using this model, it is possible to explain both the initial condensation reaction, the formation of disilicic acid, and the subsequent oligomerization process. As in the previous models, the initial catalytic step involves a nucleophilic attack (S_N2 type) of the (electronegative) oxygen atom from the hydroxy group of the active site Ser to the (electropositive) silicon atom of a silicic acid molecule. It is proposed that this reaction is facilitated by hydrogen bridge formation between the Ser hydroxy and the His imidazole within the catalytic center, a reaction that increases the nucleophilicity of the Ser hydroxy group. Then, a proton transfer from the His imidazole nitrogen (Ser · His hydrogen bond) to one of the silicon OH ligands of the pentavalent intermediate (in the transition stage) occurs, resulting in the release of a water molecule. In the next step, the silicic acid molecule which remains covalently bound through an ester-like linkage to the enzyme Ser moiety undergoes a nucleophilic attack on the silicon atom of a second ortho-silicic acid molecule, pulling it to the substrate pocket of the enzyme. Subsequently, the nucleophilicity of the attacking oxygen atom of the first (enzyme-bound) silicic acid molecule is assumed to be increased via formation of a hydrogen bond with the imidazole nitrogen of His, in analogy to the reaction proposed [57.28]. The loss of a water molecule generates a disilicic acid molecule, which remains bound to the enzyme. Now, a rotation of the ester bond allows the interaction of a second OH ligand of the enzyme-bound silicic acid unit with the imidazole nitrogen of the His in the catalytic center, giving rise to a further growth of the disilicic acid by a third orthosilicic acid molecule under a release of a further water molecule. Adopting previous data [57.29], our model proposes a final cyclization step. The products released from the enzyme by hydrolytic cleavage (cyclic trisilicic acid and higher oligomers) are much more reactive than the silicic acid monomers and promote further condensation reaction.

Enzyme kinetic studies were performed with recombinant silicatein-α and the model substrate bis(*p*-aminophenoxy)-dimethylsilane (BAPD) which contains two silicic ester-like and two silane bonds [57.24].

In this assay, the temperature optimum was in the range of 20–25 °C, revealing a value for the temperature coefficient (Q_{10}) that decreased by 2.5-fold above 25 °C (25 °C/35 °C) and increased by 2.9-fold below 25 °C (15 °C/25 °C). The Michaelis constant (K_m) was determined to be 22.7 μM. In comparison, the K' value for human recombinant cathepsin L, the closely related enzyme to silicatein, was 1.1 μM by using the substrate benzyloxycarbonyl-Phe-Arg-4-methylcoumarin-7-amide [57.30]. The turnover value (molecules of substrate converted per enzyme molecule per second) for silicatein in the silica esterase assay was 5.2, close to the value found for the human cathepsin L [57.31]. Very recently, the activity of the recombinant silicatein-α was determined in in vitro, assays and compared with the silica-forming activity during spicule formation in vivo [57.32]. The silica-forming activity in vivo, was assessed by calculating the amount of biosilica formed from the growth rate of the spicules of the demosponge *Ephydatia fluviatilis*. The growth rate of those spicules with an average size of 200–350 μm and a thickness of 15 μm was determined to be 1–10 μm h^{-1} in length [57.33]. This implies that the total weight of one spicule is approximately 88.3×10^{-9} g, which is equivalent to about 4.4×10^{13} molecules of biosilica being formed during 1 h in one spicule. Adopting a protein content of about 5% (mainly silicatein-α) in a siliceous spicule [57.21], it follows that 2.2×10^{-10} g of protein catalyze the synthesis of 4.4×10^{-9} g of biosilica per hour. Since the molecular mass of mature silicatein is 25 kDa, it follows that 8.8×10^{-15} mol of silicatein (5.3×10^{9} molecules) synthesize 4.4×10^{-9} g (equivalent to 7.33×10^{-11} mol (4.4×10^{13} molecules)) of biosilica per hour. In turn, and referring to native silicatein, the turnover number of silicate per silicatein on a molar basis is 8×10^{3} molecules of converted substrate per silicatein molecule per hour in spicules. With respect to recombinant silicatein-α, a value of 18.7×10^{3} molecules per molecule of silicatein-α per hour (substrate BAPD) had been determined for the esterase activity [57.24] and 5.4×10^{3} molecules per molecule of silicatein-α per hour using ortho-silicic acid as a substrate [57.32]. This value can even increase by the addition of silintaphin-1 in a 4 : 1 molar stoichiometric ratio (silicatein-α : silintaphin-1) by > 5-fold. Based on these calculations, it is most likely that the silica polymerization activity during spicule formation is mediated enzymatically by silicatein only [57.34].

57.5 Self-Healing Property of Silicatein Embedded in Spicules

The central canal of the spicules contains the proteinaceous axial filament (Fig. 57.5b) that is laterally assembled by about 10 nanofibrils [57.35]. By completion of the formation of the mature spicules, the axial filament remains embedded in this central structure. After treatment of the spicules with HF, the proteinaceous filaments can be recovered [57.35] and stained with Coomassie brilliant blue. With progressing time, the silica is dissolved, leaving behind only the organic axial filament. It is surprising that the proteinaceous axial filament remains preserved in the spicules, even in spicules of an age of about 2 Ma, as demonstrated for the Baikalian sponge *Lubomirskia baicalensis* [57.36]. The silicatein in these axial filaments remains functionally active as silica-binding protein [57.13], or as enzyme [57.32] after dissolution of the biosilica shell. This functional activity of the axial filament can be demonstrated by incubating them with prehydrolyzed TEOS (orthosilicate). If the incubation is performed in the presence of 6 μg/mL of axial filament protein in a 50 mM Tris/HCl buffer (pH 7.4; 150 mM NaCl), containing 200 μM orthosilicate bulky depositions of biosilica onto the axial filament structures can be visualized. Those structures cover almost completely the axial filaments after an incubation period of 180 min. Axial filaments do not exist not only as rods, like those isolated from the tylostyles from *S. domuncula* but also appear as radial tufts, as in asters from *G. cydonium* [57.37].

After indenting and partial cracking the spicule, the silicatein molecules become exposed to the environment (Fig. 57.5b) and the silica mantel crumbles. In case if orthosilicate is present in the aqueous environment at sufficiently high concentrations, the silicatein can associate with those monomers and can form biosilica. The concentrations of silica in the present-day oceans, with about 5–20 μM [57.38], might be sufficient to realize an extensive biosilica deposition as the K_m value for silicatein was determined to be 22.7 μM [57.24]. In turn, even spicules directly exposed to the aqueous environment have the capacity to self-heal (partially) broken spicules. If existing as skeletal elements in the body, the high concentrations of silica in the spicule-synthesizing cells, the sclerocytes,

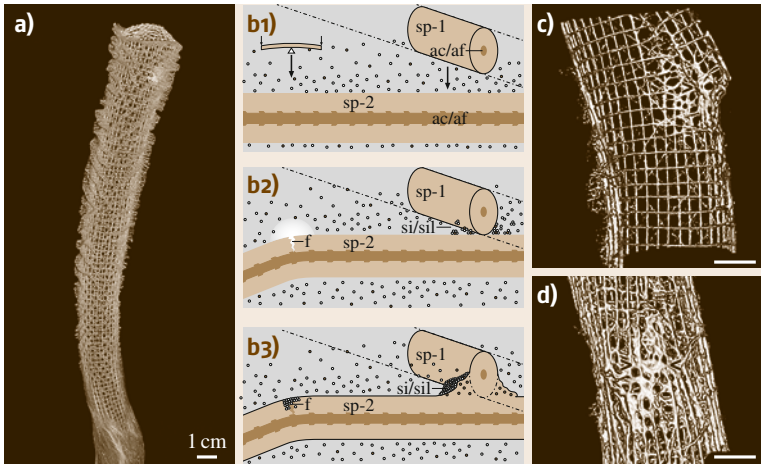

Fig. 57.5a–d Silicatein-mediated self-healing process in sponge spicules. **(a)** *Euplectella aspergillum* skeleton, formed of spicules that are fused to a biosilica continuum. The spicules are regularly organized in a modular segments. **(b)** Schematic outline of the silicatein-driven self-healing process in sponge spicules. **(b1–b3)** In sponge tissue (primmorphs) that has been treated with Mn-sulfate, biosilica is un-physiologically deposited outside of two crossing spicules, spicule sp-1 and sp-2. The fusion of the two spicules occurs by biosilica deposition; biosilica is enzymatically formed from silicatein (sil) and its substrate ortho-silicate (si). This fusion process is catalyzed by silicatein that is released by cells into the extracellular space; there it becomes associated with the surface of the spicules. In the second self-healing process, silicatein oozes out from the axial canal (ac), which harbors the axial filament (af). This axial filament is composed of silicatein. During this process of self-healing, silicatein is oozing out into the fissure (f) that happened in the silica mantel of the spicule. In the presence of ortho-silicate this rupture is resealed again. **(c,d)** Very rarely the biosilica formed spicular skeleton had been broken and reunified (><)

are sufficient to provide silicatein with the appropriate level of silica for the enzymatic synthesis of biosilica.

Repair, self-healing, processes taken place within spicules can be frequently recognized within hexactinellid spicules in their lamellar regions, for example, within the giant basal spicules of *M. chuni* or in the skeleton of *Euplectella aspergillum* (Fig. 57.5a–c). Fractures that have been introduced into the spicules, followed by a stay in a dry environment for a short time, remain nonsealed. However, fractures within the lamellae that occur in spicules during the growth phase fuse almost completely together (Fig. 57.5c,d).

57.6 Bio-Silica: The Osteogenic Bioinorganic Polymer

The formation of the skeletal system from the earliest metazoans, the sponges (phylum Porifera) [57.36] to the crown taxa, the mammalians [57.39], and the insects [57.40], is dominated by a tuned communication between cells controlling anabolic processes and cells executing catabolic reactions. Basically, two kinds of inorganic scaffold materials had been applied in the metazoan kingdom to form skeletons: calcium (calcium-based skeletal systems) and – only found in siliceous sponges – silica (silica-based skeletons).

A breakthrough in the understanding of the siliceous spicule formation of the demosponges and the hexactinellid sponges came from the discovery that the axial filaments of the spicules, the skeletal elements of these sponges (demosponges [57.13]; hexactinellids [57.20]) contain an enzymatically active protein which synthesizes polymeric silicate, the biosilica. This enzyme, silicatein, has been found to catalyze/polycondensate biosilica during the axial and radial growth of the spicules. In contrast to plant phytoliths and diatom frustules, where biosilica is deposited from a supersaturated solution onto organic templates, the siliceous spicules of sponges are formed in a hypo-saturated intraorganism environment following an enzymatic mechanism by lowering the activation energy of the polycondensation reaction. In in vitro, systems,

orthosilicic acid or tetraethyl orthosilicate (TEOS) has been used as substrate/biosilica precursor for the enzymatic reaction. During the latter process, ethanol is released [57.13]. The silicatein-mediated formation of silica proceeds at silica substrate concentrations of around 200 μM, far below the concentrations which are required for the chemical condensation of 1 mM or higher at neutral pH [57.34, 41, 42].

Silica is an essential nutrient both for the natural ecosystem in general [57.43] and for humans and other vertebrates in particular [57.44, 45]. Importantly, silicon deprivation results in severe skeletal malformations [57.46]. The experimental studies showed that in avian connective tissue, the highest silicon concentrations are found, in contrast to heart or muscle tissue, where the silicon concentrations are much lower. Moreover, a spatial correlation could be established between the areas of bone formation within animal tissue and the accumulation of silicon. Based on these data, it has been concluded that a burst of silicon accumulation occurs around the osteoid and osteoid–bone interfaces, implying that this inorganic component is essential for bone formation. Consequently, we studied the effect of biosilica on the activity of osteoblasts and osteoblasts in vitro. SaOS-2 cells were grown in the activation cocktail on a support, coated either with hydroxyapatite (HA) or with biosilica. The cell layers that were grown on HA did not form HA crystals on their surfaces, while the cells that had been cultivated for 5 days on biosilica well formed HA crystals that are often fusing to clusters [57.47]. This observation which had been supported by alizarin red S staining assays underscores that biosilica displays an inductive effect on SaOS-2 [57.47, 48].

The tuned interaction of osteoblasts and osteoclasts during bone formation is well established. Furthermore, the decisive role of the osteoclastogenic ligand RANKL (ligand of the receptor activator of NF-κB) had also been discussed. This ligand is processed by metalloproteases to a soluble form and interacts with RANK (receptor activator of NF-κB) and finally induces osteoclastogenesis [57.49]. This molecular cross-talk that coordinates osteoclastogenesis is controlled by the third component, OPG (osteoprotegerin); this osteotropic effector acts as a soluble bone protector [57.50]. In turn, the molecular triad, OPG/RANK/RANKL (NF-κB ligand) [57.51], is not only crucially controlling osteoclast differentiation, but is also involved in cell differentiation pathways of the immune and vascular systems [57.49]. Vice versa, recent results provided experimental evidence demonstrating that osteoclasts contribute with their cytokines to the fine-tuning of the osteoclast/osteoblast balanced functions [57.52].

Previous experimental evidence showed that silicate/silicon is an essential trace element in vertebrate nutrition [57.46, 53, 54]. In continuation, we showed that biosilica induces HA crystallite formation in SaOS-2 cells [57.55]. More recently, we demonstrated that in SaOS-2 cells, after exposure to biosilica, a differential gene expression is seen that strongly up-regulates the steady-state level of OPG transcripts and leaves the level of RANKL transcripts almost unchanged. Based on the observed increase of the OPG/RANKL ratio, it was intriguing to suggest that silica/silicate has a favorable biomedical potential for the treatment and/or prophylaxis of osteoporotic disorders [57.48]; Fig. 57.6. Since silica was found to elicit in vitro, an increased (^{3}H)dThd incorporation into DNA and a likewise increased HA formation, an osteogenic potential of silica had been deduced [57.47]. Finally we discovered that SaOS-2 cells, after exposure to biosilica, release a soluble factor, the *osteoblasts-derived inhibitory factor*, which causes an inhibition of RAW 264.7 cell growth [57.56].

57.7 Future Design of Novel Bioinspired, Silica-Based Materials

The longest-lived animals, existing at present on Earth, are the species of the hexactinellid *M. chuni*, which reach an age of 11 000 years [57.57]. During this period, the biosilica-based giant basal spicule persisted as the main skeletal element in the bodies of this sponge. This observation reflects one of the properties of sponge biosilica to be durable and simultaneously flexible and resistant against breakage. Even more, since the major organic component of the biosilica nano-hybrid particles is silicatein, it can be assumed that the biosilica material comprises a self-repair property as well [57.58]. The biosilica-based sponge spicules, as intelligent structures, do not always function in the same way; they have the adaptive ability to adjust their degree of polycondensation to reach the preferred physical and chemical properties. Through the genetic and biological means provided by the silicatein-based system, the polycondensation/gelation process can be controlled and in turn the level of polymerization, coarsening, and phase trans-

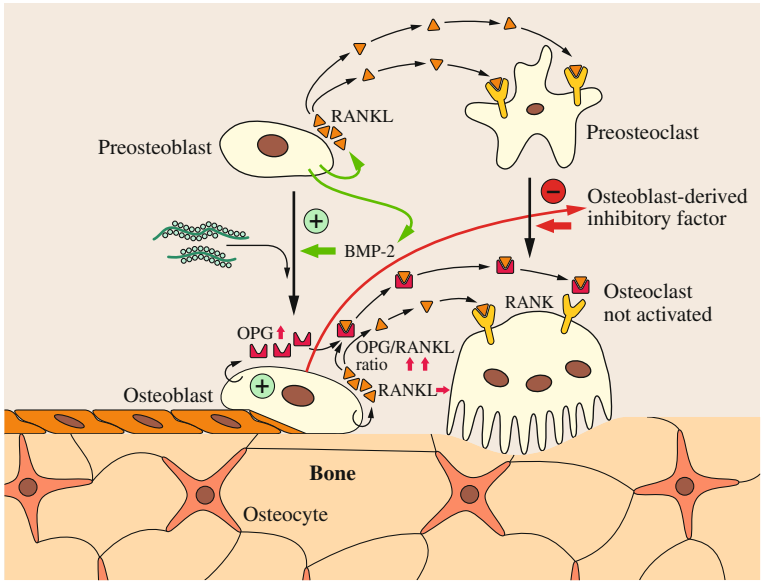

Fig. 57.6 Proposed effects of biosilica on osteoblasts, osteoclasts, and their progenitor cells; schematic representation. Biosilica causes an increased expression of OPG in osteoblasts. In addition, the differentiation of osteoblasts is induced and accelerated by BMP-2 (bone morphogenetic protein 2). It is assumed that in turn the osteoblasts acquire the potential to differentiate to osteocytes and to lining cells. Furthermore, OPG counteracts various effects of RANKL, a cytokine that induces preosteoclast maturation and osteoclast activation. Finally, it could be identified that the osteoblasts release a factor, the osteoblasts-derived inhibitory factor that strongly inhibits the proliferation of osteoclasts; the nature of this factor is not yet known

formation can be adjusted. Since it is common in biological systems that reactions are reversible, a feedback control might also exist in the silicatein-based biosilica reaction, allowing a proof-reading process of the quality of the product (here the polymeric silica) formed. This property, a characteristic feature of all living systems, provides them with high flexibility and robustness. It should be mentioned that also the silicatein reaction is reversible [57.24]; the action of this usually acting enzyme, the silicase [57.14]. Dynamic feedback control mechanisms are crucial elements adjusting the dynamic homeostasis of the internal environment of living systems [57.59] and are very difficult to realize and to adapt for artificial bionic processes.

The work on biosilica in sponges already provides us with strategies to fabricate nano-hybrid particle-based glass materials which are moldable and might be used as potential biomaterials accelerating bone-repair processes [57.60]. In the near future, it is expected that intelligent materials based on biosilica can be produced in cell culture systems, by controlling the gene expression of the key molecules, silicatein and silintaphins. A first success has already been achieved by the demonstration that the process of aging and syneresis in the sponge cell system, the primmorphs, can be modulated by inhibition of the function of the aquaporin membrane pores [57.61, 62]. Through this approach, a fusion of individual biosilica spicules can be induced that had not been seen in nature before.

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58. Biomineralization in Marine Organisms

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This chapter describes biominerals and the marine organisms that produce them. The proteins involved in biomineralization, as well as functions of the biomineralized structures, are treated. Current and future applications of bioinspired material synthesis in engineering and medicine highlight the enormous potential of biomineralization in marine organisms and the status, challenges, and prospects regarding successful marine biotechnology.

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58.1 Overview

Marine biotechnology has huge potential across a broad spectrum of applications, ranging from biomedicine to the environment. However, marine biotechnology has not yet matured into an economically significant field [58.1]:

> *Fundamental knowledge is lacking in areas that are pivotal to the commercialization of biomedical products and to the commercial application of biotechnology to solve marine environmental problems, such as pollution, ecosystem disease, and harmful algal blooms.*

One of the recommendations of the 2002 report of the Ocean Studies Board and the Board on Life Sciences is that better tools should be developed for the use of marine biotechnology to help solve environmental problems such as biofouling, pollution, ecosystem degradation, and hazards to human health.

This chapter gives a functional approach to biomineralization in marine organisms. It presents the materials that are biomineralized (which include simple precipitated minerals, biologically produced perfect crystals, and composites of minerals and an organic matrix with interesting new properties) and the proteins that are important in biomineralization, gives an overview of some of the thousands of marine organisms that produce such materials, including information on the respective biomineralized struc-

tures and their functions, and finally presents current and possible future applications of bioinspired material synthesis in engineering (including mining) and medicine.

One of the amazing properties of biomineralization in organisms is that material, structure, and function are strongly correlated. Biominerals are highly controlled in structure, composition, shape, and organization, and can yield new, more benign approaches in engineering. The complex shapes of biominerals cannot be explained with simple mechanistic models of crystal growth [58.2].

Biotechnologists, material scientists, biologists, geologists, engineers, and medical doctors have long been fascinated by mineral structures in organisms. Now, with highly developed measurement devices at our disposal, we can investigate and understand biological materials, structures, and processes, and increasingly produce related bioinspired analogs [58.3].

Minerals are usually stiff, brittle, and cheap energy wise. Organic materials are soft and pliable. The synergistic combination of both yields biominerals with amazing functionalities, with a lightweight organic frame (which saves metabolic energy), filled with *cheap* inorganic material (e.g., calcium carbonate), yielding inorganic-organic hybrids (biocomposites) with well-defined mechanical properties [58.4].

Biominerals have functional structures and shapes, e.g., curved teeth and light baskets. The organic matrix acts as a meditator of mineralization and as crystal modifier. Characteristics of materials produced by controlled biomineralization are uniform particle sizes, well-defined structures and compositions, high levels of spatial organization, complex morphologies, controlled aggregation and texture, preferential crystallographic orientation, and higher-order assembly into hierarchical structures.

In contrast to most other biological transformations, biomineralization leaves far-reaching effects on the biosphere and lithosphere, including traces such as bones, shells, and fossils, but also mountain ranges and cliffs [58.5]. Biomineralization has implications on the global scale, via the Earth sciences. It is important in the global cycling of elements, in sedimentology, in fossilization (paleontology and taxonomy), in marine chemistry, and in geochemistry [58.4].

In the course of biomineralization, mineral products (biominerals) are created in organisms. Biomineralization has been around since the first Prokaryota appeared in the Archaean, the geological aeon from about 4 to 2.5 billion years ago. Biomineralization is of high interest to biologists and also to engineers, material scientists, and tissue engineers.

Biomineralization is characterized by interesting chemical reactions involving proteins, the creation of perfect crystals, the control of crystal growth and inhibition depending on the crystallographic axis, as well as the production of composite materials with properties that are of high value to engineering. Many biological fluids are supersaturated with respect to certain inorganic minerals, but crystals do not form spontaneously. An example of such crystal growth inhibition is saliva; it is supersaturated with respect to hydroxyapatite formation, yet teeth do not grow continuously. The overgrowth is prevented by phosphoprotein macromolecules that bind to enamel crystals.

Solubility controls biomineralization. Organisms produce hard parts by exceeding the solubility of the mineral component. Increased CO_2 in the oceans increases carbonate mineral solubility, making biomineralization of calcium carbonate structures more difficult. Many of these calcium carbonate biomineralizing organisms are important parts of the marine food chain.

The number of marine biomineralizers is vast. There are 128 000 species of molluscs, 700 species of calcareous green, red, and brown algae, more than 300 species of deep-sea benthic foraminifera, and 200 000 diatom species [58.6].

Biomineralization describes the formation of organized mineral structures through highly regulated cellular and molecular processes. Examples of biomineralized materials are enamel (97% mineral) and dentin (70% mineral), as well as bone (70% mineral). Crystal formation takes place in two steps: crystal nucleation (requires a high degree of saturation) and crystal growth (requires lower degree of saturation).

58.1.1 Marine Biomining

Current methods of mining are, in many cases, not environmentally sustainable. It might be interesting to focus on bio-assisted ways of obtaining resources such as Fe, Al, and Ti from marine environments (Table 58.1) [58.7]. All kinds of microbes contribute actively to geological phenomena, and central to many such geomicrobial processes are transformations of metals and minerals. Bioremediation is the use of biological systems for the clean-up of organic and inorganic pollution, with bacteria and fungi being the most important organisms for reclamation, immobilization, or detoxification of metallic and radionuclide pollutants [58.8].

Table 58.1 Concentration of transition metals and zinc in seawater (after [58.11], original references after [58.12–14])

Element	Seawater (M) × 10^8
Fe	0.005–2
Zn	8.0
Cu	1.0
Mo	10.0
Co	0.7
Cr	0.4
V	4.0
Mn	0.7
Ni	0.5

Biomining is the use of microorganisms and plants (phytomining) to aid in the extraction and recovery of metals from ores [58.9]. The microorganisms that grow in this aerobic, lithotrophic, and acidic environment are usually chemolithoautotrophic, using reduced forms of sulfur and iron, and acidophilic [58.10, p. 307].

Microorganisms themselves might be very beneficial in this kind of approach or processes inspired by biological processes that go on in biomineralizing organisms [58.15, 16]. The usage of controlled microbial cultures in order to concentrate certain minerals and ores was discussed already in 1972 [58.17]. It might be worth reconsidering such ideas, with the increased knowledge of biomineralization and environmental impact, as well as the novel methodologies that we now have at our disposal.

Bacteria that are active in marine microbial corrosion are promising with respect to marine mining. *Acidithiobacillus ferrooxidans*, for example, lives in pyrite deposits, metabolizes iron and sulfur, and produces sulfuric acid. *Acidithiobacillus thiooxidans* consumes sulfur and produces sulfuric acid. Both of these bacteria are already used as catalysts in bioleaching, whereby metals are extracted from their ores through oxidation. *Sulfobacillus* sp. are ferrous-iron and mineral-sulfide-oxidizing bacteria. *Sulfurivirga caldicuralii* is a microaerobic, thermophilic, thiosulfate-oxidizing chemolithoautotroph that is related to pyrite, arsenical pyrite, and chalcopyrite. The archaea *Sulfolobus shibitae, Metallosphaera* sp. and *Acidianus infernus* are related to chalcopyrite [58.18].

> *In low-temperature, aqueous habitats with oxygen, bacteria can affect the dissolution or precipitation of minerals through their reduction or oxidation of compounds containing Mn, Fe, S, C, U, Cu, Mo, Hg, and Cr* [58.10, p. 306].

Certain organisms are involved in the deposition of marine minerals, e.g., bacteria in deep-sea polymetallic nodules and coccoliths in seamount crusts [58.19]. Biosynthesis and bioleaching, i. e., extraction of specific metals from their ores through the use of bacteria and further organisms, are of increasing importance.

Limestone and other fixed carbonates represent 1.8×10^{22} g carbon in the Earth's lithosphere [58.20]. 40% of the Earth's limestone deposits that were previously thought to be of abiotic origin are, in fact, the consequence of heterotrophic bacterial metabolism [58.21], which thus emphasizes the role of microbial mineralization (carbonation) in the process of locking atmospheric and organic carbon as carbonate rocks back to the Earth's lithosphere, a long-term carbon storage compartment. While the precipitation of carbonate leads to CO_2 release in the ocean, this reaction must be considered in terms of final balance, as the buried sedimentary layers of carbonate, which no longer interact with the ocean water, constitute a long-term carbon sink [58.22]. The oceans absorb about half of the carbon dioxide that is generated when burning fossil fuels. As a consequence of this increased amount of carbon dioxide, the oceans are becoming increasingly acidic, which is resulting in less calcium carbonate biomineralization and a potential collapse of the marine ecosystem.

58.2 Materials – Biominerals

Many organisms build inorganic structures in various shapes and forms. The synthesis, as well as the size, morphology, composition, and location of these biogenic materials is genetically programmed and controlled. Detailed investigations and descriptions of biomineralization have only become possible with the modern methods that are now at the disposal of biologists. Many of the crystals and composite materials made up of proteins and amorphous inorganic parts are still unknown in current inorganic chemistry. Nowadays we know more than 70 minerals that are produced by organ-

Table 58.2 Non-exhaustive list of biominerals produced by marine organisms (after [58.4, 23–27])

Biomineral	Chemical formula	Marine biomineralizers of the respective biomineral
Carbonates		
Calcite	$CaCO_3$	Foraminiferans (e.g., *Rosalina leei* and *Spiroloculina hyaline*), coccolithophorids, brachiopod and mollusc shells, crustaceans, mammals, birds, corals, Archaeocyatha, bryozoans, echinoderms (brittle star, sea urchin, star fish, sand dollars, sea cucumbers…), Serpulidae (tube-building annelid worms in the class Polychaeta), barnacles, cyanobacteria, sponges, algae
Mg-calcite	$Mg_xCa_{1-x}CO_3$	Foraminiferans, calcareous and hypercalcified sponges, calcareous sponge spicules, octocorals, crustaceans, echinoderms, corals, bryozoans
Magnesite	$MgCO_3$	Tropical coralline alga *Hydrolithon onkodes*, cyanobacteria
Aragonite	$CaCO_3$	Hypercalcified demosponges, scleractinians, brachiopods, molluscs, teleosteans, bryozoans, Serpulidae (tube-building annelid worms in the class Polychaeta), algae, bacteria
Vaterite	$CaCO_3$	Ascidians
Monohydrocalcite	$CaCO_3 \cdot H_2O$	
Protodolomite	$CaMg(CO_3)_2$	Sea urchin teeth (protodolomite: a crystalline calcium-magnesium carbonate with a disordered lattice in which the metallic ions occur in the same crystallographic layers instead of in alternate layers as in the dolomite mineral)
Hydrocerussite	$Pb_3(CO_3)_2(OH)_2$	
Amorphous calcium carbonate (at least five forms)	$CaCO_3 \cdot H_2O$ or $CaCO_3$	Vascular plants, crustaceans
Dolomite	$CaMg(CO_3)_2$	Tropical coralline alga *Hydrolithon onkodes*, sea urchin teeth, embryos of the Nudibranch gastropod *Aplysia punctata*
Strontianite	$SrCO_3$	Marine snail shells, microbes, cyanobacteria
Siderite	$FeCO_3$	Bacteria
Phosphates		
Octacalcium phosphate	$Ca_8H_2(PO_4)_6$	Vertebrates (bone/teeth precursor)
Hydroxyapatite	$Ca_5[OH\|(PO_4)_3]$	Vertebrate bones, vertebrate teeth (enamel), brachiopods, conodonts (teeth), fish (scales), in the radular apparatus of the mollusc *Falcidens* sp.
Brushite	$CaHPO_4 \cdot 2H_2O$	Tropic bivalve species *Codakia orbicularis* and *Tivela mactroides*
Francolite	$Ca_{10}(PO_4)_6F_2$	Linguliformea (brachiopods)
Carbonated hydroxyapatite	$Ca_5(PO_4,CO_3)_3(OH)$	Bones
Carbonated apatite (dahlite, dahllite)	$Ca_5(PO_4,CO_3)_3F$	Bones, dental enamel, dentin
Whitlockite	$Ca_{18}H_2(Mg,Fe^{2+})_2(PO_4)_{14}$	Dental plaque
Struvite	$Mg(NH_4)(PO_4) \cdot 6H_2O$	Bacteria, embryos of the Mediterranean mussel *Mytilus galloprovincialis*
Vivianite	$Fe_2^{3+}(PO_4)_2 \cdot 8H_2O$	Bacteria
Amorphous calcium phosphate (at least six forms)	Variable	Vertebrates, bivalves, crustaceans, chitons (teeth), gastropods (gizzard plates)
Amorphous calcium pyrophosphate	$Ca_2P_2O_7 \cdot 2H_2O$	Granules in the hymenostomatidian ciliate *Tetrahymena pyriformis*

Table 58.2 (continued)

Biomineral	Chemical formula	Marine biomineralizers of the respective biomineral
Oxides and hydroxides		
Magnetite	Fe_3O_4	Eubacteria, Archaebacteria, teleosteans, polyplacophorans (chitons)
Amorphous ilmenite	$Fe^{+2}TiO_3$	Foraminifera, snail radulae
Maghemite	γ-Fe_2O_3	Magnetotactic bacteria
Amorphous iron oxide	Fe_2O_3	In the radular apparatus of the mollusc *Falcidens* sp.
Amorphous manganese oxide	Mn_3O_4	Bacteria
Manganese(III) oxohydroxide	MnOOH	Bacterial spores of the marine *Bacillus*, strain SG-1
Goethite	α-FeOOH	Gastropods, limpet teeth, marine sponges
Akaganeite	β-FeOOH	Bacteria
Lepidocrocite	γ-FeOOH	Polyplacophorans (chitons), marine sponges
Ferrihydrite	$(Fe^{3+})_2O_3 \cdot 0.5H_2O$	Lamprey *Geotria australis*, chitons, snails
Todorokite	$(Na,Ca,K,Ba,Sr)_{1-x}(Mn,Mg,Al)_6O_{12} \cdot 3{-}4H_2O$	Hydrothermal vent microbes
Birnessite	$(Na_{0.3}Ca_{0.1}K_{0.1})(Mn^{4+},Mn^{3+})_2O_4 \cdot 1.5H_2O$	Microbes
Sulfates		
Gypsum	$CaSO_4 \cdot 2H_2O$	Cnidarians, statoliths of certain medusae, Desmidae (algae), cyanobacteria
Bassanite	$CaSO_4 \cdot 0.5H_2O$	Statoliths of certain medusae
Barite	$BaSO_4$	Cyanobacteria, Spirogyra (alga), Loxididae (protozoa), *Chara fragilis* (higher alga), Xenophyophorea (large deep-sea protists), diatoms, foraminifera, Loxodes (gravity receptor)
Celestite	$SrSO_4$	Radiolarians, Acantharia, algae, foraminifera *Rosalina leei* and *Spiroloculina hyaline*, snail shell
Jarosite	$KFe_3^{3+}(SO_4)_2(OH)_6$	*Purpureocillium lilacinum* (an acidophilic fungus), *Acidithiobacillus ferroxidans*
Sulfides		
Pyrite	FeS_2	Magnetotactic bacteria
Amorphous pyrrhotite	$Fe_{(1-x)}S$ ($x = 0$ to 0.2)	
Hydrotroilite	$FeS \cdot nH_2O$	Sulphate-reducing bacteria, *Desulfovibrio* spp.
Sphalerite	(Zn,Fe)S	Magnetotactic bacteria
Galena	PbS	Sulfate reducing bacteria
Greigite	Fe_3S_4	Magnetotactic bacteria
Mackinawite	$(Fe,Ni)_{1+x}S$ ($x = 0$ to 0.11)	
Wurtzite	(Zn,Fe)S	
Cadmium sulfide	CdS nanoparticles	Marine cyanobacterium *Phormidium tenue*
Acanthite	Ag_2S	Polychaete worm *Pomatoceros triqueter*
Arsenates		
Orpiment	As_2S_3	Bacteria
Native elements		
Sulfur	S nanoparticles	
Gold	Au nanoparticles	Tropical marine yeast *Yarrowia lipolytica*, marine sponge *Acanthella elongate*, marine alga *Sargassum wightii*
Silver	Ag nanoparticles	Marine fungus *Penicillium fellutanum*, *Fusarium oxysporum* (a fungus that is reported to infect marine mammals)

Table 58.2 Continued

Biomineral	Chemical formula	Marine biomineralizers of the respective biomineral
Native Elements		
Selenium	Se nanoparticles	Bacteria *Sulfurospirillum barnesii* and *Pseudomonas marina*
Uranium	U nanoparticles	*Shewanella oneidensis* (bacterium); reduction of Uranium by *Desulfovibrio vulgaris*, a bacterium that builds marine aerobic biofilms
Silicates		
Silica (opal)	$SiO_2 \cdot nH_2O$	Radiolarians, diatoms, demosponges, hexactinellid sponges, most sponge spicules, Opal teeth in copepods and limpets, mollusc penial structures, heliozoan spines and scales
Sepiolite	$Mg_4Si_6O_{15}(OH)_2 \cdot 6H_2O$	Microorganisms (?)
Halides		
Fluorite	CaF_2	Vertebrate and invertebrate skeletons, fish skin, mollusc shell, gizzard plates of gastropods, statoliths of marine mysid crustaceans
Hieratite	K_2SiF_6	Gravity receptors
Amorphous fluorite	CaF_2	*Archidoris* (sea slug) spicules
Atacamite	$Cu_2(OH)_3Cl$	Jaws of the marine bloodworm *Glycera dibranchiata*
Organic minerals		
Weddellite	$CaC_2O_4 \cdot 2H_2O$	*Thalassia testudinum* (turtle grass), sea grass, vascular plants, Bornetella (marine alga) skeleton, gizzard plates of the deep water gastropod *Scaphander cylindrellus*, renal sac of the ascidian tunicate (marine filter feeder) *Mogula manhattensis*, molluscs
Whewellite	$CaC_2O_4 \cdot H_2O$	
Manganese oxalate	$MnC_2O_4 \cdot 2H_2O$	
Calcium tartrate	$CaC_4H_4O_6$	Chiton
Calcium malate	$C_4H_4CaO_5$	
Earlandite	$Ca_3(C_6H_5O_7) \cdot 4(H_2)O)$	
Guanine	$C_5H_5N_5O$ (one of the four main nucleobases found in the nucleic acids DNA and RNA)	Fish scales (fish silver), *Tetragnatha* spiders (silver color)
Uric acid	$C_5H_4N_4O_3$	
Paraffin hydrocarbon	C_nH_{2n+2}	
Wax	organic compounds that characteristically consist of long alkyl chains	
Magnesium oxalate (glushinskite)	$Mg(C_2O_4) \cdot 2(H_2O)$	
Copper oxalate (moolooite)	$Cu(C_2O_4) \cdot 0.4H_2O$	
Anhydrous ferric oxalate		
Sodium urate	$C_5H_3N_4O_3Na$	

isms in various ways (Table 58.2). Biomineralization takes place at normal temperatures and pressures, both much lower than those required to form the same mineralized structures by conventional chemical synthesis.

Biomineralized products comprise metals and alloys, ceramics, polymers, and composites. Examples are Ba, Ca, Cu, Fe, K, Mn, Mg, Na, Ni, Pb, Sr, and Zn; as hydroxides, oxides, and sulfates or sulfides, carbonates, and phosphates [58.23, p. 25]. Some bacteria

(e.g., from the species *Geobacter* and *Citrobacter*) accumulate and passivate toxic metal ions, such as UO_2^{2+}, Pb^{2+}, and Cd^{2+}. Bacteria have learnt to cope with most elements of the periodic table, e.g., with encoded resistance systems for toxic metal ions. For many chemical elements, bacteria have found uses either in ultralow concentrations in functional biomolecules (e.g., vanadium), or in vast amounts (e.g., calcium for the formation of shells) [58.28]. Gold, silver, uranium, palladium and CdS nanocrystals are produced by various organisms [58.29], Chaps. 25 and 55. The size of nanocrystals strongly determines fundamental properties such as their color or the external field required to switch a magnetized particle in hard disk drives. The perfect control of the size of the biomineralized nanocrystals is one major reason why bioassisted nanocrystal production is widely viewed as highly promising regarding base materials for new man-made optical and electrical materials. Zinc (Zn) is present in the jaws of the marine worm *Nereis* sp. [58.30], and copper (in the form of the biomineral atacamite, $Cu_2(OH)_3Cl$) is present in the jaws of the marine bloodworm *Glycera dibranchiate* [58.31]. The metals reinforce protein fibres in worm jaws.

The three different types of biominerals are either produced by simple precipitation, as perfect crystals,or as composites.

58.2.1 Biominerals Produced by Simple Precipitation and Oxidation Reactions

Calcium carbonate, iron(III)-oxide hydrate (FeOOH), manganese(IV)-oxide (MnO_2), and pyrite, as well as marcasite (both FeS_2) are generated in organisms by precipitation and oxidation reactions, i. e., relatively simple reactions in which solved substances are translated into insoluble ones via the metabolism of organisms. Example: $CaCO_2$ in stromatolites that were built by autotrophic cyanobacteria as far back in time as 3.5 billion years ago.

Calcium Carbonate $CaCO_3$

$[Ca^{2+} + 2HCO_3^-]$ (carbonate) $\rightarrow$ $CaCO_3$ (stromatolite) + CO_2 (insertion to the biomass) + H_2O.

The advantage for the cyanobacteria might be mechanical fixation of the biofilm in water.

Iron(III)-Oxide-Hydroxide FeOOH Goethite

Gallionella ferruginea is a chemoautotrophic iron-oxidizing chemolithotrophic bacterium that has been found in a variety of different aquatic habitats. It uses the energy it obtains from oxidization, remains of its activities are drains clogged with iron depositions, iron(III)-oxide (Fe_2O_3) and iron(III) oxide-hydroxide ($HFeO_2$). This bacterium or some of its biochemicals related to the oxidization of iron might be of high interest for an alternative way of mining iron in the sea.

Manganese Dioxide MnO_2

Manganese(IV)-oxide (MnO_2) is precipitated via microorganisms such as *Leptothrix discophora* [58.32]. The sheaths of the organisms of the Sphaerotilus-Leptothrix group often are coated with $Fe(OH)_3$ or MnO_2 [58.33].

The Mn and Fe oxidizing/depositing bacteria in freshwater habitats belong to the genera *Sphaerotilus*, *Gallionella*, and *Leptothrix* [58.34, 35].

> *In marine environments, the direct evidence for Fe-oxidizing bacteria is not well documented. The one notable exception was the finding of abundant Gallionella-like stalk material and microscopic identification of putative G. ferruginea cells from a shallow water volcanic system near Santorini Island in the Mediterranean Sea.* [58.36]

Pyrite and Marcasite FeS_2

Sulfate reducing bacteria gain energy from the reduction of sulfates. When iron(II) ions are present, FeS_2 can be produced,

$$2SO_4^{2-} + 3.5C + 2H^+ + Fe^{2+} \rightarrow FeS_2 + 3.5CO_2 + H_2O$$

(the C comes from organic substances).

58.2.2 Biological Production of Perfectly Crystallized Minerals

Ice H_2O

Ice is a mineral with a relatively low melting point. Organisms actively protect themselves against this unwanted mineral in their bodies via two strategies: either they prevent freezing of their bodily fluids via freeze protection proteins (Sect. 58.3) or they enrich ice nucleation proteins in their blood, which promote and control freezing so that their cells do not suffer. Some bacteria such as *Pseudomonas syringae* produce proteins that promote freezing; they are used for the production of artificial snow. Various organisms such as arctic fish, plants, fungi, microorganisms, and bacteria build an-

tifreeze and ice structuring proteins. With the help of these proteins, the organisms can survive in temperatures below the freezing point of water.

Antifreeze proteins bind to ice crystals and prevent their growth and recrystallization, thereby preventing the cells of the organisms from being destroyed by ice crystals. Due to their ice binding properties, antifreeze proteins in living nature work at very low concentrations (300–500 times less than conventional man-made antifreeze agents). Frost-tolerant species survive the freezing of their body fluids [58.37].

Calcium Carbonate $CaCO_3$ (Calcite, Aragonite, and Vaterite)

Calcite is the most stable polymorph of calcium carbonate. It is transparent, with a refractive index of 1.6584 and 1.4864 (depending on the crystallographic axis). These properties make it a good material for the production of optical lenses. The brittle star *Ophioma wentii* has calcite eyes of optically corrected microlenses all over its body (Fig. 58.1).

Already 350 millions of years ago some trilobite species used calcite lenses; up to 15 000 lenses making up one single eye! Calcium carbonate is also present in the ear: zebra fish use Starmaker proteins to biomineralize aragonite otoliths for hearing and their vestibular sense [58.39].

Magnetite Fe_3O_4

Magnetite is a ferromagnetic black iron oxide that can, e.g., be found in a chain of magnetosomes, i. e., single magnetic domain crystals in magnetotactic bacteria (Fig. 58.2), e.g., *Magnetospirillum magnetotacticum*. The anaerobic bacterium uses the magnets to orient itself along the magnetic field lines of the Earth, and thereby determine up and down (it cannot do this via gravity, because as a small bacterium it lives a life at a low Reynold's number, like a piece of dust in honey – which is no way to rely on gravity). Magnetosomes were also found in migratory birds, trout, and salmon. Magnetite particles with molecular precision have even been identified in the human brain (Fig. 58.3). Biogenic magnetite in the human brain may account for the high-field saturation effects observed in magnetic resonance imaging and, perhaps, for a variety of biological effects of low-frequency magnetic fields.

The formation of bacterial magnetite crystals is as follows: first, the bacterium takes Fe(III) ions from the environment. Then it reduces Fe(III) to Fe(II) ions during the transport across the cell membrane. Sub-

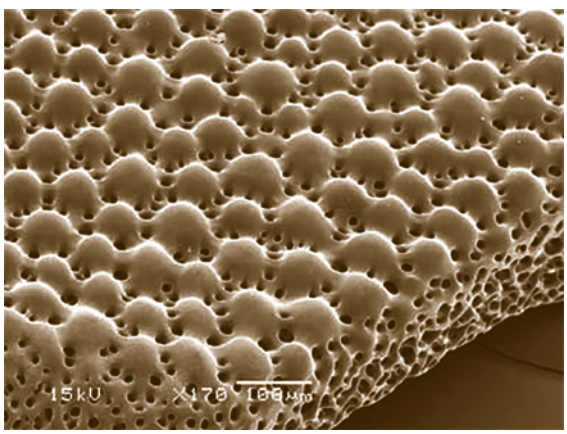

Fig. 58.1 The calcite microlenses of the brittle star. Each of the lenses is only some tens of micrometers in diameter. Scale bar 100 µm

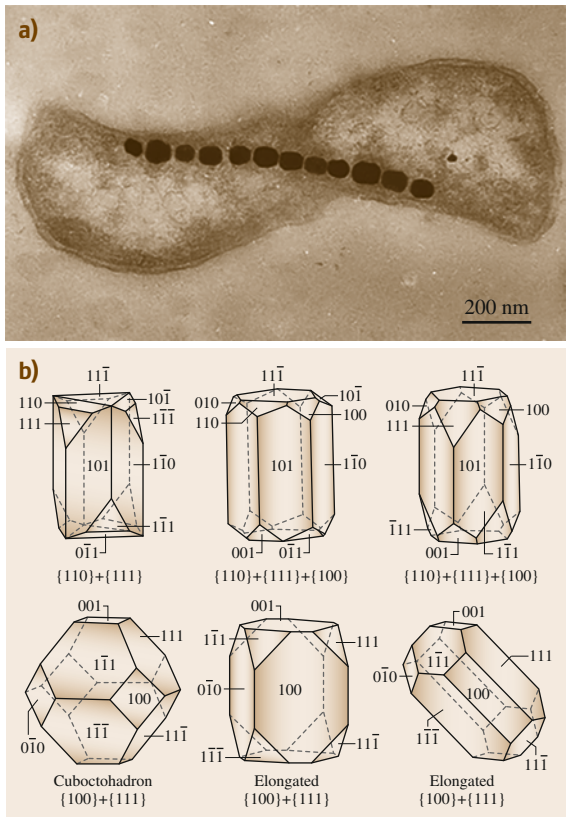

Fig. 58.2 **(a)** A magnetotactic bacterium. **(b)** Magnetosome crystal morphology. The magnetosomes are built with atomic precision (after [58.38], with permission)

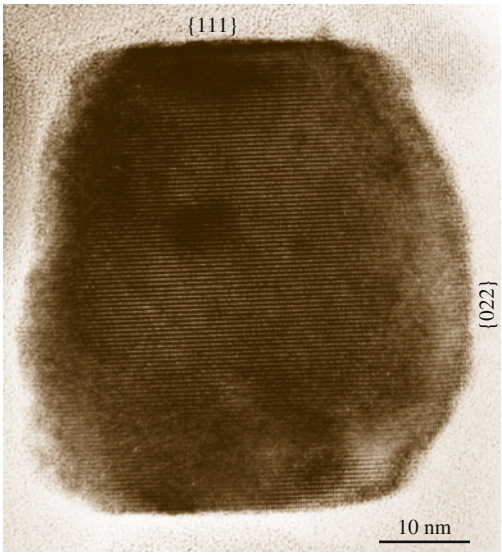

Fig. 58.3 A magnetite particle from the human brain (after [58.42], with permission)

Fig. 58.4 Pearls

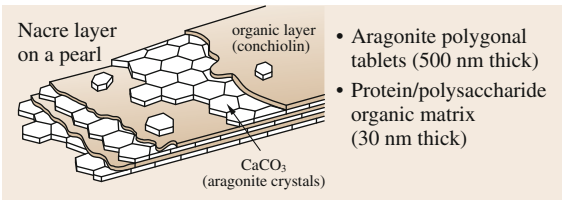

Fig. 58.5 Nacre layer on a pearl. The conchiolin protein/polysaccharide matrix is about 30 nm thick, the aragonite polygonal tablets are about 500 nm thick. The beautiful luster of the pearl comes from optical interference effects of light on the thin tablets. Permission pending

sequently, Fe(II) ions are transported to and across the vesicle membrane (the magnetosome membrane), amorphous hydrated Fe(III) oxide is precipitated within the vesicle, and the amorphous phase is transformed to magnetite by surface reactions involving mixed-valence intermediates [58.40, 41].

58.2.3 Composite Biomaterials

Composite materials combine two or more materials yielding new materials with interesting properties. Many biomaterials are composites. In most cases, the functionality of the biological composites is based on nanoscale structures. Examples for such nanocomposites (also known as hybrid biomaterials) are nacre (the beautifully iridescent layer in, e.g., abalone shells or pearls, Figs. 58.4 and 58.5), bones and enamel, as well as egg or mollusc shells. In many cases, the mechanical properties of such nanocomposites are outstanding: abalone nacre, for example, has a fracture toughness that is 3000 times higher than that of calcite crystals [58.43].

Calcium Carbonate $CaCO_3$ Nanocomposites

Calcium carbonate is an important ingredient in the nanocomposite that makes up the shells of foraminifers [58.44], eggs of birds and mollusc shells. Calcium carbonate is the material that is biomineralized most: remnants of calcium carbonate biomineralizing organisms yielded whole mountain ranges. The calcium carbonate shell serves as mechanical protection for the soft bodies of the organisms, be they foraminifers, other single-celled organisms, mussels, molluscs, or other animals.

Nacre is an iridescent form of aragonite (which is a calcium carbonate with orthorhombic symmetry as opposed to the trigonal symmetry in calcite) that is made by certain marine animals. Its protein content is about 5%.

Sea urchins biomineralize calcium carbonate in their shells, teeth, and spines. Sea urchin spines can reach a length of 10 cm, with their toughness by far outnumbering the toughness of pure calcium carbonate. Sea urchin teeth are single crystal calcites [58.45].

In corals, sensitive little animals (polyps) biomineralize with the help of algae, with whom they live in symbiosis, an exoskeleton of calcium carbon-

ate. The algae consume the CO_2 and aid in $CaCO_3$ precipitation.

Nanostructured Silicon Dioxide SiO_2 in Diatoms and Glass Sponges

Diatoms are unicellular algae that live in fresh or saltwater and on moist surfaces, and that at normal temperature and pressure biomineralize an exoskeleton of rigid, tough, and hard silica [58.46]. These exoskeletons are nanostructured and of exquisite beauty (Fig. 58.6). Sometimes, single diatom cells are connected via hinges and mechanical interconnecting devices. Such linkages have a size on the order of several hundreds of nanometers. No signs of wear in diatoms have ever been reported, not even when they fossilized and were alive tens of millions of years earlier (as is the case for the fossil diatom depicted in Fig. 58.6). Diatoms can serve as inspiring natural microsystems when it comes to tribological or mechanical aspects of microengineered devices, especially 3D microelectromechanical systems (MEMS) [58.47, 48].

Proteins that are involved in the biomineralization of silica are called silicatein, silaffin and silicase. Silicateins are high molecular weight proteins that strengthen the silica structure; silaffins are low molecular weight proteins that yield silica precipitation within minutes.

Diatomaceous earth is a natural resource that is mined in various places on the Earth. It contains the remnant shells of diatoms. Because of the porous nanostructured shell of the diatom, diatomaceous earth has huge surface area and is, therefore, used for filtering drinks such as beer and apple juice, or for the production of explosives such as dynamite.

A second type of marine organisms that biomineralize silica are the about 500 species of glass sponges (Hexactinellida) [58.50]. Glass sponges are exclusively marine organisms; they comprise about 7% of all sponges currently known. Their skeleton is made from amorphous hydrated silica needles (spicules) of exquisite shapes (Fig. 58.7). One single species can have up to 20 different types of needles. *Monorhaphis chuni* produces a silica needle of up to 3 m in length and 8 mm in diameter, which it uses to stabilize itself at the bottom of the Indian and Pacific oceans.

Glass sponges are an important ecological factor in the Antarctic; spicules of dead animals can build woolly layers up to 2 m high. The single spicules have concentric layers of silica, arranged around a hollow central canal. The spicules are a composite material of silica and proteins and are in many cases highly elastic.

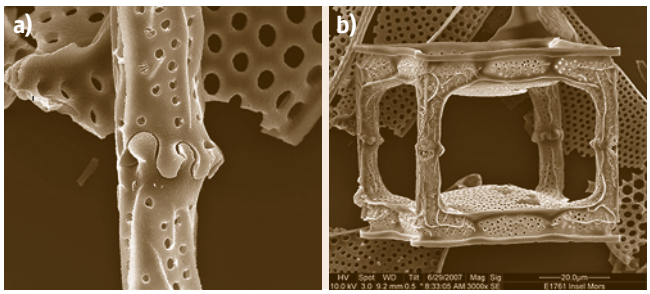

Fig. 58.6a,b Diatoms are silica biomineralizing phytosynthetic single-celled organisms. The fossil diatom *Solium exsculptum* from the island of Mors in Denmark depicted in the image lived 45 millions of years ago. It beautifully shows an expedient nanostructured shell, reinforcement ribs, connections, and primary mechanical structures. Panel **(a)** is a zoom into the most left junction in panel **(b)**. The sample is from the Hustedt Collection in Bremerhaven, Germany, # E1761 (Courtesy of F. Hinz, AWI Bremerhaven). Image reproduced with kind permission

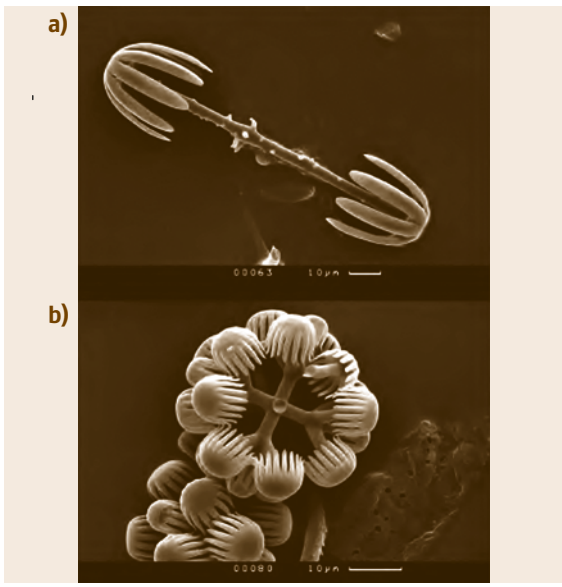

Fig. 58.7a,b Hexactinellida (glass sponge) spicules. Glass sponges consist of a network of such spicules covered by a thin layer of living cells. In this way they are heavily defended both inside and out. Single glass sponges have up to 20 different types of spicules in various shapes, some of them needle like **(a)**, some ball-like **(b)**, some in star shapes. Scale bars 10 μm

Hydroxyapatite $Ca_5[OH|(PO_4)_3]$

Animal bones consist of about 65% inorganic components, mainly hydroxyapatite (a calcium phosphate with the chemical formula $Ca_5[OH|(PO_4)_3]$), providing compressive strength, and about 35% inorganic components, mainly collagen, providing high tensile strength. Further ingredients in bones are proteins and fats. The high strength of bone is due to the fact that hydroxyapatite crystals are ordered mainly along the lines of tension and compressive stress, which results in a strut-like arrangement. See Fig. 58.8 for a drawing of the seven layers of hierarchy in bone.

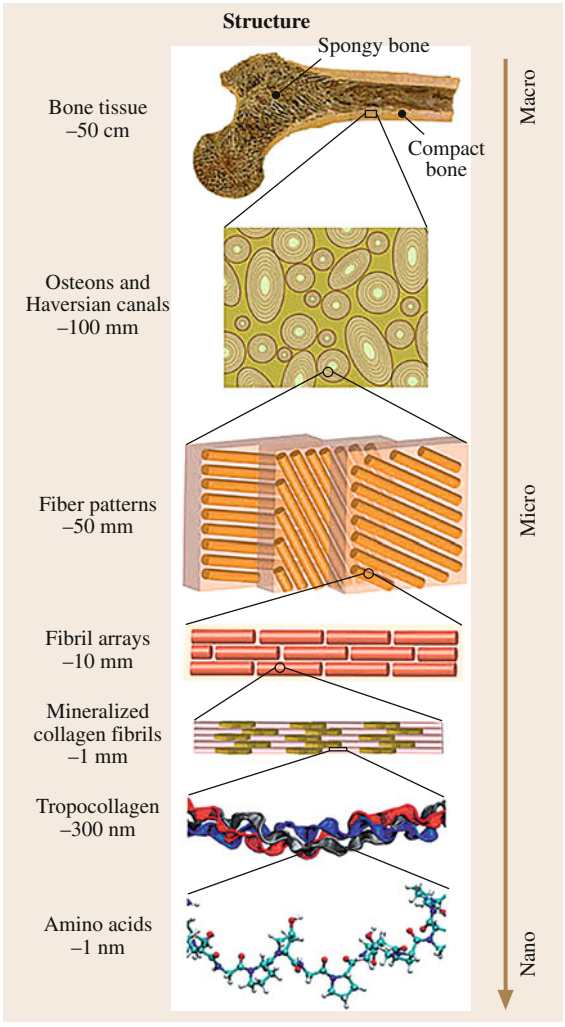

Fig. 58.8 The hierarchical levels of bone, a biomineralized structure with added functionality on each level of hierarchy, resulting in a tough and strong, yet lightweight material [58.49], with permission

Fluorapatite $Ca_5(PO_4)_3F$

Enamel has 95% inorganic components, mainly hydroxyapatite. In fluorapatite the OH group is replaced with F – this makes enamel more resistant against acids and provides a better protection against caries.

58.2.4 Example of Uptake and Conversion of a Very Rare Element: Selenium

In 1983, *Foda* et al. [58.51] described uptake and conversion of the very rare element selenium by the marine bacterium *Pseudomonas marina* in seawater containing either selenite or selenate. *Pseudomonas marina* bioconverts selenite into water-soluble non-Se(IV) metabolite(s) and subsequently releases them back into the medium. It is also capable of reducing Se(IV) to elemental Se; this pathway becomes increasingly evident at higher concentrations of selenite.

In 2011, selenium-reducing microorganisms that produce elemental selenium nanoparticles were reported [58.52]. This study identified high-affinity proteins associated with such bionanominerals and with non-biogenic elemental selenium. Proteins with an anticipated functional role in selenium reduction, such as a metalloid reductase, were found to be associated

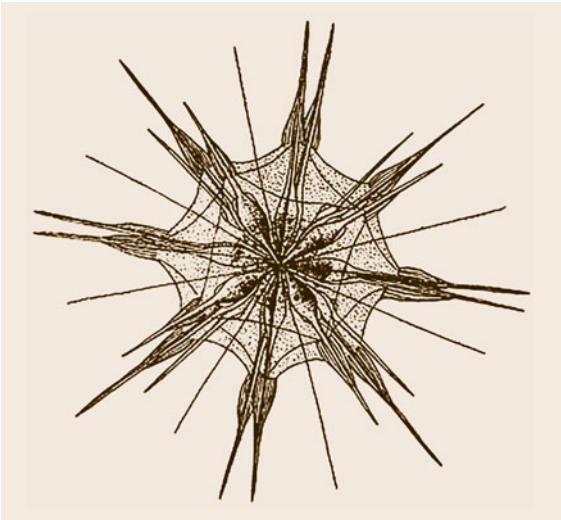

Fig. 58.9 An Acantharea exoskeleton. These planktonic, free living, exclusively marine protozoa range in size from 0.05–5 mm in diameter

with nanoparticles formed by one selenium respirer, *Sulfurospirillum barnesii*.

58.2.5 Example of Strontium Mineralization in Various Marine Organisms

Strontium is an alkaline earth metal that occurs naturally in the minerals celestine ($SrSO_4$) and strontianite ($SrSO_3$). These two minerals also occur as biominerals in radiolarians, acantharia, algae, the foraminiferae *Rosalina leei* and *Spiroloculina hyaline* [58.53], and in snail shells.

Acantharea are one of the four types of large amoebae that occur in the marine water column. Acantharea have a very regular exoskeleton and are of high importance for strontium circles in the sea. Their sizes range between 0.05–5 mm (Fig. 58.9).

58.2.6 Example of Biomineralization of the Unstable Calcium Carbonate Polymorph Vaterite

Vaterite is a polymorph of calcium carbonate and is less stable than calcite or aragonite. Vaterite occurs naturally in some organisms, such as in the spicules of sea quirts [58.54], in turtle eggshells [58.55], and in gastropod (e.g., snail, abalone, limpet) shells (e.g., [58.56]). In those circumstances, impurities such as metal ions or organic matter may stabilize the vaterite and prevent its transformation into calcite or aragonite.

58.3 Materials – Proteins Controlling Biomineralization

We are just beginning to understand the role of proteins in biomineralization [58.57]. Proteins have several important active roles in biomineralization: they inhibit spontaneous mineral formation from solution (e.g., the protein statherin in the mouth inhibits spontaneous precipitation of calcium phosphate), they inhibit the growth of existing crystals, and they are responsible for directing crystal nucleation, phase, morphology, and growth dynamics. The crystal shape is, for example, affected by proteins with specific structures and sequences that adsorb to different faces of the crystal, leading to regulation of shape (crystal faces have different charges and arrangements of atoms so proteins can selectively adsorb). Furthermore, proteins can self-assemble into ordered arrays that guide the formation of organized mineralized structures.

Proteins ultimately contribute to the extraordinary mechanical, optical, etc., properties of the biomineralized material and structures. Chitin and collagen seem to be universal and alternative templates in biomineralization.

Table 58.3 gives a non-exhaustive list of the proteins involved in biomineralization and the respective biomineralized material.

The three main organic structuring and scaffolding polymers are chitin, cellulose, and collagen. All three have common principles in their organization: they form nanofibrils with 1.5–2 nm diameter, they have the ability to self-assemble, they produce fibrillar and fiber-like structures with hierarchical organization from the nanolevel up to macrolevels, they have the ability to act as scaffolds and as templates for biomineralization, and they form rigid skeletal structures [58.23].

Certain proteins provide active organic matrices that control the formation of specific mineral structures; others act as catalysts that facilitate the crystallization of certain metal ions [58.58].

58.4 Organisms and Structures That They Biomineralize

Marine vertebrates, invertebrates, and plants biomineralize more than 70 different substances (Tables 58.2 and 58.4); some specific crystals produced by living nature cannot be produced by conventional chemical synthesis (e.g., the defect-free magnetosomes in magnetotactic bacteria or some crystal classes that obviously need proteins for their build-up – they do not exist in geological crystal formations). The number of marine biomineralizers, and with them the range of different structures, materials, processes, and functions, is tremendous: 128 000 species of molluscs have been described, 700 species of calcareous green, red, and brown algae, more than 300 species of deep-sea benthic foraminifera, and 200 000 diatoms species [58.23, p. 26].

Many biomineralized structures are built from nanostructured, hierarchical materials, and sometimes even functional gradient materials. Bone, for example,

Table 58.3 Biominerals and the respective proteins involved in biomineralization (non-exhaustive list)

Biomineral	Proteins
Apatite	Collagen (controls apatite) (in Porifera, coelenterates, molluscs, echinoderms)
Bone	Biglycan (a small leucine-rich repeat proteoglycan (SLRP) found in a variety of extracellular matrix tissues, including bone, cartilage, and tendons; essential for the structure and function of mineralized tissue) Osteonectin (a bone-specific protein linking mineral to collagen) Osteopontin (an extracellular structural protein and, therefore, an organic component of bone) Sialoprotein (BSP) (a component of mineralized tissues such as bone, dentin, cementum, and calcified cartilage) Osteocalcin, phosphophoryn, bone sialoprotein, proteoglycans, glycoproteins, glycosaminoglycans
CdS nanoparticles	C-phycoerythrin (from the marine cyanobacterium *Phormidium tenue*)
Calcium carbonate	Calcified cartilage sialoprotein (BSP) (a component of mineralized tissues such as bone, dentin, cementum, and calcified cartilage) Orchestin (a calcium-binding phosphoprotein in the calcified cuticle of a crustacean) Snail shells conchiolin (conchin) Calmodulin-like protein (from pearl oyster *Pinctada fucata*) Eggshell matrix proteins Otoconin (in octoconia, small crystals of calcium carbonate, also called statoconia, as gravity and acceleration sensors) Statherin (growth *inhibiting* protein, inhibits spontaneous precipitation of calcium phosphate in the mouth) Pancreatic stone protein (PSP) *inhibits* calcium carbonate precipitation in pancreatic fluid Starmaker proteins (in zebrafish, aragonite biomineralization)
Calcium phosphate	Serum protein fetuin-A (inhibition of calcium phosphate precipitation, *inhibiting* smooth muscle cell calcification)
Dentin	Dentin sialoprotein (BSP) (a component of mineralized tissues such as bone, dentin, cementum, and calcified cartilage)
Enamel	Amelogenin (a series of closely related proteins involved in amelogenesis, the development of Tuftelin) Enamelin and amelogenin (tooth enamel proteins) Tuftelin (enamel) Transcription factor FoxO1 (essential for enamel biomineralization) Phosphoprotein (inhibits hydroxyapatite formation)
Goethite	Chitin (in limpet teeth, as template for goethite growth)
Gold	Cytochrome C Lysosome
Ice	Antifreeze proteins (control of ice crystal growth) Ice interaction polypeptides
Magnetite	MamA (required for the activation of magnetosome vesicles) MamJ (directs the assembly and localization of magnetosomes) Mms6 (regulates magnetite crystal morphology)
Nacre	Nacre proteins (perlucin, n16N,...)
Silica	Polyamines, silicatein and silaffine (in diatoms) Frustulin, pleuralin (in diatoms)
Uranium	Cytochrome c3 (reduction of uranium, from *Desulfovibrio vulgaris*)
Vaterite	Eggshell pelovaterin (turtle eggshells, vaterite crystals)

Table 58.4 Examples for biomineralizing organisms in all six biological kingdoms

Kingdom	Examples
Plants	*Thalassia testudinum* (turtle grass), sea grass, vascular plants
Animals	Avian egg shell ($CaCO_3$) Archaeocyatha ($CaCO_3$) Brachiopod and mollusc shells ($CaCO_3$) Sea-mats (Bryozoans) ($CaCO_3$) Calcareous sponge spicules ($CaCO_3$) Calcareous tunicate (marine filter feeders) spicules ($CaCO_3$) Conodonts (apatite (phosphate carbonate)) Enamel (vertebrate teeth) (apatite (phosphate carbonate)) Echinoderms ($CaCO_3$) Corals ($CaCO_3$) Glass sponges such as the Venus flower basket *Euplectella aspergillum* (amorphous hydrated silica) Lanternshark (mineralized spines) Crystal structures in the inner ear in zebrafish Japanese pearl oyster *Pinctada fucata* Cephalopods such as the Giant Pacific octopus (beak) Sea urchins (needles, larval skeleton) Fanworms (Serpulidae) ($CaCO_3$) Vertebrate bone (apatite (phosphate carbonate)) Whale teeth Narwhal whale *Monodon monoceros* tooth (up to 9 feet long) Shells, snail shell (strontium, calcium) Rhopaliophoran medusae (Cnidaria) statoliths (calcium sulfate hemihydrate) Demosponges (silica) Brittle star crystal eyes (Calcite) Most sponge spicules (silica) Hypercalcified sponge (calcium carbonate basal skeleton in addition to their spicules) *Petrobiona massiliana* (Calcarea, Calcaronea), *Ceratoporella nicholsoni*, *Goreauiella auriculata* (Astroscleridae), *Astrosclera willeyana*, *Hispidopetra miniana*, *Stromatospongia norae* (Ceratoporellidae: Porifera), *Calcifibrospongia actinostromarioides*
Protista	Coccolithophores ($CaCO_3$) Diatoms, radiolarians and silicoflagellates (hydrated silica) Foraminifera ($CaCO_3$, $SrSO_4$)
Fungi	Fungi in corals (pearl-like skeletons)
Bacteria	Chemosynthetic marine organisms Magnetotactic bacteria (e.g., *Magnetospirillum gryphiswaldense*, *Magnetospirillum magneticum*) Cynobacteria in stromatolithes Marine bacteria ($CaCO_3$, Selenium nanoparticles)
Archaea	*Sulfolobus shibitae*, *Metallosphaera* sp., *Acidianus infernus* (iron, sulfur, uranium)

has seven layers of hierarchy, spanning from the components of collagen fibrils to the whole bone. Level 1 consists of the components themselves, including proline and hydroxyproline. On level 2, one step up in size, in hierarchy, and with added functionality, comes the mineralized collagen. On level 3 there are arrays of fibers, which on level 4 build patterns, e.g., spirals. On level 5 the osteons, cylindrical motifs in bone structure, appear. Level 6 brings in as added functionality, integrated into the whole bone structure, spongy *vs.* compact bone, and level 7 is the whole bone (Fig. 58.9).

Nature's nanostructures are built by benign chemistry and via self-assembly and templating. Human chemical synthesis is slowly reaching the efficiency and

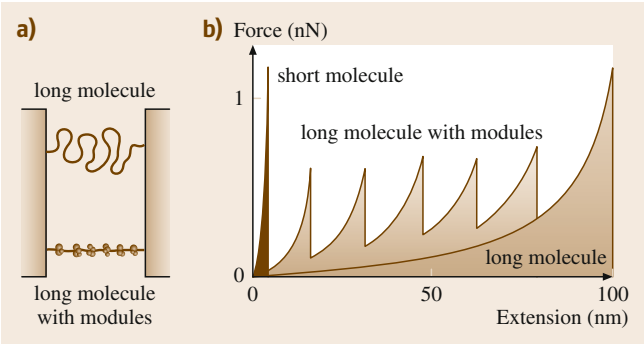

Fig. 58.10a,b Concept for a self-repairing adhesive [58.43]. **(a)** Two ways to attach two particles: with a long molecule or with a long molecule with nodules. **(b)** When stretched, a short molecule can only be extended a little and would then break. A long molecule would be stretched much more and finally break. However, a long molecule with nodules, with sacrificial bonds that break before the backbone of the molecule breaks, increases the toughness of the adhesive. Such a strategy is applied in the abalone shell and also in diatom adhesives (after [58.43], with permission) ►

purity of natural biomineralized crystals, and in terms of controlling morphology and hierarchy, there is still a lot for us to learn [58.59].

From nanometer small crystals that serve as nucleation centers up to meter-long silica spicules in certain marine glass sponges – biomineralized structures span many orders of magnitude in length.

58.4.1 Example: Molluscan Shells

The main material in molluscan shells is calcium carbonate: it amounts to about 95–99% of the weight. The remaining 1–5% are made up of proteins. The complex biocomposite (Fig. 58.10) of molluscan shells is far more fracture resistant than a calcite crystal: in the case of the abalone shell, this factor amounts to 3000 [58.43]. The proteins controlling the biomineralization (for an overview, see Sect. 58.3) allow for calcium carbonate (and/or strontianite, celestite, and fluorite, see Sect. 58.2) synthesis at ambient conditions, and control the crystal nucleation, phase, morphology, and growth dynamics. The abalone shell is an example of a biomineralized material with high fracture toughness. The blue-rayed limpet *Ansates pellucida* has a shell that shows stripes of green-blue dynamic structural coloration (photonic structures). It exerts different control over the calcium carbonate crystal phases in different parts of its shell, utilizing different proteins and making different structures. Several phases of $CaCO_3$ can exist locally next to each other, each containing its characteristic proteins [58.60].

58.4.2 Example: Coccolithophores

Coccolithophores (Fig. 58.11) are small unicellular photosynthesizing protists that produce exoskeletons of elaborate calcite plates with a size of about

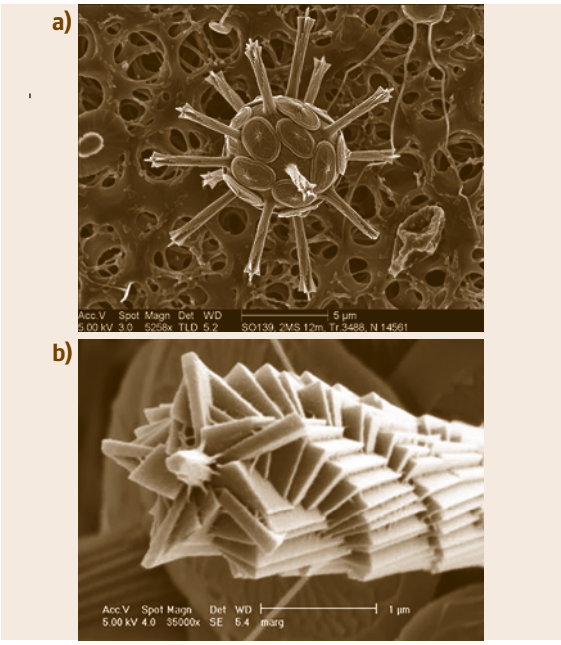

Fig. 58.11a,b The coccolithophorid *Rhabdosphaera clavigera*. **(a)** Whole organism. **(b)** Detail of the tip of a single spine showing spiral structure formed from consistently aligned crystal units with rhombic faces (after [58.61], permission pending)

1–10 μm. These microscopic organisms control the nucleation as well as the growth of the plates. Coccolithophores form a significant proportion of total marine primary production and carbon fixation; the biomineralized structures of dead coccolithophores are the largest single component of deep-sea sediments and they also make up the white cliffs of Dover [58.61, 62].

58.5 Functions

Most structures in biological systems are multifunctional. Their integrated approach allows for highly compact, cheap solutions to various chemical, dynamic, structural, mechanical, or physical demands *in one go*. Structures in organisms can be great teachers for engineers and inspire novel, sometimes even revolutionary, new approaches. However, because of their intrinsic multifunctionality, not always can one single aspect of an organism be successfully isolated in pure form for man-made applications, sometimes not even concept-wise. One fascinating example of the multifunctionality of living nature is biomineralized structures and their respective functions. Especially more complex arrangements, such as the silica micro- and nanostructures in diatoms, are envisaged to provide substantial inspiration in micro- and nanotechnology, especially tribology (regarding friction, adhesion, lubrication, and wear of small rigid interacting parts in relative motion) [58.63]. Further examples of tribological optimization in biomineralized structures are chitons, where iron is stored close to their magnetite-coated teeth, and the teeth can easily be renewed as they wear [58.64], and the jaws of certain marine worms that contain copper and zinc-reinforced proteins [58.30, 31].

The generalized principle *use structure rather than material* can be identified in various organisms. Especially for micro- and nanoscale organisms with rigid interacting parts in relative motion (e.g., some diatoms) straightforward biomimetic principle transfer to novel MEMS can be envisaged [58.47, 48].

Examples of functions of biomineralized structures are given in Table 58.5 and contain, for example, the calcite cell wall scales in coccolithophorids that are used as exoskeleton, the calcite shells of foraminifera and molluscs, the aragonite cell walls of scleractinian corals, aragonite mollusc shells, and vaterite gastropod (e.g., snail, abalone, limpet) shells. Calcite was used for optical imaging in the now extinct trilobites (it constituted their eye lens), and provides mechanical strength to the cuticle of crustaceans, e.g., crabs. In eggshells it provides mechanical protection. Mg-calcite provides mechanical strength to the spicules of octocorals, and strength as well as protection to the shell and spines of echinoderms (marine invertebrates with tube feet and five-part radially symmetrical bodies, such as sea stars and sea urchins). Chitin is the material of the poison dart in certain marine gastropods. Aragonite provides buoyancy devices in cephalopod (e.g., octopus) shells, as well as gravity receptors in fish heads. Vaterite spicules provide protection for ascidians (sessile coral-looking animals, e.g., sea squirts), and amorphous calcium carbonate in the crab cuticle provides these crustaceans with mechanical strength, whereas in the leaves of marine plants it serves as calcium storage. Iron-silica biominerals provide cyanobacteria with an effective UV screen [58.65].

58.6 Applications

58.6.1 Current Applications of Bioinspired Material Synthesis in Engineering and Medicine

Micro- and nanoengineers profit from the fact that in biological systems very often structure rather than material is used to achieve certain functionalities. In many cases, the structures are solely responsible for the respective functions, allowing for simple biomimetic transfer of principles from the inspiring structure to the respective application in engineering. This is of relevance in current MEMS design (where only a handful of base materials can be used, and the MEMS designer therefore has to work with structure rather than material), and will be even more relevant in the future when we finally mass-produce various 3D MEMS.

The abundance and distribution of chemical elements in seawater is a function of their solubility, reactivity, and involvement in biotic and abiotic processes, as well as oceanic circulation; it shows similarity to the composition of the human body (Fig. 58.12). Animals have a similar composition; in plants the first four most abundant elements are the same as in people or animals (O, C, H, and N), but the fifth most abundant element is P. The elemental composition of the cell phone (which shall serve here as a representation of a technical device) is completely different (Fig. 58.12). Potential future marine biotechnology might provide materials and structures for new technologies that are less focused on plastics and metals. For each such marine biotechnology attempt, careful considerations regarding the potential benefit of

Table 58.5 Non-exhaustive list of already identified functions in biomineralized structures of marine organisms, sorted by chemical, dynamic, mechanical, physical, and structural functions

Chemical functions	Dynamic functions	Structural functions
Calcium storage High performance nanocomposites Molecular glue in composite structures Storage (biominerals are ion reservoir for cellular functions) Mineralized holdfast (byssus of the jingle shell *Anomia*) Underwater adhesives Waste disposal (inside, like some nanocrystals or pearls, perhaps also outside)	Lubrication (bearing-like structures) Mobility Motion Movable rigid parts (ensuring a certain maximum and minimum distance) Pumps	Sclerites (a component section of an exoskeleton, especially each of the plates forming the skeleton of an arthropod) Spicules Structure building (corals) Surface texturing for optimized mechanical properties

Mechanical Functions	Physical Functions
Click-stop mechanism Crack redirection Distance holder Fixation (e.g., click stop in diatoms) Fracture resistance Hinges Injection Interlocking devices Mechanical connection Mechanical fixation Mechanical protection Mechanical strength Protection Reinforcement Scaffolds Skeleton (endoskeleton, exoskeleton) Springs Stability Strength and Integrity Teeth for cutting, rasping and grinding (e.g., iron in the teeth of chitons) Toughness (abalone) Weapons (defensive: e.g., sea urchin needles, aggressive: e.g., pistol shrimp dactyl club, conch snail harpoons) Wear protection (addition of zinc or copper for reinforcement)	Buoyancy Co-orientation mechanisms in biominerals (control of crystal orientation) Dynamic colors (diatoms, polychaete worms) Electrically conductive bacterial nanowires (nanowiring in microbial communities) Energy dissipation Gravity sensing (otoconia, statoliths) Lenses (optical) Magnets (for navigation, location) Optical components (antireflective layers, lenses, transparent containers for photosynthesizing organisms) Ossicles Photonic components (e.g., light guiding in deep-sea organisms) Photoprotective coatings Reflectivity (fish silver) Gravity sensing Magnetic sensing Optical sensing Balance sensing UV protection Whiteness

biomimetic and biomineralization approaches need to be made, taking energy and other factors into account. One of the advantages of technology as opposed to functional entities in organisms is the freedom of choice in materials. Organisms are stuck with a certain material as soon as they start using it. Examples are $SrSO_4$ in the exoskeleton of Acantharea (Sect. 58.2.5), or SiO_2 in diatoms (Sect. 58.2.3) – with the abundance of Sr and Si in ocean water being about 8 and 2 ppm, respectively [58.66].

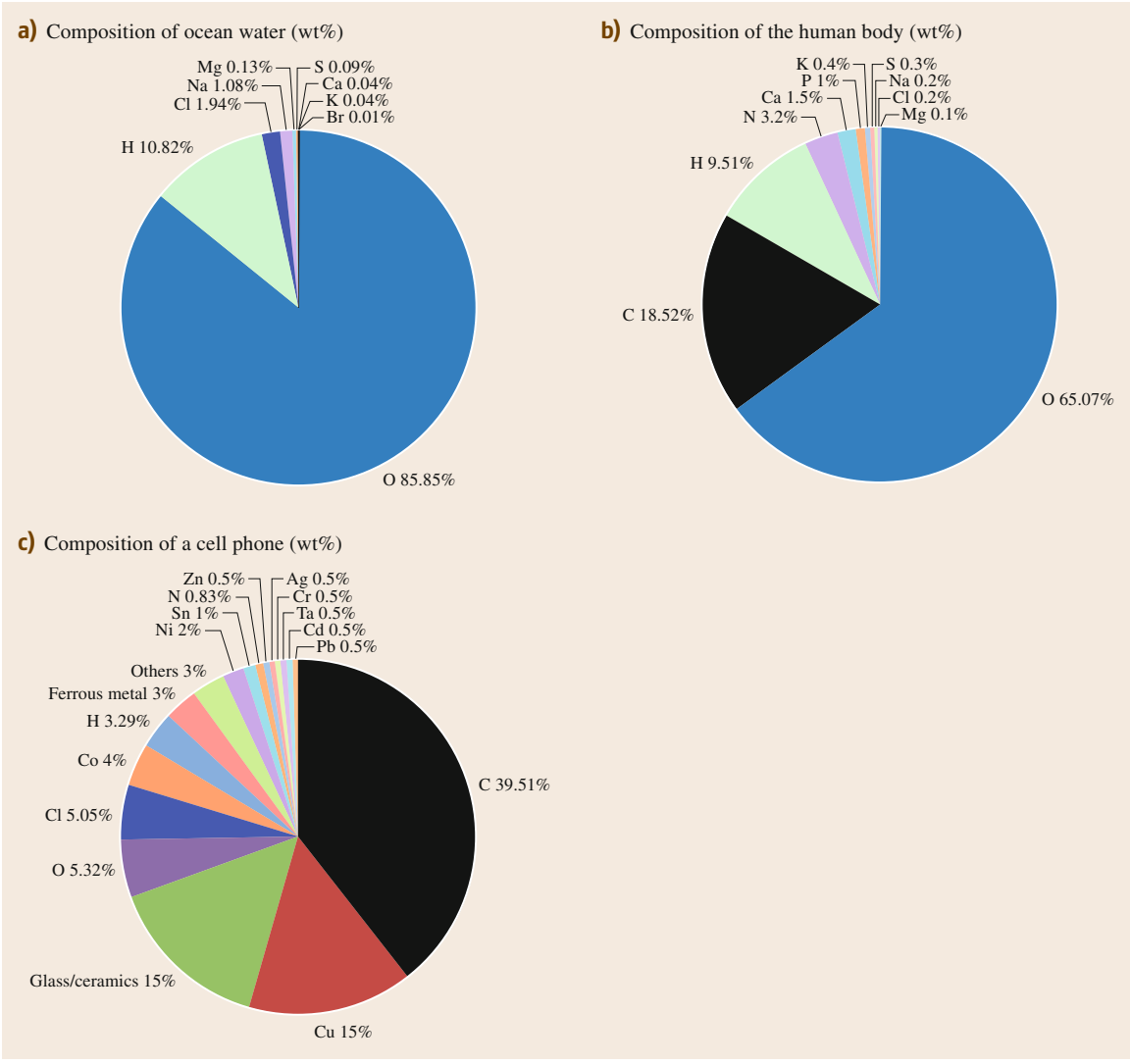

Fig. 58.12a–c The elemental compositions of ocean water, the human body, and a cell phone. The elemental composition of ocean water **(a)** is similar to the elemental composition of the human body **(b)** and very different from the composition of current engineering devices such as a cell phone **(c)**. Novel disruptive engineering approaches that learn from living nature (e.g., by using the principle *use structure rather than material*) might in the future yield marine biotechnology inspired machines and devices that need less metal and plastics, but rather achieve the needed functionality mainly from their structure (e.g., a navigation device inspired by homing sea turtles, with no dependence on satellites or metal parts)

Bioinspired material synthesis is also of increasing importance in tissue engineering, especially regarding applications for scaffolding (Chap. 53). Important factors are similar in a scaffold and in a biomineral, and relate to the chemical composition, the pore structure and architecture, the degradation rate and mechanical properties. The strength of the scaffold must be high enough to resist fragmentation before the cells synthesize their own extracellular matrix. The modulus of elasticity (i. e., the stiffness) must be high enough to resist compressive forces that would collapse the pores, must transmit stress (and strain) in the physiological range to surrounding tissues, and must prevent concentrated loading and *stress shielding*.

Also the synthesis of scaffold materials using principles and processes underlying biomineralization is of high interest. Biomineralized materials can be used as biomaterial scaffolds, either as they occur naturally or after treatment for modification. Further applications are bioinert and biodegradable materials, in medical devices, for tissue engineering, and for the coating of implants.

We have already started to initiate chemical reactions by printing reagents directly into a 3D reactionware matrix (i. e., printing of molecules and tissues with a commercially available 3D printing platform), and time will tell how far we can get with printing whole organs or machines, from basic ingredients, simple base materials, in our homes [58.67, 68].

Current biotechnology allows the usage of proteins that are important in biomineralization across species. *Natalio* and coworkers used silicatein-α (from sponges) to guide the self-assembly of calcite *spicules* similar to the spicules of calcareous sponges. As opposed to the rather brittle natural spicules, the synthetic spicules show greatly enhanced bending strength, and furthermore waveguiding properties even when they are bent [58.69].

58.6.2 Possible Future Applications of Bioinspired Material Synthesis in Engineering and Medicine – Outlook

The future of marine biotechnology is dependent upon the development of an enhanced understanding of the physical, chemical, and biological properties of marine organisms and ecosystems. Materials, structures and processes related to marine environments can provide valuable contributions to engineering, resource management, medicine, and various other fields. A prerequisite for this is the understanding of the connection between functionalities of biomineralized materials and structures from the nanoscale to the macroscale, including the effect of hierarchy and structuring. Especially interesting are models where macroscale properties can be understood from distinct functionalities at various length scales and subsequent implementation in technological processes. Efficient successful synthesis of biomineralized materials and structures, where form follows function, will pave the way towards tailored multiscale marine biotechnology, which starts at the molecular level and incorporates the hierarchical functionalities of biological materials through the length scales up to the macroscale, perfectly integrating nanotechnology and biology.

Higher-order organization by mesoscale self-assembly and transformation of hybrid nanostructures as proposed by *Cölfen* and *Mann* [58.70] is a key challenge in the design of integrated materials with advanced functions. Macromolecules and surfactants could be used to significantly increase the scope for controlled materials synthesis.

Biomimetic silica biosynthesis opens a new route to semiconductor nanofabrication [58.71, 72]. In 2008, *Jeffryes* and coworkers succeeded in metabolically inserting nanostructured Ge into a patterned silica matrix of the diatom *Pinnularia* sp. at levels ranging from 0.24 to 0.97 wt % Ge [58.72]. Embedding nanoscale germanium (Ge) into dielectric silica is of high importance for optoelectronic applications. The same group succeeded in incorporating amorphous titania into the frustule, which maintained its native structure even when local TiO_2 concentrations within the nanopores approached 60 wt %. Similar to germanium-silica nanocomposites, titanium dioxide nanocomposites are of high interest for optoelectronic, photocatalytic, and solar cell applications.

Genetic approaches to engineering hierarchical scale materials, engineered virus and protein cage architectures for biomimetic material synthesis, and bone-like materials by mineralization of hydrogels are further promising areas of research and development.

Biomineralization processes will play increasingly important roles in biology, biotechnology, medicine, chemistry, interfacial science, materials science, and nanotechnology [58.73]. With cellular and genetic controls of mineral formation, we could shape the minerals exactly as we need them. In vitro models of mineralization would allow us to understand in great detail the interactions and molecular contacts between macromolecular and mineral components. Macromolecular scaffolds for materials synthesis and organization would open up new directions in tissue engineering. A detailed understanding of the complex matrix–mineral relationships from the molecular level to the skeletal tissue, its properties and structure–function relations will open completely new approaches in engineering and medicine. For jewelery applications, we might even think about growing facetted crystals by spatially controlling growth inhibition and promoting proteins (control of crystal orientation and shape by interface engineering). Just imagine crystals grown in gels, with exactly the shape and size needed!

Ehrlich proposed bamboo corals as living bone implants [58.23, pp. 195–199]. The high content of calcium carbonate scaffolds of the commonly used coral resembles bone in terms of structure and mechanical properties. The coral exoskeleton is biocompatible, osteoconductive, and biodegradable, and allows for attachment, growth, spreading, and differentiation of bone cells. Coralline hydroxyapatite can be manufactured by hydrothermal conversion of the calcium carbonate coral skeleton.

Nature can furthermore serve as a teacher concerning the nanofabrication of crystalline materials, optical structures, nanoscale attenuators (for micro- and nanoelectromechanical systems (MEMS/NEMS)), and dynamic and hierarchical structures [58.74].

Microbe–mineral interactions that lead to sedimentary structures such as microbially induced sedimentary structures (MISS) and stromatolites, banded iron formations, cherts, sandstones, and carbonate rocks might one day be reproduced in the laboratory or in the field, and allow a more benign, environmentally friendly way of mining.

Although synthetic bone replacement materials are now widely used in orthopaedics, much research and development is still needed before we will be able to replicate both the structure as well as the exquisite mechanical properties of natural bone. Synthetic biomimetic materials fabricated according to biological principles and processes of self-assembly and self-organization are promising new approaches for a new generation of biologically and structurally related bone analogs for tissue engineering [58.75]. Current strategies for bone repair have accepted limitations. Marine organisms with naturally occurring porous structures as templates for bone growth might be an inspiration for novel ex vivo bone tissue engineering approaches [58.76].

Marine invertebrate cell cultures are currently not well established. They could serve as tools for biomineralization studies [58.77] and ultimately yield a new generation of advanced, high-performance composites required for new construction materials, new microelectronic, optoelectronic and catalytic devices, chemical and biological sensors, energy transducers and harvesters, smart medical implants, and faster and higher capacity biochips. Marine biotechnology regarding applications of bioinspired material synthesis in engineering and medicine has enormous potential.

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59. Functional Feeds in Aquaculture

Jorge Olmos Soto, José de Jesús Paniagua-Michel, Lus Lopez, Leonel Ochoa

The development of functional feed (FF) represents a great opportunity in the aquaculture industry. FF must promote the growth and health of cultivated organisms, improve their immune systems, and induce physiological benefits beyond traditional feeds. FF must be economically attractive and environmentally friendly. In this sense, the inclusion of animal products in FF formulations must be partially or totally eliminated, increasing the inclusion of alternative economical vegetable products. However, the kind and amount of vegetable protein, carbohydrates (CHO), and lipids added to formulations are of great concern for growth, health, environmental, and economical issues. Therefore, deficiencies in proteases, carbohydrolases, and lipases in shrimp/fish are a major impediment to the digestion and assimilation of vegetable sources, limiting its high-level inclusion in formulations. In this sense, the utilization of probiotic bacteria has emerged as a solution with huge applications in the aquaculture industry. Today, *Bacillus* species are the most investigated bacteria for animal probiotic development due to:

a) The versatility of their growth nutrients
b) High level of enzyme production
c) Secretion of antimicrobial compounds.

In addition, *Bacillus subtilis* (Bs) is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA), meaning that it is harmless to animal and humans. In this work, we present the potential benefits of the utilization of FF in the aquaculture industry.

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59.1 Overview

59.1.1 Importance of Aquaculture Development

Aquaculture is the fastest growing sector of the world food economy, increasing by more than 10% per year and currently accounts for more than 50% of all shrimp/fish consumed. The United Nations Food and Agriculture Organization (FAO) defines aquaculture as the farming of aquatic organisms including fish, mollusks, crustaceans, and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies that individual or corporate ownership of the stock being cultivated [59.1].

The global population's demand for aquatic food products is growing in importance; however fisheries' capture production has leveled off and most of the main fishing areas have reached their maximum potential [59.2, 3]. Because of this, fishmeal and oil prices have increased considerably in recent years (Fig. 59.1). In addition, concern has also arisen about the negative impact of fishmeal production on the ecology of global fisheries and on the environment [59.4, 5]. Aquaculture, which is probably the fastest growing food-producing sector, represents the greatest potential to meet demands for aquatic food supply. However, instead of helping to ease the crisis in wild fisheries, unsustainable aquaculture development may exacerbate the problems and create new ones, damaging our important and already-stressed coastal areas [59.6]. In order to accomplish these goals, the sector will face significant challenges to increase aquaculture profitability and, environmental and ecological sustainability.

59.1.2 Development of Functional Feed

Today, feeding represents 40–60% of the total production costs in shrimp/fish farming, making the aquaculture industry a great challenge for future generations; therefore, new feed formulations must be directed to be well-balanced and less expensive [59.7]. Use of animal protein sources, such as fish meal (FM) in shrimp/fish feeds, is expected to be considerably reduced or totally eliminated as a consequence of increasing economical, environmental and sanitary regulations [59.8–11]. Partial or complete FM substitution by vegetable protein and complex carbohydrates sources has been a major concern to the field for more than 10 years, without promising results [59.12, 13]. In this sense, functional feeds development as *foods with dietary ingredients that provide growth, health, environmental and economical benefits beyond traditional feeds* represents a great opportunity to guarantee the future of Aquaculture [59.14]. FF must be primarily supplemented with high levels of vegetable protein, complex carbohydrates and specifically selected innocuous probiotic bacteria (Fig. 59.2). A well formulated FF could transform aquaculture in a sustainable and competitive industry for new generations [59.15]. The present work evaluated a FF supplemented with high levels of soy bean meal (SBM), high levels of complex carbohydrates and a *Bacillus subtilis* probiotic strain on; *Litopennaeus vannamei, Oreochromis niloticus* and *Atractoscion nobilis*. Excellent results were obtained opening great opportunities to new functional feeds development for shrimp and fish.

59.2 Food Formulation Ingredients

Feeds considered *traditional* are mainly made from meal and fish oil ingredients and with vegetable flours as a carbohydrate source. These vegetable flours must be kept in the formulation of FF and, in fact, must be increased; besides carbohydrates, FF must contain protein and oil from vegetable origin, preferentially from soy [59.15]. Soy contains a large enough quantity of proteins and lipids to substitute both animal ingredients in shrimp and fresh-water fish diets. Additionally, *Litopenaeus vannamei* and *Oreochromis niloticus* require or tolerate protein and carbohydrate (starch) concentrations of around 30% [59.16, 17]. However, carnivorous marine fishes require or tolerate carbohydrate concentrations no greater than 10%, but they need 50% protein concentration for adequate development [59.18]. It is logical to speculate that such extreme demands are due to the fact that this kind of fish does not contain adequate types and/or amounts of carbohydrolases to digest starches; therefore their energy requirement must be obtained from protein, increasing the cost for its cultivation.

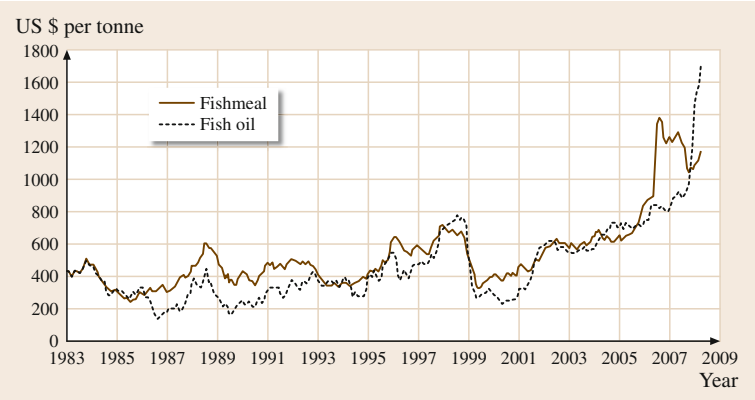

Fig. 59.1 International market price for fish oil and fish meal

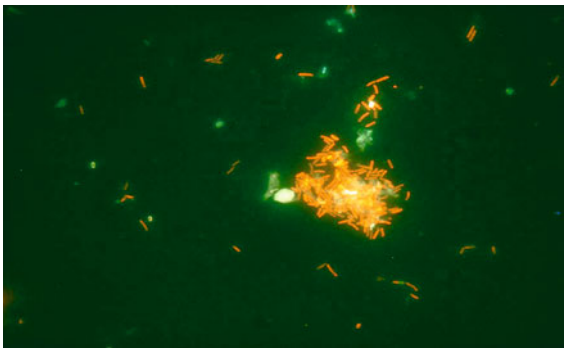

Fig. 59.2 Functional feed particle formulated with SBM, CHO's, vegetable oil and *Bacillus subtilis* multifunctional probiotic strain

On the other hand, it is important to point out that there are 20 most represented amino acids in the majority of proteins present in nature, coming from animal or vegetable origin. In this sense, the conformation structure is the greatest difference between proteins [59.19]. Furthermore, if added probiotics bacteria are capable of transforming the proteins into amino acids, there will be no problems for shrimp and/or fish to assimilate and incorporate them into their biosynthetic pathways.

Similar situations occur with carbohydrates regarding digestion. Although this is more marked in marine fishes, also fresh-water fishes and shrimp present enzymatic deficiencies in their digestion, but in smaller magnitude [59.20–22]. This is logical if we consider that aquatic animals did not evolve with starch, the main component used in formulations, therefore, they did not have the need to produce carbohydrolases to digest it, producing severe digestive, health and contamination problems. Hence, in the last 10 years, many ingredients of animal and vegetable origin have been used to substitute meal and fish oil, without the expected success [59.23]. Subsequently, with the increasing knowledge of the digestive physiology, we have tried to adapt the diets to the enzymatic capability of animals, which has not been practical or economically viable for commercial aquaculture. In this sense, our innovation has been focused on understanding the enzymatic deficiencies in animals and the benefits of vegetable ingredients, to general knowledge we will generate FF that contains enzymatic profiles adequate for digestion and assimilation of proteins, carbohydrates, and lipids, independently from the source that they come from.

59.2.1 Fish Meal and Oil

Several decades ago small pelagic fish, were the greatest economical and nutritional source of protein and oil that existed. However, the growing aquaculture and livestock activities in general have provoked an excessive demand, and consequently the over-exploitation of fish stock used for this purpose, causing an ecological imbalance and an increase in prices, making it unaffordable to producers. Worse than the elevated prices is the fact that the capture of fish has been decreasing since 1996, and human need for consumption of aquatic products has been increasing [59.3]. Reduced inclusion of fish meal and oil in the formulations is a tendency for almost a decade. Furthermore, it has been predicted that by 2020, the amount of fish meal and oil in diets used in aquatic cultures will be minimal or null [59.3]. It is evident that in times of abundance, excessive quantities of fish meal and oil were used, without having a clear idea of the real nutrient necessities for these animals. Now that we have enough knowledge, we know that if the amount of protein and oil is reduced be-

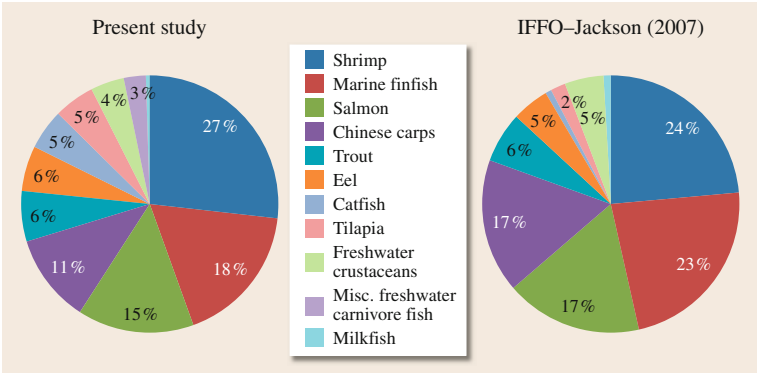

Fig. 59.3 Estimated global use of fish oil within compound aquafeeds

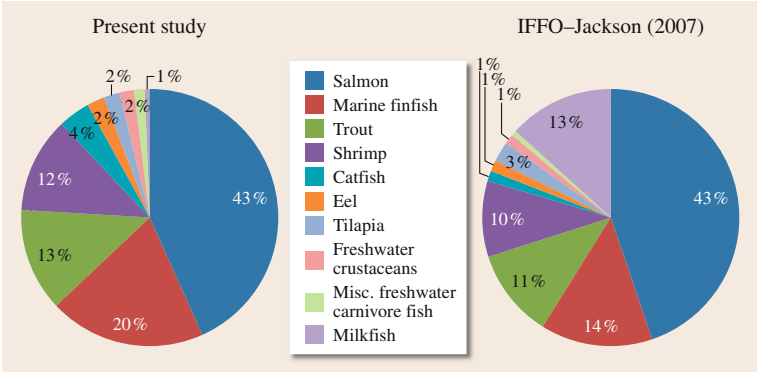

Fig. 59.4 Estimated global use of fish oil within compound aquafeeds

low the nutritional requirements, the animals will not grow and gain weight properly. From a ton of processed fish, approximately 25% of meal is obtained, containing an average of 60% digestible protein. In shrimp and marine fish feeds the fish meal is added in concentrations of about 20–50%, respectively (Fig. 59.3). Nowadays, we know that most carnivores require between 20–30% protein and similar amounts of carbohydrates. Regarding this, the aquaculture industry has had to search for viable alternative digestible and economical ingredients to satisfy the nutritional requirements of cultivated animals. Soy is the most probable candidate due to its high concentration of protein, carbohydrates, and lipids [59.24].

In addition, the limited capacity of fish and shrimp to digest starch has induced fish meal to be used by animals as a source of protein and energy, making feeding less efficient and more expensive [59.20–22, 25–29].

Regarding fish oil, the problem is even greater than with fish meal, because the amount generated by a ton of processed fish is less than 10%, and the requirements in shrimp and fish diets are between 10 and 40%, respectively (Fig. 59.4) [59.3, 18]. In fact, all the produced oil is used in aquaculture feeds, increasing the price of this ingredient even higher than fish meal (Fig. 59.1). The possible candidates to replace fish oil are vegetable oils with profiles of saturated and unsaturated fatty acids, similar to those present on fish. In this sense, the oil of some microalgae species fulfills all the requirements necessary to replace fish oil. Analyzing fatty acids, especially the unsaturated ones contained in fish oil, most come from marine phytoplankton, since the animals does not produce them de novo (Table 59.1). Presently, the technology to produce fatty acids from microalgae is well established, due to the interest in the use of these lipids to generate biodiesel. This technology can facilitate lipid production in the required volumes for aquaculture, assure its availability, and reduce its cost [59.30].

59.2.2 Soybean Meal and Oil

SBM is the most studied ingredient for the substitution of fish meal in aquatic animal feeds, because it contains high levels of proteins and carbohydrates and may represent half or less of the FM price. However, un-

Table 59.1 Fatty acids comparison between fish oil and algae oil

Fatty acids	Formula	Fish oil	Algae oil	SL
Lauric	C12:0	0.16; 0.01	0.32; 0.00	***
Myristic	C14:0	7.04; 0.35	9.09; 0.00	***
Palmitic	C16:0	17.33; 0.59	22.86; 0.00	***
t-Palmitoleic	C16:1t	0.57; 0.18	0.07; 0.00	***
Palmitoleic	C16:1	7.96; 0.40	0.21: 0.00	***
Stearic	C18:0	3.50; 0.07	0.57; 0.00	***
Elaidic	C18:1t	1.17; 0.11	0.01; 0.00	***
Oleic	C18:1(ω-9)	8.69; 0.29	1.11; 0.02	***
Vaccenic	C18:l(ω-7)	3.11; 0.11	0.13; 0.0 1	***
t-Linoleic	C18:2t	0.06; 0.02	0.06; 0.00	sl
Linoleic	C18:2(ω-6)	1.26; 0.05	0.46; 0.02	***
Arachidic	C20:0	0.20; 0.01	0.00; 0.00	***
γ-Iinolénico	C18:3(ω-6)	0.22; 0.02	0.22; 0.00	s1
α-linolenic acid	C18:3(ω-3)	1.16; 0.07	0.09; 0.00	***
Behenic	C22:0	0.05; 0.00	0.03; 0.00	***
Brassidic	C20:1t	1.84; 0.02	0.41; 0.00	***
Erucic	C22:1	0.05; 0.00	1.71; 0.00	***
Arachidonic	C20:4(ω-6)	1.14; 0.10	0.51; 0.01	***
Eicosapentaenoic	C20:5(ω-3)	16.92; 2.03	1.25; 0.01	***
Docosapentaenoic	C22:5(ω-6)	0.00; 0.00	15.44; 0.00	***
Nervonic	C24:1	0.60; 0.00	0.00; 0.00	***
Docosapentaenoic	C22:5(ω-3)	2.23; 0.00	0.22; 0.00	***
Docosahexaenoic	C22:6(ω-3)	13.44; 0.58	42.41; 0.02	***

SL: level of significance; ***: $p < 0.001$; s1: no significance: $p > 0.05$

satisfactory results have been obtained because SBM contains compounds that are toxic and antinutritional to monogastric animals, and no more than 20% can be included in shrimp/fish formulations without causing health problems [59.31, 32].

Furthermore, flour from oleaginous seeds offer annually more than 200 million metric tons (MT), soy being the most abundant and in expansion. Soybean meal has a natural resistance to oxidation and in storage it stays fungus and bacteria free, that can be pathogenic to shrimp and fish development [59.33, 34]. The protein fraction of soybean meal is between 45–48% for common meals, while the *premium* grade exceeds 50%.

Concerning its carbohydrate composition, soybean meal contains approximately 32% from which 12% are soluble sugars, distributed as follows; 4–5% sucrose, 1–2% raffinose, 3.5–4.5% stachyose, as well as small amounts of melibiose and verbascose. 20% the remaining fraction which is difficult to digest are known as anti-nutritional compounds, due to the toxic effects they causes in monogastric animals (Table 59.2) [59.34].

Soybean oil is another possible candidate to replace fish oil, since it also contains enough quantities of essential polyunsaturated fatty acids (omega 3 and 6), to satisfy aquaculture and consumer needs. The growing demand of soybean oil in homes around the world is due to its low cost and the amounts of omega 3 (linolenic 7.5%), omega 6 (linoleic 54%), and omega 9 (oleic 22%). It is important to comment that oleic acid is a monounsaturated fatty acid, which can be transformed to polyunsaturated acid by the enzymatic machinery of animals. Furthermore, soybean oil also contains saturated fatty acids such as palmitic acid (11–12%) and stearic acid (3–5%).

59.2.3 Complex and Most Used Carbohydrates

Today starch present in corn and wheat flour is the most utilized source of energy; for economical and availability reasons. Additionally, it has been shown that starch may replace protein as an energy source without de-

Table 59.2 Non-starch polysaccharide (NSP) content from cereals and soybean meal

Dry matter %	Carbohydrates	Corn	Wheat	Barley	Soybean meal
NSP		9.9	11.9	18.7	21.9
Arabinoxylans	Arabinose	2.2	2.9	2.8	2.6
	Xylose	3	4.7	5.6	1.9
	Uronic acid	0.7	0.4	–	4.8
Pectins	Mannose	0.3	0.3	0.4	1.3
	Rhamnose	–	–	–	0.3
	Galactose	0.5	0.4	0.3	4.1
β-glucans	Glucose	0.1	0.8	4.2	–
Cellulose	Glucose	2.2	2	4.3	6.2

creasing growth [59.20, 35]. However, starch is one of the less digestible ingredients in fish and crustaceans, and high-level inclusions represent an important obstacle to shrimp/fish aquaculture; the carbohydrate digestion capabilities of animals are limited to no more than 10% in carnivorous fishes and 30% in tilapia and shrimps [59.20, 21, 36]. Nevertheless, higher carbohydrate levels are included in feeds because they are the most economical and for this reason the most used ingredient in the aquaculture industry. The identification of glucosidases producing probiotic bacteria and their addition to low-cost diets as a digestive promoting supplement should lead to better assimilation, lower production cost, faster animals growth, and less pollution and diseases [59.22].

59.2.4 Probiotic Bacteria

Recently, beneficial microorganisms (probiotics) were utilized to improve nutrient assimilation and to enhance the growth performance and health of monogastric animals [59.22, 37, 38]. Additionally, pollution of shrimp effluent ponds was also treated with microorganisms [59.39]. In this sense, we have defined a probiotic as a living microbial supplement that:

a) Positively affects hosts by modifying the host-associated microbial community and immune system.
b) Secretes a variety of enzymes to improve feed degradation, enhancing its assimilation.
c) Improves the quality of environmental parameters [59.14].

Bacillus probiotic strains have been used to improve growth performance, digestive enzyme activity, and immune response, with good results being obtained in all the parameters measured [59.22, 40–42]. The genus *Bacillus* constitutes a diverse group of rod-shaped, Gram-positive bacteria, characterized by their ability to produce a robust spore. Most *Bacillus* species are not harmful to mammals, including humans, and are commercially important as producers of a high and diverse amount of secondary metabolites, antibiotics, heterologous proteins, fine chemicals, and enzymes [59.43–47]. *Bacillus* enzymes are very efficient in breaking down a large variety of proteins, carbohydrates, and lipids into smaller units. *Bacillus* species grow efficiently with very low-cost carbon and nitrogen sources [59.43]. They also degrade organic accumulated debris in ponds of shrimp cultures [59.14, 48–50]. Taking into account the advantageous characteristics of *Bacillus* strains, these bacteria are good candidates for consideration as probiotics in diets of crustaceans and fish [59.14, 15]. Additionally, *B. subtilis* is generally recognized as safe (GRAS) by the FDA, meaning that this bacterium is not harmful to animals or humans (Fig. 59.5).

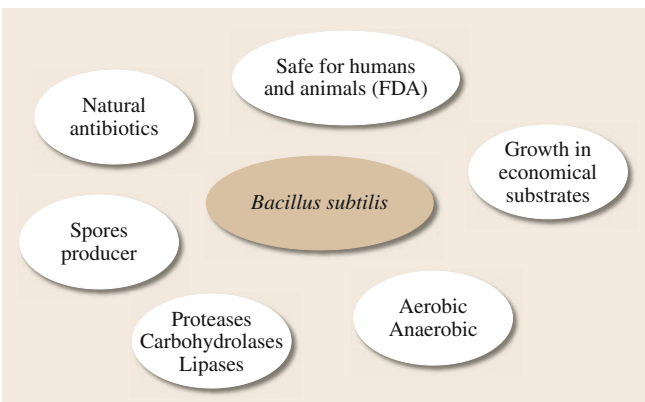

Fig. 59.5 *Bacillus subtilis* multifunctional probiotic capacities

59.3 Conventional Feeds Versus Functional Feeds

Currently, the cultivation of shrimp and fish is very important, socially and economically speaking, being *Litopenaeus vannamei* and *Oreochromis niloticus* the most produced around the world. However, apart from the questions about which culture is the most important or which generates the greatest economical dividends, the diets utilized for the aquaculture of any animal are developed with mainly three ingredients:

1) Fish meal, because it contains high concentrations of easily digestible protein, which promotes optimal growth and weight gain in cultivated animals.
2) Carbohydrates that come from vegetable flour, specially those present in corn and wheat starches, which give the necessary energy for vital functioning.
3) Lipids that are obtained as a by-product from the processing of fish meal and are the structural base for the production of hormones and other important bioactive macromolecules.

However the use of fish meal and oil in the production of feeds is limited due to costs, principally since these ingredients come from fish that are at risk due to unmeasured exploitation [59.3, 51]. In this sense, the production of feeds that does not depend on the utilization of fish products has been widely investigated during the last 10 years, and there has been experimentation with a wide range of animal and vegetable ingredients [59.52]. Unfortunately, up to now even the high cost of fish products and by-products, feeds are for aquaculture still being formulated with them. This means that alternative formulations that can compete with the benefits of survival, weight gain, and growth that fish meal and oil ingredients generate has not yet been found. Alternative feeds produced and sold in a commercial manner as a consequence of the high prices, are mixtures reduced in fish meal and oil content and increased in the amount of vegetable protein and oils. This has brought as a consequence low food conversion factors (FCF), poor growth, intoxication and health status detriment. Also, the use of vegetable products has generated an increase in environmental contamination due to the low digestibility and, consequently, pathogen proliferation and massive death of the cultured animals.

For these reasons, our research group has been trying for more than 10 years to understand the most important strengths and weaknesses of cultivated animals [59.14, 19]. Additionally, alternative vegetable ingredients economically viable to substitute fish meal and oil have been investigated [59.15, 22]. Also, the animal-environment-pathogen relationship had been studied to generate innovations that can fully satisfy aquaculture necessities [59.53, 54]. After all this time, and after having experimented with crustacean and fish cultivations, we believe that we have found healthy formulas that are sustainable with the environment and that are economically viable, that we have named *functional feed*.

According to our concept, FF must be formulated with little or no fish meal and oil, instead vegetable ingredients increase is recommended; this will decrease its price and ammonium pond contamination considerably. Furthermore, it will relax the fish population and environmental stress. However, vegetable ingredients are not recommended by most nutritionists, due to their low digestibility, contents of highly toxic compounds, and mortality increase. We believe that FF must contain high levels of vegetable flour and oils; rich in proteins, carbohydrates, and lipids. Micronutrients must be supplemented for the optimal development of the animals. In this sense and in our particular point of view, the most important components of FF are probiotic bacteria, since they produce enzymes with several capacities to amplify the digestive spectrum and help the animals to assimilate the ingredients, even if they come from vegetable origin. Likewise, the enzymes must have the capacity to degrade the toxic compounds contained in vegetable flour [59.55]. The enzymatic diversity and activity of proteases, carbohydrolases, and lipases must be maintained, even in adverse physicochemical or environmental conditions such as temperature, pH, oxygenation, etc.; so the enzymes will not be inhibited in the digestive system of animals or in cultivation ponds once they are excreted. This also will help with the effluent bio-remediation. Probiotics must be harmless to animals and humans, but must have the capacity to selectively inhibit pathogen growth.

59.4 Aquaculture Regulations

Recently, the massive development of aquaculture has promoted the establishment of sanitary and environmental regulations in most countries where this activity is practiced. This is particularly due to the spread of diseases and environmental contamination generated by poor cultivation management (Fig. 59.6). It is important to emphasize that the majority of these regulations have been generated in little or in great measurement due to the feed; starting with the ingredients and concentrations utilized in them, up to the amount of feed administered during cultivation. Furthermore, the time period of water exchange in ponds is also a consequence of the feed and the enzymatic limitation of animals must digest and assimilate them. Therefore, poorly formulated feeds can be the main reason of environmental deterioration due to the contamination generated, resulting in the spread of diseases and death of cultivated animals (Fig. 59.7).

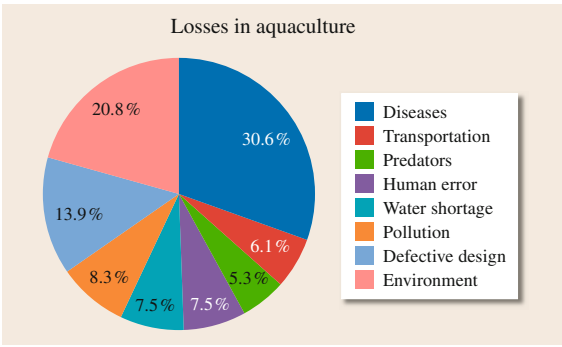

Fig. 59.6 Schematic representation of loss percentages in aquaculture

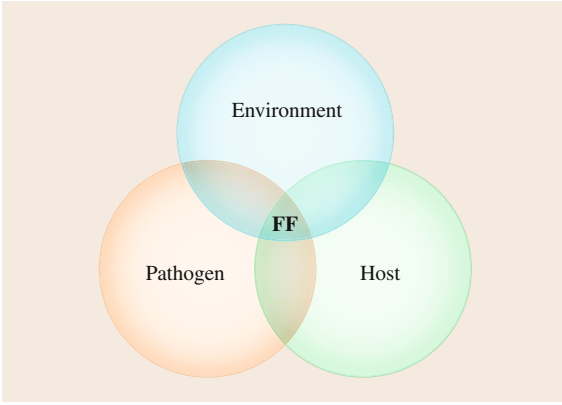

Fig. 59.7 Illustration of the balance involved in the state of health and disease of a cultivated organism with or without FF

59.4.1 Sanitary Regulations

The formulation of feeds with animal origin ingredients can be a factor in the spread of viral as well as bacterial diseases, that can be contained in animal-based ingredients. The feed utilized in aquaculture is sometimes distributed around the world and can generate global pandemics and epizooties, like the ones produced by *Vibrio* genera and the white spot syndrome virus [59.56, 57]. Therefore, animal products and by-products used for the formulation of feed must be certified by the sanitary authorities of each country to verify that it does not contain pathogen microorganisms above the concentrations permitted by the official norms. This will assure its innocuousness but raises the production costs of these ingredients, and thus, the cost of the formulated feed. Furthermore, cysts and spores are forms of life that are resistant to extreme conditions presented by some pathogenic bacteria [59.58]. This characteristic allows them to survive the conditions used in the preparation of products and by-products of animal origin, only to germinate once the product is in contact with water and the cultivated animal. In this sense, the prevention of diseases has also generated an unmeasured use of antibiotics, which has induced the development of pathogenic bacteria stock resistant to a great variety of these compounds. It is important to keep in mind that human beings can be susceptible to the antibiotics used in aquaculture, as well as to pathogens present in cultivated animals, especially when that person has an immune compromised system. *Vibrio parahemoliticus* is an opportunistic pathogen that can cause death to individuals with problems such as liver cirrhosis, if they consume contaminated oysters. The transmission of the disease known as Mad Cow Disease and other diseases that come from poultry stock are examples of the importance of the innocuousness and sanitary regulations that have to be implemented in products from animal origin [59.59]. In this sense, the use of vegetable ingredients in FF can eliminate the pathogen transference contained in animal products, as well as the unmeasured use of antibiotics.

59.4.2 Environmental Regulations

Environmental regulations with respect to aquaculture, even when they are stipulated in the official norms and are very clear regarding the compounds and quantities that can be thrown into rivers or seas; in most of the countries where massive production of fishes and crustaceans is being practiced, are not being complied. Therefore, high concentrations of organic contaminant compounds are indiscriminately thrown into rivers and seas each year, generating constant contamination and deterioration of the environment and ecosystem that surrounds farms. Unfortunately, most countries where aquaculture is being practiced, even with the existence of official norms, do not count with mechanisms to carry out those norms. In addition, the formulation and usage of FF of easy digestion and assimilation by cultivated animals will generate low concentrations of organic waste, and can be a direct mechanism to reduce contamination of ponds and waste that is thrown into rivers and seas [59.14, 60]. In this sense, the probiotics used in FF must also have the capacity to degrade the contaminants generated in farms.

59.5 Functional Feeds in Aquaculture

Autochthonous bacteria of crustaceans and fishes can be recommended as probiotics, only if they have the capacity of secreting a wide variety of enzymes that will allow them to take adequate advantage of different sources of protein, carbohydrates, and lipids. Also, they must be capable of producing antimicrobial compounds to inhibit the growth of different pathogens, without having any effect on beneficial bacterial flora. They must also be able of surviving sudden nutritional and environmental changes, such as temperature, pH, oxygenation, etc. Furthermore, autochthonous strains are, in general, bacteria that adhere to the cells of the animal's intestine and, therefore, are circumscribed to scarcely variable nutritional and environmental conditions. For this reason, most of them do not have the capacities mentioned before. On the other hand, free life strains like *Bacillus subtilis* have all these capacities, hence it can be considered as a potential probiotic. Also, this bacterium is classified by the FDA as GRAS, which means that is not a pathogen to animals or humans [59.22].

59.5.1 Advantages and Disadvantages of Adhesion

The advantage of adhesion is that probiotics should only be used a few times until they have colonized the digestive tract, which would reduce the cost and would increase the profitability of producers. Otherwise, the capacity of adhesion means that the strains are adapted to live within the host, which assures a high percentage of interaction, as long as the conditions of the environment are satisfactory. Unfortunately, most pathogens have a highly developed capacity of adhesion as an invasive mechanism. In this sense, it is almost a general rule that when cultivation is in its initial stage, the bacterial flora present is composed by certain groups. However, once cultivation advances and conditions deteriorate, more capable bacterial groups surge, taking advantage of the reigning nutritional and environmental conditions. Most proliferating bacteria are opportunistic pathogens with a high capacity of adhesion. Therefore, if we isolate bacterial strains from aquatic cultivated organisms with the purpose of utilizing them as probiotics, it is recommended to isolate them from the first stages of the cultivation or from finalized healthy and high-productivity cultures.

In the late stages of cultivation, the possibility of the isolation of opportunistic pathogenic bacteria rises [59.54, 60]. Lately, it has been very complicated to keep healthy cultures with economically income, mainly due to food expenses and the great amount of energy used for water exchange in the required percentages. This is one of the reason for the huge proliferation of pathogens and the consequent mortality in cultures. Generally, when an opportunistic pathogenic bacterium has adequate conditions it takes advantage of it to proliferate.

If we take probiotics from humans as an example, most of them are *Lactobacillus* species with adhesion capacity to epithelial intestinal cells, which means that a daily dose must be taken to maintain their active growth and probiotic capacity. In this sense, we know for a fact that up to this date there is no commercial strain that is taken once and maintains its activity during the rest of the host life. For example, daily or almost daily consumption of *Lactobacillus*-based products is needed to keep its positive effects; this is due

to the feeding conditions and stress factors that restrict the proliferation of probiotic strains on cultivated animals. Yet, it is more complicated to maintain probiotic strains that are permanently in contact with a water flow in the digestive tract, as is the case for fish and crustaceans. If we add the fact that most of enzymes and antimicrobial compounds are produced in a short window of the stationary phase from most bacteria, the protection due to the production of these compounds will also be restricted to when the probiotic remains inside the animal. Additionally, due to its evolutionary environment the autochthonous bacteria of crustaceans and fish have never been in contact with carbohydrates of terrestrial origin such as starch, which is the main ingredient in used formulations. Therefore, the physiological and economical conditions to utilize an autochthonous probiotic strain added to the epithelium cells, do not appear, in theory, to have advantages with respect to those free living bacteria. Otherwise, if we have a probiotic that adheres well to epithelium cells, we need to take into consideration that the elimination of these bacteria from the digestive tract of the animals is more complicated. In this sense, if the probiotic is not recognized as safe and is not approved by the FDA, it can generate problems when the time comes to commercialize the animal for human consumption. This also happens with unauthorized antibiotics and hormones.

Even though the diverse marine and aquatic systems represent 85% of all forms of life on earth, it is important to point out that if we are going to use FF especially formulated with nutrients of land origin, we should select probiotic strains with the capacity to digest them without any problem. Therefore, if we develop free living bacteria probiotics strains accustomed to surviving in extreme conditions, with an abundance as well as limitations of nutrients, salts, temperature, and variable pH, they will have a greater adaptive response to sudden changes in the culture. Moreover, if these strains count with the capacity of enzyme secretion to degrade any kind of nutrient, as well as to produce compounds to solely kill pathogenic microorganisms and do not present toxic effects in animals and humans, these strains can be considered as a potential probiotics. The only inconvenience is that good fortune and capacity is needed to isolate and characterize them adequately. Additionally, continuous probiotic supplementation must be carried out to obtain an effective and therapeutic dose, and consequently, maintain its probiotic properties. In this sense, FF are the adequate vehicle to introduce probiotics into the animal, considering stability and price.

59.6 Results Obtained in Crustaceans and Fish Using Functional Feeds

59.6.1 Functional and Commercial Feed Proximal Composition Used in *Litopenaeus vannamei* and *Oreochromis niloticus*

Table 59.3 shows the proximal composition of the ingredients in diets utilized for white shrimp and tilapia cultivation. The formulated FF (basal diet + probiotic), was the same for *L. vannamei* as for tilapia because the nutritional requirements are similar for both animals. In this sense, analyzing the commercial diet for tilapia we could see its similarity regarding the formulated basal diet. The origin of the ingredients contained in the commercial diet for tilapia are not known to us, although the basal diet ingredients were reported previously [59.14]. It is important to point out that the protein in the basal diet comes from 100% soybean meal. In both diets, the carbohydrate concentration is around 50%, which could originate health problems in shrimp, since this amount surpasses the nutritional requirements of this ingredient [59.61]. This is not the case for tilapia because it is an omnivore with the capacity to tolerate high concentrations of carbohydrates.

The commercial diet for shrimp has 10% more protein than the basal diet formulated by our research group. The origins of the ingredients of the commercial diet for shrimp are unknown to us, although

Table 59.3 Functional and commercial feed proximal composition used in *Litopenaeus vannamei* and *Oreochromis niloticus*

Items %	Basal + P FF	Commercial feed to *L. vannamei*	Commercial feed to *O. niloticus*
Crude protein	27.41	36.39	32
Total lipid	6.46	3.98	5
Carbohydrates	49.50	38.42	50
Moisture	11.34	9.88	5
Ash	5.29	11.33	8
Probiotic (P)	1 kg t^{-1}	–	–

it is a fact that the protein used is of animal origin. The commercial diet for shrimp contains 10% less carbohydrates than the basal diet, which is the closest to what is required and/or tolerated for this crustacean [59.61].

59.6.2 Basal and Functional Feed Proximal Composition Used in *Atractoscion nobilis*

Table 59.4 shows the basal diet containing the required ingredients and concentrations for *Atractoscion nobilis*, a strictly carnivorous fish. From the diets CHO10 to CHO22, the corresponding carbohydrate percentage was increased and the lipid concentrations reduced, as indicated in the table. The probiotic was added to all the CHO diets in concentrations of $1\,kg\,t^{-1}$. It has been widely reported that *A. nobilis* presents a health problem when the carbohydrates contained in the diet surpass 10%. Additionally, the development for marine fishes is limited when the lipid concentrations in the diet is reduced. Moreover, it is most important to maintain the high levels of fish protein in this species, which is why this ingredient was maintained at 50% in all the formulated diets.

59.6.3 Functional Feed Effects on Survival and Growth Performance of *L. vannamei*, *O. niloticus* and *A. nobilis*

Once the animals were fed in controlled conditions, biometry studies were carried out to determine the dietary effects on growth, survival, etc. In experiments with *Litopenaeus vannamei* (Fig. 59.8), Table 59.5 shows that the parameters analyzed improved considerably in animals fed with the basal diet containing the probiotic (FF), with respect to the commercial diet and the basal diet without the probiotic. The food conversion ratio (FCR) was the parameter showing the best result, which confirms that *L. vannamei* has difficulties digesting vegetable sources of proteins and high levels of CHO. These results also confirm that the probiotic is the most important ingredient in the formulation of FF, since the inclusions of the bacteria allows the use of alternative, economical, healthy and available nutrients [59.14, 22].

Table 59.4 Basal and functional feed proximal composition used in *Atractoscion nobilis*

Items %	Basal feed	CHO 10 FF	CHO 14 FF	CHO 18 FF	CHO 22 FF
Crude protein	56.5	56.1	56.0	54.2	55.9
Total lipid	19.3	18.3	15.0	15.0	14.3
Carbohydrates (CHO)	10.3	10.5	13.9	18.8	21.4
Ash	9.8	10.5	10.2	10.8	8.9
Energy Cal g^{-1}	5076	5102	5037	5071	5066
Probiotic (P)	–	$1\,kg\,t^{-1}$	$1\,kg\,t^{-1}$	$1\,kg\,t^{-1}$	$1\,kg\,t^{-1}$

Fig. 59.8 *Litopenaeus vannamei* Pacific Ocean white shrimp

Fig. 59.9 Effect of FF on *Oreochromis niloticus* development. From *left* to *right*: animals fed with basal diet + probiotic (FF), commercial diet + probiotic, commercial diet, and basal diet

Table 59.5 FF effects on survival and growth performance in *Litopenaeus vanammei*

Items	Basal feed	Basal + P FF	Commercial feed
Initial weight (g)	5.98 ± 0.22^a	5.96 ± 0.20^a	6.06 ± 0.18^a
Final weight (g)	9.48 ± 0.13^c	10.71 ± 0.11^a	10.38 ± 0.16^b
Daily weight gain ($g\,d^{-1}$)	0.125 ± 0.003^c	0.169 ± 0.003^a	0.154 ± 0.001^b
Food conversion ratio (FCR)	2.49 ± 0.15^c	1.54 ± 0.07^a	2.06 ± 0.22^b
Survival (%)	96.67 ± 3.87^a	100^a	96.67 ± 3.87^a
Probiotic (P)		$1\,kg\,t^{-1}$	

Means in the same row with different superscripts are significantly different ($P < 0.05$)

Table 59.6 shows that the values obtained for *O. niloticus* using the basal plus the probiotic bacteria (FF), where almost twofold greater than the results obtained using the basal and the commercial diet, with the exception of the survival rates, which were similar in the three assays. These results mean that the basal diet formulated for this experiment and the commercial diet are very similar with respect to the energy content. Moreover, it also means that even though *O. niloticus* is an omnivore it shows certain problems digesting vegetable protein and high concentrations of carbohydrates. When the probiotic was added to the vegetable diet (basal diet), all parameters improved almost 100% (Fig. 59.9). The biomass and its size doubled in the same period of time and with the same amount of food, which opens great possibilities and hopes for the development of healthy, sustainable, and profitable feeds in the immediate future.

Table 59.6 FF effects on survival and growth performance in *Oreochromis niloticus*

Items	Basal feed	Basal + P FF	Commercial feed
Initial weight (g)	3.46^a	3.90^c	3.63^a
Final weight (g)	5.40^a	9.40^c	6.74^b
Absolute growth (g)	1.94^a	5.50^c	3.11^b
Biomass gained (g)	55^a	165^c	81^b
Specific growth rate (SGR)	3.16^a	8.12^c	5.40^b
Efficiency feed consumption	0.21^a	0.49^c	0.30^b
Food conversion ratio (FCR)	4.71^a	2.04^c	3.37^b
Protein efficiency ratio (PER)	4.95^a	10.78^c	6.92^b
Survival (%)	100^a	100^a	90^b
Probiotic (P)		$1\,kg\,t^{-1}$	

Means in the same row with different superscripts are significantly different ($P < 0.05$)

With respect to the results obtained in *Atractoscion nobilis*, the CHO22 diet tripled its weight and duplicated its size in comparison with the basal diet (Fig. 59.10). According to studies on this marine

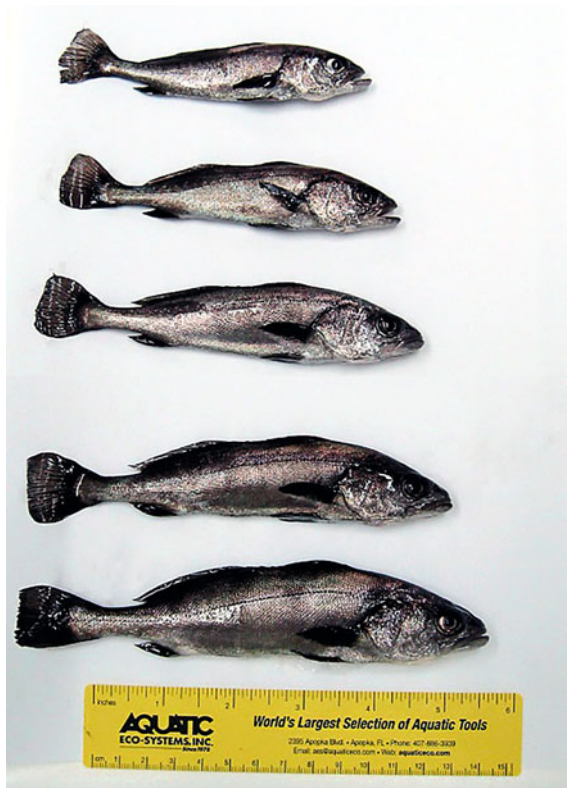

Fig. 59.10 Effect of FF on *Atractoscion nobilis* development. From *top* to *bottom*: animals fed with basal diet, CHO10, CHO14, CHO18, and CHO22 FF

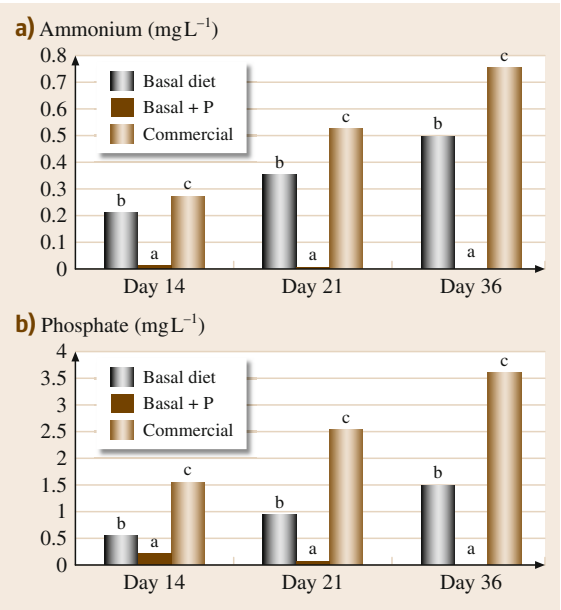

Fig. 59.11a,b FF environmental parameter reduction. **(a)** Ammonium evaluation and **(b)** phosphate evaluation

and strictly carnivorous species, it tolerates a maximum of 10% carbohydrates in its diet without having health problems. However, the results in Table 59.7 show that it is possible to increase the carbohydrate levels up to 22% without producing toxic effects in the animal. These results also demonstrate that if these animals have sources of energy that are easier to assimilate than protein, they will prefer them because the energy wasted to metabolize them is less significant [59.19]. In this sense, the CHO22 FF diet was very successful in all the parameters evaluated, due to this diet, the animal used protein to grow and carbohydrates as a source of energy. Because the weight was tripled using FF, regarding the basal diet, it is probable that the extra energy from digested-assimilated carbohydrates was canalized to generate protein, as happened in assays carried out with *L. vannamei* [59.14]. The interconnection of the biosynthetic pathways gives the possibility to redirect the carbon flow and send it to where it is required most, as long as the system is in balance between the nutrient entry and the output waste discarded from the animal cells. All this shows, as demonstrated in this experiment and in the one with tilapia and shrimp, that it is possible to use complex sources of proteins, carbohydrates, and lipids, when probiotic bacteria are included to carry out processes to control the liberation of nutrients independently of their origin.

According to the results obtained in this study (Tables 59.5–59.7), the FF effects were more impressive in fish for the basic and simple reason that fish eat the pellet complete and shrimp chew their food. This small but great difference induces fish to obtain a complete dose of FF and its consequent benefits. This is not the same for shrimp, which because of the way they feed, only take advantage of half or less of the FF.

Table 59.7 Effect of FF on survival and growth performance of *Atractoscion nobilis*

Items	Basal feed	CHO 10 FF	CHO 14 FF	CHO 18 FF	CHO 22 FF
Initial weight (g)	9.63 ± 0.07	9.52 ± 0.04	9.50 ± 0.20	9.37 ± 0.20	9.51 ± 0.11
Final weight (g)	18.26 ± 1.04^{ed}	21.37 ± 0.15^{dc}	21.67 ± 0.59^{c}	27.45 ± 1.88^{b}	33.77 ± 1.47^{a}
Gained weight (g)	8.63 ± 1.02^{e}	11.85 ± 0.11^{dc}	12.16 ± 0.78^{c}	18.08 ± 1.84^{b}	24.26 ± 1.38^{a}
Initial length (cm)	9.02 ± 0.06	9.03 ± 0.04	8.87 ± 0.16	8.99 ± 0.09	9.04 ± 0.05
Final length (cm)	11.66 ± 0.05^{ed}	11.90 ± 0.02^{dc}	12.12 ± 0.10^{c}	12.91 ± 0.22^{b}	13.65 ± 0.13^{a}
Gained length (cm)	2.64 ± 0.07^{ed}	2.88 ± 0.02^{d}	3.26 ± 0.18^{c}	3.92 ± 0.16^{b}	4.62 ± 0.08^{a}
Specific Growth Rate (SGR)	1.06 ± 0.09^{e}	1.35 ± 0.01^{dc}	1.37 ± 0.08^{c}	1.79 ± 0.11^{b}	2.11 ± 0.06^{a}
Efficiency of feed conversion (EFC)	0.62 ± 0.07^{ed}	0.81 ± 0.01^{c}	0.74 ± 0.05^{dc}	1.00 ± 0.10^{ba}	1.17 ± 0.07^{a}
Protein efficiency ratio (PER)	1.09 ± 0.13^{e}	1.44 ± 0.01^{d}	2.36 ± 0.06^{a}	1.85 ± 0.19^{cb}	2.10 ± 0.12^{ba}
Survival %	88.0 ± 0.88	98.0 ± 0.00	90.0 ± 0.58	93.0 ± 0.58	100.0 ± 0.00
Probiotic (P)	–	$1\ kg\ t^{-1}$	$1\ kg\ t^{-1}$	$1\ kg\ t^{-1}$	$1\ kg\ t^{-1}$

Means in the same row with different superscripts are significantly different ($P < 0.05$)

Table 59.8 Functional feed effects increasing energy sources and immunology parameters in *Litopenaeus vannamei* and *Atractoscion nobilis*

Parameters	*L. vannamei*				*A. nobilis*
	Basal feed	Basal + P FF	Commercial feed	Basal feed	CHO 22 FF
Glucose (mmol L^{-1})	0.470 ± 0.02^b	0.675 ± 0.02^a	0.452 ± 0.03^b	80 ± 3.7^b	168 ± 3.6^a
Lactate (mmol L^{-1})	0.261 ± 0.02^b	$0.385^a \pm 0.03^a$	0.249 ± 0.03^b		
Total cholesterol (mmol L^{-1})	0.134 ± 0.07^b	0.323 ± 0.08^a	0.163 ± 0.03^b		
Haemocytes (cell mL^{-1})	$9.41 \times 10^6 \pm 0.15^b$	$2.02 \times 10^7 \pm 0.08^a$	$9.63 \times 10^6 \pm 0.12^b$		
Red cells (cell mL^{-1})				1.5 ± 0.3^b	2.0 ± 0.3^a
Hb (G dL^{-1})				12.5 ± 1.8^b	14.3 ± 2.3^a

Means in the same row with different superscripts are significantly different ($P < 0.05$)

Table 59.9 FF effects on stress tolerance in *Litopenaeus vannamei*

Parameters	Survival %		
	Basal feed	Basal + P FF	Commercial feed
Stress tolerance to high ammonium concentrations	0.0^b	83.33 ± 0.11^b	0.0^b
Stress tolerance to low oxygen concentrations	66.67 ± 2.22^b	91.67 ± 3.20^a	41.67 ± 4.18^c

Means in the same row with different superscripts are significantly different ($P < 0.05$)

59.6.4 Functional Feed Effects on *L. vannamei* and *A. nobilis* – Health Status

The capacity of the probiotic used to inhibit the growth of the pathogens due to the production of antimicrobial compounds was demonstrated previously [59.15]. Additionally, the improvement in the health status of the animals was evidenced with the increase of blood cells and energy sources like glucose and lactate; these results were obtained exclusively in treatments where FF had been used (Table 59.8). This means that animals assimilate nutrients in greater amounts, due to the increase in the digestibility processes that was made possible by the probiotic enzymes. Because of the increase in weight and size, it was evident that if the animals have a greater nutrient concentration in the bloodstream, a greater generation of biomass and energy will be obtained in animals that consume FF. Conventional nutrition classifies the increase in glucose, lactose, etc. as stress indicators in animals. However, if we consider that the animals are in perfect shape, are bigger, weigh more, and are more vigorous, it is logical to translate these increases in blood parameters as a direct measurement of health status improvement, rather than a state of stress in animals consuming FF. A greater number of blood parameters evaluated in *O. niloticus* and *A. nobilis* will be shown in studies published in specialized journals in the near future.

59.6.5 Functional Feed Effects on Environmental Parameters in *L. vannamei*

The environment where cultivated animals develop is as important as their nutrition and/or state of health, since the three parameters are in close relationship with each other (Fig. 59.7). Because the conditions of the environment are directly related with the quality of food, our conception of a FF take into account the decrease in water contamination; due to the characteristics of the food with vegetable ingredients, and/or the intrinsic capacity of bioremediation by the probiotic added to the FF. In this sense, all the contaminants evaluated in the culture shrimp ponds that used FF showed a considerable decrease in water concentration, in comparison to the assays where no FF were used (Fig. 59.11). Regarding the results obtained with the basal diet without the probiotic, this is due in part by the nature of the food, which is less pollutant than the commercial diet [59.14, 22].

59.6.6 Functional Feed Effects on Stress Tolerance in *L. vannamei*

Ammonia stress experiments carried out by *Olmos* et al. [59.14] showed that shrimp fed with FF tolerated well high levels of ammonia throughout the 24 h of the experiment (Table 59.9). Taking into account that basal and commercial diet do not presented any survival rate and that glucose and lactate were higher and statistically different in animals treated with FF (Table 59.8), we can hypothesize that:

a) Shrimp survived due a higher energy level availability on the circulatory system, giving them a greater response capacity,
b) by a direct ammonia conversion-transformation reaction accomplished by the probiotic bacteria [59.14].

On the other hand, even when oxygen depletion stress-experiments do not presented such drastic mortality results as the one presented by the ammonia experiments, 35 and 60% shrimp mortality was occurred with the use of basal and commercial diets, with respect to 10% mortality showed by the FF treated animals (Table 59.9). These results clearly indicate that:

a) Shrimp fed with FF have the capacity to tolerate well oxygen depletion.
b) The basal diet without the probiotic bacteria, in comparison with the commercial diet, presented less oxygen consumption by itself or by less ammonia contamination [59.14].

The results obtained in the stress experiments indicate that FF utilization in shrimp/fish aquaculture would help them to tolerate extreme variation of environmental parameters in a better way than commercial diets do.

59.7 Conclusions

The benefits of FF are the following:

1. FF promotes the digestion of ingredients and assimilation of nutrients, improving growth, weight gain, and FCR.
2. FF increases the health *status* of animals, eliminating pathogens and stimulating the immune system.
3. FF improves the quality of water in culture ponds, making them environmentally sustainable.
4. FF eliminates ecological stress in seas by using economical and abundant vegetable ingredients.
5. FF are cheaper than traditional feeds, which improves the competitiveness and profitability of the producers.
6. There is no alternative than the development of FF to assure the future of aquaculture.
7. The *movie star* is the probiotic, and therefore we need to isolate a multifunctional strain.
8. *Bacillus subtilis* is an ideal probiotic bacteria.

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60. Mussel-Derived Bioadhesives

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Marine mussels use MAPs (MAPs) for their adhesion. MAPs have fascinating properties, including strong adhesion to various material substrates, water displacement, biocompatibility, and controlled biodegradability. In this work, among six types of MAPs, including fp-1–fp-6, biosynthetic constructs of MAPs are considered; hybrid type recombinant MAPs are designed to improve productivity and purification. Hybrid recombinant fp-151, which comprises six decapeptide repeats of fp-1 at both N and C-termini of fp-5, was successfully overexpressed in a bacterial system, showing approximately ≈ 1 g/L production yield in a pilot scale fed-batch bioreactor culture. For industrial applications, it was attempted to use MAPs in tissue engineering fields as coating extracellular matrix (ECM) through surface modification and constructing nanofibrous scaffolds. As a result, the MAP-based coating strategy could be generally applied for facile and efficient surface modification of negatively charged bioactive molecules for tissue engineering. The use of MAP-based nanofibers could provide bioactive peptides efficiently onto the scaffold surface, enhancing the cell attachment and proliferation on the nanofibers fabricated using RGD peptide-conjugated MAPs compared with bare polycaprolactone (PCL) polymer nanofibers as well having a four times higher mechanical strength. Also, easy fabrication through blending with diverse types of synthetic polymers and significant bone regeneration was observed. In addition, there was a trial for utilization of MAPs in pharmaceutics, cosmetics, and food industries with encapsulating active molecules such as chemical drugs, proteins, cells, and flavor ingredients through a complex coacervation technique based on MAPs.

Finally, MAPs have been suggested as immobilization material for biosensors due to their unique adhesive property on various materials, including biomolecules, glass, polymers, and metals. MAP was genetically fused with C-termini of the BC domain of protein A and can be successfully used as a functional material for the development of various immunosensors and immunoassays. The MAP-based whole cell biosensor can be successfully used for industrial applications, including environmental monitoring of chemicals and heavy metals, and food screening. Collectively, MAPs might be a useful and applicable biomaterial in diverse industrial fields due to their superior adhesion properties (even in water), biocompatibility, and controlled biodegradable properties.

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60.1 Marine Mussel Adhesion

Marine-fouling invertebrates such as mussels, barnacles, and sandcastle worms endure continuous mechanical stresses due to the tide, buoyancy, and drag through a strong attachment to the marine substratum with their specialized physical and chemical underwater adhesives [60.1–3]. These marine adhesions have given us many unveiled lessons to learn; especially because the materials for attachment used by these marine organisms have revealed their potential, the investigation of the underwater adhesives from these marine organisms has proceeded with much effort to develop real-world underwater adhesives. The adhesion of mussels is one of the best model systems. Marine mussels use mussel adhesive proteins (MAPs) for their adhesion, and MAPs have fascinating properties including strong adhesion to various material substrates, water displacement, biocompatibility, and controlled biodegradability [60.5]. Importantly, MAPs contain a high level of 3,4-dihydroxy-L-phenylalanine (DOPA), which is transformed by the hydroxylation of tyrosine residue via post-translational modification. DOPA is regarded as a key factor for strong adhesion [60.6]. DOPA has the ability to form hydrogen bonds and complexes with metal ions and oxides by chelation or covalent bond formation, which can explain the rapid, strong adhesion to various surfaces [60.7, 8]. In the meantime, DOPA-quinone, an oxidized form of DOPA, can form cross-links with other DOPA-quinone molecules via aryl coupling and a thiol group of cysteine residue, providing cohesive interactions [60.9–13]. As a result, DOPA plays dual roles in surface adhesion with the substrate and in cohesive cross-linking [60.13].

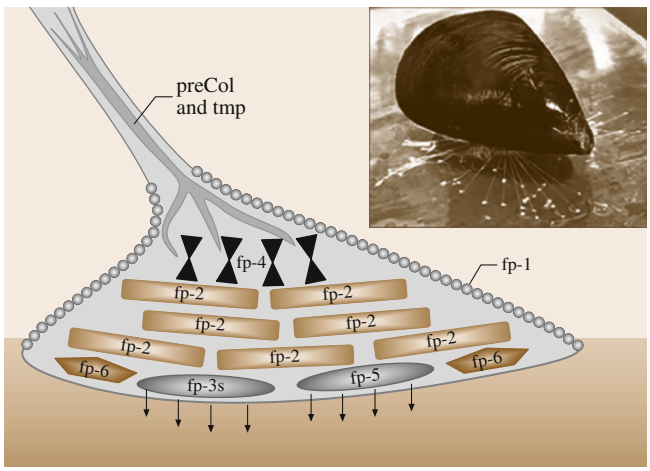

Fig. 60.1 Mussel adhesive proteins of *Mytilus* at the byssal plaque. The mussel (*Mytilus galloprovincialis*, *inset*) attached to a mica sheet is shown. One of its plaques (*red circle*) is enlarged as a schematic illustration of the approximate distribution of proteins (after [60.4], courtesy of the American Society for Biochemistry and Molecular Biology)

60.1.1 Mussel Adhesive Proteins (MAPs)

To adhere to the substratum, the mussel fabricates and utilizes byssus, which is a bundle of threads. The individual thread is called a byssal thread, and there is an adhesive plaque called a byssal plaque at the end of individual byssal thread, where underwater adhesion occurs between the byssal thread and the substratum [60.4]. Byssus is a proteinous structure, primarily composed of proteins (slightly greater than 90%); therefore, the adhesive proteins have been focused as the main research material. To date, six types of MAPs, including foot proteins type 1 (fp-1) to type 6 (fp-6), have been discovered, and each protein is distributed at a specific location and plays a different role (Fig. 60.1) [60.4, 14–16].

Composed of 50–80 repeats of a tandem decapeptide, fp-1 (≈108 kDa), the first identified and the most studied MAP is a coating protein that covers the entire exposed surface of the byssus. This protein is highly posttranslationally modified so that around 60–70% of amino acids are hydroxylated [60.17]. fp-3 (5–7 kDa) and fp-5 (≈9 kDa), which are abundant in the adhesion interface, are very small proteins containing a high DOPA concentration (10–30 mol %). It is proposed that they have a primer-like function in adhering to substrata, mediating strong surface interaction with a high DOPA content [60.12, 18]. fp-6 (≈11 kDa) is cysteine rich with an abundant thiol group, modulating interprotein thiol-mediated redox of DOPA [60.11]. fp-3, fp-5, and fp-6 form a thin primer layer on the substrate surface. Above this layer, the bulk of the byssus plaque is formed with fp-2 and fp-4. fp-2 (≈45 kDa); fp-4 (≈94 kDa) has a low DOPA content, and these proteins are relatively resistant to proteolysis. fp-2 is an adhesive protein that has repetitive motifs similar to the epidermal growth factor, and play a stabilization role [60.19]. fp-4 has high levels of histidine, lysine, and arginine, which seem to serve as coupling agent and thread collagen anchor, connecting with the byssus thread made up of fp-1 and collagen [60.20].

60.1.2 Production of Recombinant MAPs

Natural extraction, initially used for the isolation of MAPs, is an expensive and inefficient method. For 1 gram of fp-1, around 10 000 mussels are required [60.21]. To obtain large amounts of MAPs for bulk-scale adhesives and practical applications, recombinant DNA technology has been proposed as a solution. However, the recombinant MAP production was faced with difficulties with low production and purification yields [60.22, 23]. Herein, synthetic constructs of MAPs are considered; hybrid recombinant MAPs have been designed to improve the productivity and purification yields. The hybrid recombinant fp-151, which comprises six decapeptide repeats of fp-1 at both N and C-termini of fp-5, was constructed and successfully overexpressed in *Escherichia coli* [60.24]. The production yield showed approximately 100 mg/L in 5 L laboratory-scale cultures and ≈1 g/L in a pilot-scale fed-batch bioreactor culture. This approach has improved the productivity greatly and has expanded the practical applications of MAPs. In addition, low production yields of individual MAPs, such as fp-3 and fp-5, were recently overcome by sequence optimization based on *E. coli* codon preference and the application of diverse vector systems [60.25].

60.2 Application of MAPs to Tissue Engineering

One of the most promising areas for recombinant mussel adhesive protein is the field of tissue engineering. The central issue of tissue engineering has concentrated on designing structurally and biologically similar materials to the extracellular matrix (ECM) through surface modification [60.26]. Because ECM is mainly composed of several proteins and glycosaminoglycans (GAGs), tremendous efforts have been made to modify the surface of material using ECM components. Surface modifications for materials with ECM components are usually accomplished by two approaches.

One strategy is to incorporate ECM components into materials through physical modifications [60.27, 28]. The physical immobilization method is advantageous in terms of simplicity, but desorption of coating materials can easily occur. The other strategy is to provide biomaterials with ECM components by chemical modifications [60.29, 30]. Although this approach is a very powerful method to immobilize bioactive components, modification procedures are generally complicated; in addition, potentially cytotoxic chemical cross-linkers are commonly used for immobilization. General characteristics of MAPs show that they can be a powerful alternative to overcome the problems of previous immobilization methods. Herein, applications of MAPs in tissue engineering will be discussed as coating materials to immobilize various ECM components.

60.2.1 Protein Components

Various protein components derived from ECM and growth factors have been directly utilized to modify the material surface via covalent conjugation or physical adsorption [60.31–33]. Even though they showed good biological activity in in vitro systems, several complications, such as uneconomical production, random folding on the surface, undefined characteristics, and immunogenicity, hamper their biomedical application [60.34, 35]. The usage of bioactive peptides derived from proteins can overcome these complications, so it can be effectively applied to modify the surface of materials in tissue engineering [60.36, 37]. The protein-derived bioactive peptides immobilized or incorporated onto well-defined surfaces act as receptor binding motifs, and, therefore, the artificial surfaces efficiently promote various cellular behaviors, including adhesion, proliferation, survival, and differentiation [60.35, 38–40].

Recombinant MAP, fp-151, is a hybrid protein, so that various types of bioactive peptides can be introduced into it through genetic fusion strategy. Several ECM-derived bioactive peptides have been conjugated with fp-151 to make self-adhesive artificial ECM mimics [60.41]. Self-adhesive artificial ECM mimics can be effectively used to immobilize ECM-derived short peptides on surfaces (Fig. 60.2); the strong adhesion

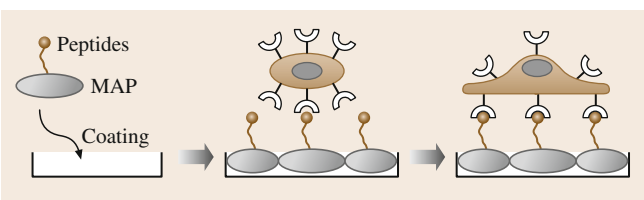

Fig. 60.2 A scheme for the usage of fp-151-peptides as self-adhesive ECM mimics (after [60.41], courtesy of Elsevier)

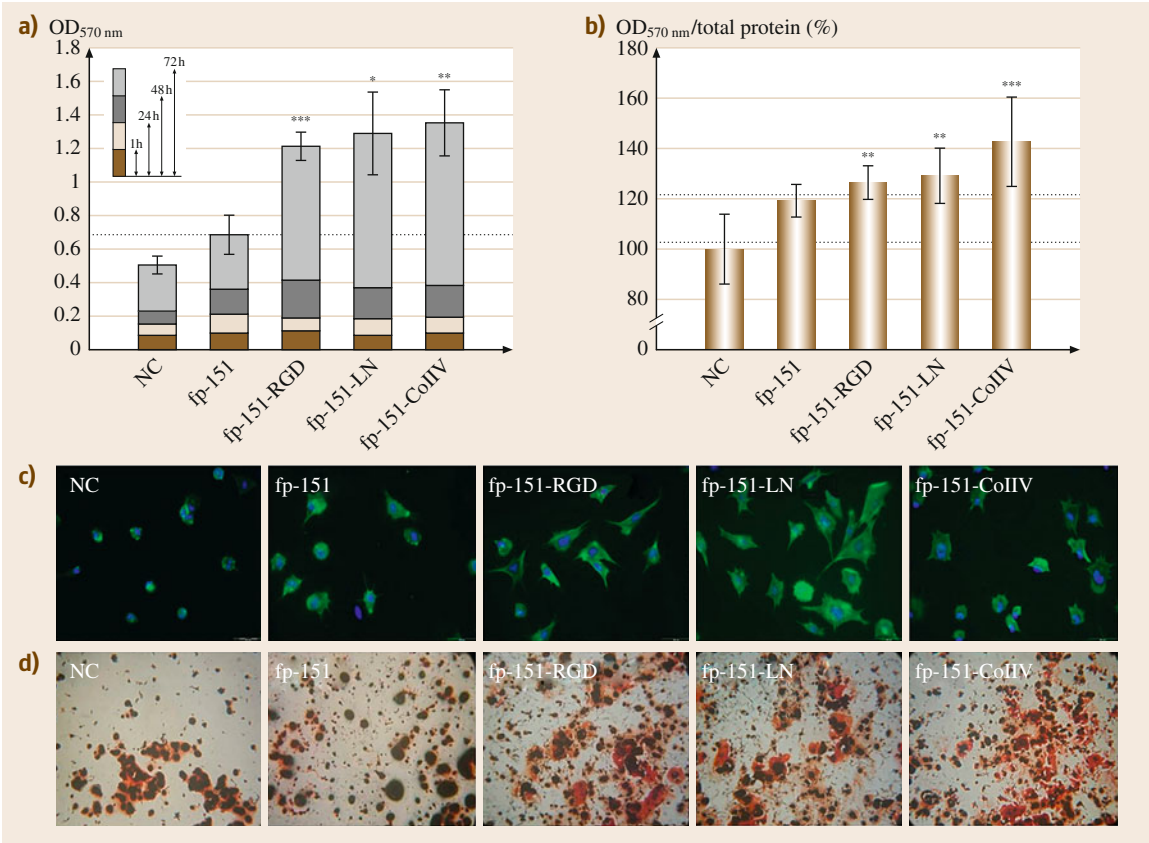

Fig. 60.3 (a) Proliferation, (b) alkaline phosphatase activity, (c) spreading, (d) alizarin red S staining results of MC3T3-E1 pre-osteoblast cells on self-adhesive artificial ECM mimics-coated tissue culture plate (after [60.41], courtesy of Elsevier)

ability of MAPs facilitates efficient surface modification of ECM-derived active peptides on scaffold materials without protein and/or surface modifications, and signaling-mediated ECM-derived active peptides efficiently promote diverse cellular events, including adhesion, spreading, proliferation, differentiation, and survival.

In three mouse cell lines of pre-osteoblasts, chondrocytes, and pre-adipocytes, immobilized ECM-derived active peptides via MAP coating significantly enhanced the essential cell behavior patterns of adhesion, proliferation, and spreading through interactions between ECM-derived active peptides and cellular receptors. Moreover, ECM-derived active peptides conjugated with MAP substantially improved survival and differentiation of pre-osteoblast cells (Fig. 60.3). Based on these data, fusion strategy using MAP successfully immobilized ECM-derived active peptides on the surface of material, and self-adhesive artificial ECM mimics can be usefully employed in cell and tissue engineering.

60.2.2 Glycosaminoglycan (GAGs) Components

Glycosaminoglycans (GAGs), mainly composed of heterogeneous carbohydrate, have also contributed to many cell signaling events [60.43, 44]. Because of their sulfated or carboxylated disaccharides, GAGs are strongly hydrophilic and negatively charged ECM components [60.18]. In contrast, MAPs are highly positive charged proteins (≈10 pI) because their composition includes DOPA (modified from tyrosine), positively charged lysine, and flexible glycine residues [60.24]. Therefore, immobilization of GAG components was performed by general characteristics

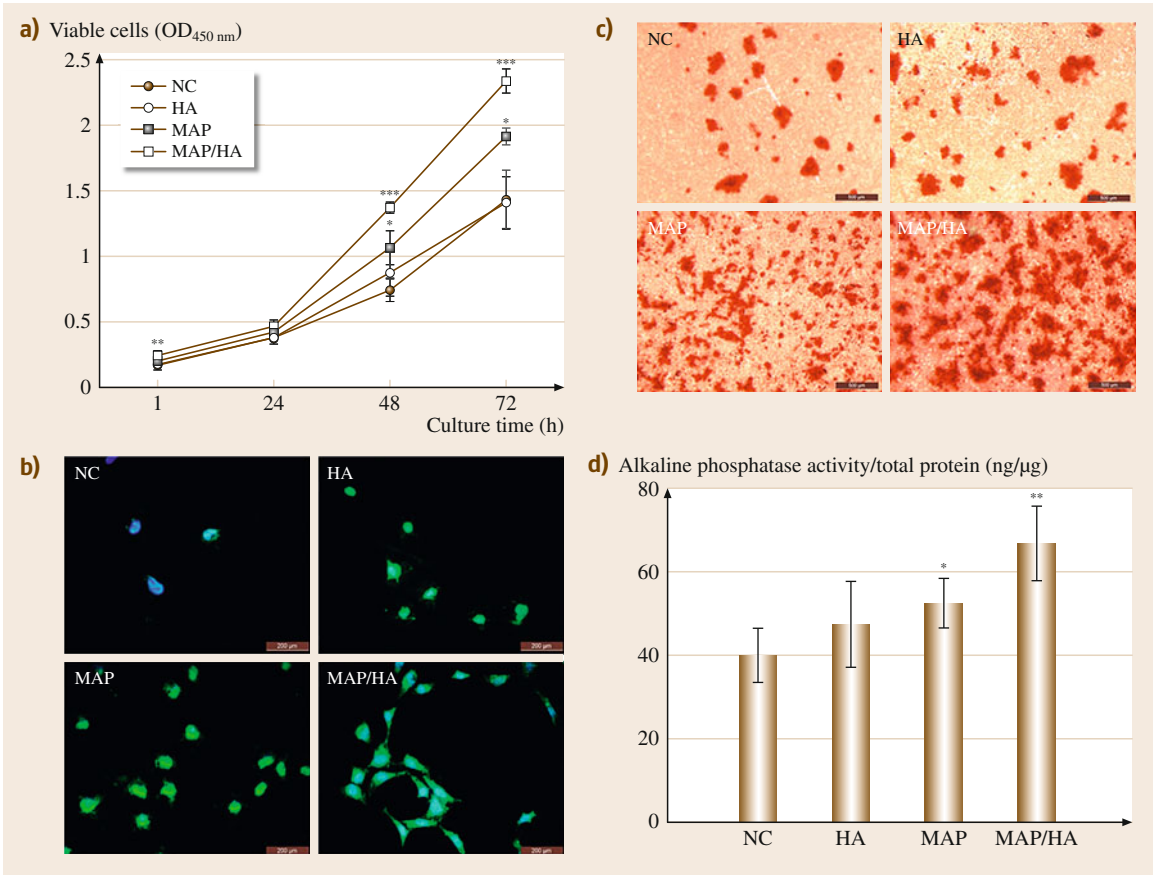

Fig. 60.4 **(a)** Proliferation, **(b)** spreading, **(c)** alizarin red S staining, **(d)** alkaline phosphatase activity of MC3T3-E1 pre-osteoblast cells on HA-immobilized titanium surface using MAP-based coating strategy (after [60.42], courtesy of Mary Ann Liebert, Inc.)

of MAP, such as adhesive property and highly positively charged protein [60.42]. This strategy using MAP is a new and facile method for the preparation of an artificial ECM via the immobilization of GAG molecules without any chemical modifications in a wet environment.

Titanium has been widely used in diverse medical applications due to its biocompatible property, although it has several limitations such as the insufficient osteointegrative property [60.45]. In order to overcome these problems, surface treatment and functionalization using bioactive molecules, such as hyaluronic acid (HA), have mainly been utilized to improve the biological activity of titanium-based materials [60.46, 47]. HA immobilized by the MAP-based coating strategy brought an increase in diverse pre-osteoblast behaviors, such as proliferation, spreading, and differentiation (Fig. 60.4).

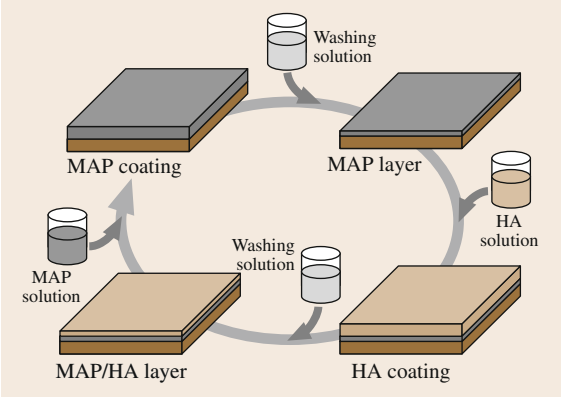

Fig. 60.5 A scheme for HA surface immobilization using the MAP-based coating strategy (after [60.42], courtesy of Mary Ann Liebert, Inc.)

Various negatively charged GAG molecules, including HA, heparin sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS), were successfully immobilized on a titanium surface using the MAP-based coating strategy. Moreover, because the MAP-based coating strategy uses two counter-ionic molecules, it can be applied to fabricate a multilayer film on to a titanium surface using MAP and counter-ion partners (Fig. 60.5). The MAP-based coating with counter-ion partners offers a facile and efficient surface modification of negatively charged bioactive molecules for tissue engineering applications.

60.3 Application of MAP to Tissue Engineering Scaffolds

Tissue engineering is an interdisciplinary field that utilizes principles of biology and various types of engineering technologies for the purpose of tissue regeneration [60.48]. Since the needs of patients for the treatment of their tissue loss or organ failure are becoming bigger and bigger, tissue engineering has been recognized as an important field to many scientists and surgeons. The main strategy of tissue engineering utilizes the combinatorial treatment of cells, scaffolds, and bioactive factors. Among these three factors, the scaffold is considered as the most important one; thus, the development of scaffolds that have bioactive factors and can support cells should be followed for successful tissue engineering applications.

Nowadays, the roles of tissue engineering scaffolds often require a three-dimensional architecture that can modulate specific cell functions, which goes beyond just being a supporting structure for seeded cells. In this manner, an ideal tissue engineering scaffold needs to provide a similar structure to the natural ECM, structurally, mechanically, and chemically. MAPs could be considered as potential biomaterial candidates for tissue engineering scaffold components due to their biocompatibility, biodegradability, and superior cell adhesive and bioactive properties. In this section, several examples where MAPs have been utilized as blending materials of nanofibrous scaffolds and three-dimensional solid freeform scaffold coating materials for tissue engineering application will be discussed.

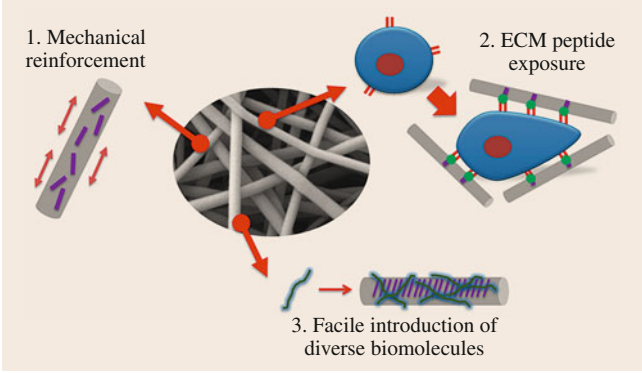

Fig. 60.6 Schematic representation of MAP-based reinforced multifunctional nanofibrous scaffolds (after [60.49], courtesy of Wiley-VCH)

60.3.1 MAP-Based Reinforced Multifunctionalized Nanofibrous Scaffolds

Nanofiber technology has been greatly recognized due to its ability to fabricate multiple nanosized fibers that are structurally similar to collagen fibrils of natural ECMs [60.50]. The large surface area of nanofibers is a major advantage, since these are the sites at which cell-substrate interactions occur. Therefore, rendering the nanofiber surface to be bio-functional is critical for its successful application in tissue engineering [60.51].

Recently, mechanically-reinforced multifunctional composite nanofibrous scaffolds were developed using MAPs, which showed outstanding adhesive and coating performance (Fig. 60.6) [60.49]. Via simple blending MAP solutions with various biodegradable synthetic polymers and an electrospinning process, the composite nanofibers based on MAPs were easily fabricated. With the help of the peculiar adhesive and coating ability of MAP-based nanofibers, various forms of biomolecules that can be useful in the medical industry, such as proteins, nucleic acids, and a variety of biomaterials, can be easily combined on the surface of composite nanofibers without physical and chemical pretreatment. Generally, in order to introduce bioactive molecules, such as ECM proteins, ECM carbohydrates, ECM-derived peptides, and growth factors, onto the nanofiber surfaces, typical conjugation chemistry and physical adsorption procedures are used after the completion of surface activation steps, including plasma and wet chemical treatments or surface graft polymerization [60.52]. However, these strategies are limited in availability due to the multiple, complicated procedures that are required to complete them. Therefore, this novel, multiple biofunctional

coating nanofiber platform based on MAPs may be a promising surface functionalization tool for successful tissue engineering applications.

Moreover, using MAPs fused with bioactive peptides, these MAP-based nanofibers could provide bioactive peptides efficiently onto the surface. It was identified that through MC3T3-E1 cell culture experiments, cell attachment and proliferation was mostly enhanced on the nanofibers fabricated using RGD peptide-conjugated MAPs, compared with bare PCL polymer nanofibers. In addition, it was also confirmed that the MAP-based nanofibers had four times higher mechanical strength than polymer nanofibers. Because easy fabrication is possible through blending with diverse types of synthetic polymers, MAP-based nanofibers can be successfully applied for various purposes.

60.3.2 Bone Regeneration Using MAP-Coated Three-Dimensional Scaffolds

Artificial tissue engineering scaffolds have been fabricated using biodegradable synthetic polymers through diverse methods, such as solvent casting/particulate leaching, gas foaming, emulsion freeze-drying, phase separation, and fiber bonding using synthetic polymers [60.53]. However, controlling interconnectivity and scaffold geometry is quite difficult when these methods are used. Furthermore, practical tissue engineering applications are limited due to the toxic organic solvents that can remain during the fabrication process.

Alternatively, the solid freeform fabrication (SFF) method, which is able to construct a fully interconnected and well-defined porous structure, was recognized [60.55]. In addition, highly permissive transportation of oxygen and nutrients throughout the scaffolds is considered as another advantage. A multihead deposition system (MHDS) has been developed as one form of SFF technology, which has high mechanical strength and biocompatibility because of its organic solvent-free environment during the fabrication process [60.56]. Indeed, several studies reported that cell growth and tissue regeneration were significantly promoted by controllable architectures of SFF-based tissue engineering scaffolds fabricated using MHDS [60.57, 58]. However, the lack of cell-recognition motifs and hydrophobic surface properties of synthetic polymer-based scaffolds still remain limitations of this method. In addition, the introduction of natural polymers or biomolecules (e.g., ECM proteins, carbohydrates, ECM-derived peptides, and growth factors) that can enhance cellular function is also limited in several scaffold fabrication methods due to thermal or solvent-employed procedures.

The development of bio-functionalized SFF-based scaffolds by simple coating of MAPs for the purpose of bone regeneration was reported recently [60.54]. In that work, MAPs were efficiently coated on the surface of SFF-based poly(D,L-lactide-co-glycolide)/polycaprolactone (PLGA/PCL) scaffolds via simple dipping of the MAP solution. RGD-fused MAPs were also applied with same method to obtain RGD peptide-exposed SFF-based scaffolds. Those MAP coating performances were assessed as a powerful coating tool for modifying synthetic polymer-based surfaces into biofunctional scaffolds and for other diverse biomedical fields.

The biofunctionality of MAP-coated SFF-based scaffolds was evaluated by in vitro human adipose-derived stem cell (hADSC) culture experiments. Through investigating diverse cell behaviors such as cell attachment, growth, and bone cell differentiation, it was identified that all of the aspects were signif-

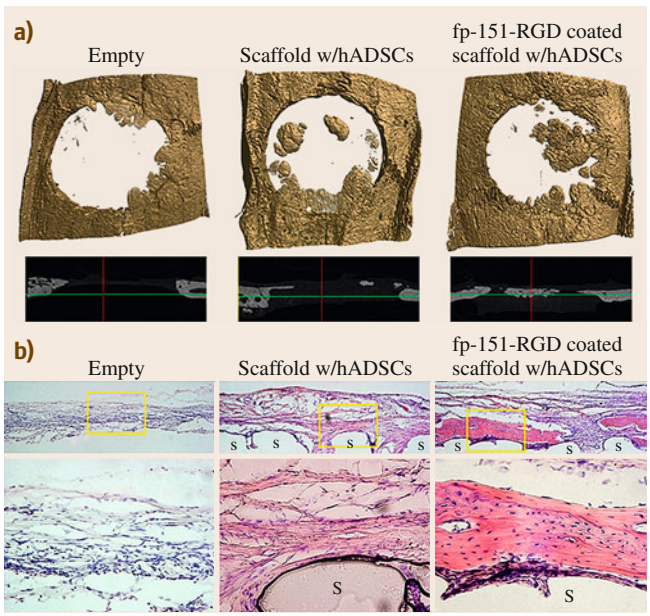

Fig. 60.7a,b Bone regeneration effect of MAP-coated SFF-based scaffolds implantation using a rat calvaria defect model **(a)** micro CT scan 3-D (*upper panel*) and X-ray 3-D axis images (*lower panel*) of rat calvaria. **(b)** Histological analysis of in vivo bone regeneration in the defect area of the (*left*) empty group and implanted groups of (*middle*) uncoated scaffold with hADSCs and (*right*) fp-151-RGD-coated scaffold with hADSCs (after [60.54], courtesy of Elsevier)

icantly enhanced on the surface of RGD-conjugated MAP-coated scaffolds compared to polymer and sole MAP-coated surfaces. Moreover, in order to validate the actual bone regeneration effect in vivo, a rat calvaria defect animal model study was performed. In this experiment, significant bone regeneration was observed in the sample where an hADSC-seeded MAP-RGD-coated scaffold was implanted after 8 weeks (Fig. 60.7).

60.4 Application of MAP Using Complex Coacervation

60.4.1 General Features of Complex Coacervation

Complex coacervation is a liquid–liquid phase separation process where two oppositely charged polyelectrolytes are met. After processing, a condensed coacervate phase and a diluted phase are separated, and they are immiscible (Fig. 60.8). The phenomenon was defined by *Bungenberg de Jong* [60.59] and theoretically investigated by *Voorn* and *Overbeek* [60.60]. Complex coacervation depends on ambient pH and salt concentration, mixing ratio, molecular weight, and ionic strength of componential polyelectrolytes [60.61, 62]. Generally, the coacervation phase has low interfacial tension in water [60.59]. Thus, the coacervation phase easily surrounds various particles in water. Moreover, coacervates can be gelated and cross-linked by pH or temperature change such as casein and gelatin. Because of those behaviors, a complex coacervation technique is utilized in the pharmaceutics, cosmetics, and food industries with encapsulating active molecules such as chemical drugs, proteins, cells, and flavor ingredients [60.62–65].

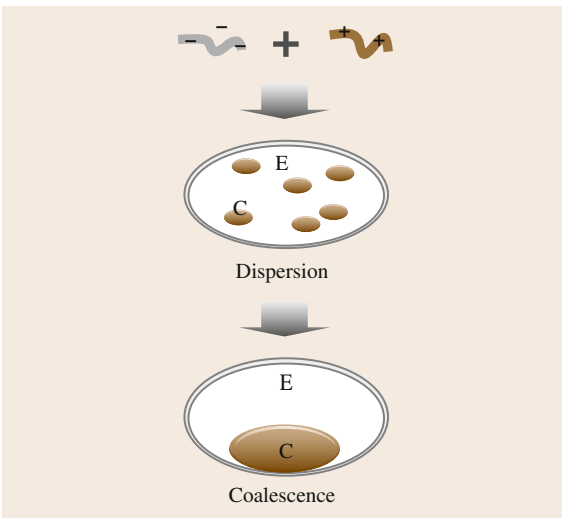

Fig. 60.8 Conceptual figure of complex coacervation

60.4.2 Complex Coacervation in Nature

Complex coacervation is naturally found in the case of the California sandcastle worm, *Phargmatopoma californica*. Such a marine polychaete builds a protective tube made of minerals, such as sand and shells, and then these minerals stick together through a coacervation mechanism with secreted cement proteins from the core body to seawater [60.77, 78]. Similarly, an aquatic insect, the caddisfly larvae, is another candidate for exploiting the coacervation process using its sticky underwater silk, which contains a high density and balanced ratio of positive to negative charges [60.79]. Condensation of MAPs to levels as high as 30 w/v % in vacuoles remain poorly understood. Complex coacervation was suggested to explain this condensation [60.80], but it has not been experimentally confirmed with the natural type of MAPs due to their limited availability.

60.4.3 Applications Using Complex Coacervated MAPs

Complex Coacervation of MAPs

Recently, complex coacervations using recombinant hybrid MAPs, fp-151, and fp-131 were investigated (Fig. 60.9). Complex coacervation was confirmed by observing morphology where positively charged fp-151 and fp-131 were mixed with the negatively

Fig. 60.9 Complex coacervated fp-151 in distilled water

Table 60.1 Microencapsulation using a complex coacervation system

Complex coacervation systems	Encapsulated products	Fields of application	References
Gelatin-acacia gum	Olive oil, squalane, oleic acid, indomethacin, sulfamethoxazole, liposomes, acetyl salicylic acid, vitamin A	Biotechnology, cosmetic, pharmaceutics	[60.66–72]
Gelatin-pectin	Sulfamerazine	Pharmacy	[60.73]
Gelatin-gellan gum	Paraffin oil, sunflower oil, aluminum powder, dowex ion-exchange resin	Biotechnology, food industry	[60.74]
Collagen-chondroitin sulfate	Albumin	Pharmaceutics	[60.75]
Whey proteins-maltodextrins, starch, corn syrup solids	Milk fat	Food industry	[60.76]

charged partner, HA. The optimal condition for complex coacervation was determined by turbidity measurement among various combined conditions of pH, salt concentration, molecular weight of HA, and mixing ratio between MAPs and HA. It was found that the optimal mixing ratio of MAP : HA was irrelevant to salt concentration and HA molecular weight [60.81].

Microencapsulation Using Complex Coacervation of MAPs

Complex coacervation has been utilized in the food industry, pharmaceutics, cosmetics, and biotechnology by microencapsulation (Table 60.1). Microencapsulation is the most common method for hydrophobic molecules and PLGA/PCL systems [60.82]. The microencapsulation process is generally composed of four steps:

1) Formation of a three-immiscible chemical phase (liquid manufacturing vehicle phase, core material phase, and coating material phase)
2) Deposition of the coating
3) Solidification of the coating (thermally, cross-linking, desolvation)
4) Collection by filtration, or centrifugation, washing and drying by spray drying or fluidized bed drying [60.83, 84].

Oil, the representative hydrophobic material, was microencapsulated using coacervation with fp-151/HA and fp-131/HA, which confirmed the formation of oil-encapsulated coacervates by fluorescence monitoring [60.81]. Oil microencapsulation allows a proposal that complex coacervation systems based on combinations of adhesion properties of MAPs and bioactive property of HA can be used as superior adhesive biomaterials, including self-adhesive drug carriers.

Adhesive Ability Enhancement of Complex Coacervated MAPs

The bulk-scale adhesive properties of a recombinant hybrid mussel protein, fp-151, were measured and exhibited significant adhesion strength where the weight and cross-linking process were added ($\approx$1 MPa) [60.85]. These large-scale measurements indicate that recombinant fp-151 MAP holds potential for the many biomedical applications in which water-resistant adhesives are needed. However, the sole recombinant MAP needs to be changed in order to obtain more suitable formula and enhanced adhesive strength.

Coacervated fp-151 and fp-131 were highly condensed like naturally stockpiled foot proteins in mussel foot cells ($\approx$800–1000 g/L), and their density was more increased than the maximal solubility of sole fp-151 and fp-131. The bulk adhesive strengths of sole hybrid MAPs and sole fp-5 were $\approx$2 MPa and $\approx$1 MPa in a dry condition, respectively. The coacervation process enhanced the adhesive strength approximately twofold (to $\approx$3–4 MPa for hybrid MAPs and $\approx$2 MPa for fp-5) on aluminum adherends [60.24, 81]. It was assumed that this improvement in the bulk adhesive strength came from the significant density increment and better wettability from low interfacial energy (0.1–3 mJ/m^2) in MAP-based complex coacervates [60.81, 86]. Therefore, in practical applications, coacervated adhesives can be utilized for a wet environment, such as human tissues that are full of body fluid, because the coacervate phase is immiscible, the coacervate adhesive has a strong adhesive ability, and the coacervate is well-spread over on diverse substrates underwater. In the meantime, the coacervation process with MAPs has extremely enhanced the MC-3T3 cell proliferation on a titanium surface [60.86]. Therefore, coacervated MAPs are also expected to be utilized for cell adhesion materials.

60.5 MAP-Based Biosensors for Industrial Applications

MAPs have been suggested as immobilization material for biosensors [60.87, 88] as they are able to adhere to various materials, including biomolecules, glass, polymers, and metals [60.89]. However, as quantities of mussel-extracted proteins are extremely small, and attempts to overproduce recombinant MAP have failed [60.90–92], the application of MAPs to immobilize biomolecules (or whole cells) on surfaces has been limited. Recently, hybrid versions of recombinant MAPs were designed and successfully overexpressed in *E. coli* [60.24, 41, 93].

Antibody-binding proteins, such as proteins A and G, have been used to enhance the sensitivity and specificity of immunosensors [60.94]. For efficient immobilization of antibody-binding proteins onto solid supports, proteins fused with severalbiomolecules [60.95–99], such as cysteine residue(s), gold-binding peptides, hexahistidine $(His)_6$ peptide, oligonucleotides, and glutathione-S-transferase, have been developed. However, they have limitations for the preparation of immunosensors: (1) they can only be immobilized onto a corresponding reactive group-derived surface, and (2) modifying the surface is time consuming and sometimes difficult for nonexperts.

To overcome the limitations, recombinant MAP fp-5 was genetically fused with C-termini of the BC domain of protein A, which consists of five domains (A–E) (Fig. 60.10) [60.93]. The recombinant BC-MAP (fp-5) fusion protein was overexpressed in *E. coli* and purified with high purity. The BC-MAP fusion proteins were successfully immobilized onto diverse surfaces, including glass, polymer, and metal, and the fusion protein-immobilized surfaces, showed antibody binding, followed by selective detection of antigen on the surfaces. The BC-MAP fusion protein presented an excellent antibody-binding ability compared to that of the BC protein. This was due to a more oriented exposure of the BC domain of the fusion protein but not the adhesive ability of the MAP. The MAP of the fusion protein contributed to direct and efficient attachment of the fusion protein onto diverse surfaces without any surface treatment, and the BC domain enabled oriented immobilization of antibodies on the surfaces. The BC-MAP fusion protein can be successfully used as a functional material for the development of various immunosensors and immunoassays.

Whole cell-based biosensors have been widely utilized in high-throughput drug discovery, environmental

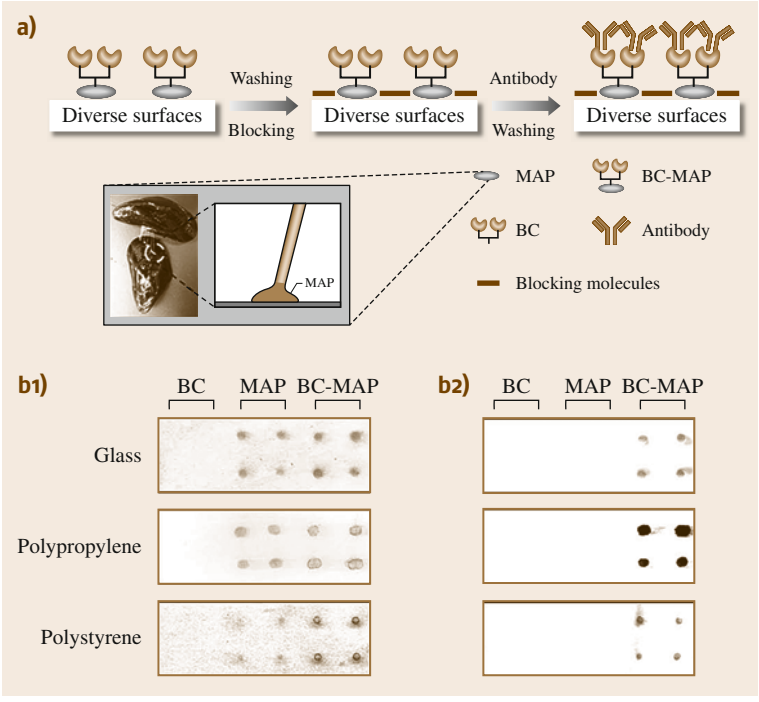

Fig. 60.10 **(a)** A schematic representation of simple and efficient antibody immobilization on diverse surfaces coated with BC-MAP. **(b)** Direct surface-coating analyses of BC, MAP, and BC-MAP on three transparent surfaces **(b1)** and antibody binding analyses of BC, MAP, and BC-MAP-spotted transparent surfaces **(b2)** (after [60.93], courtesy of Wiley-VCH)

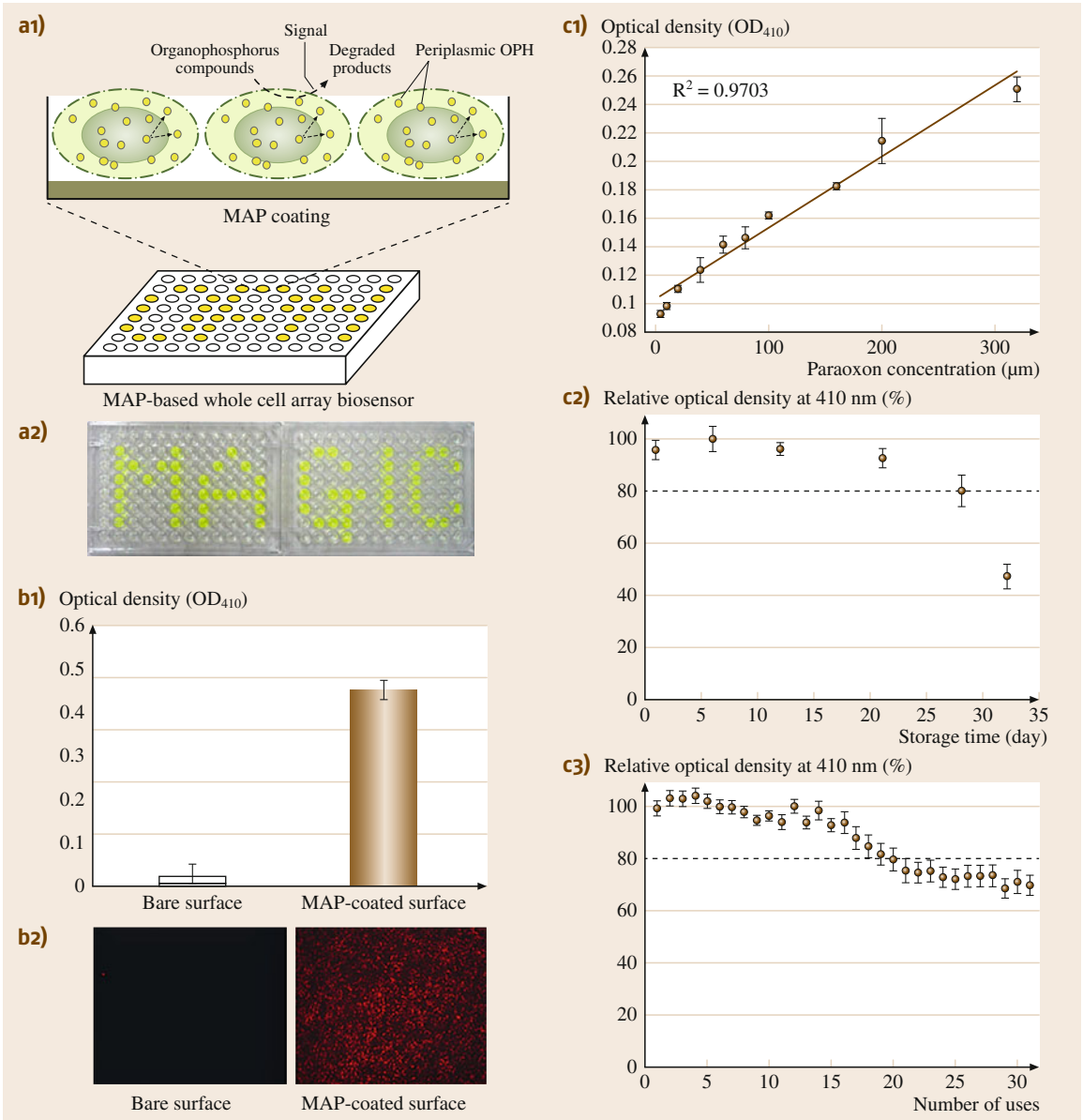

Fig. 60.11 **(a1)** A schematic representation of the whole cell array biosensor for the detection of OPs and **(a2)** demonstration of paraoxon detection as the word "MAGIC". **(b1,b2)** Comparison of the MAP-based cell immobilization method with simple physical adsorption-based cell immobilization. **(c1,c2)** Quantitative detection, reproducibility, long-term stability, and reusability of the MAP-based whole cell array biosensor. This research was originally published in [60.100], courtesy of Elsevier Advanced Technology

monitoring of chemicals and heavy metals, and clinical diagnosis [60.101–105]. Efficient surface immobilization of cells is critical because it affects both sensitivity and stability of whole cell-based biosensors [60.104, 106–110]. Physical and chemical immobilization methods have been widely used for cell immobilization, but they still have several limitations, including limited mass transfer, loss of immobilized cells, and nega-

tive cell viability [60.106, 107, 111, 112]. Recombinant MAPs can improve the limitations of the method due to their efficient adhesion ability and biocompatibility; they have been used as a cell adhesion material for mammalian and insect cells [60.24, 41, 113, 114]. Recently, an organophosphorus hydrolase (OPH)-expressing *E. coli*-based whole cell biosensor was developed by using MAP as a cell-immobilizing material for the detection of organophosphorus compounds (OPs) (Fig. 60.11) [60.100]. The MAP-based immobilization method showed enhanced cell-immobilizing efficiency and stability compared to the simple physical adsorption-based immobilization method. This result is attributed to strong adhesion ability of MAPs. The whole cell biosensor, where *E. coli* expressing OPH in periplasmic space was immobilized onto MAP-coated 96-well microplates, showed a low detection concentration of 5 μM paraoxon with high reproducibility, a good long-term stability for ≈1 month with 80% retained activity, and a proper reusability of up to 20 times. MAP-based whole cell biosensors can be successfully used for industrial applications, including environmental monitoring of chemicals and heavy metals, and food screening.

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61. Marine Silicon Biotechnology

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The discovery of the catalytic capability in silicatein, a constituent of the protein axial filament occluded in biosilica of the marine demosponge *Tethya aurantia*, for hydrolysis and polycondensation of silicon alkoxides to yield silica, as well as its role as a template for silicon biomineralization brought about emergence of a new field, *silicon biotechnology*, as a part of marine biotechnology. This new discipline focuses on understanding the mechanisms controlling biomineralization of silicon elements and its application to the development of new routes to the synthesis of silicon-based materials under environmentally benign conditions. However, as demonstrated by recent studies, the capability of silicatein is not limited to the synthesis of silicon-based materials but is also applicable to the production of various metal oxide materials and organic polymers. Thus, silicon biotechnology represents technology involved in or inspired by silicatein and actually covers the synthesis of silicon-based materials as well as materials consisting of other elements. In this chapter, the molecular biological properties of silicatein and its possible mechanisms on silica polycondensation are summarized, and then a variety of examples of silicatein for the synthesis of silica as well as other materials in environmentally benign routes are described.

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61.1 Overview

61.1.1 Silicon in Biological Systems

The element silicon, the second most abundant element in the Earth's crust, exists as silicon dioxide or silicates in minerals. Some silicon is dissolved as silicic acid from minerals by weathering and is carried to aqueous environments such as rivers, lakes, and ocean. The concentration of silicic acid in the world's oceans averages about 70 μM, which is much lower than saturated concentration of silicic acid (at pH <10 and room temperature, ≈ 1 mM) [61.1]. Our life is full of silicon-based materials. Clay and stone are used without processing in bulk. Industrial products ranging from traditionally manufactured glasses and ceramics to highly technological semiconductors and elastic polymers are also silicon-based.

Silicon exists in the biosphere as a mineralized structure containing silicon or silicon biomineral in the form of amorphous hydrated silica. Living organisms that produce silicon biominerals include higher plants,

diatoms, radiolarians, sponges, and mollusks. After organisms die, silicon biominerals (mainly diatoms and radiolarians) are dissolved at the surface or become sediments at the sea floor, where biogenic silica are recycled to the lithosphere.

Silicon biominerals made by these organisms are exquisitely controlled in a species-specific manner from the nanoscale to the macroscopic scale. The morphological appearance of silicon biominerals have been well documented for a long time because of their importance for the classification of the species. However, information on the mechanisms underling silicon biomineralization remains limited. Most of our knowledge on silicon biomineralization has been obtained from studies on diatoms and sponges.

Diatoms are eukaryotic unicellular photosynthetic algae. These organisms contribute to one-third of global production. At the same time, they produce 240×10^{12} mol Si each year [61.1]. Silicon is deposited with species-specific intricate ornaments in the organic matrix of diatom cell walls. Diatoms are experimentally advantageous for elucidation of silicon biomineralization in many aspects, as follows: a number of diatom species can be cultured in the laboratory; whole genome sequences of two species *Thalassiosira pseudonana* [61.2] and *Phaeodactylum tricornutum* [61.3] have been accomplished; methods for genetic manipulation have been established [61.4–10]; and cell cycles can be synchronized by depletion of silicic acid in the culture media [61.11, 12]. Organic molecules have been identified from silica structures of diatoms. These molecules are silaffin [61.13, 14], long-chain polyamines [61.15], and singulin [61.16], which involved in silica production as acceleration of silica deposition.

Sponges or Porifera are aquatic sessile animals living at various depths from the intertidal zone to several thousand meters in marine or freshwater environments. Sponges lack distinct tissues and organs and are thought to be the most primitive among multicellular animals. Two classes of sponges, demosponges and hexactinellid sponges, produce silicon biominerals as needle-like spicules that are usually millimeters, and exceptionally meters in length. Spicules in some species, the hexactinellid sponge *Euplectella aspergillum*, for example, are fused by cementing to construct a skeleton.

61.1.2 Marine Biotechnology with Silicon

The manufacture of commercial silicon-based products often requires high energy-consuming conditions and processes that have harsh impacts on the environment. By contrast, biological silicon-containing materials are produced through physiological activities under mild conditions with remarkable exquisite structural complexities and high fidelities. These conspicuous properties of biological silicon-containing materials indicate the involvement of genetic control in the underlying mechanisms. Recent advances in molecular biological approaches have accumulated information on the genes, proteins, and molecular mechanisms controlling silicon biomineralization. Harnessing the knowledge of biology to the technology offers the prospect of developing environmentally benign routes to synthesize new silicon-based materials. We call this biotechnological discipline *silicon biotechnology* [61.17]. Most of the recent knowledge on silicon biomineralization was obtained from marine organisms, especially from diatoms and sponges, making silicon biotechnology an important field in marine biotechnology.

Since the identification of silicatein from the demosponge *Thetya aurantia* [61.18], several organic substances associated with silicon biominerals of diatoms and sponges have been identified and characterized. Among these molecules, silicatein is one of most frequently utilized for application in vitro. In this chapter, marine biotechnology of silicon biominerals is described by focusing on the structure and the function of silicateins.

61.2 Silicateins: Structure and Molecular Mechanisms

61.2.1 Structure of Silicateins

Silicateins were first identified as protein constituents of the axial filaments of silica spicules in *T. aurantia* (Fig. 61.1) [61.18]. Three subtypes, α, β, and γ were isolated, and then complementary DNAs (cDNAs) of α and β were cloned. Sequence analyses revealed that silicateins are highly similar to cathepsin L, animal lysosomal proteases, belonging to the papain-like cysteine protease superfamily (Fig. 61.2). The sequence identity between *T. aurantia* silicatein α and human cathepsin L is 52%, and correspondence of

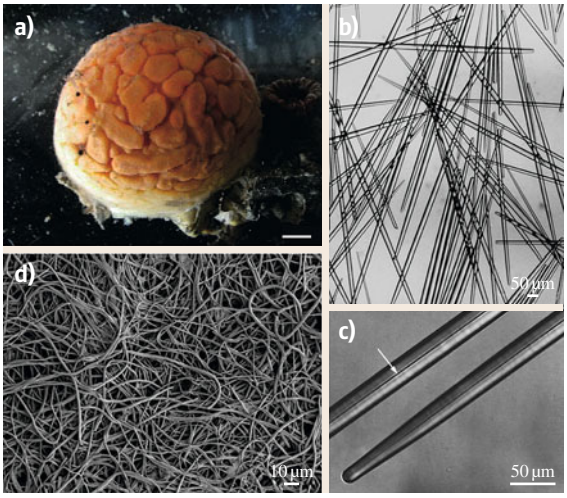

Fig. 61.1 **(a)** The marine demosponge *T. aurantia*. The *bar* indicates 1 cm. **(b)** An optical micrograph of strongyloxea from *T. aurantia*. **(c)** Enlarged optical micrograph of strongyloxea. The *arrow* indicates the axial filaments. **(d)** Scanning electron micrograph of axial filaments (**(b–d)** after [61.19], courtesy of Wiley-Liss)

residues with biochemically identical or similar side chains is 75%. The same degree of similarity was found between silicatein α and cathepsin L from the marine demosponge *Suberites domuncula* (50% identity; 78% similarity) [61.20]. Identities include the six cysteine residues that form intramolecular disulfide bridges in the proteases, making it likely that the three-dimensional structure of silicatein α is similar to that of cathepsin L (Fig. 61.3).

The catalytic cysteine (sulfhydryl) residue at the active site of the proteases is replaced in silicatein α with a serine(hydroxyl). The silicateins from *T. aurantia* did not display proteolytic activity as seen with a mutant form of cathepsin L, in which the catalytic cysteine replaced by serine is also inactive as a protease. However, the silicateins extracted from the demosponge *S. domuncula* under mild conditions with Tris-glycerol solution and the silicatein filaments of the freshwater sponge *Lubomirskia baikalensis* displayed proteolytic activity [61.21, 22], indicating that proteolytic activity was not completely lost, at least in these species.

Although the calculated isoelectric points (pIs) of silicatein α and cathepsin L are identical, there are fewer charged amino acids in silicatein α and somewhat more hydroxy amino acids than in the protease (20.3% in mature silicatein versus 17.2% in mature cathepsin L). This observation is consistent with our finding that silicateins are released from the filament by treatment with sodium dodecylsulfate (SDS) or urea, indicating that the subunits associate by means of nonionic, noncovalent interactions. The assembly of macroscopic filaments exhibiting a highly ordered nanostructure from protein subunits that are small and presumably globular appears to be a distinctive feature of silicateins, which has not been reported previously for cathepsin L or related proteases.

X-ray diffraction studies of oriented spicules from *T. aurantia* demonstrated some degree of order between the silicatein proteins comprising the central protein filament, in common with other demosponge spicules [61.24]. Although the three-dimensional structure of silicatein α was similar to that of cathepsin L, which was a highly soluble monomer with mixed α-helix and β-sheet, Fourier transform infrared spectroscopy of the protein filament of spicules of *T. aurantia* showed that the β-sheet structure was predominant. The analysis with the soluble silicatein α/cathepsin L

```
silicatein α  AYPETVDWRT KGAVTGIKSQ GDCGASYAFS AMGALEGINA LATGKLTYLS EQNIIDCSVP  60
silicatein β   YPESLDWRT KGAVTSVKNQ GDCGASYAFS AIGSLEGALS LAQGKLTYLS EQNVIDCSVA  59
h_cathepsin L  APRSVDWRE KGYVTPVKNQ GQCGSCWAFS ATGALEGQMF RKTGRLISLS EQNLVDCSGP  59

silicatein α  YGNHGCKGGN MYVAFLYVVA NEGVDDGGSY PFRGKQSSCT YQEQYRGASM SGSVQINSGS 120
silicatein β  YGNHGCQGGN MYNTYLYILS NDGIDTSDGY PFKGKQTSCT YDRSCRGTSI SGSIAITSGS 129
h_cathepsin L QGNEGCNGGL MDYAFQYVQD NGGLDSEESY PYEATEESCK YNPKYSVAND TGFVDIPK.Q 128

silicatein α  ESDLEAAVAN VGPVAVAIDG ESNAFRFYYS GVYDSSRCSS SSLNHAMVIT GYGI....SN 176
silicatein β  ESDLQAAVAS AGPVAVAVDG DSRAFRFYDY GLYNLPGCSS YQLSHALLIT GYGS....FN 175
h_cathepsin L EKALMKAVAT VGPISVAIDA GHESFLFYKE GIYFEPDCSS EDMDHGVLVV GYGFESTESD 178

silicatein α  NQEYWLAKNS WGENWGELGY VKMARNKYNQ CGIASDASYP TL                  218
silicatein β  GNQYWLVKNS WGTNWGMSGY IMMTRNNYNQ CGIATDAAYP TL                  217
h_cathepsin L NNKYWLVKNS WGEEWGMGGY VKMAKDRRNH CGIASAASYP TV                  220
```

Fig. 61.2 Comparison of the amino acid sequence of *T. aurantia* silicatein α, β, and human cathepsin L. *Shaded* amino acids indicate identical residues among the three proteins; *open triangles*, the active site residues; *closed triangles*, the cysteine residues (C) responsible for the formation of disulfide bonds. A *bar* above 155–162 of silicatein α represents a typical *Ser cluster*

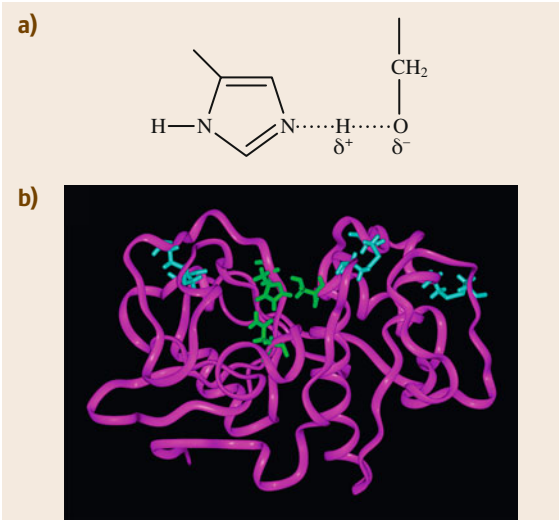

Fig. 61.3 (a) Schematic drawing of the hydrogen bond between serine and histidine in the silicatein α active site. (b) Ribbon model of silicatein α from an energy minimization program. The catalytic site and the disulfide bonds are highlighted in *green* and *blue*, respectively (after [61.23], courtesy of the National Academy of Sciences)

chimeric proteins demonstrated immediate conformational transition from mixed α-helix/β-sheet structure to β-sheet structure at neutral pH [61.25], which explains that the β-sheet structure was dominant in the silicatein filaments of spicules of *T. aurantia*.

A comparison of the hydrophobicity of silicateins α and β versus human cathepsin L and K reveals five regions that differ appreciably between the soluble cathepsins and the insoluble silicateins, identifying hydrophobic regions that may be important in the formation and stabilization of the silicatein filament [61.26]. Superimposition of those unique hydrophobic patches on a three-dimensional model of silicatein α, constructed with energy minimization using the structure of cathepsin L as a template, shows that these regions are present on the solvent-accessible surface of the silicatein α monomer. The interaction of these hydrophobic patches on the surfaces is suggested to produce the driving force for self-assembly of the silicatein subunits to the oligomers, which then assemble into a fractal network that subsequently condenses and organizes into a filamentous structure.

Clusters of the hydroxy amino acids – serine, tyrosine, and threonine – constitute one of the most distinctive features of silicatein α. Serine-rich sequences including Ser-Ser-Cys-Thr-Tyr and Ser-Ser-Arg-Cys-Ser-Ser-Ser-Ser sequences, and the Ser-Tyr sequence at the site of the serine-replaced active site of the protease are notable examples of the localized high concentrations of hydroxyls in this protein. These clusters of hydroxyls were conserved among silicateins from various species, although the extent varied.

Since silicateins were identified from *T. aurantia*, the corresponding proteins and genes have been isolated from the axial filaments of various demosponges. To date, 27 complete cDNAs of the silicateins have been cloned from 10 demosponge species, and cDNAs from 19 species in the demosponges have been described (Table 61.1). Whole genome shotgun sequencing of the marine demosponge *Amphimedon queenslandica* revealed that the species carries six silicatein-like genes [61.27] (GenBank Accession No. NW_003546256, NW_003546249, NW_003546859). Molecular phylogenetical analysis suggested that the ancestral silicateins were separate from cathepsin L [61.28], and, in addition, demonstrated that the silicateins of marine sponges and silicateins of freshwater sponges form two clearly distinguished clusters on the tree [61.29].

Recently, silicateins were identified in the hexactinellid sponges *Euplectella aspergillum*, *Monorhaphis chuni*, and *Crateromorpha meyeri* as proteins recognized with anti-silicatein antibodies [61.30, 31] followed by isolation of partial silicatein cDNA from these species [61.32, 33]. *Veremeichik* et al. [61.34] attempted to isolate silicatein genes from the three glass sponges (*Pheronema raphanus*, *Aulosaccus schulzei*, and *Bathydorus levis*) by polymerase chain reaction (PCR) with the degenerated primers targeting conserved regions within silicatein/cathepsin proteins. Although three clones from *A. schulzei* were found to represent transcripts that can be related to silicateins, the deduced protein sequences contained a catalytic cysteine instead of the conventional serine. It is not clear whether the corresponding protein is involved in the production of spicules. These results demonstrate that silicateins are commonly expressed in the silica producing sponges for biomineralization of silica through common mechanisms.

61.2.2 Roles of Silicateins in Silicon Biomineralization

Silicateins bear more than single role in spicule formation. It has been proposed that these axial filaments play an essential role in the determination of the gross

Table 61.1 Silicatein genes

Species	Subtype	Region	Length	Source	Accession
Marine demosponge					
Tethya aurantia	Alpha	Complete	1360	RNA	AF032117
	Beta	Complete	1418	RNA	AF098670
Tethya aurantium red var.		Complete	1324	RNA	FR748154
Tethya aurantium yellow var.		Complete	1307	RNA	FR748155
Suberites domuncula	Alpha	Complete	1200	RNA	AJ272013
	Alpha	Complete	2280	DNA	AJ877017
	Beta	Complete	1372	RNA	AJ547635
	Beta	Complete	1152	RNA	AJ784227
	Beta	Partial	1740	DNA	AJ877016
		Complete	1200	RNA	AJ272013
Geodia cydonium	Alpha	Complete	1167	RNA	AM500857
Acanthodendrilla sp.	Alpha	Partial	345	RNA	FJ013044
	Beta	Partial	315	RNA	FJ013043
Halichondria okadai		Partial	569	RNA	AB071667
Hymeniacidon perlevis	1	Partial	503	RNA	EF174599
	2	Partial	220	RNA	EF174600
	Alpha	Complete	1296	RNA	DQ364228
Petrosia ficiformis		Complete	1121	RNA	AY158071
Discodermia japonica		Partial	454	RNA	FR748157
Freshwater demosponge					
Latrunculia oparinae	Alpha 1	Complete	1195	RNA	EU888269
	Alpha	Partial	506	RNA	EU909155
	Alpha 2	Complete	1209	RNA	EU909156
	Alpha 3	Partial	819	RNA	EU909157
	Beta	Partial	775	RNA	EU909158
Baikalospongia fungiformis	Alpha	Partial	977	DNA	GU289403
	Alpha 4	Partial	780	DNA	GU289404
Baikalospongia intermedia	Alpha 1	Partial	1003	DNA	FJ812083
	Alpha 1	Partial	500	RNA	FM160555
	Alpha 4	Partial	781	DNA	FJ812084
	Alpha 4	Partial	678	RNA	FM160556
Lubomirskia baicalensis	Alpha	Complete	1167	RNA	AJ872183
	Alpha	Complete	2040	DNA	AJ877018
	Alpha	Partial	1668	DNA	GU222667
	Alpha 2	Complete	1227	RNA	AJ968945
	Alpha 3	Complete	1154	RNA	AJ968946
	Alpha 3	Partial	1657	DNA	GU222668
	Alpha 4	Complete	1123	RNA	AJ968947
	Alpha 4	Partial	1540	DNA	GU222669
		Partial	506	RNA	AJ786771
Lubomirskia incrustans	Alpha 1	Partial	1225	DNA	FJ812082
	Alpha 4	Partial	780	DNA	FJ812081
Swartschewskia papyracea	Alpha 4	Partial	452	RNA	FM160565
	Alpha 4	Partial	779	DNA	FJ812085

Table 61.1 (continued)

Species	Subtype	Region	Length	Source	Accession
Freshwater sponge					
Ephydatia fluviatilis	Alpha 1	Partial	518	RNA	AM167900
	Alpha 2	Partial	506	RNA	AM167901
	Alpha 4	Partial	670	DNA	FJ812077
	G1	Complete	1179	RNA	AB370209
	G2	Complete	1161	RNA	AB370210
	M2	Complete	1119	RNA	AB370206
	M3	Complete	1113	RNA	AB370207
	M4	Complete	1192	RNA	AB370208
		Complete	1179	RNA	AB219573
Ephydatia muelleri	Alpha 2	Complete	1198	RNA	FM160558
	Alpha 3	Complete	1166	RNA	FM160559
	Alpha 4	Partial	757	DNA	FJ812080
	Alpha 4	Partial	451	RNA	FM160560
Ephydatia sp. n. *1 PW-2008*	Alpha 4	Partial	569	RNA	FM160557
Ephydatia sp. n. *2 PW-2008*	Alpha 2	Partial	569	RNA	FM160561
	Alpha 4	Partial	569	RNA	FM160562
Spongilla lacustris	Alpha 3	Partial	754	DNA	FJ812078
	Alpha 3	Complete	1086	RNA	FM160563
	Alpha 4	Partial	705	DNA	FJ812079
	Alpha 4	Complete	1108	RNA	FM160564
Hexactinellid					
Monorhaphis chuni		Partial	378	RNA	FN394978
Crateromorpha meyeri		Partial	475	RNA	AM920776
Euplectella aspergillum		Partial	561	RNA	FR748156

morphology of the spicules, meaning that silicateins, constituents of the axial filaments, were in charge of this role. *Mohri* et al. [61.51] isolated six isoforms of silicateins from the freshwater sponge *Ephydatia fluviatilis*, which has two types of morphologically and functionally different spicules, called megascleres and gemmoscleres, and demonstrated the spicule-type-specific messenger RNA (mRNA) expression of these isoforms and differential expression during spicule development. The marine demosponge *Geodia cydonium* possesses two types of spicules, megascleres and microscleres. Polyacrylamide gel electrophoresis (SDS-PAGE) analysis with the isolated axial filaments demonstrated that the axial filaments of the microscleres contained only one form of silicatein, termed silicatein-α/β, with a size of 25 kDa in contrast to the composition of the silicateins in the megascleres (isoforms: -α, -β, and -γ) [61.52]. These two cases suggested that the different composition of the axial filaments with respect to silicateins, contributes to the morphology of the different types of spicules.

The second role of silicateins was demonstrated with the filaments as well as the silicateins dissociated from the filaments [61.53]. The silicateins accelerated the in vitro polymerization of silica from tetraethoxysilane (TEOS) at neutral pH. The formation of a dendritic silica precipitate was confirmed under electron microscopy. Normally, under these conditions, polymerization of silica from TEOS requires either an acid or a base catalyst. The activity of the silicateins was abolished by thermal denaturation, which indicates a dependence on the native three-dimensional conformation of the subunit proteins. Denaturation with the detergent SDS also abolished the activity. Recombinant silicatein α, when expressed in *Escherichia coli* from a recombinant DNA template and subsequently purified and reconstituted, proved to be sufficient to accelerate the polymerization of silica from TEOS at neutral pH. In this case also, thermal denaturation abolished the reactivity with the silicon alkoxide. The intact silicatein filaments were also active, promoting the condensation of silicon alkoxides

Table 61.2 Environmentally benign synthesis of materials with silicateins

Catalyst	Template	Substrate	Product	References
Silicatein filament	Silicatein filament	Ti(BALDH)	TiO_2	[61.35]
	Silicatein filament	$Ga(NO_3)_3$	GaOOH	[61.36]
	Silicatein filament	$BaTiF_6$	$BaTiOF_4$	[61.37]
	Silicatein filament	Siloxane-functionalized NCN-Pt, PCP-Pd	Silicatein filament with NCN-Pt,PCP-Pd	[61.38]
r-silicatein		Alkoxysilanes	Silica, polydimethyl-siloxane	[61.39]
	Gold, polystyrene, silicon wafer	TMOS	Silica	[61.40]
	Silicon wafer (microcontact printing)	TEOS	Silica microstructures, films	[61.41]
r-silicatein (*E. coli*)	Cell surface display	Ti(BALDH)	Titanium phosphate	[61.42]
	Cell surface	Silicic acid	Silica	[61.43]
r-silicatein, His-tag	Gold surface	TEOS	Silica	[61.44]
	Gold surface	Ti(BALDH) hexafluorozirconate	Titania, zirconia	[61.45]
	WS_2 nanotube	Ti(BALDH)	Titania	[61.46]
	Glass surface	Na_2SnF_6	SnO_2	[61.47]
	Magnetite nanoparticle	TEOS	Silica	[61.48]
	Polystylene	TEOS	Silica	[61.49]
r-silicatein, Glu-tag	Hydroxyapatite	Sodium metasilicate	Silica	[61.50]

r-silicatein: recombinant silicatein, Ti(BALDH): titanium (IV) bis(ammonium lactato) dihydroxide, TMOS: tetramethoxysilane, TEOS: tetraethoxysilane, NCN-Pt: platinum-dimethylamino pincer complex, PCP-Pd: paradium-diphenylphosphino pincer complex, His-tag: hexahistidine-tag, Glu-tag: octaglutamic acid-tag

to form the corresponding polymerized silica at neutral pH. The macroscopic filaments serve as scaffolds to organize the deposition of the resulting silica and silsesquioxanes. The activity of the silicatein filaments is abolished by thermal denaturation, indicating a dependence on the native conformation of the constituent proteins.

Proteolytic activity is the additional role of silicateins. As mentioned above, silicateins from *T. aurantia* did not display proteolytic activity as seen with a mutant form of cathepsin L in which the catalytic cysteine is replaced by serine is also inactive as a protease. However, silicateins of the marine sponge *S. domuncula* and the freshwater sponge *Lubomirskia baikalensis* displayed proteolytic activity, which was specific to cathepsin L [61.21, 22]. In addition, protease activity was displayed with silicatein(-related 27 kDa protein) extracted from the giant spicules of the deep sea hexactinellid sponge *M. chuni* [61.54]. Although the substrate of silicateins as proteases has not yet been identified, there is a possibility that silicateins digest the pro-peptide for maturation of the proteases, as seen in cathepsin L and other proteases of this class.

61.2.3 Catalytic Mechanism of Silicateins in Silica Polycondensation

Comparison of the silicatein α and cathepsin L sequences suggested that the overall three-dimensional structures of the two proteins are quite similar. The activity of silicatein to accelerate silica production was abolished by thermal denaturation of the enzyme, demonstrating that the activity was dependent upon the tertiary conformation of silicatein as the activity of the proteases.

Of the three residues of the *catalytic triad* of the active site in cathepsin L, two, His and Asn, were conserved in silicatein α, but the third active-site residue in cathepsin L, Cys, was replaced in silicatein by Ser. The three amino acids of silicatein α are identical to the catalytic triad of the other major class of proteases, the serine proteases, which include trypsin and chymotrypsin as typical members. Site-directed mutagenesis results confirmed the requirement for Ser26 and His165 residues of *T. aurantia* silicatein α for catalysis of the siloxane polymerization [61.55].

The cleavage of peptide bonds by the proteases proceeds through a hydrolysis reaction accelerated by

general acid–base catalysis. Similarly, the condensation of silicon alkoxides promoted by silicateins may occur through hydrolysis reaction and accelerated by general acid–base catalysis. Therefore, the mechanism of action of silicatein α may be related to that of the well-characterized serine proteases carrying the Ser/His active-site. The enzymatic mechanism of the serine proteases involves nucleophilic attack of the alcohol functionality on the peptide amide bond at the carbonyl carbon. In the proposed enzyme mechanism for silicatein, hydrogen bonding between the hydroxyl group of the active site serine and the imidazole nitrogen of the conserved histidine is postulated to increase the nucleophilicity of the serine oxygen, facilitating nucleophilic attack on the substrate silicon center (Fig. 61.4). Nucleophilic attack is followed by elimination of ethanol and the formation of a protein-O-Si intermediate that is potentially stabilized by nitrogen donation from the imidazole to form a pentacoordinate silicon center. The addition of water completes the hydrolysis of the alkoxide bond to form a silanol, or Si-OH, with subsequent condensation occurring to build up the silica framework [61.53].

Fairhead et al. [61.56] made a series of cathepsin L mutants that increasingly match the sequence features unique to silicatein α. These cathepsin L/silicatein α chimera molecules were water-soluble and showed the ability to condense silica from silicic acid solution, although any of the mutant proteins did not precipitate silica from TEOS. The most active mutant carried mutations matching catalytic serine and residues flanking catalytic serine. The authors suggested that the mutation at the residues flanking catalytic serine enlarged the volume of the active site just enough to accommodate the silicic acid substrate. The three-dimensional structure of the chimera was determined at 1.5 Å resolution by X-ray crystallography to confirm its structural similarity to cathepsin L. Furthermore, the crystal structure indicated that the distance between serine hydroxyl and imidazole nitrogen of the histidine of the active site was 3.6 Å, which is beyond the H-bonding distance, which presumably decreases the nucleophilicity of the serine. Instead, the authors proposed an alternative mechanism that the catalytic histidine deprotonated one molecule of silicic acid, which then further condensed with other silicon centers. It is important to note, however, that neither cathepsin L nor cathepsin L/silicatein α chimeras hydrolyzed TEOS, suggesting that there was a fundamental difference at some level between the structures of these mutants and the native and recombinant forms of silicatein α.

61.3 Silicatein-Mediated Synthesis of Inorganic Materials

61.3.1 SiO_2

Since the catalytic capability of silicateins for hydrolysis and polycondensation of silicon alkoxides to produce silica was demonstrated, the capability has been applied in various ranges from silica to organosilicons, metal oxides, and biopolymers. Some of the applications are introduced in this section.

Living cells and proteins have been encapsulated in silica gels, maintaining their biological activities. However, the cells were not stable for a long time due to remaining alcohols and other additives in silica gels made by sol-gel methods. The introduction of silicatein improved this problem. The bacteria *Escherichia coli* transformed with the silicatein gene and produced enzymatically active recombinant silicatein proteins [61.43]. Electron microscopic analysis revealed that the bacteria that expressed silicatein were covered with silica around them when growing in the presence of silicic acid.

Biological silica produced through the activity of silicateins can be useful for medical applications, especially the area of orthopedics, because silicon is thought be essential for mineralization of vertebrate bones [61.57, 58].

Human osteosarcoma SaOS-2 cells grow and produce calcium phosphate biomineral on culture plates precoated with type I collagen. When the cells were cultured on the plates precoated with type I collagen and silicatein, and subsequently modified by coating with biosilica produced with TEOS as a precursor, SaOS-2 demonstrated a marked enhancement of its capability to produce calcium phosphate, although the growth of SaOS-2 cells was not affected by these treatments [61.49].

Recombinant silicateins with Glu-tag, which is known to have high affinity to hydroxyapatite, were immobilized on the surfaces of microcrystalline hydroxyapatite, synthetic hydroxyapatite nanofibrils, and dental hydroxyapatite. These silicateins on the hydrox-

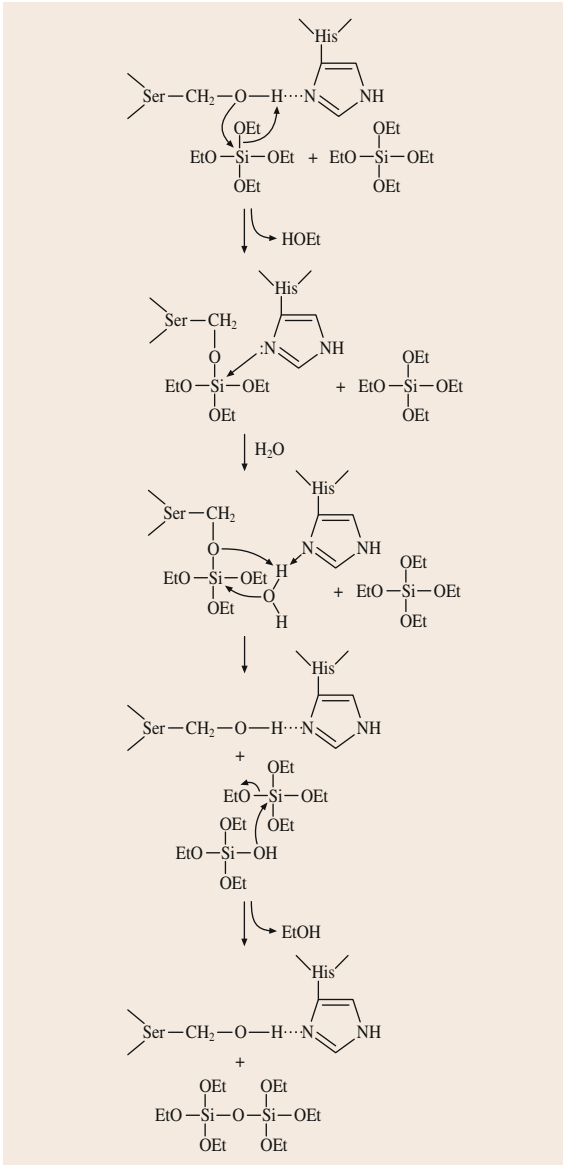

Fig. 61.4 Proposed reaction mechanism of silicon ethoxide condensation catalyzed by silicatein α, based on the mechanism of catalysis by the serine proteases (after [61.53], courtesy of the National Academy of Sciences)

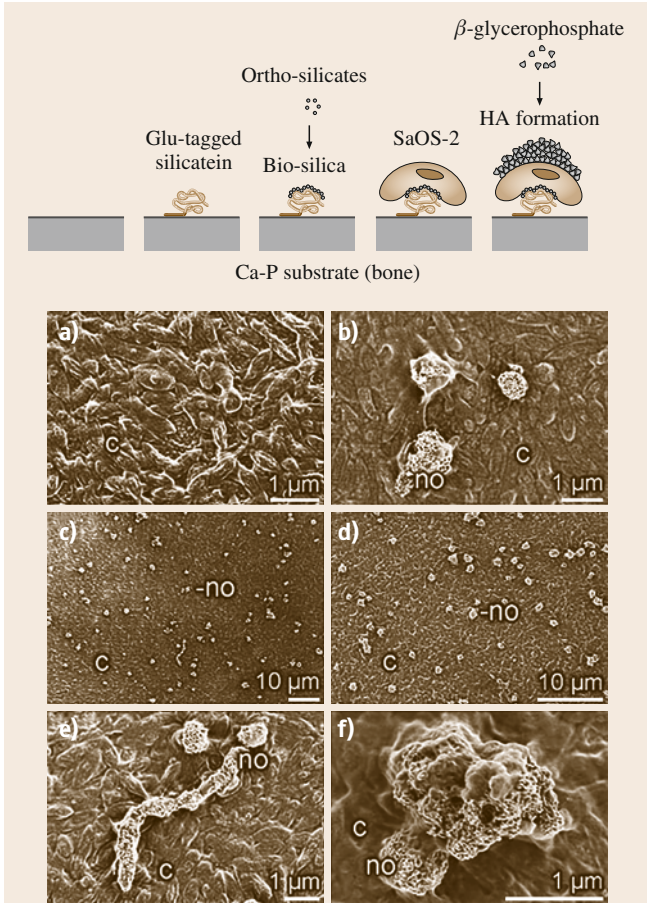

Fig. 61.5 *Top*: schematic outline of the silicatein/biosilica-coating process of Ca–P substrates. Glu-tagged recombinant silicatein immobilized on a Ca–P substrate facilitates biosilica synthesis in the presence of a silica source. The matrix thus modified is bioactive and, consequently, enhances hydroxyapatite (HA) formation by mineralizing cells. *Bottom*, formation of HA nodules by SaOS-2 cells on bone HA. **(a)** Cells (c) cultivated on untreated bone HA. After 5 days few HA nodules were detected. **(b–f)** HA nodules (no) formation by cells (c) grown on silicatein/biosilicacoated bone HA. **(b)** Clusters of cells seem to contribute to the formation of HA nodules. **(c,d)** Lower magnification shows numerous HA nodules. **(e,f)** Higher magnification reveals that the nodules are arranged longitudinally **(c)** or spherically **(d)** (after [61.60], courtesy of Springer)

yapatite materials catalyzed synthesis of biosilica coatings from sodium metasilicate [61.50]. This technology indicates that Glu-tagged silicatein reveals considerable biomedical potential with regenerative and prophylactic implementations. The silica surface induced proliferation of SaOS-2 cells that expressed the genes involved in osteogenesis and produced a hydroxyapatite structure (Fig. 61.5) [61.59, 60].

Wiens et al. [61.61] prepared the silicatein/bio-silica-based implant materials aiming at the application in the area of the regenerative medicine. Microspheres

containing both silicatein and silica were prepared by encapsulating silicatein together with sodium metasilicate in poly(D,L-lactide)/poly(vinyl pyrrolidone)-based microspheres. Then, the microspheres were embedded in a poly(vinyl pyrrolidone)/starch-based matrix. A blend of two components formed a biocompatible, moldable, and biodegradable functional implant material that hardened at a controlled and clinically suitable rate within approximately 30 min to 6 h to implants that were tightly integrated in artificial defects of rabbit femurs. No toxic reactions were observed in vitro or in vivo.

Silica layers with electrically-insulating properties are especially useful as gate dielectrics for field-effect transistors and sensing devices, which are currently attracting increasing interest in the field of organic electronics. *Polini* et al. [61.41] reported a novel approach for realizing layers of silica exploiting the enzymatic activity of immobilized recombinant silicatein and demonstrated the properties of biomineralized films as electrical insulators potentially usable in bio and microelectronic applications. The recombinant silicatein was patterned on silicon substrates by microcontact printing-mediated physisorption and then incubated in a silica precursor TEOS solution. Biosilica layers were formed with an estimated thickness of 200–220 nm after 120–132 h of incubation. By applying voltages in the range 0–10 V to a two-terminal configuration, biosilica layers exhibit currents down to the nA range, which corresponds to leakage current densities around 10^{-5} A cm^{-2} through the dielectrics. Patterning recombinant silicatein to realize silica dielectrics can be particularly useful for producing electrical insulating elements in microelectronics under mild conditions and bioactive materials for biomedical applications.

Polini et al. [61.62] also showed the use of recombinant silicatein α to direct the formation of optical waveguides through soft microlithography. The recombinant protein was first patterned by microfluidics on a hydroxylated Si/SiO_2 substrate, exploiting an elastomeric mold, and then incubated in a TEOS solution for 120 h after peeling off. The patterned recombinant silicatein layer mostly exhibited linear, parallel-oriented protruding features, which indicated assembled filaments aligning along the flow direction. The artificial biosilica fibers mimicked the natural sponge spicules, exhibiting refractive index values suitable for the confinement of light within waveguides, with optical losses in the range of 5–10 cm^{-1}, which is suitable for application in lab-on-chips systems. Biosilification can be extended to the controlled fabrication of optical components by physiological processing conditions.

The formation of uniform silica films with controlled thickness, roughness, and hydrophilicity was achieved by silicatein immobilized on gold-coated surfaces [61.40]. Recombinant silicateins were immobilized by glutalaldehyde with the gold surface amine-functionalized with cystamine or cysteamine. Silica coating of the silicatein-immobilized gold surface was achieved by treatment with prehydrolized tetramethoxysilane. The thickness (20–100 nm), roughness (1.2–5.2 nm), and water contact angle 48–16° of the gold films covered with silica could be controlled by varying the amount of silicatein absorbed (10–30 ng/cm^2) and time of treatment with silica precursors (30–120 min).

The capability of silicateins to produce silica was used for preparation of the nanoparticles composed of the maghemite core with the silica shell [61.48]. Maghemite nanoparticles were coated with poly(pentafluorophenyl acrylate) polymer functionalized with both dopamine and nitrilotriacetic acid (NTA) through the binding capability of dopamine to maghemite. His-tagged silicatein was immobilized on the surface of the polymer-coated maghemite particles in the presence of Ni^{2+}. Incubation of the particles with TEOS facilitated synthesis of the maghemite nanoparticles covered with silica.

61.3.2 Organo–Silicon

Silicateins can be useful for synthesis of various and industrially important organo-silicon molecules under environmentally benign conditions. *Cha* et al. [61.53] demonstrated that intact silicatein filaments promote the condensation of organically modified silicon alkoxides to form the polymerized silsesquioxanes $(RSiO_{3/2})_n$ (silicones in which R represents an organic side chain such as methyl and phenyl) at neutral pH in addition to silica condensation from silicon alkoxides. If silicateins can catalyze hydrolysis and polymerization of a wide variety of organically modified silicon alkoxides (mono-, di-, tri-alkoxides), we will be able to synthesize numerous organo-silicon materials that have not been obtained due to harsh reaction conditions.

Recombinant silicatein α was shown to catalyze condensation of alkoxy silanes at neutral pH and ambient temperature to yield silicones like the straight-chained polydimethylsiloxane [61.39]. Silicatein α dissolved in buffer solution at neutral pH was covered with dimethoxydimethylsilane dissolved in diethyl ether. In

control assays, heat-denaturated silicatein or bovine serum albumin, or no protein was added. After incubation periods of up to 5 h at 20 °C under intense shaking, an extensive increase in the formation rate of oligomers and in the chain length of the silicones in the presence of silicatein was observed, but not heat-denaturated silicatein or bovine serum albumin, or no protein. This biocatalytic reaction may have an impact for the sustainable synthesis of silicones.

Organo-metallic pincer complexes (NCN-Pt, PCP-Pd) bearing siloxanes were immobilized on the surface of the silicatein filaments under mild conditions (Fig. 61.6) [61.38]. A suspension of the silicatein filaments in water was mixed with a solution of the siloxy functionalized organo-metallic pincers NCN-Pt or PCP-Pd complexes in toluene and TEOS. The

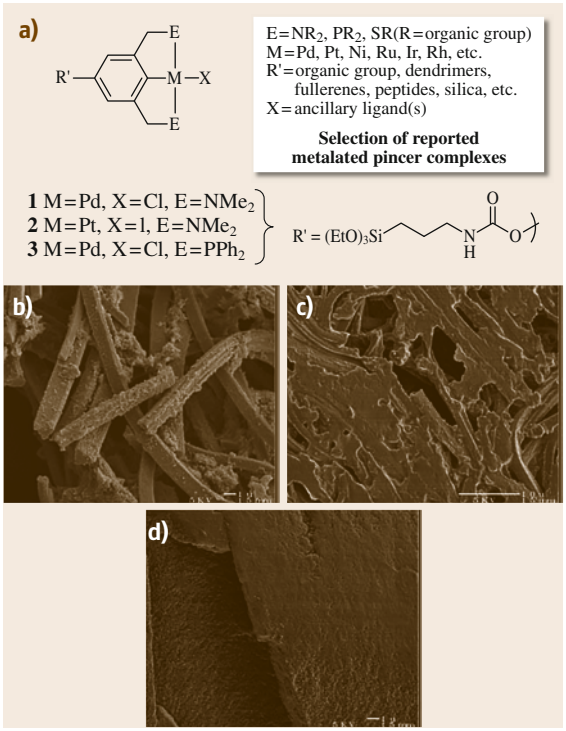

Fig. 61.6 **(a)** Pincer metal complexes. *Bottom*, Scanning electron microscopy images of products. **(b)** The NCN-Pd pincer alkoxysilane 1 in the presence of protein filament with no TEOS added. **(c)** The PCP-Pd pincer alkoxysilane (3) and TEOS in the presence of the silicatein filaments. **(d)** The NCN-Pt pincer alkoxysilane (2) and TEOS in the presence of silicatein filaments (after [61.38], courtesy of the Royal Society of Chemistry)

filament immobilized NCN-Pt pincer changed colors with reversible binding of SO_2, which indicates that this material can be used as a gas sensor. The PCP-Pd pincer immobilized on the silicatein filament was shown in the reaction profile as precatalyst, similar to those of the immobilized catalysts obtained by chemical means. This observation confirmed that silicatein-mediated immobilization of organometallic pincer complexes was practically useful.

61.3.3 Metal Oxides

The catalytic activity of silicatein filaments and isolated silicateins for hydrolysis and polycondensation of silicon alkoxides inspired us to utilize the catalysts for synthesis of metalloid oxides and metal oxides in addition to silica and organosilicons. Titanium dioxide (TiO_2 or titania) is used in a wide range of applications, including in pigments, solar cells, and photo-catalysts. Most titanium alkoxides are very reactive and spontaneously react with water at room temperature to form an amorphous TiO_2 precipitate, which indicates that these compounds are not suitable as precursors for use with silicatein. To circumvent this problem, titanium bis(ammonium lactato) dihydroxide (Ti(BALDH)) was identified as a water-stable, alkoxide-like target substrate that could potentially be converted to TiO_2 by silicatein-mediated hydrolysis and polycondensation [61.35]. Indeed, silicatein catalyzed the hydrolysis and subsequent polycondensation of this substrate. Incubation of TiBALDH with silicatein filaments at neutral pH and 20 °C for 24 h resulted in a layer of TiO_2 covering the surface of the protein filaments. Unexpectedly, TiO_2 produced with silicatein was nanocrystalline anatase. Crystalline polymorphs of TiO_2, such as rutile and anatase, are usually only accessible through high temperature routes (>500 °C) or very low pH conditions (<pH 3) at lower temperatures.

This technology was used for modification of the surfaces of inorganic materials [61.64]. A titania coating was obtained on the WS_2 nanotubes, which was functionalized with the polymer carrying NTA side chains by complexation through Ni^{2+}, followed by attaching the silicatein-containing His-tag to the NTA ligands on the surface of the nanotubes by complexation of Ni^{2+} ions through the His-tag. The formation of the titania coating was mediated by the immobilized silicatein onto the surface of WS_2 nanotubes with TiBALDH as a substrate.

Titania and zirconia layers were obtained on the gold surface functionalized with recombinant silicateins through Ni-NTA from Ti(BALDH) and hexafluorozirconate as substrates, respectively [61.44, 45].

The gold surface was also coated with titania as follows (Fig. 61.7) [61.63]. Recombinant silicatein, genetically engineered to carry a polycysteine sequence, which possessed an affinity to gold, was patterned by microcontact printing methods on gold surfaces. The silicatein-patterned gold surface was subjected to precursor solution TiBALDH, resulting in the formation of photocatalytically active titania on the gold surface. The gold surface with the silicatein-mediated TiO_2 micropattern enhanced the decrease of the color in a methylene blue solution by UV irradiation through the photocatalytic activity of TiO_2.

When TiBALDH was incubated in phosphate buffer saline with a surface-displayed recombinant silicatein α at the exterior of a bacterial cell, a layered titanium phosphate was synthesized [61.42]. The synthesis of titanium phosphate instead of TiO_2 indicated that during the enzymatic hydrolysis and subsequent polycondensation of TiBALDH, sodium and phosphate groups from the isotonic reaction buffer were incorporated into the titanium-based product.

Perovskite and perovskite-like materials have considerable technological value, which makes innovative low cost and/or low energy fabrication routes to kinetically controlled nanostructures extremely desirable. Nanocrystalline $BaTiOF_4$, a multimetallic perovskite-like material, was synthesized and templated along the silicatein filaments by low temperature silicatein-mediated hydrolysis/condensation of $BaTiF_6$ in the presence of H_3BO_3 (Fig. 61.8) [61.37]. The $BaTiOF_4$ grew as floret microstructures composed of nanostructured petals along the silicatein filaments. In addition, $BaTiOF_4$ with silicatein filaments was transformed to $BaTiO_3$ with a 1 : 1 ratio of Ba and Ti by pyrolysis. The result is significant because nanocrystalline perovskite-like materials was obtained from single-source molecular precursors with silicatein.

In addition to SiO_2, TiO_2, and $BaTiO_3$, nanostructured cassiterite SnO_2 was synthesized from Na_2SnF_6

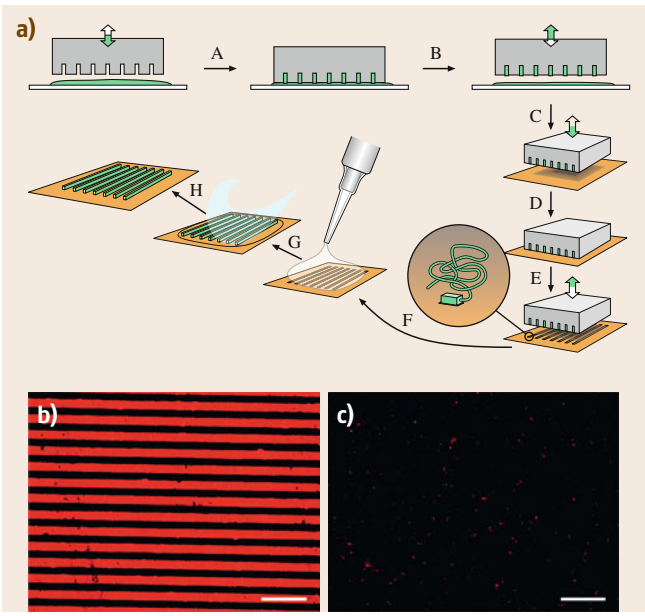

Fig. 61.7 **(a)** Microcontact printing of silicatein onto gold surfaces and subsequent silicatein-mediated synthesis of titania micropatterns. (A and B) The PDMS replica is wetted with a solution of Cys-tagged silicatein (green). (C–E) The loaded replica is placed for 10 s in conformal contact with a Au surface. (F) The enzyme is area-selectively anchored via the Cys-tag's thiol groups, according to the relief pattern of the replica (*inset*; the affinity tag is depicted as a *box*). (G and H) The microcontact printed surface is covered by precursor solution (TiBALDH), resulting in the formation of titania, according to the printed pattern of silicatein. *Bottom*, fluorescence micrographs of silicatein printed onto gold surfaces observed under a laser scanning microscope. *Red area* indicates silicatein immunodetected with the specific antibodies. **(b)** Cys-tagged silicatein. **(c)** Silicatein without the tag. *Bars* 40 mm (after [61.63], courtesy of the Royal Society of Chemistry)

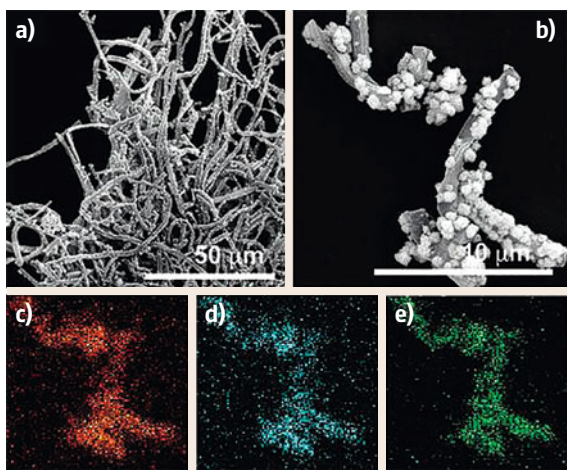

Fig. 61.8 **(a,b)** $BaTiOF_4$ florets catalytically grown and templated by silicatein filaments at 16 °*C*. **(c–e)** EDX elemental maps (of image shown in **(b)**) for **(c)** barium, **(d)** titanium, and **(e)** fluorine (after [61.37], courtesy of the American Chemical Society)

through the catalytic activity of silicatein α immobilized on glass slides through a histidine-tag chelating anchor at room temperature and neutral pH in a buffered system [61.47]. The glass slides after the catalytic deposition of SnO_2 remained colorless and transparent in the UV range. The result demonstrated that technologically interesting transparent conductive oxides were obtained by this simple method, which can be applied to the development of new composite materials.

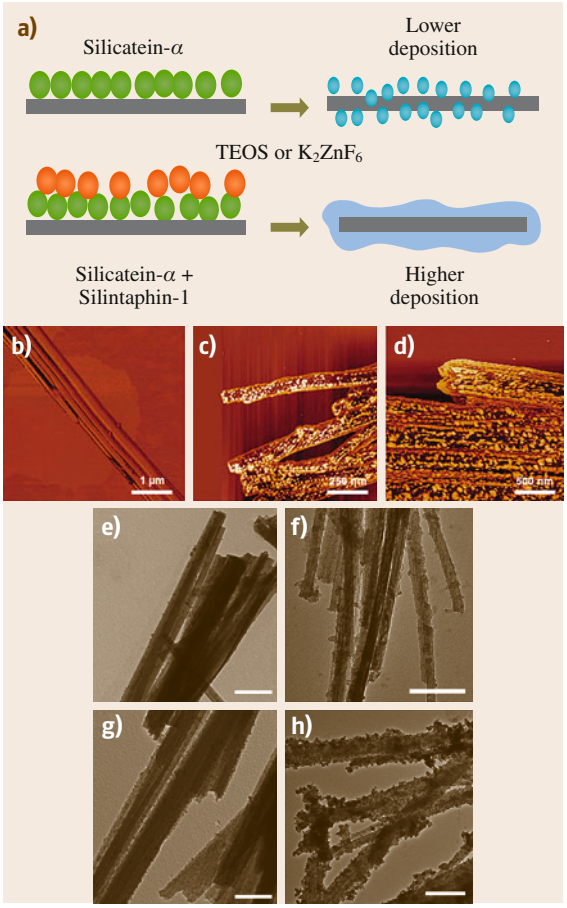

Fig. 61.9 **(a)** Schematic drawing of mineral deposition by immobilized silicatein α with/without silintaphin-1 on TiO_2 nanowires. **(b–d)** AFM phase-contrast images of TiO_2 nanowires; **(b)** as synthesized; **(c)** with immobilized silicatein α; **(d)** with immobilized silicatein a and silintaphin-1. *Bottom*, TEM overview image of mineral deposition on TiO_2 nanowires. **(e)** Silica on immobilized Glu-tagged silicatein α. **(f)** Silica on immobilized Glu-tagged silicatein α/silintaphin-1. **(g)** Zirconia on immobilized Glu-tagged silicatein α. **(h)** Zirconia on immobilized Glu-tagged silicatein α/silintaphin-1. Scale bars: **(e,f)** 100 nm, **(g)** 50 nm, **(h)** 100 nm (after [61.65], courtesy of the American Chemical Society)

The silicatein filaments were capable of hydrolysis and polycondensation of a gallium oxide precursor, hydrated gallium nitrate (GNO), to yield GaOOH or spinel gallium oxide (γ-Ga_2O_3) with confined structure at room temperature [61.36]. With an aqueous solution of highly concentrated GNO at 16 °C, GaOOH was formed in the presence of native silicatein filaments but not heat-denatured filaments, as observed in the case of the formation of silica from TEOS. At lower precursor and silicatein concentrations with the same precursor/silicatein molar ratio as in the case above, a dispersed coating of nanocrystallites adhering to the surface of the filaments were observed, while no reaction product was seen with heat-denatured filaments. Analysis of high-resolution transmission electron microscopy demonstrated that the nanocrystallines were spinel polymorphs of gallium oxide, γ-Ga_2O_3, which formed typically at more than 400 °C. In addition, the particles were single crystals with (311) planes preferentially oriented relative to the surface of silicatein filaments, suggesting that the orientation of the crystal growth was directed with the surface of silicateins, especially the array of hydroxyls at the serine clusters.

Recently, silintaphin-1, a minor constituent of the axial filament, was identified as a protein with high affinity to silicateins [61.66], and it was demonstrated that the protein can modify the capability of silicateins to deposit metal oxides [61.65]. A template was prepared by grafting silicatein α onto a TiO_2 nanowire followed by co-assembly of silintaphin-1 through its specific interaction domains (Fig. 61.9). The organic-inorganic hybrid nanowires were incubated with TEOS or K_2ZrF_6, resulting in nanofibers with a TiO_2 core with SiO_2 shells or a TiO_2 core with ZrO_2 shells, respectively. In the presence of silintaphin-1, silicatein α facilitated the formation of a dense layer of SiO_2 or ZrO_2 on the protein-covered TiO_2 backbone template. The experiment indicated that the coassembly of silicatein α and silintaphin-1 contributed to the efficient synthesis of metal oxides in the proper conditions.

61.3.4 Biocompatible and Biodegradable Polymers

Biocompatible and biodegradable polymers have been used for medical applications and rapidly commercial-

ized for industrial uses such as packaging. Polylactide (PLA) is one of the most popular polymers and is also known to be derived from renewable resources and recyclable.The catalytic capability of silicateins was expanded to synthesis of PLA from the cyclic precursor L-lactide through ring-opening polymerization [61.67]. Native silicatein filaments mixed with L-lactide in an organic solvent were seen to accumulate PLA at the filament surface under scanning electron microscopic observation. Over longer timescales, coagulated structures were seen in which product formation was sufficient to join individual filaments. The heat-denatured filaments showed little change in surface morphology after incubation with L-lactide for 16 h.

PLA was also synthesized through recombinant silicatein displayed on the surface of bacteria cells (Fig. 61.10). The bacteria *Escherichia coli* carrying the gene encoding silicatein α inserted into the first extracellular loop of the *E. coli* outer membrane protein A (OmpA) expressed the recombinant silicatein at the external surface of cells. Solubilized L-lactide was introduced to a suspension of silicatein-displaying cells in isotonic phosphate-buffered saline solution. After

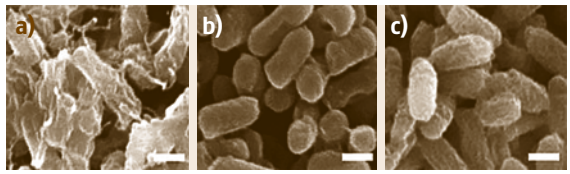

Fig. 61.10a–c Cell-based synthesis of poly(L-lactide). **(a)** *E. coli* cells displaying silicatein α. Polymeric products were observed as thread-like structures between cells. **(b)** Control cells preincubated with the protease inhibitor PMSF. **(c)** Control cells expressing recombinant OmpA only. The product was significantly reduced in **(b)** and **(c)**. Scale bar = 0.5 mm (after [61.67], courtesy of Wiley-VCH)

overnight incubation, polymeric products were apparent at the cell surface and were characterized by an increase in cell surface roughness and as *threads* between cells. These products were significantly reduced when cell suspensions were preincubated with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and in cell preparations that expressed recombinant OmpA only.

61.4 Genetically Engineered Silicateins

Recombinant DNA technology is a powerful tool to produce numerous variants of molecules thatcan be useful for the determination of structure and elucidation of the molecular mechanisms and that have capabilities exceeding those of the wild type proteins. Silicateins have been shown to have unique capabilities to produce silica and other metal oxide compounds and, at the same time, to self-assemble into insoluble filaments. The latter property often makes recombinant silicateins insoluble and causes limitation in analysis of molecular mechanisms for silica polycondensation and application for material synthesis. The production of the water-soluble cathepsin L/silicatein α chimera molecules was allowed to analyze the three-dimensional structure [61.56] and conformational transition at neutral pH [61.25] as described above.

Capabilities of silicateins were further developed by an in vitro gene evolution system coupled with in vitro compartmentalization for the expression of proteins and trapping of the expressed proteins on the

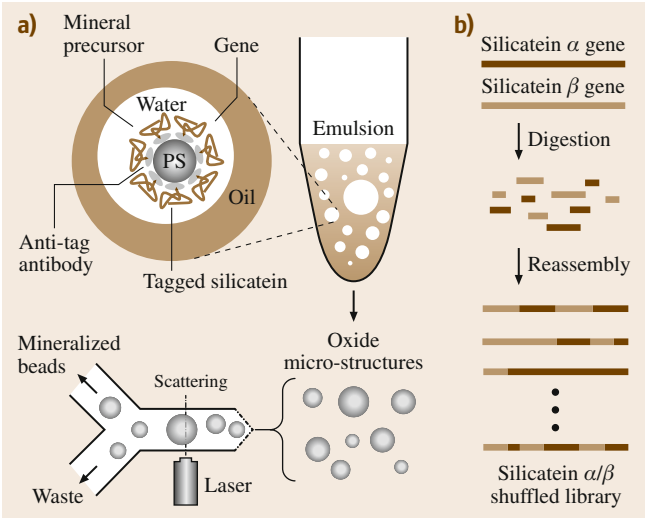

Fig. 61.11a,b Overview of the biomimetic mineralization platform for in vitro evolution by gene shuffling and selection. **(a)** In vitro compartmentalization and separation of mineral-producing beads. **(b)** Construction of a chimeric library of variant silicateins by gene shuffling. After [61.68], courtesy of the National Academy of Sciences ◄

surface of polystyrene microbeads (Fig. 61.11) [61.68]. The gene library of silicatein variants originating from silicatein α and β was constructed by gene shuffling and the introduction of random point mutations. Each silicatein variant was linked to the beads (less than one DNA molecule per bead), which equipped the antibody against the tag sequence. Variant proteins corresponding to the attached DNA were synthesized by mixing the bacteria extracts for protein expression from DNA and absorbed to the bead through the tag on the proteins and the antibody against the tag in water-in-oil emulsions. The capability of the protein variants for biomimetic mineralization was tested by mixing the beads with precursor solutions (TEOS or TiBALDH). Mineralized beads were separated from non-mineralized ones by a light scattering-equipped flow cytometer. The DNA sequence of the protein variants that produced minerals can be obtained by PCR amplification of the DNA attached to the beads followed by sequencing. When TEOS was mixed, one of the silicatein variants catalyzed the formation of crystalline silicates that the wild type silicateins never produced. The other variants demonstrated efficient production of crystalline TiO_2 from TiBALDH.

61.5 Prospectives

Since silicatein was surprisingly discovered as a catalyst for silica polycondensation as well as a template for silicon biomineralization, information on the protein has accumulated and a variety of applications has been exemplified. Also amazing is the broad range of substrates from precursors of metal oxides to organic polymers beyond silica. The capability of silicatein's hydrolysis of precursor molecules to synthesize various species of metal oxides and organic polymers in environmentally benign conditions must make it possible to manufacture energy-reduced and hazardous chemical-free products in a wide range of fields. In addition, the development of genetic engineering has made it easy for us to produce the recombinant form of silicatein and its variants, including chimeras, mutants, and tagged proteins. Therefore, silicon biotechnology with silicatein is a promising field as the development of novel routes to synthesis of various materials. Meanwhile, the discovery of silicatein tells us to learn more about marine organisms to shed light on hidden properties and then to be inspired to bring about novelties in marine biotechnology.

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62. Microalgal Biotechnology: Biofuels and Bioproducts

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In the twenty-first century, energy is considered as the most important issue for human sustainability. The world's dependence on unsustainable fossil fuels (almost 90%), and the increasing population demand new sources of energy for sustainable human activities. Algae, and particularly microalgae have nowadays become of enormous importance as a new potential source of feedstock for renewable bioenergy production. As photosynthetic microorganisms, microalgae may potentially be produced as carbon neutral and can be produced on non-arable land and cultured in marine and wastewater effluents. Furthermore, microalgae can be used to produce a range of products such as protein-rich animal feed in aquaculture, high-value products, viz, polyunsaturated fatty acids, bioactive and functional pigments and natural dyes, health foods, cosmetics, and pharmaceuticals. In this chapter, an update of the advances in microalgal biotechnology is presented as a new biomass for the potential development of biofuels, and as a realistic source of highly valuable molecules of industrial interest. The potential to harness endogenous carbon storage compounds, triacylglyceride (TAGs) and starch, as products of photosynthesis, including the photoproduction of hydrogen, can contribute to diversify the sources and yields of feedstocks for biofuel production. Even if the production of microalgae for biofuels is highly promissory and clearly has potential for contributing to environmental, social, and economic sustainability, presently this alternative is unsustainable. Definitely, the combination of biofuel production by microalgal biotechnology with co-products may contribute to the sustainability of biofuels, a condition with null or less impact on natural resources and biodiversity. The integration of all the components of the uses of microalgae, i. e., high-value compounds, aquaculture, and bioremediation coupled to the production of biofuels will play an important role in the near future to make the production of biofuels from microalgae sustainable. The integration of genomics, metabolic engineering, nanotechnology, and other areas to the aforementioned issues shall lead to a wide range of benefits for the tasks demanded by the forthcoming bioenergy industries.

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62.1 Sustainable Biofuels from Marine Microalgae: Closer to Reality than Fiction

Even when terrestrial plants are employed for biofuel production, the strategies are recognized as functional at the small scale and highly controversial when scaled up by the conflict with food production, extended surface agricultural land, and uses of potable water, which make this and other hybrid technologies unsustainable to supplant a significant fraction of petroleum for the global demand for liquid fuels [62.1, 2]. Aquatic and marine biomass has been proposed as a very promising source for biofuel production; however, in order to realize this, a complete analysis of the full life cycle impact of algal biofuel production in the context of issues such as water resource management, energy balance, and the type of algae is very necessary [62.3]. According to [62.3], biofuel production will be sustainable only if it guarantees eco-friendly processes and respective interactions with other fields. In this eco-context, the reduction of the greenhouse gas (GHG) emission, can be achieved without effecting air, water, soil, or biodiversity, etc. These issues can be accomplished by using microalgae; however, other aspects such as social acceptability and economical viability still need to be overcome. Even though all these potentials can be met theoretically, in practice many scientific and technological aspects must still be applied in order to fully consider this biotechnology as sustainable. Nevertheless, in our concept and criteria, microalgae are one of the most promising renewable feedstocks for biofuel production and biorefineries.

Microalgae can be grown almost anywhere, even in sewage, manure, salt water, ice, and in aerial conditions [62.4, 5] and do not require fertile land or food crops. Their processing requires less energy than the algae provides [62.6]. These indicators reflect the potential that renewable technologies have to supplement or replace liquid fossil fuels by biofuels and microalgae, which are still in their early developmental stages. According to specialized expertise, the expectative to expand will depend on whether underdeveloped petroleum fields are not sustainable, as reported by the International Energy Agency [62.7]. According to The National Research Council of the US National Academies, large-scale production of biofuels from algae is unsustainable using existing technologies.

The expectations and promises of the sustainable production of biofuels from microalgae are very high. However, present challenges of biofuels need to be overcome, mainly in issues related to the uses of industrial wastewater and flue gases, as well as CO_2 sequestration, under carbon neutral conditions. It is expected that several major impacts will be able to influence the sustainability of biofuels, such as its contribution to the change in land use, their feedstock, and aspects of technology and scale [62.3].

The production of different biofuels has its own profits, uncertainties, and risks for sustainability, with many more still to come. It is only in a concerted action among governments, researchers, and companies that the benefits of biofuels from microalgae will become feasible for society. The production of sustainable liquid biofuels from microalgae will need to combine fuel production with co-products and bioprocesses, a strategy which could definitely contribute to the achievement of environmental and economic viability, as well as the adoption of this potential fuel source. In this chapter, we will address the issue of microalgal biotechnology and biofuel production, considering the aspects and conditions needed to achieve sustainability, viz, combining the production of compounds and processes of industrial interest with the production of biofuels. In all situations this alternative is closer to reality than to fiction when compared to other biotechnologies and sources of biomass.

62.2 Why Microalgae is Promissory for Biofuel Production

The autotrophic algae drive photosynthesis to harness sunlight and fix the inorganic carbon from CO_2. There are many algal species that are heterotrophic and able to take up small organic molecules and perform bioconversion into the building blocks of their own. Certain algae can perform mixotrophy by using either inorganic carbon from the atmosphere or organic carbon from the environment. Any of these processes confers to algae the ability to produce carbohydrates, lipids, and proteins that can be processed to produce biofuels. Microalgae are recognized as fast-growing photosynthetic organisms and have been reported to reach transforma-

tion of 5–6% of incoming light energy into biomass. Such figures, as well as its productivity place microalgae as the most promissory candidates for providing unlimited amounts of cheap biomass as food, fodder, or energy. It is recognized that microalgae have the potential to displace other feedstocks for biodiesel owing to their high vegetable oil content and biomass production rates. The potential of microalgae as a feedstock for biofuels production has led to bioprospection and bioscreening programs, hoping to find the ideal microalga. Figure 62.1 shows that in *Chlamydomonas* JSC4, nitrogen depletion can trigger the lipid content (from 15.3 to 41.1%), whereas the protein decreased from 45.4 to 17.9% (after [62.7]). The following main advantages can be mentioned: uniqueness to thrive in seawater at growth rates five to ten times higher than that of higher plants and than any other source; efficient sunlight photo conversion for active growth and photosynthesis; and the massive accumulation of lipids and carbohydrate production, which do not compete with arable land and potable water. The property of microalgae in performing photosynthesis is considered the only process that uses sunlight as the energy source and carbon dioxide as the carbon source for the production of biomass.

In general terms, it is recognized that sustainable production of microalgal biofuels still faces challenges to overcome. Its economic feasibility could be achieved if combined with bioprocesses and production of chemical compounds coupled to the use of low cost products and nutrients. According to [62.8], microalgae can be suitable feedstock for biofuels because certain species are among the most efficient biological producers of oil on the planet and a versatile biomass source. This is why some authors pointed out that microalgae may soon be one of the Earth's most important renewable fuel crops [62.9]. Moreover, they are grown in variable climates and on non-arable land, including marginal areas unsuitable for agricultural purposes (e.g., desert and seashore lands), in non-potable water or even as for waste treatment purposes, use far less water than traditional crops, and do not displace food crop cultures; their production is not seasonal and can be harvested daily [62.8, 10]. Moreover, CO_2 fixation by algae is also an important advantage for removing gases from power plants or other polluting activity and thus can be used to reduce greenhouse gases with a higher production of microalgal biomass and, consequently, a higher potential for biodiesel production [62.11]. A flow diagram of the carbon cycle pathway, biofixation, and the remarkable steps of algal biomass technologies for the production of biofuels is shown in Fig. 62.2.

Several authors have emphasized that the key for large scale production of biofuels is to grow suitable biomass species in an integrated biomass production conversion system (IBPCS). In principle, a profit in the cost of operation of the overall system should be pursued. A conceptual model for integrated biomass production is shown in Fig. 62.2 which can be adopted for microalgal biodiesel production [62.13]. The design and implementation of such systems requires the integration and optimization of several components such as biomass culture, growth management, transport to conversion plants, drying, product separation, recycling, waste management, transport of saleable products and marketing. This model may has several options, for instance the conversion plants may be located in or near the biomass growth areas to minimize the cost of trans-

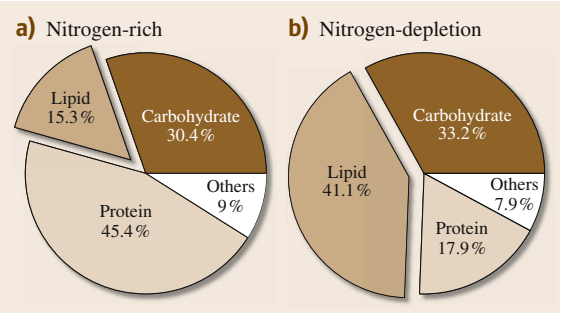

Fig. 62.1a,b Changes in biochemical composition of Chlamydomonas sp. JSC4 cultivated on nitrogen- rich **(a)** nitrogen-rich (70 to 80 % nitrogen consumed) and **(b)** in nitrate-depleted conditions (after [62.7]).

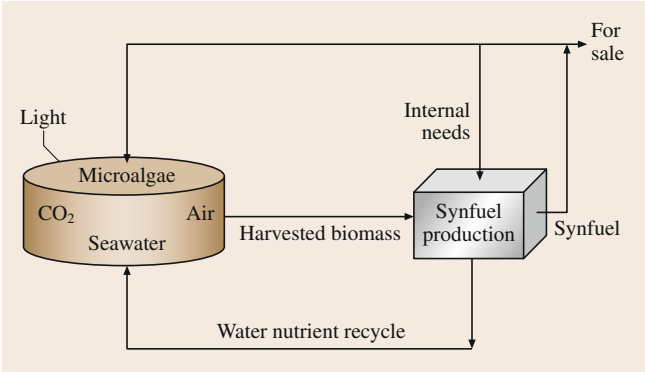

Fig. 62.2 Diagrame of the concept of culture of microalgae biomass integrated into a conversion system for synfuel production (after [62.12])

porting biomass to the plants, of which all the non-fuel effluents are recycled to the growth areas (Fig. 62.2). Based on the versatility of that kind of models, the feasibility to simultaneously integrate microalgae to synfuel production is not so far from reality in the near future.

62.3 Biodiesel Production by Microalgal Lipid Transesterification

In recent years considerable attention has been focused on methods to convert biomass to competitive liquid biofuels from various biomass materials, which may offer a promising alternative to petroleum based transportation fuels. In an epoch when fossil hydrocarbon are likely to become scarce and costly, two liquid transportation fuel at global scale from land plants are emerging. Among these emerging feedstocks, jatropha seems that can be converted to biodiesel with commercial processes, while processes capable of converting algae, are still at pre-commercial stages, but not far from outperform existing terrestrial plant sources into a comprehensive concept of sustainability. Most of bio-oils produced presently are derived from the following main sources: crop seed, animal feed, algal biomass, among others, through a transesterification process. Table 62.1 shows the yield of plant oils compared to microalgae, according to [62.10]. One of the undeniable advantage of microalgae species is it high content of oils in the cytosols and chloroplasts. Oil is extracted from microalgal biomass, which during the process of esterification is converted into biodiesel, a chemical reaction between triacylglycerols (TAGs) and alcohol in the presence of a catalyst. Finally, monoesters are produced, which are termed as biodiesel [62.3]. As a multiple step reaction, transesterification, is composed of three reversible steps in series, where triglycerides are converted to diglycerides, then diglycerides are converted to monoglycerides, and monoglycerides are then converted to esters (biodiesel) and glycerol (a by-product) [62.15].

Table 62.1 Yield of main plant oils compared to microalgae

Crop	Oil yield (L/ha)
Corn	172
Soybean	446
Canola	1190
Sunflower	952
Jatropha	1892
Palm	5950
Microalgae	100 000

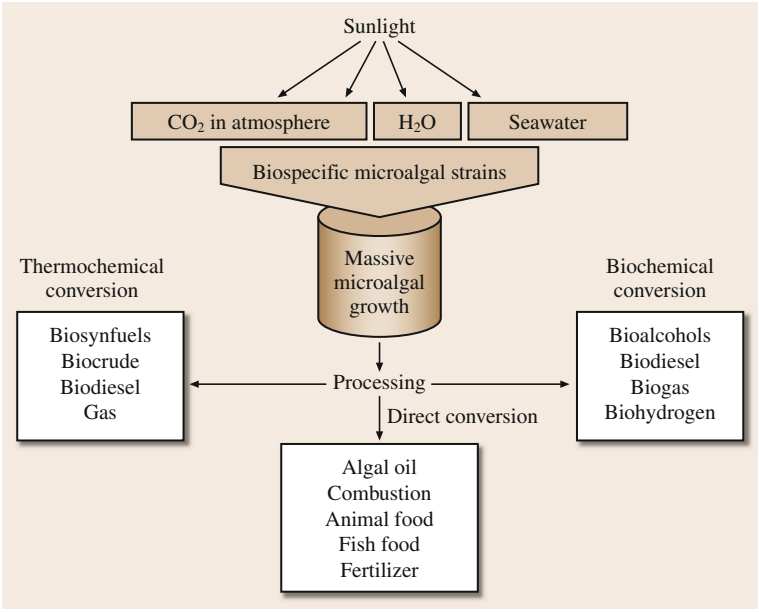

Fig. 62.3 Flow diagram of main steps of bioconversion and processing of algal biomass for the production of biofuel (after [62.14])

The fact that microalgal or bio-oils are characterized by high viscosity, high molecular weight, a higher flash point (above 200 °C), and low volumetric heating values compared to diesel fuels [62.3] imposes challenges that need to be overcome. The associated problems with substituting triglycerides for diesel fuels are: high viscosities, low volatilities, and the polyunsaturated character [62.16]. It is generally accepted that the refinement of these molecules, is an essential step for turning bio-oils into quality fuel (biodiesel) [62.17]. Moreover, biochemical and engineering processes are applied in vegetable oil derivatives in order to approximate the properties and performance of hydrocarbon-based diesel fuels. As shown in Fig. 62.3, biomass conversion processes fall into three major categories: chemical, biological, and thermochemical, but the most efficient processes may be those that combine two or more processes and use the entire plant [62.14]. Present calculations on biofuels from algae reported that one hectare algae farm on wasteland can produce over 10–100 times of oil as compared to any other known source of oil-crops. While a crop cycle may take from three months to three years for production, algae can produce oil and can be harvested all year-round using sea water and non-potable water. These facts firmly reinforced and corroborates that *algae for biofuels* shall provide an ending point solution to food vs. fuel battle. An exemplification to that approach is conceptualized in Fig. 62.3, exhibiting the main steps of algal biomass technologies coupled to biofuel production [62.14].

62.4 Bioethanol from Microalgae: A Simpler Procedure

Algae have a high photon conversion efficiency and can synthesize and accumulate large quantities of carbohydrate biomass for bioethanol production, from inexpensive raw materials. This is an effective and promissory alternative to the low yield of lignocellulosic biomass materials as a feedstock and the high cost of the hydrolysis process based on current technologies [62.19]. Even when bioethanol can be produced from several different types biomass feedstock, the algal alternative has gained acceptation, as will be described in the following. The structural composition of microalgal cells (lacking hemicelluloses and lignins) confers advantages for the production of ethanol, by eliminating the chemical and enzymatic pre-treatment steps to transform these polymers in sugars [62.19]. Microalgae (*Chlorella, Dunaliella, Chlamydomonas, Scenedesmus*) are recognized as having a high content (> 50% of the dry weight) of starch, cellulose, and glycogen, which are the basic components for ethanol production [62.20]. The starch biosynthesized by microalgae can be extracted from the cells with enzymes and then separated by extraction with water or an organic solvent and used for fermentation to yield bioethanol. Besides starch, several algae, especially green algae, can accumulate cellulose as the cell wall carbohydrate, which can also be used for ethanol production. The algal photosynthesis is mainly based on the Calvin cycle, in which ribulose-1,5-bisphosphate (RuBP) combines with CO_2 to produce 3-phosphoglyceric acid (3-PGA), which is utilized for the synthesis of glucose and other metabolites to make carbohydrates (CH). The CH molecules are vital source of storage of energy and provide the precursors to make the organic molecules and macromolecules of nearly all living cells. The process of ethanol from microalgae is more based on the carbohydrate conten of the biomass. The possibilities to utilize algal biomass for the production of biofuels, can follow different life cycle (LCA) as exemplified in Fig. 62.4 [62.6]. The assessment of the LCA options is based on indicators to compare alternative energy routes in terms of environmental impact and indirect natural resource costs towards different services and commodities. Among these, the life-cycle water and nutrients usage of microalgae-based biofuels production, which revealed that water footprint and nutrients usages during microalgae biodiesel production is an important aspect. From that, was calculated that 3726 kg water is required to generate 1 kg microalgae biodiesel if freshwater is used without recycling [62.6]. When seawater and wastewater were used for algal culture, a reduction up to 90 % was reported in nitrogen usage, which eliminates the need of other ions such as potassium, magnesium, and sulfur. The geographic position of each country play a critical role when the life-cycle assessment is performed on the overall water footprint of microalgae-based biodiesel, which production gradually decreases from north to south as solar radiation and temperature increase. Nevertheless, the potential of microalgae as an energy source is confirmed, but emphasizes the need of decreasing the energy and fertilizer consumption. This aspect is actually considered in similar initiatives, and present models had reduced

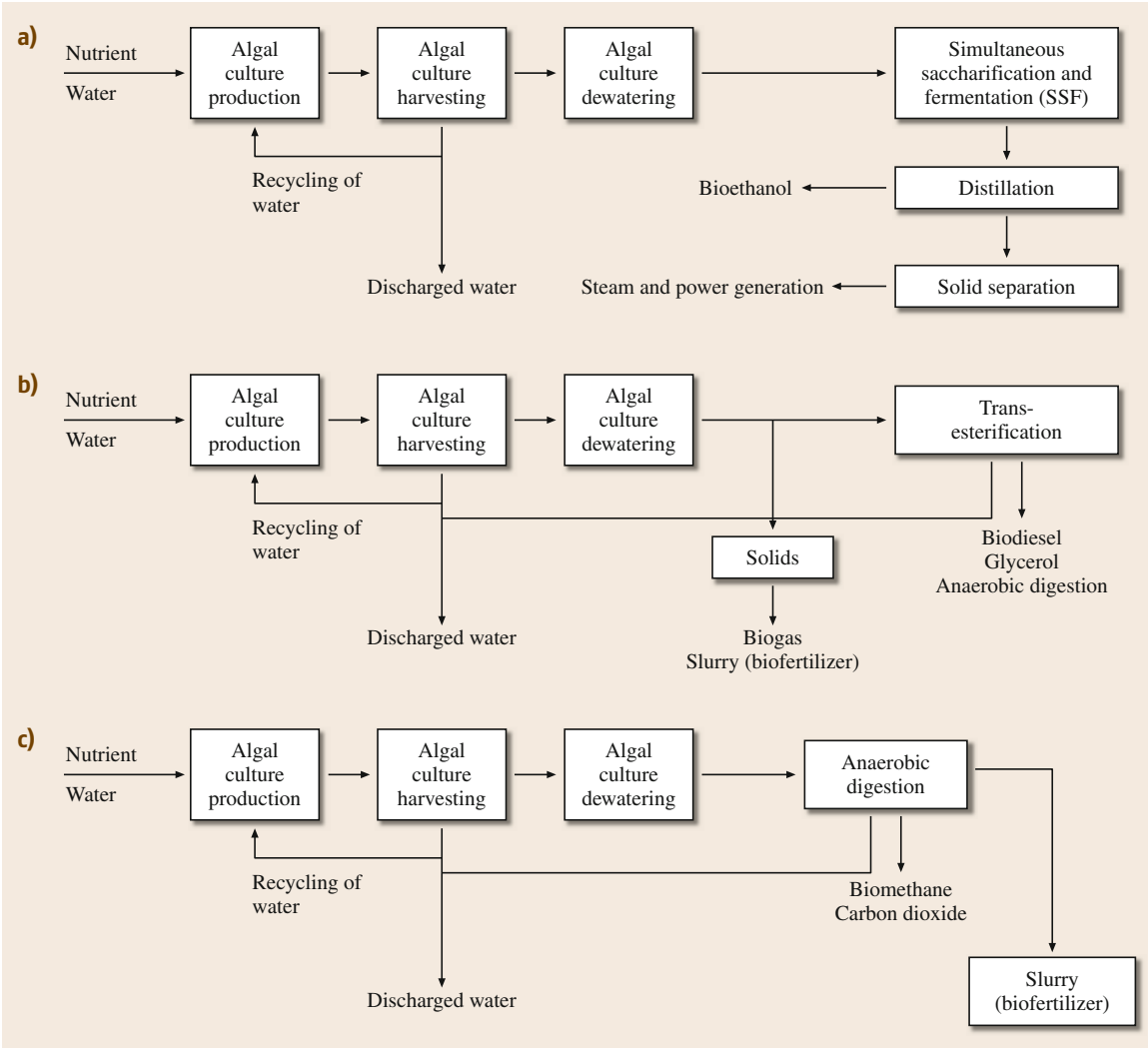

Fig. 62.4a–c Schemes of the life cycle stages of the production of biodiesel, bioethanol, and biomethane from algal biomass **(a)** exemplifies the production of biodiesel from microalgal glycerol; **(b)** shows the culturing process of microalgae to produce bioethanol; and **(c)** main steps to generate biomethane from cultured microalgae. In all three cases the produced water is recycled and bio-fertilizers are obtained (modified after [62.3, 18])

dependence on energy and fertilizers by using algae innate attributes such as CO_2 sequestration and nutrients recovering from wastewater bioremediation initiatives. Hence, algal biomass can be utilized for the production of a range of different biofuels; their life cycles are presented in Fig. 62.4.

62.5 Microalgal Biohydrogen Production Through Sunlight and Seawater

The production of biohydrogen from microalgae is not new; this process has been known for more than 65 years and was first observed in the green alga *Scenedesmus obliquus* [62.3, 15]. More recently, this

mechanism was corroborated in many other prokaryotic photosynthetic species, including cyanobacteria [62.6]. The microalgal photoproduction of hydrogen from water is a promising mechanism and has been explored as a potentially emission-free fuel stream for the future. It is expected that the desired sustainability in biofuels from microalgae can be accomplished by the photoproduction of biohydrogen because of its natural feasibility of atmospheric CO_2-sequestration [62.15]. The green microalga *Chlamydomonas reinhartii* is a successful model that has been studied for algal hydrogen production [62.21].

One of the most attractive characteristics of the bio-H_2 process is that it uses sunlight to convert water to hydrogen and oxygen, which are released in a two-phase process occurring in all oxygenic photosynthetic organisms, such as marine microalgae. A second reaction by special iron-containing chloroplast-hydrogenase enzymes also occurs in a specific group of microalgae. Cyanobacteria also exhibit a natural production of H_2 from water in a different and alternative photo-biochemical process, involving a water-splitting reaction under light and aerobic conditions. In this process, hydrogen and electrons from the water-splitting reaction of photosynthesis are used for the synthesis of adenosine triphosphate (ATP) and nicotine adenine disphosphonucleotide (NADPH), reduced). In the absence of O_2, both ATP production and the formation of NADH/NADPH are inhibited, and the stored energy in carbohydrates (starch, glycerol) is re-oriented [62.22] to the chloroplast hydrogenase to facilitate ATP production via photophosphorylation. Thus, under fermentative conditions hydrogenase plays the role of a releasing valve of protons/electrons, a condition in which protons from the medium and e- from reduced ferredoxin are balanced to produce hydrogen gas that is excreted from the cell [62.23]. *C. reinhardtii* is one of the microalgae most studied for solar-driven bio-H_2 production from water and can also use other fermentative processes. The fact that hydrogen production does not accumulate in the culture but instead is quickly released into the gas phase, makes photoproduction of hydrogen one of the most preferred biofuels.

62.6 Genomics and Metabolic Engineering of Microalgae for Biofuels Production

The gene reservoir of microalgae is only beginning to be explored. In general, microalgal genomes are structurally complex, the reported size ranges from 12.6 Mbp for Chlorophyte *Ostreococcus tauri* while 10.000 Mbp for *Karenia brevis*. The present development in genetic manipulation in microalgae has been expanded in algal biofuels which is limited a very small number of alga models. The significant advances achieved until now include the efficient expression of transgenes, the mechanisms of gene regulation, and the action of inducible nuclear promoters as well as inducible chloroplast gene expression. The nuclear genomes of a number of algae have been transformed, with a variety of reporter genes, as well as drug-resistance genes; however, extensive analysis of transgene expression has only been performed in *C. reinhardtii* [62.1]. *C. reinhardtii* has been extensively studied, and presently, abundant genomic biological and physiological data are available from this alga [62.24], which is probably not the best species for biofuel production, but as a model of study has potential for application in other suitable algal species. Microorganisms are especially attractive for biofuel production because the content in lipids is higher, 8–24 times more lipids, than in any plant [62.25]. Presently, more than 25 complete genomes have been sequenced in lipid producing cyanobacteria. In eukaryotic microalgae having significant amounts of lipids, the nuclear genome of only 10 species has been sequenced, viz, *Chlamydomonas reinhardtii*, *Volvox carteri*, *Ostreococcus marinus*, *Phaeodactylum tricornutum* and *Thalasiosira pseudonana* [62.24].

The growing interest in biofuels has led to the overexpression of acetyl-CoA carboxylase (ACCase) in the diatom *Cyclotella cryptica* to improve lipid content by increasing the first enzyme in the lipid biogenesis pathway. The result of these studies evidenced discrete results as the alga did not yield increased lipid production. The first successful transformation of microalgal strains with potential for biodiesel production was achieved in 1994, *C. triptica* and *Navicula saprophila* [62.26, 27]. Currently there are only a few species of microalgae that fulfill even some of these crucial requirements. Apart from *C. reinhardtii* and *P. tricornutum*, only *Chlorella kessleri*, *Porphyridium*, and fairly recently *Nannochloropsis* and *Dunaliella salina* have been successfully transformed [62.28]. Acetyl CoA carboxylase and other enzymes of the lipid

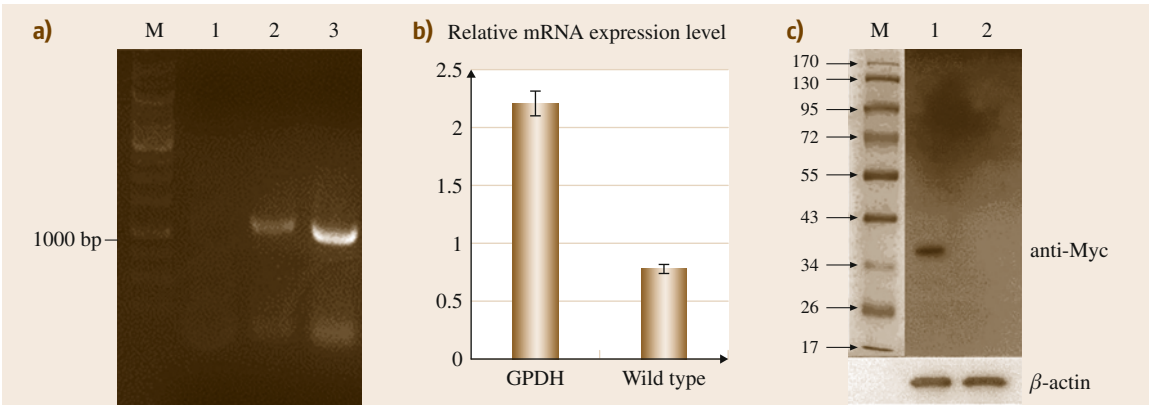

Fig. 62.5 Analysis of glycerol-3-phosphate dehydrogenase transgenic *P. tricornutum* cells. **(a)** transgenic diatom cells testes by genomic polymerase chain reaction (PCR). Lane M: 1 kb plus DNA ladder; lane 1: PCR of wild type with primers on the transformation vector; lane 2: PCR of transgenic line with primers on the transformation vector flanking GPDH; lane 3: PCR of transgenic line with primers on both ends of GPDH. **(b)** GPDH mRNA expression in the diatom cells determined by quantitative PCR. **(c)** Protein expression of introduced GPDH detected by western blotting with anti-myc antibody (Invitrogen, USA). M: Protein molecular weight marker; lane 1: transgenic *P. tricornutum* overexpressing GPDH tagged with myc; lane 2: wild type (after [62.26])

biosynthesis pathway have been used as targets for improving oil production. Lipid metabolism and the biosynthesis of fatty acids, glycerolipids, sterols, hydrocarbons, and ether lipids in eukaryotic algae were recently reviewed in the context of optimization for biodiesel production. Enhancement of the algal potential for the genetic modification of their lipid pathways can be followed either by up-regulation of fatty acid biosynthesis or by down-regulation of β-oxidation. By knocking out or modifying the enzymes responsible for the synthesis of polyunsaturated lipids in the cell, it should be possible to enhance the proportion of monounsaturated lipids. In this aspect, the specificity of algal species will drive its own biosynthetic pathway of lipids, which is why it is highly recommended to utilize species that have a suitable lipid profile for biodiesel production. At present, the mechanisms involved in the fatty acid biosynthetic pathway in microalgae have not been extensively studied [62.29]. Genes encoding the key enzymes involved in fatty acid biosynthesis have been identified in *Ostreococcus tauri*, *T. pseudonana*, *C. reindhardtii*, and in *P. tricornutum*. Recently [62.26] studied lipid metabolism and glycerol biosynthesis in the diatom Phaedactylum tricornutum (genome sequence available at: http://genome.jgipsf.org/Phatr2/Phatr2.home.html). Overexpression of Glycerol-3-phosphate dehydrogenase (GPDH) involved in lipid metabolism and glycerol biosynthesis led to the successful conversion of dihydroxyacetone phosphate to glycerol-3-phosphate and indicates that the GPDH transgene was incorporated into the transgenic diatom cells. The transcriptional expression of the introduced transgene consequently increased the GPDH transcript abundance in the transgenic diatom cells. The obtained results reported glycerol content, 6.8 fold in GPDH-overexpressing cells of *P. tricornutum*, compared with the wild type (Fig. 62.5). Moreover, genomics, transcriptomics, proteomics, and metabolomics are elucidating aspects of metabolic pathway regulation and integration linked to targets to optimize biofuel production. The recent progress in metabolic engineering toward biofuel production has incorporated random insertional mutagenesis and targeted gene disruption in microalgae. However, further research is required to better understand the partitioning of fixed carbon toward the increased production of starch for subsequent fermentation into H_2 or ethanol, or the redirection of photosynthate from starch into lipids for conversion to diesel fuels. New developments in metabolic engineering toward enhanced carbon storage in unicellular microalgae capable of synthesizing a range of biofuels are actually under development, as in the case of lipids and carbohydrates (the main energy storage molecules in algae). These approaches are basic for the understanding of primary metabolism to manipulate electron flux toward these products or H_2 for bioenergy applications. But at the level of organelles, the situation becomes more complex, such

in the case of isoforms of phosphoenolpyruvate carboxylase (PEP-C). This enzyme that carboxylates PEP, have been identified in *C. reinhardtii*, both of which are responsive to inorganic carbon and nitrogen levels. Additionally, six pyruvate kinase and five malate dehydrogenase homologs are present in the *C. reinhardtii* genome exhibiting different level of expression. These results emphasizes the complexity of algal metabolism and the need to characterize the systems on an enzymatic level.

62.7 Microalgal Culture Systems: A Contribution to the Sustainability of Biofuels

62.7.1 Culture Conditions and Better Yields of Biomass for Biofuels

It is recognized that the optimization of the microalgal production system is an important factor for the achievement of sustainability of microalgal-based biofuels. Successful histories of high-value products from microalgaehave been reported with the use of conventional open-pond algal production systems, although closed algal bioreactors have already achieved economic viability in the case when the production of high-value products such as astaxanthin and nutraceuticals are aimed for human consumption. Moreover, many interrelated factors affecting strains can each be limiting in the final yield of biomass, and hence in the outcome of biofuels.

Another critical and important factor, is media formulation, which is important to ensure a sufficient and stable supply of nutrients to attain maximal growth acceleration and cell density, and ultimately to produce biofuels at higher efficiencies [62.30]. There are many different recipes and formulations, depending on the species and particularities of the objective being sought. In the process of biomass optimization, the culture mode plays a preponderant role in the yield and biofuel production. It has been recognized that batch feeding of heterotrophic algal cultures and CO_2 enrichment of photoautotrophic algal cultures can significantly increase the biomass. On the other hand, the proper selection and optimization of mineral nutrients increases culture productivity. Two important nutrients that are constitutive of the microalgal metabolism are nitrogen and phosphorous, mostly at early stages in the mineral optimization of the media formulations. Other equally important components of the growth media are minerals, which are vital elements for support of the structural and metabolic biochemistry of the cell and in modulating the molecular configuration of photosynthetic complexes and enzymatic reactions. Unless otherwise stated, vitamins are generally omitted in massive cultures because of the susceptibility of the large-scale level, which in general are more fragile and hard to control, apart from economic reasons.

The use of organic substances is a common task for the improvement of the growth and strain preferences. Organic nutrition can sometimes be supplied with the use of domestic waste water, after secondary treatment. The dilemma is how to conciliate sanitation with water shortage due to the increasing scarcity of freshwater resources in many countries. In such situation, the use of wastewater bioremediated is an attractive presently explored for biofuel production. Maintenance of an acceptable pH range throughout culturing is of utmost importance as it impacts all aspects of media biochemistry and culture progression. Both ionic absorption from the media and the metabolic biochemistry of the cell exert significant pressure upon the pH. In high-performance cultures their effect is powerful enough to overcome the neutralizing capacity of exogenous buffering agents. Currently, microinjection of strong acids and alkalis, metabolic balancing in heterotrophic cultures, and regulated CO_2 dissolution in both photoautotrophic and heterotrophic cultures are the most practical and economical strategies for pH control.

62.7.2 Open Ponds and Raceways: Low-Cost Production Systems

Most of the microalgae produced presently are cultured in open ponds. Open ponds and raceways are built in whatever shape best suits the location. According to the needs and objectives, these are usually not mechanically mixed but driven by gravity flow or provided with paddle wheels. Traditional open ponds are highly diverse in shape, size, and technology, but the most commonly used design is the raceway pond. In these, the whole area is sectioned into a rectangular grid, with each divided rectangle containing a chan-

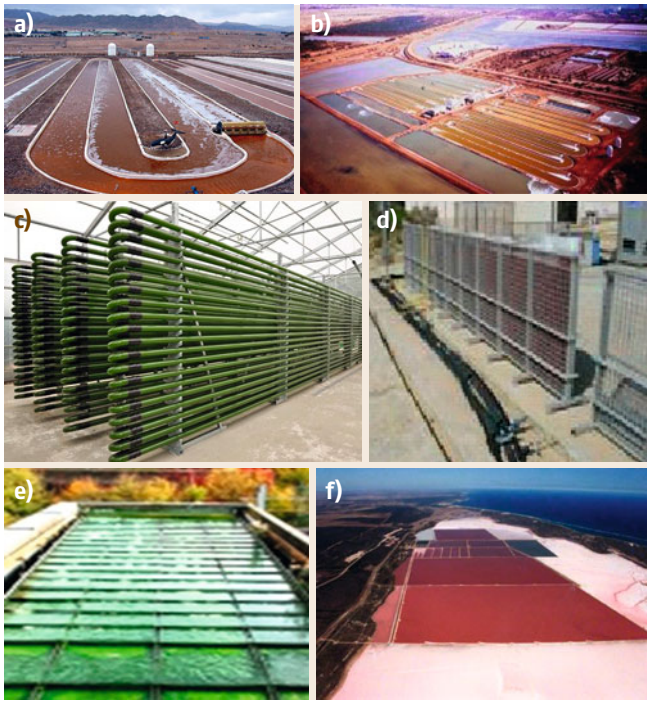

Fig. 62.6a–f Different culture systems for the industrial production of microalgae. **(a)** raceway facilities (NBT, Eliat Israel); **(b)** closed bioreactors; **(c)** alveolar panels (Beer Sheva, Israel); **(d)** cascade systems (University of Liege, Belgica); **(e,f)** open ponds (*Dunaliella salina* plant at Hutt Lagoon Western Australia, Cognis)

nel in the shape of an oval. The water flow is driven continuously by a paddle wheel all around the circuit. The reported functional and operational water depth of 15–20 cm are preferred for obtaining biomass concentrations of 1 g dry weight per liter productivities of 60–100 mg/L/day (i. e., 10–25 g/m_2/day) [62.31]. However, differences and year round productivities cannot be maintained. Raceway ponds are more expensive to construct due to the extra technologies required (paddle wheel) and the need to keep pond integrity and cell properties, a condition imposed by the flow rates. Probably, open ponds are the simplest facilities to construct, since no transparent material is required in their construction and they are relatively easy to maintain considering the large open access, which facilitates the cleaning of overgrowth and biofouling of unwanted microorganisms that builds up on surfaces. The conditions of an open culture system to the atmosphere are one of the main disadvantages that faces this system, because it is a constant exposition to unwanted species and the loss of water by evaporation, and the consequent increase of salinity. These kinds of ponds are suitable for the growth of extremophile microalgal species that tolerate and outcompete other species in conditions imposed by its particular requirements (e.g., high/low pH or salinity, or alkalinity). Examples of these conditions can be found in strains of *Arthrospira platensis/maxima* and *Dunaliella salina/bardawil*. Figure 62.6 shows the different culture systems of the massive production of microalgae worldwide.

62.7.3 Closed Bioreactors: Improving Feedstocks for Biofuels

There are different and unlimited designs of closed photobioreactors for the production of high yields of biomass. The most common shapes and designs are tubular reactors, plate reactors, or bubble column reactors. These different bioreactors have several advantages, such as saving water, energy and chemicals, and many other advantages that are increasingly making them the reactor of choice for biofuel production. Probably the most important advantages are the high control and quality of products that can be obtained [62.32].

It is to be mentioned that the most important among these aspects is that they support up to fivefold higher productivity with respect to reactor volume and consequently have a smaller *footprint* on a yield basis. The latter advantage is reinforced by the fact that they can collect as much solar energy as possible per square meter. The higher yields per square meter obtained in photobioreactors contributes to compensating for, or in some cases amortizing, the cost of photobioreactors [62.33]. Nevertheless, there are structures and operations of bioreactors that need to be optimized in order to design a performing reactor according to the needs of the algal cells at the lowest cost, ensuring the economic viability of the process [62.32]. According to the current energy cost and productivity, bioreactor costs should not exceed US$ 15 per m^2 [62.32]. Sunlight energy is another important parameter to control when dealing with photobioreactors, since most microalgae exhibit growth/light kinetics at which light saturation occurs. Sunlight may exert photoinhibition or even photobleaching in microalgae, and as a consequence, low figures in productivity and final biomass yields.

Another important factor in bioreactors is mixing, which prevents sedimentation of the cells and allows efficient homogenization of CO_2 and O_2. Recently, the use of more economical material for bioreactor construction, viz, polyethylene bags on annular reactors

or plate reactors, has contributed to refine the challenges of productivity by footprint bioreactors. The algal productivity registered based on the theoretical maximum average accounts for a yield of 100 $g/m^2/d$. According to the present expectative for the development of second-generation microalgal biofuelsystems, higher microalgal yields and photobioreactor optimization can lead to optimal and economically viable systems [62.33]. It is emphasized that the yield of terrestrial crops for the production of biodiesel cannot compete with that of algae, as exemplified from the case of soybean: 450 L of oil/ha/year, while microalgae, depending on their oil composition, can produce up to 100 000 L/ha/year [62.33, 34].

62.8 Products of Industrial Interest from Microalgae

Microalgae are an important source of food and chemicals for different industries (Table 62.2).

The demand for shellfish feed is increasing for human consumption, because of the environmental concerns over open-ocean fishing and because of the high demand for aquaculture markets. Microalgal biomass is rich in proteins, fatty acids, oils, and carbohydrates. These biomass and respective primary and secondary metabolites are of paramount importance for biotechnology and are among the most promising sources for new products and applications. With the development of sophisticated culture and screening techniques, microalgal biotechnologycan already meet the high demands of both the food and pharmaceutical industries [62.31].

In the last 20 years, most of the relevant developments in microalgal biotechnology were performed in four microalgae, viz, the green algae (Chlorophycea) *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Dunaliella salina*, and the cyanobacteria *Arthrospira (Spirulina) maxima* or *platensis*, which have been commercialized worldwide and applied as ingredients and supplements for humans and animal feed additives. The characteristics and attributes of each of these microalgal species, emphasizing their functional pigments and respective industrial applications, are described in the following.

Spirulina (Arthrospira) under the tribal name of tecuitlatl [62.35] was part of the diet of the Mexican Aztec population. This Cyanophyte has attracted special attention due to its importance as a nutritious supplement and its demonstrated (in vitro and in vivo) functional properties. The high protein content of this microalga, up to 70% dry weight, and its amino acid composition is of great interest, not only because *S. platensis* possesses all of the essential amino acids, but also because these amino acids have a great bioavailability [62.36, 37]. The phycobiliproteins (allophycocyanin and c-phycocyanin) are one of the major compounds in *Arthrospira* [62.37]. Important medical and pharmacological attributes such as hepatoprotective, anti-inflammatory, and antioxidant properties have been described and are thought to be basically related to the presence of phycobilins. Besides, phycobiliproteins might have an important role in different photodynamic therapies of various cancer tumors and leukemia treatment [62.38]. Another equally important microalga, the green unicellular *D. salina*, is an extremophilic microalga well-known for being the most abundant natural source of β-carotene (up to 14% of its dry weight) [62.39]. This microalgae is undoubtedly one of most studied [62.40, 41] because β-carotene has important applications in the food, pharmaceutical, and cosmetics industries. Studies on carotenogenic *D. salina* samples carried-out by *Olmos-Soto* et al. [62.42], successfully differentiated carotenogenic from non-carotenogenic species based on introns within 18S rDNA.

Haematococcus pluvialis is especially important due to its ability to accumulate, under stress conditions, large quantities of astaxanthin, up to 2–3% on a dry weight basis [62.43]. The carotenogenic process in *Haematococcus* induces different changes in the cell physiology and morphology of this alga, resulting in large red palmelloid cells. Astaxanthin is present in lipid globules outside the chloroplast, although the signal for induction to the massive accumulation of astaxanthin has been reported as originating in cytosol [62.44]. The pigment arsenal in *Haematococcus* possesses powerful biological activities, including antioxidant capacity [62.45], ulcer prevention, immunomodulation, and cancer prevention [62.46]. Experimental studies carried out on *Chlorella* demonstrated its antitumor effect, hepatoprotective and antioxidant properties [62.46, 47], antibacterial effects [62.48], or even the immunostimulant activity of enzymatic protein hydrolysates from this microalgae. *Chlorella* contains many dietary antioxidants,

Table 62.2 Main microalgal species and respective products and applications of biotechnology (modified after [62.12])

Species/group	Product	Application areas
Arthrospira platensis	Phycocyanin, biomass	Health food, cosmetics
Chlorella vulgaris	Biomass	Health food, food supplement, feeds
Dunaliella salina	Carotenoids, β-carotene	Health food, food supplement, feeds
Haematococcus pluvialis	Carotenoids, astaxanthin	Health food, pharmaceuticals, feeds
Odontella aurita	Fatty acids	Pharamaceuticals, cosmetics,
Porphyridium cruentum	Polysaccharides	Pharmaceuticals, cosmetics,
Isochrysis galbana	Fatty acids	Animal nutrition
Phaedactylum tricornutum	Lipids, fatty acids	Nutrition, fuel production
Lyngbya majuscule	Immune modulators	Phamaceuticals, nutrition

which may be responsible for some of its functional activities.

62.8.1 Health Foods and High-Value Added Substances from Microalgae

During the past decades, microalgal biomass was predominately utilized in the health food market, with more than 75% of the annual microalgal biomass. Currently, most products launched to serve the health food market are supplied as tablets or in powder or liquid formulations. However, algal extracts in various product forms are creating a new market sector for each algal species (Table 62.2). The market of functional foods is believed to be the most dynamic sector in the food industry and could constitute up to 20% of the entire food market within the next few years. Food supplemented with microalgal biomass might have other positive influences, e.g., prebiotic effects or mineral fortification.

There is an increasing demand for products from microalgae, which after special biotechnological processes acquire a higher value in the market considering the uniqueness of microalgae as specialty products. Among these products, polyunsaturated fatty acids occupy a preponderant position, because according to [62.49], only plants and algae are able to synthesize omega-3 fatty acids (ω-3) (Fig. 62.7). Polyunsaturated fatty acids (PUFAs) are essential components for the growth of higher eukaryotes and provide significant health benefits, reducing cardiac diseases and having other beneficial effects for depression, rheumatoid arthritis, and asthma [62.29]. Currently, the main source of these fatty acids for human consumption is marine fish. Actually, the relevant advantages from microalgae over fish oils, such as the lack of unpleasant odor, the reduced risk of chemical contamination, and a better purification potential, place microalgae as an excellent and preferred source of PUFAs. Microalgal PUFAs are established on the market both for food and feed, e.g., health-promoting purified PUFAs are added to infant milk formulas in Europe, and hens are fed with special microalgae to produce *OMEGA* eggs. The production of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) products from microalgal biotechnology for human and other applications has already been released by biotechnological companies.

Some microalgae have also developed protective mechanisms to prevent the accumulation of free radicals and reactive oxygen species, and thus counteract cell-damaging activities. These attributes confers the properties of antioxidants to some microalgae. For functional food/nutraceuticals, the radical-scavenging capacity of microalgal products is of growing interest, especially in the beverage market segment and in pharmaceutical applications for the therapy of oxidation-associated diseases like inflammation. Microalgae, including cyanobacteria, are overcharged with a multitude of photosynthetic and accessory pigments, which improve the efficiency of light energy utilization (phycobiliproteins) and protect them against solar radiation (carotenoids). The structure and function of these pigments have acquired anthropogenic importance due to their bioactivity, especially as health promoting compounds in human health and as therapies for functional disorders, neurodegenerative diseases, and cancer.

62.8.2 Microalgae in Aquaculture: A Successful Living History

Aquaculture is a fast growing industrial food sector. Aquaculture plays an important and critical function in the food system, because supply nearly 3 billion people with at least 15 % of their animal protein intake. In many developing countries, aquaculture has

accomplished its role and has been sufficiently profitable which has enhanced and harnessed strong growth in different world regions. Microalgae as live foodis undeniably one of the most important components in aquacultural enterprises. In fact, no activity is conceived in aquacultural production systems if lacking in live microalgae. The main applications of microalgae for aquaculture are directly related with nutrition, being used fresh or as food additive of health and coloring feeds or for inducing other biological functions. Worldwide, two trends dominate the field of microalgal applications in aquaculture: (1) the production of microalgal species, as live feeds to meet the feeding requirements of invertebrate larvae, and (2) the selection and production of specific strains of microalgae into shellfish feed to increase yields at industrial scale. As the basis of the natural food chain, microalgae play a key role in aquaculture, especially mariculture, being the food source for larvae of many species of mollusks, crustaceans, and fish. In addition, microalgae serve as a food source for live zooplankton production (Artemia, rotifers, copepods), which in turn are reused as feed for rearing fish and mollusk larvae [62.50]. More than 40 species of microalgae are used in aquaculture worldwide, depending on the special requirements of local seafood production.

Concerning the microalgal species used in aquaculture, a remarkable feature is the fact that presently, almost 40 years after the initial efforts, hatcheries are still using essentially the same strains for their production. The attributes of microalgae for filter feeder invertebrates reflect their importance in production nurseries and hatcheries of aquaculture species of commercial importance. Microalgae are required for larva nutrition, directly or indirectly at least in a short period of their life cycle of most of cultured shellfish species. Microalgal strains to be used in aquaculture have to meet various criteria of efficiency and functionality. In first term, it has to be easily cultured and nontoxic and needs to be of the correct size and shape to be ingested. Moreover, microalgae for aquaculture must have high nutritional qualities and a digestible cell wall to make nutrients available [62.51]. Moreover, they should undergo fast growth rates, and be scalable to mass culture, and a high nutritional value, including absence of toxins. Among these properties, protein and highly unsaturated fatty acid (e.g., eicosapentaenoic acid (EPA), arachidonic acid (AA) and docosahexaenoic acid (DHA)) content are major factor determining the nutritional value of microalgae. Recently, the role of ratios of DHA, EPA and AA has be elucidated as more important than their absolute levels as well as microalgal vitamin content. The following microalgae genera are usually supplied in aquaculture facilities: *Chaetoceros, Thalassiosira, Tetraselmis, Isochrysis*, *Nannochloropsis*, *Pavlova*, and *Skeletonema*. However, the use and time period of uses of each can vary depending on the cultured shellfish species. For practical reasons, we have chosen the representative group of shellfish species, in which these microalgal strains are an unavoidable source of feed. Bivalve mollusks are probably the most dependent and representative group of aquaculture species, as in the case of oysters, clams, and abalones. The intensive rearing of these bivalves relies on live algae and they represent the largest biomass in the hatchery and demand the highest weight-specific rations. It is well known that the suitable algal species: *C. calcitrans*, *T. pseudonana* (3H), *I. galbana*, and *T. suecica* (for larvae $>120\,\mu m$ in length) are preferred for the larvae of bivalves, either as single monoculture (unialgal diets) or in combinations with different other microalgal strains (flagellates and diatoms).

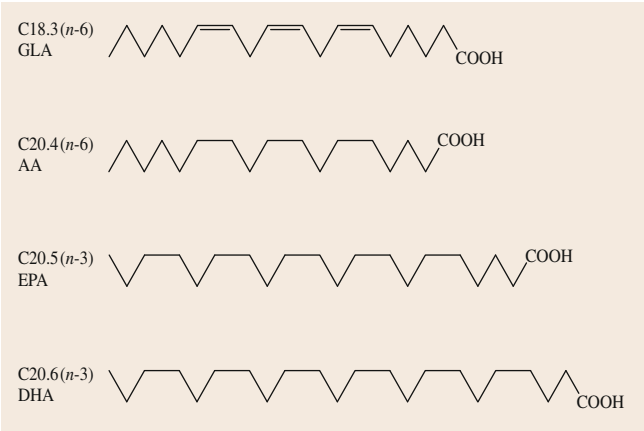

Fig. 62.7 PUFAs of high pharmaceutical and nutritional value that can be obtained from plants and microalgae (after [62.29, 50])

A different scheme of intensive feeding in hatcheries is exhibited by penaeid shrimp. The penaeid shrimp species, which is cultured worldwide, usually refers to the white shrimp *Penaeus vannamei*, the blue shrimp *P. stilirrostris*, or *P. monodon*. These crustaceans are fed with algae added during the non-feeding nauplius stage in order to have algae available upon molting into the protozoa stage. The most often used algal species are *Tetraselmis chui*, *Chaetoceros gracilis*, and *Skeletonema costatum*. As feeding preferences change from primarily herbivorous to carnivorous during the

mysis stages, the quantity of algae is reduced. Nevertheless, a background level of algae is maintained as this may stabilize water quality. In spite of all efforts to replace live algae by inert feeds, aquaculturists are still dependent on the production and use of microalgae and microinvertebrates (Zooplankton, *Artemia* and *Brachionus* sp.) as live food for commercially important fish, molluscs and crustaceans, during at least part of their life cycle. However, it is expected that technological advances in the integration among complementary approaches such as synbiotics, probiotics, bioengineered and functional feeds as well as microalgae of bioremediation activities shall lead to a more sustainable production and efficient use of microalgae. But in any situation for the purposes of industrial enterprises of microalgae, the main factor to consider is to reduce production costs while maintaining reliability in microalgae feed [62.51].

62.9 Future Needs: Making Sustainable the Unsustainable Lightness of Biofuels

The promising and clear potential of algal biofuels for contributing to environmental, social, and economic sustainability need to be transformed into a sustainable reality. Which biofuel will win: biodiesel, bioethanol, or biohydrogen? There is no doubt that an important volume of research, bioengineering, and technology is needed in order to answer these questions and to confer the sustainability tag. Microalgae are presently the most promissory renewable resource to achieve sustainable production of biofuels, considering the following aspects and uniquenesss:

a) Low impacts on the environment and in the world's food supply
b) High photon bioconversion efficiency and biomass production from photosynthesis
c) Supply energy, food and nutraceuticals for the human population
d) Can be used in bioremediation processes and simultaneously coupling CO_2-neutral fuel production with CO_2 sequestration
e) Can be cultured and harvested all-year-round
f) Can be grown on non-arable land, and in seawater and wastewater streams (avoiding the use of freshwater and recycling nutrients).

However, an assessment of the life cycle, energy and carbon balance, biofuels yield per unit area, and high efficiency production, are important issues and current limitations needed to overcome in order to make sustainable the biofuels production from microalgae.

An important strategy is the combination of fuel production with co-products, which could contribute to sustainable biofuels and reinforced by eco-friendly initiatives with minimum impact on the present reserves of air, water, and biodiversity. Moreover, it must be socially acceptable and economically viable. There is no doubt that integration of all the components of uses of microalgae: high-value compounds, aquaculture, bioremediation coupled to the production of biofuels, will play an important role in the near future to make sustainable the unsustainable lightness of biofuels from microalgae. Even with these potentialities and strategies, we believe that biofuel production from microalgae will achieve cost competitiveness in the next couple of decades, only if the aforementioned issues are considered, as well as aspects of engineering to the particular tasks demanded by bioenergy industries that are now under development. However, present innovations of new ways to make the process economically feasible shall provide many improvements and technologies contributing to the sustainability of the biofuels production from microalgae.

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63. Marine Actinobacterial Metabolites and Their Pharmaceutical Potential

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Marine actinobacteria are one of the most efficient groups of secondary metabolite producers and are very important from a pharmaceutical industry point of view. Among its various genera, *Streptomyces*, *Saccharopolyspora*, *Amycolatopsis*, *Micromonospora*, and *Actinoplanes* are the major producers of commercially important biomolecules. Several species have been isolated and screened from soil in the past decades. Consequently, the chance of isolating a novel actinobacteria strain from a terrestrial habitat, which would produce new biologically active metabolites, has been reduced. The most relevant reason for discovering novel secondary metabolites is to circumvent the problem of resistant pathogens, which are no longer susceptible to the currently used drugs. Existence of actinobacteria has been reported in the hitherto untapped marine ecosystem. Marine actinobacteria are efficient producers of new secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, antitumor, cytotoxic activities, etc. Bioactive compounds from marine actinobacteria possess distinct chemical structures that may form the basis for the synthesis of new drugs that could be used to combat resistant pathogens.

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63.1 Marine Actinobacteria

Marine actinobacteria are the most economically and biotechnologically valuable prokaryotes able to produce wide range of bioactive secondary metabolites, such as antibiotics, anticancer, antitumor, and immunosuppressive agents and enzymes. These metabolites are known to posses antibacterial, antifungal, neuritogenic, anticancer, antialgal, antimalarial and anti-inflammatory activities [63.1].

Marine actinobacteria have the capacity to synthesize many different biologically active secondary metabolites such as cosmetics, vitamins, nutritional materials, herbicides, antibiotics, pesticides, anti-parasitic ones, and enzymes like cellulose and xylanase used in waste treatment [63.2]. They are free living, saprophytic bacteria, and a major source for the production of antibiotics [63.3].

As the frequency of novel bioactive compounds discovered from terrestrial actinobacteria decreases with time, much attention has been focused on the screening of actinobacteria from diverse environments for their ability to produce new secondary metabolites. Actinobacteria isolated from the marine environment are metabolically active and have adapted to life in the sea. Streptomyces are especially prolific and can produce a great many antibiotics (around 80% of the total antibiotic production and active secondary metabolites) [63.4].

Of the total sea surface, only 7–8% is coastal area and the rest is deep sea, of which again 60% is covered by water more than 2000 m deep. The deep sea is a unique and extreme environment characterized by high pressure, low temperature, a lack

of light and variable salinity and oxygen concentrations. Although the geographical area of the deep sea is vast, our knowledge, understanding, and studies of deep-sea microorganisms are meagre. However, it is a good source of novel organisms for microbiologists and biotechnologists [63.5]. Moreover, it has been shown to be a good source of novel microorganisms for the discovery of new antibiotics [63.6]. Actinobacteria isolated from deep-sea sediments in earlier studies, however, were poorly characterized [63.7]. More recently, culture-independent studies have shown that indigenous marine actinomycetes certainly exist in the oceans [63.8]. These include members of the genera *Dietzia*, *Rhodococcus* [63.9–12], *Streptomyces* [63.13], the newly described genera *Salinispora* [63.14–16], and *Marinispora* [63.15, 17], both of which require seawater for growth and have marine chemotype signatures, and *Aeromicrobium marinum* [63.18], which also has an obligate requirement for salt. Another recently characterized genus, *Salinibacterium*, can tolerate up to 10% NaCl but does not have a salt requirement for growth [63.19]. The recently reported *Verrucosispora* strain AB-18-032 [63.20] might also qualify as an indigenous marine actinobacterium. Some of these species were found to produce unique compounds, such as salinosporamides, that are now in clinical trials as potent anticancer agents [63.21].

About 23 000 antibiotics have been discovered from microorganisms. It has been estimated that approximately 10 000 of them were isolated from actinomycetes. Actinomycetes, mainly the genus *Streptomyces*, have the ability to produce a wide variety of secondary metabolites as bioactive compounds, including antibiotics. The group has an enormous biosynthetic potential, which remains unchallenged among other microbial groups. The immense diversity, along with its underutilization is the fundamental reason for attracting researchers towards it for discovering novel metabolites. The genus *Streptomyces* is represented in nature by the largest number of species among all the genera of actinomycetes and figures over 500 species. The name *Streptomyces* was introduced in 1943 for the aerial mycelia producing actinomycetes. Actinomycetes comprise about 9% of the bacteria colonizing marine aggregates and can be isolated from various marine sources. Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry. In this book chapter, research and application of marine actinobacterial metabolites in the pharmaceutical industries are outlined in detail.

63.2 Research on Marine Actinobacteria

Marine microbiology is developing strongly in several countries, with a distinct focus on bioactive compounds. Analysis of the geographical origins of compounds, extracts, bioactivities, and actinobacteria up to 2003 indicated that $\approx 67\%$ of marine natural products were sourced from Australia, the Caribbean, Indian Ocean, Japan, the Mediterranean, and Western Pacific Ocean sites [63.22]. Interestingly the latest data [63.22] reveal a dramatic rise in reports pertaining to the China Sea, reflecting growing international attention on marine natural products.

The old impression that the diversity of actinobacteria in the oceans was small and restricted has been completely dispelled by 16S phylogenetic diversity inventories and estimates, and cultivation approaches. Thus, deep-sea sediments have been found to contain > 1300 different actinobacterial operational taxonomic units, a great proportion of which are predicted to represent novel species and genera [63.23]. Complementing this strategy are intelligent approaches to sample handling and growth conditions, which have led to the recovery in culture of many new taxa. Among newly described marine genera are: *Salinispora* (Micromonosporineae) [63.24], *Demequina* (Micrococcineae) [63.25], and others awaiting formal taxonomic description: *Marinispora* [63.17], *Solwaraspora* [63.26], and *Lamerjespora* [63.27]. New species of known actinobacterial genera are being described on a regular basis [63.28–32]; the only serious limitation in this endeavor is the availability of qualified taxonomists.

Several issues merit reiterating and updating from the perspective of effective biodiscovery. First, the question of whether to sample shallow or deep marine sites, associated with the assumption that the former can be dominated by strains originating from terrestrial runoff. Although intuition points to the deep seas (the more unusual, least explored environment) it is cautionary to note that novel genera are being recovered from surface seawater (*Serinicoccus* [63.33]) and tidal mud flats (*Demequina* [63.25]). Based on an obligate requirement for salt several indigenous genera have been identified (*Salinibacterium* [63.19], *Serinicoccus* [63.33], and

Salinispora [63.24]), whereas deep-sea polar strains of *Dietzia* and *Rhodococcus* have obligate salt requirements and psychrotolerance [63.34], and several actinomycetes taxa isolated from Palau seem to be obligate marine taxa [63.35]. To date no obligate piezophilic marine actinobacteria have been reported, but organisms isolated at atmospheric pressure can exhibit significant piezotolerance [63.12, 36].

Second, it was thought that actinobacteria were largely associated with marine sediments or occurred as invertebrate symbionts, and that the planktonic lifestyle was rare. Recent studies, however, are challenging this assumption and pointing to the need for a more comprehensive understanding of ocean dynamics and biology. *Morris* et al. [63.37] recorded significant increases in populations of planktonic actinobacteria in response to deep ocean convective mixing and, thereby, nutrient availability, thus drawing attention to the importance of temporal sampling [63.37]. In an important paper *Bouvier* and *Del Giorgio* [63.38] reported the potential effect of viruses on marine bacterioplankton composition and abundance. When these communities were incubated in virus-free ambient seawater an *unexpected and dramatic increase in the relative abundance of bacterial groups that are generally undetectable in the in situ assemblages* occurred; in open ocean regions the proportions of actinobacteria rose from zero to 35% of the bacterioplankton [63.38].

Finally, there is the question of marine actinobacterial biogeography [63.39]. In general a large body of research supports the hypothesis of biogeographical distribution of free living microorganisms and that such spatial variation reflects the proposition that *the environment selects* [63.40]. *Martiny* et al. [63.41] provide an excellent analysis of the contributions of environmental and historical effects and a framework for experimental testing of microbial biogeography; they argue that such studies should involve systematic sampling and recording of data from various distances, habitats, and environmental factors [63.41]. Such crucial data for free-living marine actinobacteria are lacking, but a study of bacterial symbionts in the sponge *Cymbastela concentrica* in tropical and temperate locations of Australia [63.42] showed that symbiotic communities had similar compositions at temperate locations, but different communities (which included actinobacteria) occurred in the temperate and tropical sponge populations, which might reflect endemic distribution or environmental selection, or both. The biogeography debate is not simply of academic interest because a greater understanding of distribution patterns is of clear importance for biodiscovery. For example, although the three currently known species of *Salinispora* co-occur at six widely separated and distinct locations [63.43], only strains of *Salinispora tropica* isolated from the Caribbean produce the potent anticancer compound salinosporamide A [63.15]. In conclusion, progress in isolating actinobacteria from the seas and oceans, especially from sediments, has been notable in the past few years.

63.3 Novel Metabolites from Marine Actinobacteria

Although more than 30 000 diseases have been clinically described, less than one third of these can be treated symptomatically and fewer can be cured [63.44]. New therapeutic agents are urgently needed to fulfill the medical needs that are currently unmet [63.45]. Natural products once played a major role in drug discovery [63.46]. Although the exploitation of marine actinobacteria as a source for discovery of novel secondary metabolites is at an early stage, numerous novel metabolites have been isolated in the past few years [63.47].

63.3.1 Antimicrobial Activity

Development of multiple drug resistant microbes revealed the need to search for new and novel antimicrobials [63.48]. Among the antibiotic producing microbes, the class Actinobacteria represents a broad range of valuable and prominent sources of pharmaceutically active metabolites in which, the members of the genus *Streptomyces* alone contributes more than half of the naturally occurring metabolites discovered up to date. Besides, 60% of antibiotics developed for agricultural purpose were isolated from the same genus [63.49]. However, the discovery of new metabolites from these common and ubiquitous actinobacteria has declined [63.50]. Hence, in the present era, search for rare actinobacteria has gained much importance in order to enhance the rate of discovery of new and potent antimicrobial agents [63.51].

Bisanthraquinone **(1)** (Fig. 63.1) is a new antibiotic isolated from *Streptomyces* sp. Biological activities were measured against clinically-derived isolates of vancomycin-resistant *Enterococcus faecium*

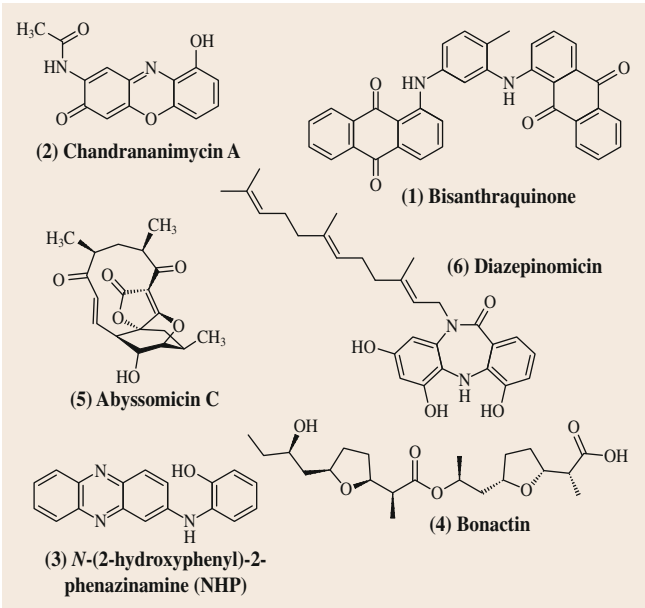

Fig. 63.1 Chemical structures of bisanthraquinone, chandrananimycin A, *N*-(2-hydroxyphenyl)-2-phenazinamine (NHP), bonactin, abyssomicin C and diazepinomicin

(VRE),and methicillin-susceptible, methicillin-resistant, and tetracycline-resistant *Staphylococcus aureus* (MSSA, MRSA, and TRSA, respectively). The most potent antibiotic displayed MIC_{50} (minimum inhibitory concentration) values of 0.11, 0.23, and 0.90 μM against a panel ($n = 25$ each) of clinical MSSA, MRSA, and VRE, respectively, and was determined to be bactericidal by time-kill analysis [63.52].

Chandrananimycin A **(2)** (Fig. 63.1) is a novel antibiotic isolated from *Actinomadura* sp. Chandrananimycin A possesses potent antifungal activity against *Mucor miehei*. It also exhibits antialgal activity against the microalgae *Chlorella vulgaris* and *C. sorokiniana*, and antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*, along with anticancer activity [63.53]. *N*-(2-hydroxyphenyl)-2-phenazinamine (NHP) **(3)** (Fig. 63.1) is a new antibiotic isolated from *Nocardia dassonvillei*. The new compound has shown significant antifungal activity against *Candida albicans*, with a MIC of 64 $\mu g\,mL^{-1}$ and high cancer cell cytotoxicity against HepG2, A549, HCT-116, and COC1 cells [63.54].

A new compound, assigned the trivial name bonactin **(4)** (Fig. 63.1), has been isolated from the liquid culture of a *Streptomyces* sp. BD21-2, obtained from a shallow-water sediment sample collected at Kailua Beach, Oahu, Hawaii. Bonactin displayed antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as antifungal activity [63.55]. Abyssomicin C **(5)** (Fig. 63.1) is a novel polycyclic polyketide antibiotic produced by a marine *Verrucosispora* strain [63.28]. It is a potent inhibitor of *para*-aminobenzoic acid biosynthesis and, therefore, inhibits the folic acid biosynthesis at an earlier stage than the well-known synthetic sulfa drugs [63.56]. Abyssomicin C possesses potent activity against Gram-positive bacteria, including clinical isolates of multiple-resistant and vancomycin-resistant *Staphylococcus aureus*. Abyssomicin C or its analog [63.57] has the potential to be developed as antibacterial agent against drug-resistant pathogens.

Diazepinomicin **(6)** (Fig. 63.1) is an unique farnesylated dibenzodiazepinone produced by a *Micromonospora* strain [63.58]. It possesses antibacterial, anti-inflammatory and antitumor activity. It has a broad spectrum of in vitro cytotoxicity and has demonstrated in vivo activity against glioma, breast, and prostate cancer in mouse models. Frigocyclinone **(7)** (Fig. 63.2) is a new angucyclinone antibiotic isolated from *Streptomyces griseus* strain NTK 97, consisting of a tetrangomycin moiety attached through a *C*-glycosidic linkage with the aminodeoxysugar ossamine. Frigocyclinone has shown antibacterial activities against Gram-positive bacteria. Chlorinated dihydroquinones **(8)** (Fig. 63.2) are novel antibiotics produced by a new marine *Streptomyces* sp. [63.59]. The compounds formally possess new carbon skeletons, but are related to several previously reported metabolites of the napyradiomycin class. Structures of the new molecules possess significant antibacterial and cancer cell cytotoxicities.

Himalomycins A **(9)** and B **(10)** (Fig. 63.2) are anthracycline antibiotics. They were obtained from *Streptomyces* sp. 6921, isolated from the marine sediments of Mauritius and exhibited strong antibacterial activity [63.60]. Glyciapyrroles A **(11)**, B **(12)**, and C **(13)** (Fig. 63.3) are a new pyrrolosesquiterpenes antibiotic isolated from *Streptomyces* sp. NPS008187. They exhibited antibacterial activity [63.61]. Tirandamycin C **(14)** (Fig. 63.3) is a novel dienoyl tetramic acid isolated from *Streptomyces* sp. 307-9. Tirandamycin C showed inhibitory activity against vancomycin-resistant *Enterococcus faecalis* [63.62]. Essramycin **(15)** (Fig. 63.3) is a novel triazolopyrimidine antibiotic isolated from *Streptomyces* sp. The compound is antibacterially active with MIC of 2–8 $\mu g\,mL^{-1}$ against Gram-positive

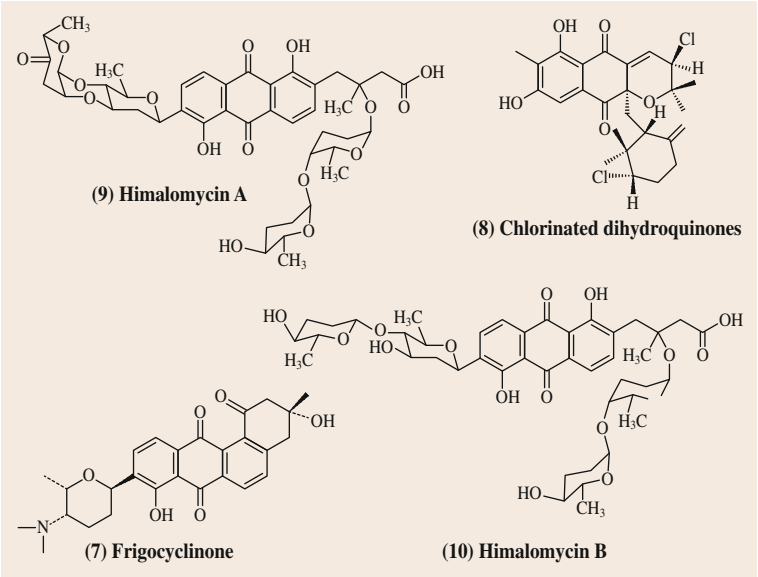

Fig. 63.2 Chemical structures of frigocyclinone, chlorinated dihydroquinones, himalomycin A and B

and Gram-negative bacteria [63.63]. Lynamicins **(16)** (Fig. 63.3) are chlorinated bisindole pyrroles, isolated from *Marinispora* sp. The antimicrobial spectrum of these compounds was evaluated against a panel of 11 pathogens, which demonstrated that these substances possess broad-spectrum activity against both Gram-positive and Gram-negative organisms. Significantly, compounds were active against drug-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* [63.64].

Caboxamycin **(17)** (Fig. 63.3) is a new benzoxazole antibiotic and was detected by HPLC-diode array screening in extracts of *Streptomyces* sp. NTK 937, another strain that was isolated from the sediments collected from the Canary Basin. The compound caboxamycin was named after the first letters of the collection site from where the organism was isolated and from letters drawn from its chemical structure. Caboxamycin showed inhibitory activity against both Gram-positive bacteria and against the tumor cell lines gastric adenocarcinoma (AGS), hepatocellular carcinoma (HepG2), and breast carcinoma cells (MCF7). The antibiotic also showed an inhibitory activity against the enzyme phosphodiesterase [63.65]. Marinopyrroles **(18)** (Fig. 63.3) are densely halogenated and axially chiral metabolites that contain an uncommon bispyrrole structure isolated from *Streptomyces* sp. The marinopyrroles possess potent antibiotic activities against methicillin-resistant *Staphylococcus aureus* [63.66].

63.3.2 Anticancer Activity

Thousands of antibiotics have been successfully applied to prevent and treat diseases caused by microbes in clinical settings over the past half-century. It is, therefore, no wonder that more and more tumor cells and bacteria have developed antimicrobial resistance or even multidrug-resistance. In the past, solutions to the problem depended primarily on the development of novel antineoplastic and antimicrobial agents from common resources.

A great number of structurally interesting and biologically active secondary metabolites produced by microorganisms have been reported, many of them being exploited by the pharmaceutical industry as potent antibiotics [63.49, 67]. However, the number of these new antibiotics is noted to have decreased dramatically in recent years due to the exhaustion of common resources [63.67]. In this regard, focus on rare resources, such as marine actinomycetes, has attracted special attention. This is due to their capability to produce biologically active secondary metabolites, with many of them as potent antibiotics and/or lead compounds that would otherwise not be discovered in terrestrial microorganisms [63.68–71]. This has facilitated the development of new and novel drugs with greater therapeutic efficiency and/or fewer side effects [63.48, 72–74], and hence led to an urgent need to explore the secondary metabolites produced by these microorganisms.

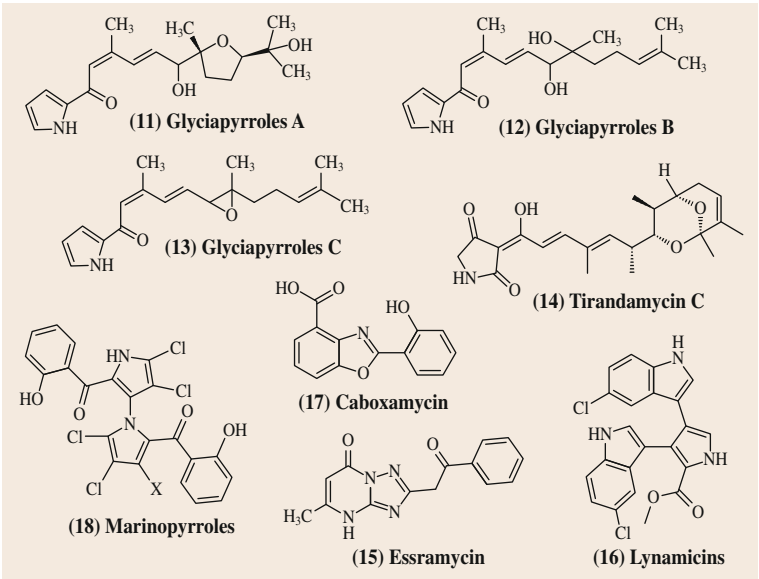

Fig. 63.3 Chemical structures of glyciapyrroles A, B, C, tirandamycin C, essramycin, lynamicins, caboxamycin, and marinopyrroles

Prudhomme et al. (2008) tested salinosporamide A for its utility as an anticancer and antimalarial drug. It was shown to have inhibitory activity against parasite development in vitro (*Plasmodium falciparum*) and in vivo (*P. yoelii*). The exact mode by which salinosporamide A inhibits *Plasmodium* erythrocytic development is unknown; however, it is likely due to the inhibition of the proteasome complex. It is interesting to note that chloroquine resistant strains are still sensitive to salinosporamide A.

Targeting the proteasome system has a huge therapeutic implication as it can restrain growth and survival of most cell types [63.75]. These attributes, along with the fact that it is already in phase I clinical trials as an antitumor agent, make it an excellent candidate for alternative therapies, such as antibacterial, anti-parasitic, antifungal, or antiviral treatments. Caprolactones **(19)** (Fig. 63.4) are new antibiotics isolated from *Streptomyces* sp., which show moderate phytotoxicity and promising activity against cancer cells with concomitant low general cytotoxicity [63.76].

Pure active compounds extracted from the marine actinobacterium, *Salinispora tropica* have shown inhibitory effects in many malignant cell types [63.75]. In particular, salinosporamide A **(20)** (Fig. 63.4) is a novel rare bicyclic beta-lactone gamma-lactam isolated from the obligate marine actinobacterium, *Salinispora tropica* [63.21, 77]. Salinosporamide A is an orally active proteasome inhibitor that induces apoptosis in multiple myeloma cells with mechanisms distinct from the commercial proteasome inhibitor anticancer drug Bortezomib [63.78]. It is being developed by Nereus Pharmaceuticals, Inc. (as NPI-0052) and was scheduled to enter clinical studies for the treatment of cancer in humans in 2006. NPI-0052 is currently being evaluated in multiple phase I trials for solid tumors, lymphoma, and multiple myeloma (http://www.nereuspharm.com/NPI-0052.shtml). NPI-0052 represents the first clinical candidate for the treatment of cancer produced by saline fermentation of an obligate marine actinobacterium [63.79].

63.3.3 Antitumor Activity

Marinomycins **(21)** (Fig. 63.4) are new antitumor antibiotics isolated from *Marinispora* sp. Marinomycins show significant antimicrobial activities against drug resistant bacterial pathogens and demonstrate impressive and selective cancer cell cytotoxicities against six of the eight melanoma cell lines in the National Cancer Institute's 60 cell line panel. The discovery of these new compounds from a new, chemically rich genus further documents that marine actinobacteria are a significant resource for drug discovery [63.17].

Manumycins constitute a class of compounds with antibiotic, cytotoxic, and other biological activities. It has been reported that manumycin A **(22)** (Fig. 63.4) and its analogs inhibit Ras farnesyl transferase and the growth of *Ki*-ras-activated murine fibrosarcoma in mice [63.80]. The side chains in

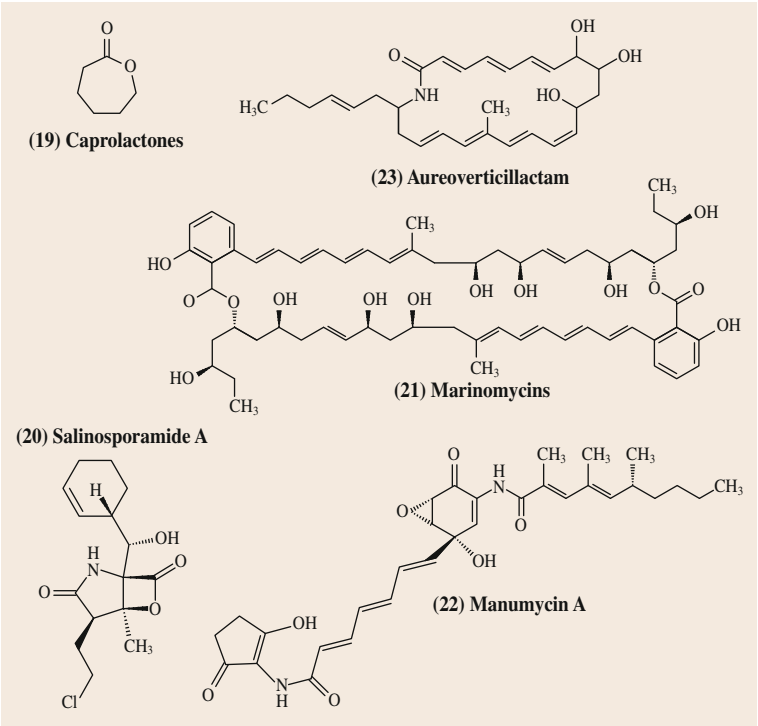

Fig. 63.4 Chemical structures of caprolactones, salinosporamide A, marinomycins, manumycin A, and aureoverticillactam

manumycins appear to be a typical polyketide-derived moiety, differing with respect to their combinations of starter and elongation units. The central cyclohexene ring may be derived from the polyketide as in the case of manumycins or from some modified amino acid-like 3-amino-5-hydroxybenzoic acid. Manumycin A and chinikomycins A and B (the quinone form of chinikomycin A) were isolated from *Streptomyces* sp. M045 derived from the sediments of Jiaozhou Bay in China [63.81].

Aureoverticillactam **(23)** (Fig. 63.4) is a 22-membered macrocyclic lactam produced by *Streptomyces aureoverticillatus* NPS001583 isolated from marine sediments. Aureoverticillactam was found to possess moderate growth inhibitory activity against human colorectal adenocarcinoma HT-29, Jurkat leukemia and mouse melanoma B16F10 cell lines [63.82]. Chinikomycins **(24)** (Fig. 63.5) are two novel antitumor antibiotics isolated from *Streptomyces* sp. They exhibited antitumor activity against different human cancer cell lines, but were inactive in antiviral, antimicrobial, and phytotoxicity test [63.81].

A higher number of type I polyketide-derived compounds with antitumor activity have been isolated from marine actinobacteria. One such compound is arenicolides **(25)** (Fig. 63.5), 26-membered polyunsaturated macrolactones, produced by the obligate marine actinobacteria *Salinispora arenicola* strain CNR-005, isolated from the marine sediments, at a depth of 20 m from the coastal around the island of Guam. In particular, arenicolide A was found to exhibit moderate cytotoxicity toward the human colon adenocarcinoma cell line HCT-116 with an IC_{50} of $30\,\mu g\,mL^{-1}$ [63.83]. Chalcomycin **(26)** (Fig. 63.5), a 16-membered macrolide, is produced by *Streptomyces* sp. M491 isolated from the Qingdao coast (China). Chalcomycin has been found to inhibit protein synthesis in HeLa human cervix carcinoma cell line [63.84, 85]. Glyciapyrroles A **(27)** (Fig. 63.5) is a new pyrrolosesquiterpenes antibiotic isolated from *Streptomyces* sp. (NPS008187). Glyciapyrrole A possesses potent antitumor activity against the pair tumor cell lines at concentration up to 1 mM [63.61].

Aureolic acid **(28)** (Fig. 63.6) (chromomycin B, A_2, and A_3) are a new antitumor antibiotics isolated from *Streptomyces* sp. WBF16. These compounds showed strong cytotoxicity against SGC 7901 (human gastric cancer cell line), HepG2, A549 (human alveolar epithelial cells), HCT116 (human colorectal carcinoma

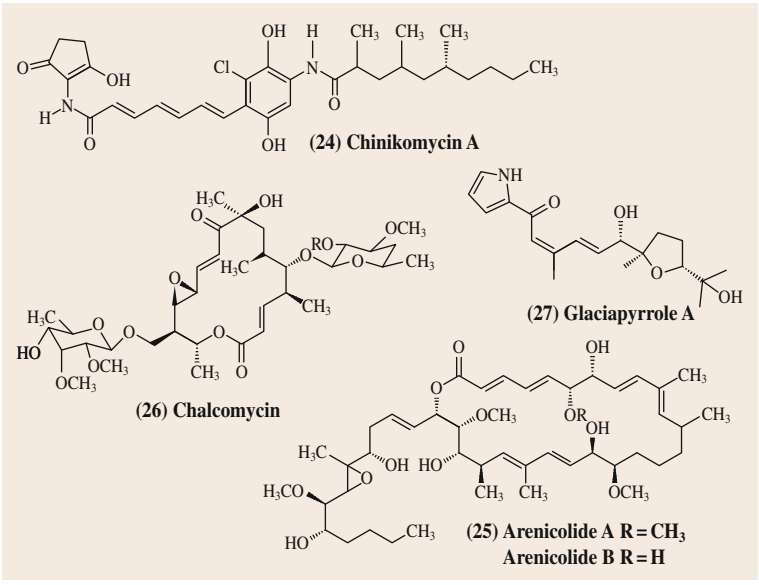

Fig. 63.5 Chemical structures of chinikomycin A, arenicolide A, B, chalcomycin, and glaciapyrrole A

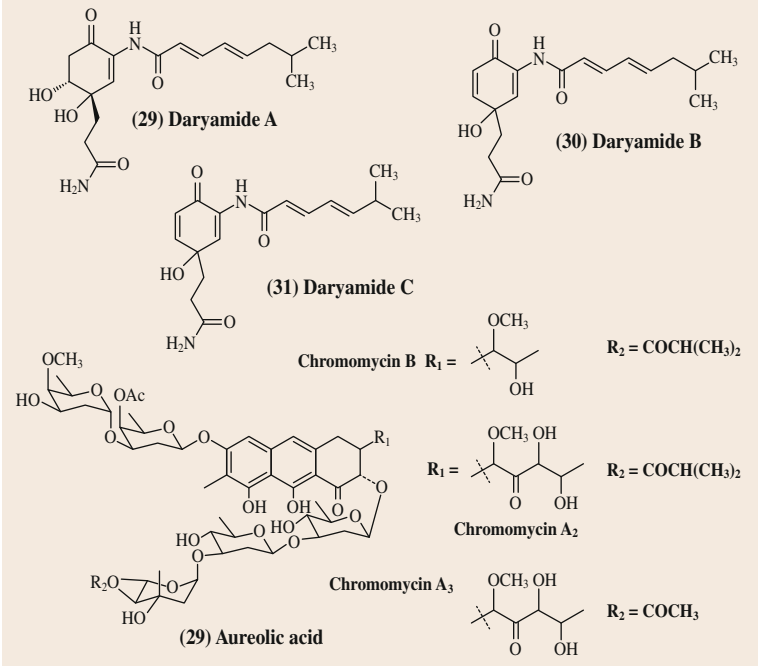

Fig. 63.6 Chemical structure of aureolic acid, daryamide A, B, and C

cell line), and COC1 (human ovarian adenocarcinoma cisplatin-sensitive cell) and HUVEC (human umbilical vein endothelial cells) [63.86]. Daryamides are new antitumor-antibiotics isolated from marine-derived *Streptomyces* strain CNQ-085. Daryamides A **(29)** (Fig. 63.6), B **(30)** and C **(31)** (Fig. 63.6) were subjected to cytotoxicity evaluation against the human colon carcinoma cell line HCT-116. Daryamide A exhibited significantly more potent cancer cell cytotoxicity, with an IC_{50} of 3.15 $\mu g\,mL^{-1}$, than daryamides B, and C and very weak antifungal activity against *Candida albicans* [63.87].

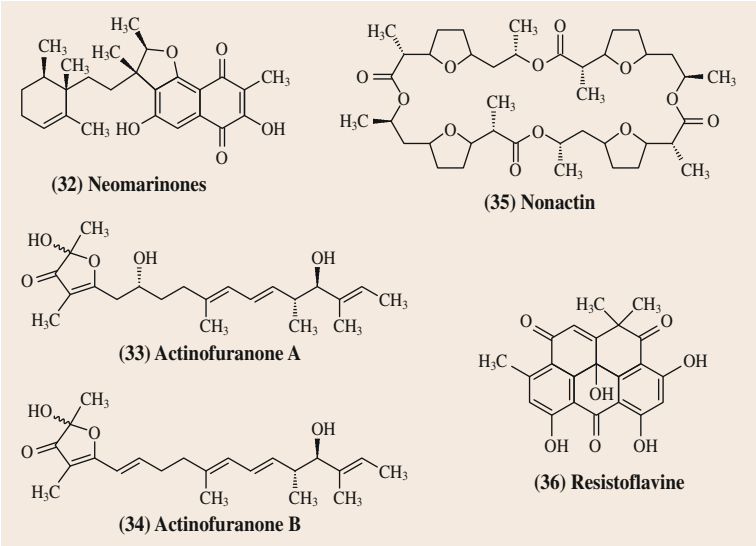

Fig. 63.7 Chemical structure of neomarinones, actinofuranone A, B, nonactin, and resistoflavine

63.3.4 Cytotoxic Activity

Neomarinones **(32)** (Fig. 63.7) are sesquiterpenoid naphthoquinones with a mixed polyketide-terpenoid origin [63.88]. Neomarinone, isomarinone, hydroxydebromomarinone, and methoxydebromomarinone were produced by the actinobacterial isolate CNH-099 obtained from sediments at 1 m depth in the Batiquitos Lagoon, North of San Diego, California. These compounds showed moderate in vitro cytotoxicity, (IC_{50} of 8 $\mu g\,mL^{-1}$) against human colon carcinoma HCT-116 cells. In addition, neomarinone generated a mean IC_{50} value of 10 μM in the NCI's 60 (human tumor cell line) cancer cell line panel [63.89, 90].

Two new polyketides, actinofuranones A **(33)** and B **(34)** (Fig. 63.7), were isolated from the culture extract of a marine-derived *Streptomyces* strain, designated as CNQ766. It showed weak in vitro cytotoxicity against mouse splenocyte T-cells and macrophages with IC_{50} values of 20 $\mu g\,mL^{-1}$ and were inactive against human colon carcinoma HCT-116 cells [63.91]. Nonactin **(35)** (Fig. 63.7), a cyclic polyether also known as macrotetrolide, has been isolated from cultures of *Streptomyces* sp. KORDI-3238, isolated from deep-sea sediments collected at Ayu Trough in the Western Pacific Ocean [63.92]. Biosynthesis of the gene cluster of nonactin has previously been isolated and characterized from *Streptomyces griseus* DSM40695 [63.93], revealing that it is synthesized by a non-iteratively acting type II PKS that involves five ketosynthases and lacks the acyl carrier protein. Nonactin exhibited significant cytotoxicity against the multidrug-resistant human erythroleukemia cell line K-562 [63.94]. Resistoflavine **(36)** (Fig. 63.7) is a cytotoxic compound, isolated from *Streptomyces chibaensis* AUBN$_1$/7. It showed cytotoxic activity against human gastric adenocarcinoma HMO2 and hepatic carcinoma HePG2 cell lines [63.95].

Lucentamycins **(37)** (Fig. 63.8), 3-methyl-4-ethylideneproline-containing peptides, are produced by *Nocardiopsis lucentensis* strain CNR-712, isolated from the sediments of a shallow saline pond from the island of Little San Salvador, in the Bahamas. Lucentamycins A and B showed significant in vitro cytotoxicity against the human colon carcinoma HCT-116 cell line with IC_{50} values of 0.20 and 11 μM, respectively. However, lucentamycins C and D were not cytotoxic in the same assay, suggesting that the presence of an aromatic ring is essential for the biological activity of this class of compounds [63.96].

Two new cytotoxic antibiotics, piericidins C7 and C8 **(38)** (Fig. 63.8), were isolated from a marine *Streptomyces* sp. [63.97]. The biological activity of piericidins was examined using rat glial cells transformed with the adenovirus E1A gene (RG-E1A-7), neuro-2a mouse neuroblastoma cells, C6 rat glioma cells, and 3Y1 rat normal fibroblast. The adenovirus E1A gene product inactivated the retinoblastoma tumor suppressor protein that plays an important role in cell-cycle and apoptosis control in mammalian cells and is inactivated during the development of

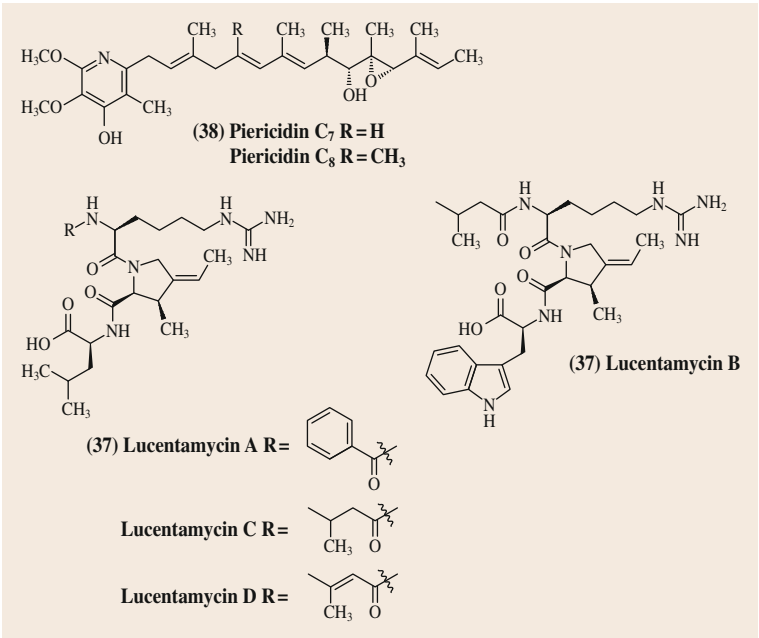

Fig. 63.8 Chemical structures of lucentamycin A, B, C, D, piericidin

a wide variety of cancers [63.98]. Piericidins C7 and C8 showed selective cytotoxicity against RG-E1A-7 cells (IC_{50} of 1.5 nM and 0.45 nM, respectively), and inhibited the growth of neuro-2a cells (IC_{50} of 0.83 nM and 0.21 nM, respectively) without cytotoxic cell death. On the other hand, C6 rat glioma cells and 3Y1 rat normal fibroblast were not affected by piericidins [63.99].

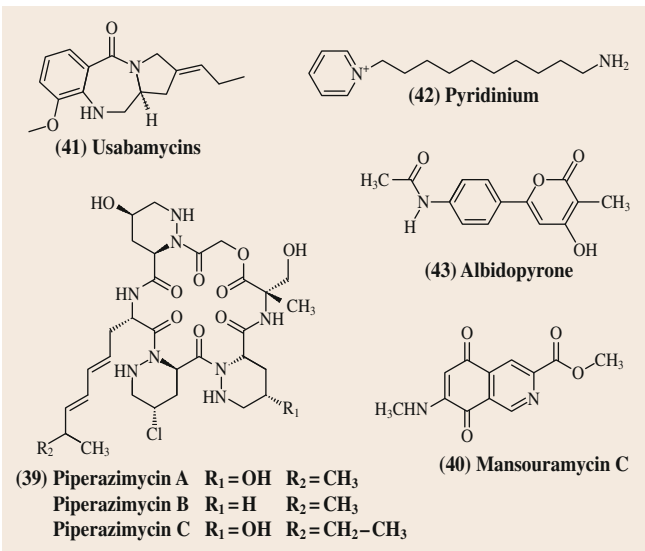

Fig. 63.9 Chemical structures of piperazimycin A, B, C, mansouramycin C, usabamycins, prydinium, and albidopyrone

Piperazimycins **(39)** (Fig. 63.9) are cyclic hexadepsipeptides isolated from the fermentation broth of a *Streptomyces* sp. strain CNQ-593, isolated from marine sediments at a depth of approximately 20 m near the island of Guam. Cytotoxic activities of piperazimycins were initially evaluated in vitro against the human colon carcinoma HCT-116 cell line. All compounds exhibited significant cytotoxicity with an average GI_{50} of 76 ng mL for each. Piperazimycin A also showed potent biological activity when evaluated against the NCI's cancer cell line panel, with mean GI_{50} (growth inhibition), total growth inhibition (TGI) and lethal concentration (LC_{50}) values for all the cell lines of 100 nM, 300 nM, and 2 μM, respectively. Overall, piperazimycin A exhibited a nearly threefold more potent activity against solid tumors (average LC_{50} of 13.9 μM) than against the leukemia cell lines tested (average LC_{50} of 31.4 μM). It was most active against the melanoma (average LC_{50} of 0.3 μM), central nervous system (average LC_{50} of 0.4 μM) and prostate cell lines (average LC_{50} of 0.6 μM) cancers [63.100]. Mansouramycin C **(40)** (Fig. 63.9) is an isoquinolinequinones antibiotic isolated from *Streptomyces* sp. Cytotoxicity profiling of mansouramycins in a panel of up to 36 tumor cell lines indicated significant cytotoxicity of several derivatives, with pronounced se-

lectivity for non-small cell lung cancer, breast cancer, melanoma, and prostate cancer cells [63.101].

Usabamycins **(41)** (Fig. 63.9) are new anthramycin-type analogs isolated from *Streptomyces* sp. NPS853. Usabamycins show weak inhibition of HeLa cell growth and selective inhibition of serotonin (5-hydroxytrypamine) 5-HT_{2B} uptake [63.102]. Pyridinium **(42)** (Fig. 63.9) is a salt antibiotic isolated from *Amycolatopsis alba*. The compound showed potent cytotoxic activity against cancer cell lines of cervix (HeLa), breast (MCF-7), and brain (U87MG) in vitro and also exhibited antibacterial activity against Gram-positive and Gram-negative bacteria [63.103]. This new α-pyrone containing secondary metabolite was detected by high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis in a culture filtrate extract of *Streptomyces* sp. NTK 227, a strain isolated from Atlantic Ocean sediments and found to be a member of the *Streptomyces albidoflavus* 16S ribosomal ribonucleic acid (rRNA) gene clade. Albidopyrone **(43)** (Fig. 63.9) showed moderate inhibitory activity against protein-tyrosine phosphatase B [63.104].

Four new polyketides, salinipyrones A **(44)** (Fig. 63.10) and B **(45)** (Fig. 63.10), and pacificanones A **(46)** and B **(47)** (Fig. 63.10) have been isolated from cultures of the obligate marine actinobacteria *Salinispora pacifica* CNS-237, found in the sediments collected from the Palau island, Western Pacific Ocean. The biological activity of these compounds is currently being examined in diverse bioassays. In the initial screening, salinipyrones and pacificanones displayed no significant activity in a cancer cytotoxicity assay using HCT-116 human colon cancer cells. In an isolated mouse splenocyte model of allergic inflammation, salinipyrone A displayed moderate inhibition of interleukin-5 production by 50% at 10 $\mu g\,mL^{-1}$ without measurable human cell cytotoxicity [63.105].

Three new cyclohexadepsipeptides, arenamides A **(48)** (Fig. 63.10), were isolated from the fermentation broth of a marine actinobacterial strain identified as *Salinipora arenicola* CNT-088, which was obtained from the marine sediments at a depth of 20 m off the Great Astrolab Reef, in the Kandavu Island chain, Fiji. Arenamides A and B exhibited weak in vitro cytotoxicity against human colon carcinoma HCT-116 with IC_{50} values of 13.2 and 19.2 $\mu g\,mL^{-1}$, respectively [63.106]. In addition, arenamides have been associated with chemoprevention of carcinogenesis by suppression of NF-κB activation. NF-κB regulates the expression of a number of genes, the products of which are involved in tumorigenesis [63.107, 108]. The effect of arenamides on NF-κB activity was studied with stably transfected 293/NF-κB-Luc human embryonic kidney cells, induced by treatment with a tumor necrosis factor (TNF). Arenamides A and B blocked TNF-induced activation in a dose and time-dependent manner with IC_{50} values of 3.7 and 1.7 μM, respectively [63.106].

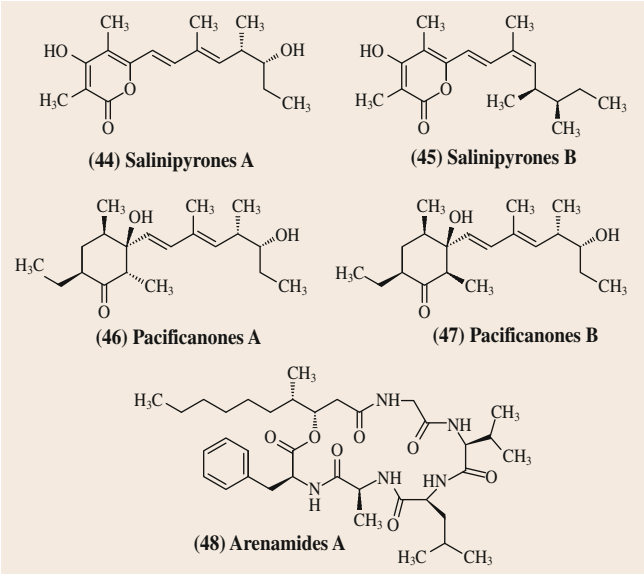

Fig. 63.10 Chemical structure of Salinipyrones A, B, Pacificanones A, B and Arenamides A

63.4 Conclusion

The marine environment has a vast biological diversity and has been recognized as a potential source of a large number of novel chemical entities. Bioactive substances derived from marine actinobacteria are just beginning to be realized. In order to improve the future exploitation of bioactive substances from marine actinobacteria and to make the process technically and economically feasible, there are numerous parameters to follow, and these include the application of appropriate isolation media (with optimal salinity, pressure, temperature, and nutrient concentration) for the various sources from the depths of the ocean and primary screening of the

fermentation broth extracts using classical methods to define the metabolites combined with techniques such as high pressure liquid chromatography with diode array detection ((HPLC–DAD)) for metabolite profiling, structural information through mass spectrometry and nuclear magnetic resonance (NMR) and two-dimensional (2-D)NMR spectra arrays.

Recent culture-independent studies [63.14] have shown that the marine environment still contains a high diversity of rare actinomycetes. Culture-independent studies of the presence and distribution of marine actinomycetes in the samples and the information obtained from these studies have also been used to design selective isolation schemes enabling successful isolation of a wide variety of marine actinomycetes including novel taxa from the same environmental samples [63.24]. Therefore, the adaptation of more efficient techniques [63.109] for the screening of new chemical compounds in the marine ecosystem and an understanding of the biology, ecology, taxonomy, and chemical biology of actinomycetes combined with polyphasic taxonomy and advanced molecular techniques [63.109] will provide detailed information on the taxonomy, ecology and chemical characteristics of uncultivable or rare actinomycetes. The development of methods for the culture of currently unculturable or rare marine actinomycetes would represent a unique and promising source for the discovery of novel secondary metabolites. It is clear from the analysis of the low or rare diversity marine actinomycete taxon (the genus *Salinispora*) that patterns of secondary-metabolite production are highly complex and that molecular studies can improve the drug discovery process.

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64. Marine Microbial Biosurfactins

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The marine biosphere offers a wealth of flora and fauna and represents a vast natural resource of imperative functional commercial-grade products. Marine bacterial natural products are predicted to become an important resource for drug discovery and enzyme mining. Among the various bioactive compounds, biosurfactants (BS) are attracting major interest and attention due to their structural and functional diversity. New techniques for the isolation of these marine bacteria are opening the possibility for the isolation of novel and potential products and processes from them. This will also help to improve our understanding of marine bacterial physiology, genetics, biochemistry, and ecology in order to provide model systems for research and production systems for commerce, and to contribute to understanding and conservation of the seas. The recent discovery of new secondary metabolites from novel bacteria and new approaches for accessing gene products from marine bacteria are contributing to the increasing development of the field. Here, we discuss the role and applications of marine microbial biosurfactants, focusing on applications for cosmeceutical and nutraceutical industry perspectives. Nutraceuticals are food products that provide health as well as medical benefits, including the prevention and treatment of disease. Few nutraceuticals are being used as pharmaceuticals and a number of others are being used and purchased by the general public as self-medication. This article outlines biosurfactants as a novel nutraceutical with their therapeutic and cosmeceutical applications.

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64.1 Overview

64.1.1 Introduction to Marine Microbial Biosurfactants

The marine environment offers a wealth of flora and fauna, which represents a vast natural resource of imperative functional commercial-grade products. Marine bacteria comprise a comparatively untapped reservoir of commercially valuable antimicrobial substances. Marine bacteria provide a luxuriant resource for screening new drugs, and several of these agents are in the process of entering clinical trials. The development of marine compounds as therapeutic agents is still in its infancy due to the lack of an analogous ethno-medical history as compared with terrestrial habitats. Over the

Table 64.1 Marine microorganisms and some types of biosurfactants (after [64.4])

Biosurfactant	Organisms	References
Glycolipids		
Glucose lipids	*Alcanivorax borkumensis*	[64.5]
	Alcaligenes sp.	[64.6]
Trehalose lipids	*Arthrobacter* sp.	[64.7]
Lipoproteins		
Ornithine lipids	*Myroides* sp. SM1	[64.8]
Phospholipids and fatty acids		
Bile acids	*Myroides* sp. SM1	[64.4]
Polymeric biosurfactants		
Lipid-carbohydrate-protein	*Yarrowia lipolytica*	[64.9]
	Pseudomonas nautica	[64.10]
Particulate biosurfactants		
Whole cells	Variety of bacteria	[64.9, 11, 12]

last few decades, significant efforts have been made, by both pharmaceutical companies and academic institutions, to isolate and identify new marine-derived, natural products. Specific programs directed towards the collection and characterization of marine natural products and evaluation of their biological activity has been established.

A surfactant is an amphiphilic agent with both lipophilic and hydrophilic structural moieties in its molecule. Surfactants are widely used in industrial, agricultural, food, cosmetic and pharmaceutical applications. Most of these compounds are chemically synthesized and potentially cause environmentally and toxically problems [64.1]. However, it is only in the past few decades that surface active molecules of microbial origin, referred to as biosurfactants, have gained considerable interest [64.2, 3].

Among the various marine bioactive compounds, BS are attracting major interest and attention due to their structural and functional diversity. The features that make biosurfactants commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability, and hence, greater environmental compatibility and better foaming properties (which are useful in mineral processing). Also unlike most synthetic surfactants, many biosurfactants function effectively in extreme conditions of pH, temperature, and salinity [64.13–19]. Some biosurfactants produced by certain species of bacteria and yeasts also exhibit effective antimicrobial, antifungal [64.20–23], antiviral, moisturizing, and antioxidant properties. Some biosurfactants have demonstrated they are potentially useful surface-active agents for emulsion [64.24–26] in various industrial processes. Moreover, they can be used for emulsification, foaming, water binding capacity, phase dispersion, de-emulsification component, and the effect of their wetting properties on viscosity and on product consistency. Thus they can be efficiently utilized by the cosmetics industry [64.27, 28]. In pharmaceutics, biosurfactants can be used for gene delivery and recovery of intracellular products. They can also serve as antimicrobial substances and emulsifying agents. The discovery of potent BS/BE (BE: bioemulsifer) producing marine microorganisms would enhance the use of environmental biodegradable surface active molecules and hopefully reduce total dependence or the number of new applications oriented towards the chemical synthetic surfactant industry (Table 64.1). The surfactant industry exceeds US$ 9 million per year. Although most are of petrochemical origin, biosurfactants are gaining importance and need to compete with chemical surfactants with respect to cost, functionality, and production capacity to meet the demand. This review provides a general overview with respect to biosynthesis, fermentative production, purification, analytical methods, and applications of surfactin (SF), an important lipopeptide type biosurfactant produced by various strains of *Bacillus subtilis*.

64.2 Methodology of Production of Marine Biosurfactants

Surfactants are amphiphilic molecules that preferentially partition at the interface between two fluid phases. In addition to lowering interfacial tension, the interfacial tension can have an impact on the interfacial rheological behavior and mass transfer. Due to these properties, surfactants are gaining importance in various industrial applications such as foam creation and stabilization in food processing, detergents for household cleaning, phase dispersion for cosmetics and textiles, or solubilization of agrochemicals. Numerous attempts have been made to reduce the cost of the production of biosurfactants. Various strategies have

been implemented to achieve improved biosurfactant production such as strain improvement, medium optimization, bioreactor design, or using agro-industrial wastes for fermentation to reduce the raw material cost.

64.2.1 Biosynthesis of Surfactins

High-Yield Mutant Biosurfactant

Surfactin was discovered by *Arima* et al. [64.29] from the culture broth of *Bacillus subtilis*. It was named thus due to its exceptional surfactant activity. Natural SFs are a mixture of isoforms A, B, C, and D with various physiological properties obtained from *B. subtilis*. They contain at least 8 depsipeptides with the number of carbon atoms between 13 and 16 as part of the ring system. Surfactin was initially identified as a potent inhibitor of fibrin clot and later found to lyse erythrocytes, protoplasts, and spheroplasts. Surfactin is a very powerful bio-surfactant; it lowers the surface tension of water from 72–27 $mN\,m^{-1}$ [64.30].

A large number of bioactive oligopeptides are produced by bacteria and fungi through a unique nonribosomal mechanism. Large modular enzymes, referred to as nonribosomal peptide synthetases, catalyze the biosynthesis of these low molecular mass peptides. All these multimodular enzymatic assemblies carry out acyl chain initiation, elongation, and chain termination, catalyzed by the protein molecules. Studies on the biosynthesis of SF began with the work of *Kluge* et al. [64.31], who proposed a nonribosomal mechanism catalyzed by multi-enzymatic thiotemplates constituting the SF synthetase. The SF synthetase complex consists of four enzymatic subunits. Three of these are enzymes SrfA (E1A, 402 kDa), SrfB (E1B, 401 kDa), SrfC (E2, 144 kDa), and the fourth is SrfD (E3, 40 kDa), which plays an important role in the SF initiation reaction [64.30, 32].

Production Strain

The bioindustrial production process is highly dependent on hyperproducing strains. Organisms that produce the final product in high concentrations are preferred, and the genetics of the producing organism is important. Apart from natural biosurfactant-producing strains, a few mutants [64.33] and recombinant strains have been reported in literature (Table 64.2). The recombinant strains of *B. subtilis* ATCC 21332 produced a lipohexapeptide, which showed less toxicity towards erythrocytes and enhanced lysis of *B. licheniformis* cells, making it useful for therapeutic applications [64.34, 35]. Thus, recombination strains not only improved the production but also gave better product characteristics.

64.2.2 Fermentative Production of Surfactin

Commercialization of any biotechnological product depends on its bioprocess economics. Although a large number of biosurfactant producers have been reported in the literature, the product enhancement has been restricted to a few organisms such as Bacillius, Pseudomonas, and Candida. Both submerged fermentation (SMF) and solid-state fermentation (SSF) have been tried for SF production [64.38].

Surfactin Production by Submerged Fermentation (SMF)

Several approaches have been tried to improve the SF yield at both flask level and fermenter level by changing environmental parameters and optimizing medium components or trace elements (Table 64.3) [64.29, 40, 41].

Surfactin Production by Solid-State Fermentation (SSF)

A cost-effective approach to produce biosurfactants is to use inexpensive raw materials, which accounts for 10–30% of the production cost. *Thompson* et al. [64.44] used high-solid (HS) and low-solid (LS) potato effluents for SF production. The growth rate of *B. subtilis* 21332 was higher in all HS and LS-based media, although the SF production was better in LS ($0.39\,g\,L^{-1}$) media compared to HS media ($0.097\,g\,L^{-1}$). The recombinant *B. subtilis* MI113 and the original strain of *B. subtilis* RB14 were also checked for their SF-producing capacity in both SSF and SMF methods [64.34, 35], a maximum of $1.8–2.0\,g\,kg^{-1}$ of SF wet mass was achieved by *B. subtilis* MI113 at 37 °C and 48 h. Among the factors affecting the results obtained in SSF processes, the moisture content of the fermenting medium plays an important role. Depending on the process, the moisture content must be maintained in a given optimum interval in order to obtain the desired results. In order to obtain higher productivity of SF, we employed a *B. subtilis* mutant strain and a semi-solid state cultivation method and used soybean as substrate. To avoid antifoaming agent interference with SF purity from the culture broth in the subsequent purification process and to improve the homogenous distribution of nutrients surrounding the substrates and initial moisture content, the semi-solid state cultivation

Table 64.2 Mutant and recombinant strains of *B. subtilis* with enhanced biosurfactant yield and improved product characteristics (after [64.36])

Mutant/recombinant strain	Characteristic feature	Yield or improved production property	References
B. subtilis ATCC 55033	Random mutagenesis with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine	Approximately 4–6 times (2–4 g L^{-1} crude SF)	[64.33, 37]
B. subtilis Th1	Random mutagenesis with ethidium bromide & UV	Approximately 3–4 times (5–7 g L^{-1} crude SF)	*Wu* and *Lu* (unpublished data)
Recombinant *B. subtilis* MI113	Incorporation of a plasmid containing *Ipa-14* gene	8 Times more SF production	[64.34]
B. subtilis SD901	Random mutagenesis with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine	4–25 Times more SF production (8–50 g L^{-1})	[64.38]
Recombinant *B. subtilis* ATCC 21332	Contains recombinantly modified peptide synthetase	Production of lipohexapeptide with reduced toxicity	[64.39]

Table 64.3 Productivity of SF in various media and culture methods (after [64.36])

Strain	Medium	Type of fermentation	γ(SF)/(mg/L)	References
B. subtilis ATCC 21322	Semisynthetic	SMF	100	[64.28]
	Synthetic	SMF	250	[64.38]
	Synthetic	SMF	800 (in foam)	[64.38]
	Meat hydrolysate	SMF	160	[64.41]
	Synthetic	SMF, aqueous two-phase	350	[64.42]
B. subtilis RB14	Semisynthetic	SMF	250	[64.34]
	Okara	SSF	200–250 (mg kg^{-1} wet mass)	[64.34]
B. subtilis MI113 (pC112)	Semisynthetic	SMF	350	[64.34]
B. subtilis MI113 (pC12)	Okara	SSF	2000 (mg kg^{-1} wet mass)	[64.34]
B. subtilis ATCC 55033	Semisynthetic	SMF	3500–4000	[64.43]
Suf-1, a mutant of *B. subtilis* ATCC2132	Synthetic	SMF	550	[64.37], *Wu* and *Lu* (unpublished data)
B. subtilis Th1	Soybean	SSSF[a]	6700	

[a] SSSF: Semi-solid state fermentation

process was carried out in a fermentative tray containing a 30% enriched MS medium. A high yield of SF biomass was achieved by SSSF. These yields were about 7.6 g kg^{-1} higher than the yield by SSF and SMF methods. In addition, after fermentation and removal of the crude biosurfactant biomass by a rinse process, the fermented soybean substrate can be re-utilized as modified raw material for soya for food or feed products.

64.2.3 Recovery of Biosurfactants

The recovery and concentration of biosurfactants account for a major portion of the total production cost. The separation strategies for biosurfactants vary according to the fermentation process and the physicochemical properties of the surfactant in question. The selection of a method for purification and recovery of surfactants depends on the nature of their charge, solubility characteristics, whether the product is intracellular or extracellular, and on the economics of recovery and downstream processing (Table 64.4) [64.45].

64.2.4 Identification of Biosurfactants

Chemical and structural analysis of lipopeptides is carried out using a broad range of techniques varying from simple colorimetric assays to sophisticated mass spectrometry (MS) and sequencing techniques. After extraction, the purification procedures of lipopeptides include chromatography methods (thin layer chro-

Table 64.4 Biosurfactant recovery methods and their advantages (adapted from [64.46])

Downstream recovery procedure	Biosurfactant property responsible for separation	Advantages	References
Acid precipitation	Biosurfactants become insoluble at low pH values	Low cost, efficient in crude biosurfactant recovery	[64.47]
Organic solvent extraction	Biosurfactants are soluble in organic solvents due to the presence of hydrophobic end	Efficient in crude biosurfactant recovery and partial purification, reusable nature	[64.48–50], *Wu* and *Lu* (unpublished data)
Ammonium sulfate precipitation	Salting-out of the polymeric or protein rich biosurfactant	Effective in isolation of certain type of polymeric biosurfactants	[64.51]
Centrifugation	Insoluble biosurfactants get precipitated because of centrifugal force	Reusable, effective in crude biosurfactant recovery	[64.52]
Foam fractionation	Biosurfactants, due to surface activity, form and partition into foam	Useful in continuous recovery procedures, high purity of product	[64.53]
Membrane ultrafiltration	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric membranes	Fast, one-step recovery, high level of purity	[64.54]
Adsorption on polystyrene resins	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvents	Fast, one-step recovery, high level of purity, reusability	[64.48]
Adsorption on wood-activated carbon	Biosurfactants are adsorbed on activated carbon and can be desorbed using organic solvent	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture	[64.47]
Ion-exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	High purity, reusability, fast recovery	[64.48]

matography (TLC) ion exchange chromatography and reversed phase high performance (RP-HPLC)). Each step of the purification will be monitored by bioassays. The bioassays could be bioautographic methods or dual culture plate [64.45, 55]. Generally, identification of the relative percentage of the lipid and protein portions is carried out using simple colorimetric assays, such as the ultraviolet absorbance spectrum and Bradford assay for protein determination, and spectroscopic methods (FTIR: Fourier transform infrared spectroscopy). The determination of the molecular mass of the compounds of interest may be facilitated by mass spectrometry using assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). This should be followed by analysis of the fatty acid portion and determination of the peptide sequence using automated Edman degradation sequencing. This combined approach would provide the necessary information required for complete structural identification [64.55].

64.3 Applications of Marine Bioactive Biosurfactants

64.3.1 Biosurfactants in the Cosmeceutical Industry

Cosmetic products using surfactants include: insect repellents, antacids, bath products, acne pads, antidandruff products, contact lens solution, hair colors and care products, deodorants, nail care products, body massage accessories, lipsticks, lip makers, eye shadows, mascaras, soap, tooth pastes and polishes, denture cleansers, adhesives, antiperspirants, lubricated condoms, baby products, foot care products, mousses, antiseptics, shampoos, conditioners, shaving and depilatory products, moisturizers, and health and beauty products [64.1]. All of these applications could be replaced by using biosurfactants. Moreover, biosurfactants as emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action in various dosage forms like creams, lotions, liquids, pastes, powders, sticks, gels, films, sprays could be used and may be replaced by biosurfactants [64.56, 57].

Cosmetics with a petroleum base may contain cancer-causing impurities. Biosurfactants are not derived from petroleum and will not have those impurities. Biosurfactants have mild toxicity and are easily biodegradable compared to synthetic surfactants. They are low or have anti-irritancy effects and are compatible with skin. Cosmetic ingredients can be contaminated with the cancer-causing impurity 1,4-dioxane, which penetrates the skin. It is considered to be a carcinogen. Those impurities of petroleum-based ingredients are found in trace amounts in products such as facial moisturizers, anti-aging products, body lotion, eye creams, hair products (relaxers, dyes, bleaches, removers), baby soap, sunless tanning products, and body firming lotion.

Marine Biosurfactants as Natural Antibiotics and Preservatives

To keep products like cleansers, hand soaps, moisturizers and toothpaste free from bacteria, parabens are used as a preservative in cosmetics. A recent study found that parabens can mimic natural hormones, including estrogen. Disruption of sex hormones may increase the risk of certain cancers. Parabens have been found in breast cancer tissue, although it is not known if the parabens caused the cancer [64.58]. Hydroquinone is banned in parts of Europe, but not in the US, as a cancer risk [64.59]. It is still used in hair dye and skin fresheners in under 2% concentrations. Formaldehyde and acrylamide are both carcinogens and irritants and are also used in cosmetics. Allergies to cosmetic products can be due to chemicals such as man-made synthetics. This can lead to a skin rash where the product is applied. If an allergy to a cosmetic product is suspected, it is important to determine which ingredients may be causing the problem.

The diverse structures of biosurfactants confer them to display versatile performance [64.60, 61]. By its structure, a biosurfactant is supposed to exert its toxicity on the permeability of the cell membrane as a detergent-like effect. A useful property of many biosurfactants is their antimicrobial activity [64.62]. Several biosurfactants have shown antimicrobial action against bacteria, fungi, algae, and viruses. A rhamnolipid mixture obtained from *Pseudomonas aeruginosa* AT10 showed inhibitory activity against the bacteria *Escherichia coli*, *Micrococcus luteus, Alcaligenes faecalis* (32 mg ml^{-1}), *Serratia arcescens*, *Mycobacterium phlei* (16 mg ml^{-1}), and *Staphylococcus epidermidis* (8 mg ml^{-1}) and excellent antifungal properties against *Aspergillus niger* (16 mg ml^{-1}), C*haetonium globosum, Enicillium crysogenum*, and *Aureobasidium pullulans* (32 mg ml^{-1}) [64.63]. Our study also indicated that marine SF is an effective substance against Gram-negative bacteria, Gram-positive bacteria (such as *Propionibacterium acnes* and *S. epidermidis*), and fungi [64.64]. *Das* et al. [64.65] reported a biosurfactant produced by marine *Bacillus circulans* that had a potent antimicrobial activity against Gram-positive and Gram-negative pathogenic and semi-pathogenic microbial strains, including multi-drug resistant (MDR). The main trump of surfactins is that they are natural compounds.

Biosurfactants in Acne Treatment

Staphylococci are the most abundant skin-colonizing bacteria and the most important causes of nosocomial infections and community-associated skin infections. Acne is the most common skin disorder of adolescence and early adulthood with a prevalence of approximately 85%. Several marine biosurfactants have a strong inhibitory effect on *P. acnes* and *S. epidermidis*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were the same (36 μM) for both bacterial species against *P. acnes* and *S. epidermidis*, respectively [64.64, 66]. In response to *P. acnes* and inflammation, the biosurfactants have the ability not only to kill *P. acnes* but also to significantly

reduce its inflammatory potential. Therefore, marine biosurfactants would be an interesting topic for further study and possibly for an alternative treatment for acne. The data presented here illustrate that marine biosurfactants have great potential as a broad-spectrum anti-infective and anti-inflammatory therapeutic for alternative treatment of dermatological conditions such as acne vulgaris and atopic dermatitis, and potential in the preservation of cosmetic preparations. The advantages of using biosurfactants as cosmetic preservatives is not only their strong spectrum and rapid cidality against wide spectrum pathogens, but also that they do not engender resistance and are not cross-resistant with other mechanisms of action. Biosurfactants have the potential to provide significant clinical benefits.

Biosurfactants as Natural Emulsifiers

Marine microorganisms such as *Acinetobacter*, *Arthrobacter*, *Pseudomonas*, *Halomonas*, *Myroides*, *Corynebacteria*, *Bacillus*, and *Alteromonas* sp. have been studied for the production of BS/BE. We are currently testing the influence of pH, temperature, and SF from *B. subutilis* spp. on emulsifier stability from diesel oil in water and common cosmetic oils in water. Results have shown that SF has emulsion activity for diesel and common cosmetic synthetic oils when dissolved (4–40 μg/ml) in saline solution and its emulsion activity remains half at 60 °C [64.64, 66].

Biosurfactants as Foaming Agents and Skin Cleansing Cosmetics

There is a growing market for formulating proteins into a wide variety of products, including laundry detergents, bath soap, conditioning shampoo, and cream hand cleanser. Foams are formed by a bubbling technique. The excellent foaming properties of SF were shown by its high ability to form density and stabilize the foam at concentrations as low as 0.05 mg mL^{-1}, in comparison with major detergents such as, sodium dodecyl sulfate (SDS), sodium laureth sulfate (SLS), disodium lauryl sulfosuccinate (DSLSS), and bovine serum albumin (BSA) where it provides good detergency combined with an excellent wetting ability [64.67]. Furthermore, SF containing a lipidic chain length of 14 carbon atoms provides lipopeptides with the best foaming properties in terms of foam density and liquid stability in foams. We are currently testing marine and aquatic biosurfactants used to partially replace detergents; they not only retained excellent foaming properties but also exhibited enhanced emulsifying properties (Table 64.3) [64.66]. These results indicate that marine biosurfactants may be used in laundry and cosmetic products to fulfill market demands.

Biosurfactants as detergents can be used advantageously to stabilize membrane proteins in the absence of a phospholipid bilayer in a manner that preserves the native conformation and permits subsequent crystallization. Cleansing cosmetics containing biosurfactants exhibit excellent washability with extremely low skin irritation. Moreover, skin moisturizing and cleansing cosmetics have been prepared in oily gel with high stability [64.68, 69].

Biosurfactant-Mediated Transdermal Drug Delivery

Transdermal drug delivery (TDD) is gaining prominence over other forms of drug delivery due to its potential advantages, such as minimal trauma induction, noninvasiveness, reduction of the enzymatic degradation associated with oral delivery, increased patient compliance, and potential for continuous, controlled delivery [64.70]. Consequently, in recent years, numerous transdermal products have been introduced into the market. The current US market for transdermal patches is over US$3 billion, and annual sales worldwide are estimated to be US$31.5 billion by 2015. The world-wide transdermal drug delivery market is quite large, but only a small number of agents have Food and Drug Administration (FDA) approval. The primary reason for such a limited development of this market is the difficulty in permeating the stratum corneum (SC) layer of human skin, which is the outermost layer of skin.

Drug delivery via the skin is not a simple task; the SC, is a formidable barrier both to water transport out of the body and to inward chemical permeation [64.71]. The SC is composed of keratin-filled, nonviable cells (corneocytes) embedded in a crystalline intercellular lipid domain. These intercellular lipids compose the continuous domain of the SC and provide its primary barrier properties. Two main pathways for transdermal penetration are normally considered (although additional alternative pathways are proposed either via the SC through the intercellular lipid lamellae or through cutaneous appendages, i. e., hair follicles and sweat glands [64.72, 73]. It is generally assumed that, under normal circumstances, the predominant route is through the intercellular spaces in the SC.

Numerous methods have been suggested to overcome the skin barrier for transdermal drug delivery. Chemical penetration enhancement methods have received the most attention, where addition of various

chemical agents, such as fatty acids, fatty esters, alcohols, terpenes, pyrrolidones, sulfoxides, and surfactants, has been tested to increase skin permeability. However, few have succeeded in delivering the relevant agents at the appropriate flux levels without causing notable skin irritation or damage [64.74, 75].

Enhancement of Penetration of Drugs and Moisturizers Through the Skin

Membrane destabilization is a key mechanism of action of SF. A conformational change in the peptide chain facilitates hydrophobic interaction, which leads to penetration of the membrane by SF [64.76–78]. Adequate penetration may occur at low concentrations ($\approx 20\,\mu\text{mol}\,\text{L}^{-1}$) and SF may be completely miscible with the phospholipids forming mixed micelles [64.79]. We have demonstrated that the lipophilic SF is capable of entrapping hydrophobic and hydrophilic drugs and increasing skin penetration of drugs (i. e., dexamethasone) and cosmetic moisturizers such as hyaluronic acid (HA) and γ-polyglutamic acid (γ-PGA) [64.64].

Enhancement of the Penetration of Gold Nanoparticles Through Skin SC and Rejuvenation of Skin

Egyptians believed that the healing powers of gold could cure all manner of physical, mental, and spiritual ailments. Romans used gold preparations for the treatment of skin lesions and sores. In ancient Chinese medicine, gold was the secret to youthful skin. The application of gold compounds to medicine, called chrysotherapy, reduces inflammation, which is beneficial in the treatment of a variety of diseases. However, this therapy often causes adverse gold-related skin reactions. Many luxury spas use 24-carat gold leaf treatments to hydrate, firm, and moisturize the skin, claiming reduced fine lines and wrinkles and resulting in smooth and radiant skin. Gold leaf also had been used for ischemic skin ulcer treatment [64.87]. The dermal application of drugs is promising due to the ease of application. In this context, nanoscale carrier systems have already been evaluated in several studies with respect to skin interaction and the impact on drug penetration. However, the data obtained do not show a clear image of the effect of nanocarriers. Especially the penetration of such particles is an open and controversially discussed topic [64.88, 89]. We are currently testing that nanogold particles using SFs as transdermal carrier can enhance penetration of Au-NP through skin SC of BALB/c (an albino, laboratory-bred strain of the House Mouse) mouse skin (Fig. 64.1). After permeation, Au-NP transformed fibroblast with multiplications of mRAN in keratinocyte growth factor (KGF) and epidermal growth factor (EGF). The application of nanogold effected the indication of balance for superoxide dismutase (SOD), metallothionein, and EGF indirectly. SOD can eliminate hydroxyl-free radicals, while metallothionein resisted skin cell damage from ultra-violet rays to achieve an anti-aging effect.

Antiwrinkle, Anti-Aging, and Rejuvenation Effects of Biosurfactants

Peptides have become very important ingredients in cosmetic products, especially in anti-aging preparations. According to their mode of action, they have been divided into three main groups: signal peptides, neurotransmitter-affecting peptides, and carrier peptides. These small sequence amino acid chains are being incorporated in cosmetic formulas to improve the signs of aging skin (Table 64.5). Chronologically aged skin demonstrates lower procollagen type I messenger ribonucleic acid (RNA) and protein, resulting in decreased production of new collagen. In addition, aging skin, and particularly aging skin that is exposed to ultraviolet (UV) light, overexpresses the proteolytic activity of the matrix metalloproteinase-1, also known as interstitial collagenase. Additionally, aging fibroblasts have a lower rate of proliferation than fetal fibroblasts [64.90]. Much of the research demonstrating the role of amino acids and peptides in reversing the cutaneous signs of aging has been a secondary benefit of research on wound healing. These peptides are small sequence amino acid chains that may stimulate angiogenesis, production of granulation tissue, and new collagen synthesis.

However, proteins and peptides are hydrophilic and are often charged molecules at physiological pH. They range in molecular weight from small peptides as small as 300 Da to proteins of size greater than 1000 kDa. These properties result in poor skin permeation and despite generally having high potency, most are therapeutically ineffective if administered transdermally because of this poor permeation. To overcome the skin barrier and facilitate the permeation of biodrugs through the skin, a number of skin penetration enhancement techniques have been investigated. Formulation and chemical enhancement techniques have potential for the local delivery of small peptides for dermatological and cosmetic applications [64.74]. The effectiveness of coupling tetrapeptide linked with a short hydrocarbon chain can enhance transepidermal delivery of protein inhibitors [64.75]. It has been demonstrated that

Table 64.5 Cosmeceutical peptides for anti-aging and antiwrinkle effects (after [64.80])

Name	Indication of topical use	Suggested Functions in cosmetic products	Efficacy	Reference
Acetyl hexapeptide-3 (Argireline)	Skin conditioning (anti-aging)	Anti-aging, relaxes facial wrinkles, reducing the degree of existing wrinkles	Significantly more reduction in the depth of wrinkles for Argireline group	[64.81]
Leuphasyl (palmitoyl pentapeptide)	Anti-aging	Antiwrinkle and collagen production stimulation		[64.82]
Palmitoyl pentapeptide-4 (Pal-KTTKS) (Matrixyl)		Healing skin wounds & tighten skins, reduces wrinkle	Antiwrinkle and lifting effects	[64.83]
Octapeptide SNAP-8		Prevention & reducing the depth of wrinkles		[64.84]
Lipopeptide (SF)		Boosts cells' natural productivity levels & functions	Significant increased sirtuin-1 and collagen I, inhibit MMP-9 mRNA	*Wu* and *Lu* (unpublished data)
GHK-Cu^{2+}	Anti-aging	Increases skin density, repairs skin cells, wound healing and revascularization of tissues	Significant improvements in all evaluations were found for both products	[64.85]
PAL-GHK (palmitoyl oligopeptide)	Anti-aging	Anti-aging and antiwrinkle effects, cutaneous restructuring and repair, stimulation of collagen repair & photoaged skin		[64.82]
Pal-GQPR (palmitoyl tetrapeptide-3)	Anti-aging	Anti-aging, wrinkle smoothing, cutaneous barrier repair, increases firmness of the skin area around the eye	Significant increase in skin thickness and firmness with active cream. AcTP1 was more effective than the placebo	[64.86]

oligopeptides linked together and attached to a fatty acid to enhance the oil solubility of peptides and thus better skin penetration.

Cutaneous absorption of interferon (INF)-a linked to various palmitoyl molecules was about 5–6 times greater than that of the parent peptides [64.91]. The lipopeptides are amphiphatic and consist of a hydrophobic chain containing between 8–24 carbon atoms and of a peptide chain that is hydrophilic or has been rendered hydrophilic. One of the applications for lipopeptides has been in antiwrinkle, anti-aging, and rejuvenation cosmetics [64.92]. Lipopeptides (such as palmitoyl pentapeptide) are at least as effective against wrinkles as retinol, but do not cause skin irritation, which is a common side-effect of retinoids. Furthermore, it has found that when added to the culture of fibroblasts (the key skin cells), palmitoyl pentapeptide stimulated the synthesis of the key constituents of the skin matrix: collagen, elastin, and glucosamnoglycans. How exactly palmitoyl pentapeptide did that remains unclear [64.80].

We have currently demonstrated the applicable in cosmetology and dermatology of using SF as an agent for stimulating skin dermis cell metabolism and more particularly, as an agent for stimulating collagen neosynthesis at low concentration in formulation. Our data indicated that SF not only up-regulated expression of the anti-aging gene (such as sirtuin I) effectively (Fig. 64.1), but also stimulated the proliferation of fibroblasts and increased the cell survival rate and repair ability after oxidative stress and UV damage. Figures 64.3 and 64.4 show that the amount of collagen and the elastin gene expression were increased and MMP-9 expression was inhibited in BALB/3T3 clone A31 *Mus musculus* embryo

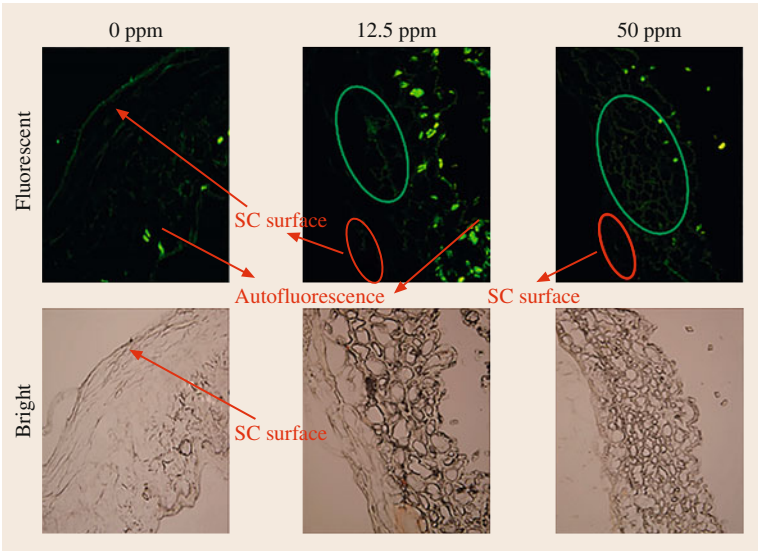

Fig. 64.1 The enhancement effect of SFs on the penetration of gold nanoparticles (2 nm) through mouse skin. The levels of Au-NP were measured with fluorescence microscopy. The *green color* represents the presence of Au-NP, which was increased in SF treatment (50 ppm) on the BALB/c dermal layer. The *green bright spot* was the fair follicle autofluorescence in BALB/c

fibroblasts by SF treatment. By encouraging the inducing extracellular matrix by efficacious stimulating the dermis cells and synthesis of new collagen fibres, purified SF can be used both as a preventive measure against aging of the skin and used as repair and restructuring products in creams for the body, and in body milks, lotions, and gels for the skin [64.66].

64.3.2 Applications of Marine Biosurfactants in the Nutraceutical Industry

Marine organisms represent a valuable source of nutraceuticals and functional compounds. The biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of novel active substances for the development of bioactive products. Recently, a great deal of interest has been expressed in marine-derived bioactive peptides because of their numerous beneficial health effects. Moreover, several studies have reported that marine bioactive peptides exhibit immunomodulatory activity, anti-inflammatory, antioxidative, and antimicrobial properties in functional foods or nutraceuticals and pharmaceuticals due to their therapeutic potential in the treatment or prevention of disease. In this chapter, we provide an overview of bioactive peptides derived from marine organisms, as well as information about their biological properties and mechanisms of action with potential applications in different areas.

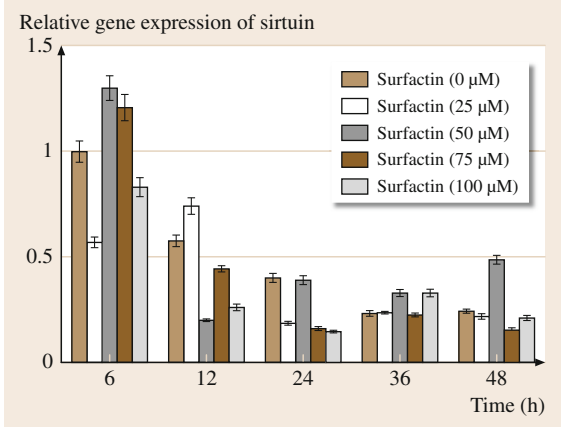

Fig. 64.2 Effect of SF on sirtuin I mRNA level in a BALB/3T3 clone A31 *Mus musculus* embryo fibroblast on treatment with different SF concentrations. The levels on sirtuin I mRNA were measured in real-time PCR. The *Mus musculus* embryo fibroblast cells were treated with varying concentrations of SF (0, 25, 50, 75, 100 μM) for 6, 12, 24, 36, 48 hours

Biosurfactants as Immunomodulators

Biosurfactants are not only useful as antibacterial, antifungal, and antiviral agents, but also have potential for use as major immunomodulatory molecules. Toll-like receptors (TLR) are critical mediators of the immune response to pathogens and human polymorphisms in this gene family regulate inflammatory pathways and are associated with susceptibility to infection [64.93].

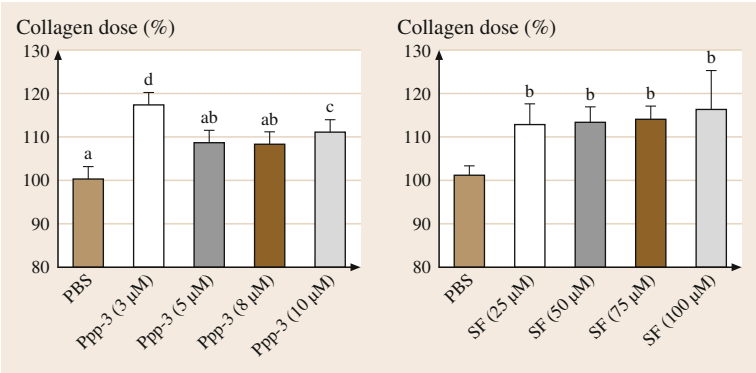

Fig. 64.3 Effect of palmitoyl pentapeptide-3 (ppp-3) and SF (SF) on collagen dose. The levels of the collagen dose were measured in the Sircol collagen assay. The *Mus musculus* embryo fibroblast cells were treated with varying concentrations of SF (0, 25, 50, 75, 100 μM) and ppp-3 (0, 3, 5, 8, 10 μM) for 24 hours

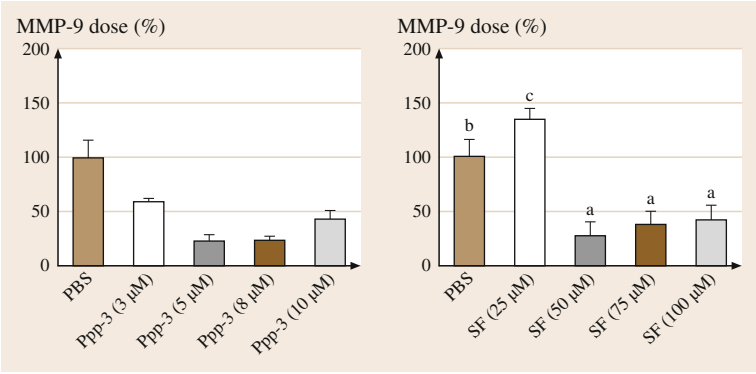

Fig. 64.4 Effect of ppp-3 and SF on matrix metalloproteinase-9 inhibitory ability. The levels of the matrix metalloproteinase-9 dose were measured in enzyme-linked immunosorbent assay (ELISA). *Mus musculus* embryo fibroblast cells were treated with varying concentrations of SF (0, 25, 50, 75, 100 μM) and ppp-3 (0, 3, 5, 8, 10 μM) for 24 hours

Lipopeptides are present in a wide variety of microbes and stimulate immune responses through TLR1/2 or TLR2/6 heterodimers [64.94]. We examined the immunomodulatory activity of marine microbial biosurfactin treatment on innate acquired immune influence and observed the expression of innate immune response in BALB/c mice. In in vivo studies we studied the stimulation of immune response in BALB/c mice after feeding them with SF for 1 week. Figure 64.5a–c shows the ratio between $CD4^+$ T cells, $CD8^+$ T cells and B cells were greatly changed in BALB/c mice. Especially, the percentage of $CD4^+$ T cells increased by over 20%. In in vivo studies, SF was shown to reduce the size of tumor in mice and prolong the survival time as well. This implies that SF has potential for the application as food additives.

Biosurfactants as Anti-Inflammatory Agents

Park et al. [64.95] demonstrated the antineuroinflammatory properties of SF in lipoteichoic acid (LTA)-stimulated BV-2 microglial cells. SF can significantly inhibit excessive production of the pro-inflammatory mediators TNF-α, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), prostaglandin E2 (PGE2), nitric oxide (NO), and reactive oxygen species (ROS), and suppressed the expression of matrix metalloproteinase-9 (MMP-9), inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2). It also showed that SF can significantly suppress lipopolysaccharide-induced expression of CD40, CD54, CD80, and MHC-II, but not of CD86 and MHC-I. *Park* and *Kim* [64.96] indicated that SF-treated macrophages also exhibited impaired phagocytosis and reduced IL-12 expression. In addition, SF inhibited the phosphorylation and degradation of IκB-alpha, and suppressed the activation of IKK (inhibitor of nuclear factor κ-B kinase subunit beta), Akt (nuclear phosphatidylinositol 3,4,5-triphosphate phosphotidyinositol 3-kinase), JNK (c-Jun N-terminal kinase), and p38 kinase. Microbial lipopeptides constitute potent nontoxic, nonpyrogenic immunological adjuvants when mixed with conventional antigens [64.97]. Low molecular mass antigens such as Iturin AL and Herbicolin A can enhance the humoral immune response [64.22]. In addition, biosurfactants represent

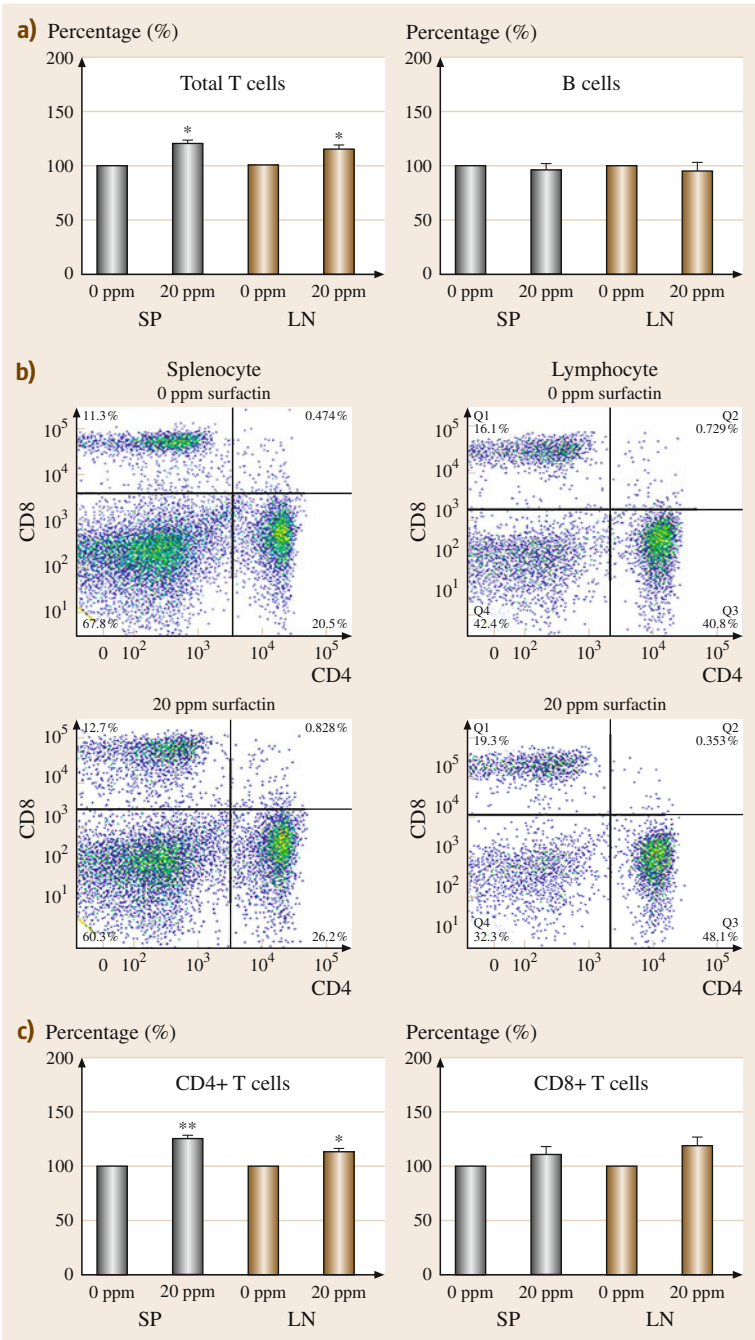

Fig. 64.5 **(a)** Effect of SF on the expression of total T cells and B cells in splenocytes (SPs) and lymphocytes (LN) (*left*). Mouse SP and LN were isolated from mice that were orally administered 20 ppm SF and PBS control for 1 week, stained with FITC-conjugated anti-CD3 monoclonal antibodies, and analyzed using flow cytometry. PBS is the only group with 100% standard (*right*). SP and LN were also stained with PE-conjugated anti-CD45R monoclonal antibodies. $*P < 0.05$ versus PBS only group. **(b)** The percentage of $CD4^+$T cells were up-regulated by SF. 20 ppm/mouse of SF was used for oral administration for 1 week with mice. $CD3^+$T cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 monoclonal antibodies, and analyzed using flow cytometry. The results are from one of the three experiments performed. **(c)** Effect of SF on BALB/c mice $CD4^+$ T and $CD8^+$T cell percentage. The percentage of CD4 and CD8 marker-positive cells in SP and LN. PBS is the group with 100% standard. $*P < 0.05$, $**P < 0.01$ versus PBS group

a promising immunomodulatory adjuvant that could enhance the therapeutic potency of vaccines in cancer therapy [64.98].

Biosurfactants as Antiviral Agents

It has been found that lipopeptides (i. e., SF) or salts or esters have a surprisingly high inactivation potential for lipid-enveloped viruses such as herpes or retroviruses, and in addition they offer the advantage of an exceedingly low in vivo toxicity, so that the step of removing the inactivating agent from pharmaceutical products or cell cultures can be omitted [64.99–101]. We have demonstrated that SF can inactivate both enveloped viruses such as the betanodavirus, and nonenveloped virus such as the iridovirus [64.66].

Biosurfactants as Antitumor Agents

Over the past years, a few studies have examined the actual effect of lipopeptides on tumor cells. *Kameda* et al. [64.102] proved that lipopeptides can inhibit the proliferation of Ehrlich ascites carcinoma cells. *Sudo* et al. [64.103] reported the ability of lipopeptides to inhibit growth and induce differentiation in HL60 human promyelocytic leukaemia cells . In addition, *Wakamatsu* et al. [64.104] discovered that lipopeptides induce neuronal differentiation in rat adrenal pheochromocytoma PC12 cells and provided the groundwork for the use of microbial extracellular lipopeptides as a novel reagent for the treatment of cancer cells. An antitumor lipopeptide purified from *Bacillus natto* TK-1 was able to inhibit the proliferation of MCF-7 human breast cancer cells in a dose and time-dependent manner [64.105]. The antitumor activity of the *Bacillus natto* SF in MCF-7 cells was associated with cell apoptosis determined by typical morphological changes and sub-G(1) peak in cell growth-phase distribution. The cell cycle was arrested at the G(2)/M phase. In addition, the caspase activity assay revealed that lipopeptide-induced apoptosis in MCF-7 cells was associated with caspase 3.

64.3.3 Development of Fermented Soy Nutraceuticals Containing Marine Biosurfactants

Nutraceuticals are food products that provide health as well as medical benefits, including the prevention and treatment of diseases. There are many nutraceutical (such as soy protein) that are beneficial in the prevention or symptom reduction of coronary heart disease (CHD). In 1999, the FDA gave permission to manufacture soy foods. At least 25 mg administration of soy protein per day reduces the risk of developing CHD [64.106].

A few studies have shown that biosurfactins have therapeutic and preventive properties against different types of cancers and pathogen-induced inflammatory causes by virus infection. Since we have developed a semi-solid-state culture technique that uses soybeans as the substrate to produce SF, these fermented soybeans might be used to make various soy food products (such as soy powder, soy beverages, etc.) (Fig. 64.6). Furthermore, after the SSSF process, the fermented soybeans contain 10 times more small peptides and 39% less phytic acid. This novel fermented soy nutraceutical, can be used within the context of chemoprevention and in therapeutics, as a main stay treatment and as adjuvant nutrition for disease prevention and the enhancement of well-being.

64.4 Conclusions

Chemically synthesized surface-active compounds are widely used in the pharmaceutical, cosmetic, petroleum, and food industries. However, with the advantages of biodegradability and production of renewable-resource substrates, biosurfactants may eventually replace their chemically synthesized counterparts. So far, the use of biosurfactants has been limited to a few specialized applications because biosurfactants have been economically uncompetitive. There is a need to gain a greater understanding of the physiology, genetics, and biochemistry of biosurfactant-producing strains and to improve process technology to reduce production costs.

Biosurfactants (SF and other lipopeptides) produced by marine microorganisms are nontoxic and inexpensive to produce and have excellent surface properties and biological activities suitable for cosmeceutical applications. Biosurfactants have good skin compatibility with low irritation and can potentially be applied as a topical dermatological product. The application of the SF approach to cutaneous delivery is relatively new and is of merit on a scientific basis. The diversity of lipopeptide activities outlines their importance as a multifunctional cosmetic ingredient.

Several studies have reported that marine bioactive peptides exhibit immunomodulatory activity, anti-in-

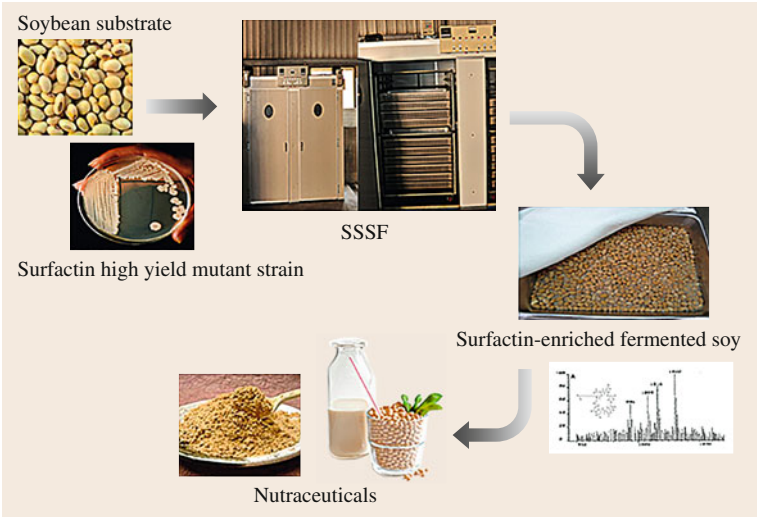

Fig. 64.6 Process of producing fermented soy nutraceutical enriched biosurfactants by SSSF culture

flammatory, antioxidative, antitumor, and antimicrobial properties, which might suggest this marine biosurfactant have therapeutic potential in the treatment or prevention of disease.

Nutraceuticals is growing healthcare industry in Asian countries. The growth of nutraceuticals is also increasing because people want treat a disease by improving their health with the help of healthcare goods. Consumers are turning massively to food supplements to improve well-being where pharmaceuticals fail. Soy and soy products have been a staple in the standard diet in China, Japan, Indonesia, and other Asian countries for centuries. In this chapter, we have proposed a possible application of biosurfactants in functional food or nutraceuticals through soy-fermented products. Soy food containing biosurfactants can be made from fermented, cooked whole soybeans. Because the fermentation process breaks down the beans' complex proteins, this soy food containing SF is more readily absorbed.

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65. Nutraceuticals and Bioactive Compounds from Seafood Processing Waste

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Seafood items are rich in several valuable nutrients and other useful components. The rising global demand for the community is witnessing an increasing quantity of processed seafood entering world markets. Commercial processing of seafood items for diverse products results in significant amounts of wastes consisting of shells, heads, intestines, scales, bones, fins, etc. Moreover, fishing operations aimed at popular species also lead to the capture of substantial amounts of fish that are commercially nonviable and, therefore, regarded as by-catch. Currently, most of these materials are discarded as landfill or converted on a limited scale into products such as animal feed and leather, which leads to serious environmental hazards. With rapid developments in biotechnology there is vast scope to make use of the wastes as sources of valuable nutraceuticals and other ingredients, which encompass proteins including collagen and gelatin, protein hydrolyzates, bioactive peptides, lipids rich in polyunsaturated fatty acids, squalene, carotenoids, polysaccharides such as chitin, chitosan, glycosaminoglycans and their derivatives, mineral-based nutraceuticals, among others. These products, depending upon their characteristics, have potential for various applications such as natural food additives, bioactive compounds, nutraceuticals, medicinal drugs, biodegradable packaging, and as encapsulation materials for diverse nutraceuticals. This chapter highlights the potential benefits of secondary processing of seafood discards, for the isolation of various valuable compounds, such as unsaturated oils, carotenoids, minerals, fine biochemicals, enzymes, proteins, nucleic acids and pharmaceuticals, antimicrobials, antioxidants, enzyme inhibitors, and other bioactive compounds.

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The food and nutritional value of fishery products have been well recognized in the last few decades. The diverse species of fish and shellfish from varied marine environments provide opportunities for the development of food products with different flavor, high nutritional value, and hence good consumer acceptance, which has resulted in a rising global demand for the commodity. In the year 2012, capture fisheries and aquaculture supplied the world with about 158 million tons (Mt) of fishery products, of which about 136 Mt was utilized as human food [65.1]. During the last 50 years, the world per capita food fish supply has almost doubled from an average of 9.9 kg (live weight equivalent) in the 1960s to 19.2 kg in 2012. In the same year, aquaculture set an all-time production high of 66 Mt. In 2008, about 39% of global seafood production at a value of US$ 102 billion entered diverse global markets, making fishery products the most internationally traded food commodity [65.2, 3]. This has resulted in huge amounts of processing discards in processing centers all over the world, causing serious environmental hazards.

65.1 Seafood as a Source of Nutraceuticals

Seafood items with their biodiversity are recognized as a functional food due to the presence of several nutraceuticals and biologically active compounds. The term *functional food*, which was coined in Japan in the 1980s, describes foods that contain nutraceutical ingredients that provide nutrition and offer protection against diseases [65.4]. The term *nutraceutical* is defined as any substance that may be considered a food or part of a food and provides medical or health benefits including the prevention and treatment of diseases. These ingredients are not identified as essential nutrients, but are considered as bioactive substances with one or more health benefits. Public health authorities consider prevention and treatment with nutraceuticals as a powerful instrument to maintain health and to act against nutritionally induced acute and chronic diseases, thereby promoting optimal health, longevity, and quality of life [65.5–7]. Over the past two decades, more than 3000 new compounds have been isolated from various marine organisms, including fishery items, seaweed species, corals, sponges, and microorganisms [65.8]. Nutraceuticals from fishery sources include calcium, unsaturated oils, carotenoids, fine biochemicals, enzymes, proteins, nucleic acids and pharmaceuticals, antimicrobials, antioxidants, enzyme inhibitors, and other specific bioactive compounds [65.9–11]. The US National Oceanic and Atmospheric Administration (NOAA), with support from the Food and Drug Administration in a study titled, *Seafood choices: balancing benefits and risks* concluded that *seafood is a nutrient-rich food that makes a positive contribution to a healthful diet* and, therefore, advises regular consumption of seafood by Americans [65.12].

65.2 Bio-Waste from Processing of Seafood

Several items of seafood such as shrimp, prawns, salmon, tuna, ground fish, flatfish, sea bass, and sea bream are commodities of international trade, involving about 200 countries, and equivalent to US$ 129.8 billion in 2011. Besides marine fish, freshwater and maricultured species such as shrimp, prawns, salmon, mollusks, tilapia, catfish, sea bass, and sea bream also constitute the trade [65.1]. Commercial processing of fish and shellfish for trade generates significant portions of the raw material as waste, which consists of heads, filleting frames, scales, viscera, gills, dark flesh, bone, and skin [65.14]. The various preprocessing operations involve beheading, skinning, gutting, descaling, filleting, etc. Processing discards are as high as 40% of whole shrimp and krill, 50% of crab, and 24% of squid and of crab and consists of heads, exoskeleton, cephalothorax, and carapace. It has been estimated that annually 500 000, 100 000, 490 000, and 60 000 t of wastes are generated from shrimp, squid, crabs, and krill, respectively [65.15]. About 250 000 and 45 000 t of processing discards are generated annually from the processing of Atlantic cod and tuna, respectively. Norwegian fisheries annually produce more than 615 000 t of waste; most of it being converted into fish silage and fish meal, while about 180 000 t of bycatch is dumped into the sea [65.16, 17]. Pre-processing of freshwater fish such as trout, carp, pike-perch, pike, and bream into various products such as gutted, headed, and free or skin on fillets results in 40–60% of the fish as waste. Frames after filleting are a good source of minced meat [65.18]. Scale constitutes $\approx 2\%$ of the weight of large fish. Freshwater fish such as perch, bream, pike-perch, and carp are rich in thick scales. Approximately 49 000 t of scales have been reported to be produced annually [65.1]. Table 65.1 indicates the proportions of waste generated during seafood processing operations. In addition to the tremendous amounts of processing wastes, about 20 Mt of fish consisting of

Table 65.1 Waste (percentage of whole fish) generated during seafood processing operations (after [65.13])

Processing operation	Waste
White fish filleting	Skin, 4–5 Head: 21–25 Bones: 24–34
Oily fish filleting	40–45
Canning	25 (head/entrails)
Scaling of white fish	2–4
Beheading of white fish	27–32 (heads and debris)
Filleting of oily fish	40 (entrails, tails, heads, and frames)
Trimming and cutting white fish	24–34 (bones and cut-off)
Precooking of fish for canning	15 (inedible parts)

various unconventional and underutilized species are landed during commercial fishing operations [65.19]. Processes for better utilization of these underutilized fish have been discussed [65.20].

65.3 Seafood Waste and Discards as Sources of Nutraceuticals

Conventionally, fishery wastes are mostly converted into low market value products, such as fish meal, ensilage, fertilizer, animal feed, and biofuel. Fish skin from larger fishes such as shark, salmon, lingcod, hagfish, tilapia, Nile perch, carp, and sea bass is used as a source of leather for making clothing, shoes, hand bags, etc. In many countries such wastes are discarded as landfill [65.17]. In 2012, about 21 Mt fish was used for fishmeal production [65.1]. In the last two decades, there has been a global awareness of the environmental, economic, and social impacts of fish processing, calling for efficient utilization of the discards [65.10, 13, 23–26]. Because seafood is nutritionally rich, as mentioned above, there is scope for the marine industry to bioprocess seafood discards and also by-catch to extract compounds that are of practical use [65.8]. Many such compounds can possess interesting bioactivities such as antihypertensive, antioxidant, antimicrobial, anticoagulant, antidiabetic, anticancer, immunostimulatory, calcium-binding, and other properties [65.8, 27, 28]. Table 65.2 shows components that can be isolated from seafood waste. For the convenience of the present discussion, these compounds can be classified into four groups (Fig. 65.1) according to their chemical origin, namely:

i) Nitrogenous compounds including proteins and peptides.
ii) Lipid and lipid-derived compounds.
iii) Polysaccharide-based compounds such as chitin, chitosan, glycosaminoglycans, and their derivatives.
iv) Mineral-based compounds.

These aspects are discussed below.

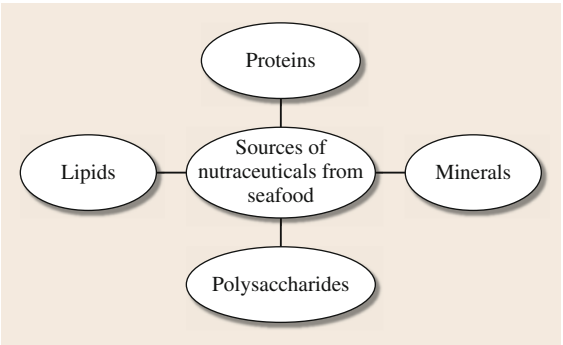

Fig. 65.1 Major sources of nutraceuticals from seafood processing waste

65.4 Nitrogen-Derived Compounds

The interest in protein goes beyond its importance to basic nutrition to specific roles in enhancing the quality of life. A portion of 150 g of fish can provide about 50–60% of an adult's daily protein requirements. In the year 2010, fish accounted for almost 16.7% of the world population's intake of animal protein and 6.5% of all protein consumed [65.1].

65.4.1 Proteins and Protein Hydrolyzates

Fish proteins are rich in all the essential amino acids, particularly methionine and lysine. While there are no significant differences in the amino acid composition of freshwater and marine fish, certain marine fish such as mackerel, tuna, etc., may be exceptionally rich in the amino acid histidine. The nutritive value of marine fish proteins is equal to or better than that of casein and red meat proteins. The protein efficiency ratio (PER) of fish proteins is slightly above that of casein, the major milk protein, and ranges from 3.1 to 3.7. The net protein utilization (NPU) of fish flesh is between 80 and 100, the values for red meat and egg, respectively. Evidently, the protein quality of most fish may exceed that of meat or are equal to that of an ideal protein such as lactalbumin [65.29]. In view of their high nutritive value, recovery of proteins from fish processing wastes for use as food ingredients has received attention. Fish proteins have been isolated by methods such as enzymatic hydrolysis, pH shifting, membrane filtration, and ohmic heating [65.30]. Different methods

Table 65.2 Components from seafood waste and uses (summarized from [65.13, 21, 22])

High-value components and nutraceuticals	Feed, color, flavor, and packaging materials	Nonfood uses
Chitin Chitosan Carotenoid pigments Glucosamine Glycosaminoglycans Chondroitin sulfate Collagen and gelatin Alkaline phosphatase Proteolytic enzymes (pepsin, trypsin, chymotrypsin, collagenases and calpains) Omega-3 fatty acids Deoxyribonucleic acid (DNA) processed as drug in Norway and used in treatment of the human immunodeficiency virus (HIV) Squalene (hydrocarbon found in fish oils useful to treat diabetes and tuberculosis)	Animal feed Crustacean meal Fish/pet food Mollusc shells (used in animal feeds as a calcium source) Encapsulation materials Edible packaging materials Coloring agents (for fish feed) Food flavoring from shrimp heads	Fertilizer Lime Filter media Leather (skin from shark, salmon, ling, cod and hagfish skins) Pearl essence from pelagic fish (iridescent chemical called guanine which gives fresh fish)

Table 65.3 Methods for isolation of proteins and their merits

Methods	Advantages	Disadvantages
Chemical hydrolysis (acid or alkali)	High recovery, inexpensive process	Potential decrease of protein functionality Bitterness Heterogeneous hydrolyzates
Enzymatic hydrolysis	High recovery yields High selectivity Low-salt final product Low contamination of wastes	Bitterness High-cost enzymes (except autolysis) Long-time process Potential decrease of protein functionality
pH shifting	High recovery	Potential decrease of protein functionality
Ultrafiltration	Simultaneous recovery and concentration of soluble proteins	Time-consuming process Expensive membranes
Supercritical fluid extraction with CO_2	Low environmental impact Easy penetration on food systems Mild processing conditions (room temperature and low pressures) Low level of solvent residues in products Inert and nontoxic technique Cost-effective technique	Equipment complexity Necessity of using high pressures Normal use of modifiers, such as ethanol
Ohmic treatment	Rapid process Relatively uniform heating	High-cost installation Possible electrolytic effect

for protein isolation and their merits are pointed out in Table 65.3.

Fish protein hydrolyzates (FPH) have been prepared from several types of fish meat, including processing wastes using various proteolytic enzymes as described in Fig. 65.2. FPHs have obvious nutritional advantages over products like fish protein concentrate or even human grade fish meal. They are useful ingredients to provide functional effects such as whipping, gelling, and texturing properties of foods [65.8, 31, 32]. Techniques for enhancing the storage stability of FPH have been pointed out [65.33]. Proximate compositions of FPHs are moisture, 1–8%, protein, 81–93% fat, 0–5.0%, and ash, 3–8%. Proteins from skin of the blue shark have been isolated using commercial proteinase at an optimal temperature of 51 °C and pH 7.1, with a recovery of 91%. The isolate, which contained all the essential amino acids was rich in low molecular weight (< 6.5 kDa) peptides having high nutritional content and antioxidant activity [65.34]. Proteins were

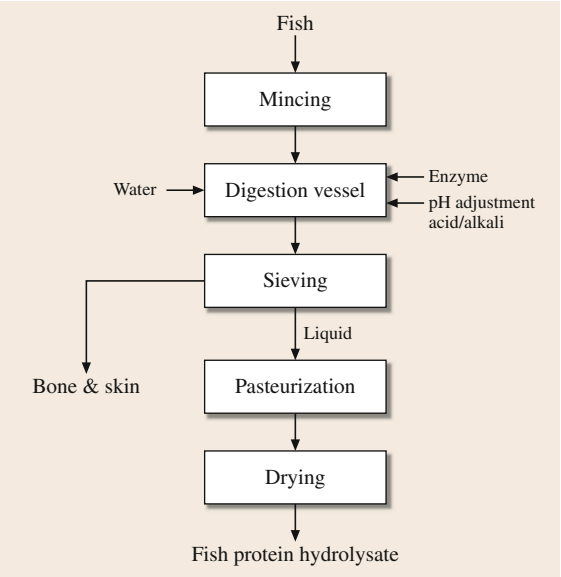

Fig. 65.2 Production of fish protein hydrolysate

recovered from the by-products of silver carp filleting waste using a combination of neutral proteases, giving 82% recovery [65.35].

A novel method is the use of glycation reaction under controlled conditions to effectively isolate functional proteins with gelling and emulsifying capacities [65.36]. Soluble powders prepared from the by-products of the processing of fish and shellfish such as Alaska pollock, herring, salmon, and arrowroot flounder had protein contents ranging between 65–79%. They are good sources of essential amino acids, meeting the requirements of FAO/WHO (FAO: food and agriculture organisation, WHO: world health organization) 1990 recommendations [65.37]. Up to 70% of the protein content of shrimp waste was recovered by boiling water extraction under controlled alkaline conditions [65.38]. Isolates from shrimp head with 12.5% protein, 6.5% chitin, and relatively low ash, can find use as aqua-feed [65.39] weight management, athletic recovery, and maintaining strength and muscle tone for good health in later life. The possible bitter taste of protein hydrolyzates may prevent their use in many products as food additives [65.31]. FPHs are good sources of peptides because they have various bioactive properties, which will be discussed later. In addition, opportunities also exist for isolation of other bioactive substances, including hydroxyapatite, taurine, and creatine [65.23].

Skin and bone waste are rich sources of collagen. Head waste in cod fisheries, yielding ≈ 20% of the whole fish, forms an excellent source of the protein [65.16]. Collagen consists of three identical or different peptide chains, each chain possessing a helical structure, together forming a triple-stranded helix consisting of repeating triplets $(\text{glycine-X-Y})_n$, where X and Y are often proline and hydroxyproline. This basic structural unit of collagen fiber, called tropocollagen, has a molecular weight of ≈ 30 kDa, a length of 280 nm, and a diameter of 1.4–1.5 nm. The process of extraction of collagen from fish bones, which form a major source of the filleting operation, involves initial acetone extraction for about 12 h at room temperature to remove lipids, drying of the treated material, decalcification using a tenfold 0.6 M hydrochloric acid for 1 day to yield collagen [65.40]. Acid-soluble collagen (ASC) and pepsin-solubilized collagen (PSC) were isolated from fish bones and scales. The molecular weights of the collagen subunits were between 110 and 130 kDa. The melting temperatures of ASC and PSC were > 34 °C. Collagen from cod and Pacific whiting surimi processing water showed better functional properties than that from skin and was similar to acid soluble collagen in whiteness, solubility, emulsifying activity, and cooking stability [65.16, 41]. The fish skin collagen was comparable to typical type-I collagens of land-based animals and has potential therapeutic effects in terms of anti-inflammatory activity and inhibition of angiogenesis. However, fish collagen has lower melting temperatures in the range of 25–45 °C compared with mammalian collagens, which melt between 60 and 70 °C.

Collagen from squid waste has also been isolated and characterized [65.42]. Collagen and another connective tissue protein, and elastin have many applications in cosmetics. They provide smoothness to the skin and prevent skin irritation. The proteins are antistatic, film forming, and humectant moisturizers. Collagen combined with the moisturizing properties of chitosan (see Sect. 65.5 on polysaccharide-derived nutraceuticals) is claimed to help restore the elasticity of the skin [65.43, 44].

Collagen is the source of gelatin. The process of isolation of gelatin from collagen involves successive extraction of fish waste at room temperature with dilute alkali and mineral acid [65.45]. Gelatin is a heterogeneous mixture of single or multistranded polypeptides containing 50–1000 amino acid residues. It is translucent, colorless, brittle, and flavorless. Because it is hydrophilic it interacts with water and undergoes changes of its physicomechanical properties depending on the moisture content. It forms transparent elastic

thermoreversible gels when heated and then cooled below ≈ 35 °C. The melt-in-the-mouth property is one of the important characteristics responsible for its wide applications in the food and pharmaceutical industries [65.46]. Fish gelatin has a lower melting point of ≈ 10 °C, compared with a melting point of 30 °C of mammalian gelatin, and hence has the potential to replace conventional porcine or bovine gelatin. It can be also used for encapsulation of heat-sensitive compounds. Fish gelatins have a comparable bloom strength (the gel strength equivalent used in the industry), viscosity, and solubility in comparison with mammalian gelatin. Their viscosities range between 15 and 75 mP [65.47–50]; gelatin hydrolyzates are sources of antioxidant and antimicrobial peptides, as will be discussed below. The food uses of gelatin are given in Table 65.4.

65.4.2 Bioactive Peptides

In addition to functioning as sources of essential amino acids, bioactive peptides play significant roles in maintaining health and in preventing diseases of the cardiovascular, nervous, or immune systems. While these peptides are inactive within the sequence of the parent protein, they become active upon release from the parent proteins. They usually contain 3–20 amino acid residues, and their activities are based on their amino acid composition and sequence. Hydrolysis of fish meat by trypsin or other pancreatic enzymes or bacterial and fungal enzymes, either alone or in combination, can give a variety of peptides depending upon the treatment conditions. The prepared hydrolyzates are then subjected to ultrafiltration and/or nanofiltration to fractionate peptides [65.8, 32, 51, 52].

The interesting bioactivities of peptides involve varied functions such as antihypertensive agents, antioxidant, immunomodulatory, antithrombotic, anticancer, and antimicrobial agents. High blood pressure is one of the major risk factors for cardiovascular diseases. The angiotensin-I-converting enzyme (ACE, EC 3.4.15.1) plays a crucial role in the regulation of blood pressure as it promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin II. Many peptides, including those from marine sources, can inhibit ACE activity, thereby controlling hypertension [65.32, 53]. The first marine ACE inhibitory peptide was isolated from sardines. Later, ACE-inhibitory peptides were isolated from other fishery items including salmon, sardines, oysters, wakame, yellowfin sole, and dried bonito [65.8, 51, 54, 55].

Some peptides function as excellent antioxidants that can control a wide array of physiological disorders such as cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases. The mechanisms involve binding of metal ions, scavenging of oxygen, converting hydroperoxides to nonradical species, deactivating singlet oxygen and, thereby, suppressing the generation of free radicals. Their antioxidant potency is suggested mostly due to the presence of hydrophobic amino acids and/or their ability to chelate metal ions [65.28, 52]. Antioxidant peptides have been isolated from seafood, including jumbo squid, oysters,

Table 65.4 Food uses of gelatin

Application	Product type
Gelling agent	Jelly confectionery
Whipping agent	Aerated confectionery
Stabilizer	Icings
Emulsifier	Fruit chews, etc.
Adhesive (sticking agent)	Various products
Coating and encapsulation agent	Various nutraceuticals

Table 65.5 Bioactive peptides from various fishery products (after [65.8, 28, 51])

Bioactivity	Fishery source
Antihypertensive action through inhibition of angiotensin-I converting enzyme activity	Sardine, salmon, big-eye tuna, Pacific hake, sea bream, oyster wakame, fish bone collagen, dried bonito digest, shrimp, hard clam, sea cucumber
Antioxidant activity	Alaska pollock, capelin, cod, eel, hake, mackerel, round scad, saithe, sole, sea cucumber, mussel, squid, gelatins from tuna, hoki, squid
Calcium-binding oligophosphopeptide	Bone from the fish hoki
Gastrin and calcitonin gene-related peptides (CGRP)	Atlantic/Greenland cod, sardine, winter flounder, industrial seafood waste
HIV-I protease inhibiting activity	Oyster
Stimulation of nonspecific immune defense system	Chub mackerel, other fish
Antimicrobial activity	Lobster, shrimp and green sea urchin, blue crab, lobster
Anti-coagulant activity	Blue mussel

Table 65.6 Enzymes from seafood wastes

Enzymes	Function	Source	Remark/benefit
Gastric proteases (e.g., pepsins, gastricsins, chymosins)	Cold *renneting* milk, Digestion aid for fish feed	Fish viscera from fishery sources[1]	Catalytic activity at lower temperatures, minimizing unwanted chemical reactions and bacterial growth
Serine and cysteine proteases (e.g., trypsins, chymotrypsins, collagenases, elastases, cathepsin B)	Inactivation of polyphenol oxidase preventing unwanted color changes in foods, low-temperature protein digestion, meat tenderization, fermentation	Pyloric ceca, pancreatic tissues, intestines, hepatopancreas (stomachless bone fish[2])	Catalytic activity at lower temperatures minimizing unwanted chemical reactions and bacterial growth
Lipases	Numerous uses in the fats and oils industry (e.g., production of omega-3-enriched triglycerides)	Various fish items[3]	Higher specificity for omega-3 fatty acids
Polyphenol oxidases (e.g., tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, catecholase)	Processing and fermentation of tea, coffee, raisins, and prunes	Crustaceans	Higher activities at lower temperatures, as compared with terrestrial counterparts
Chitinolytic enzymes	Replace HCl for conversion of chitin into oligomeric units	Digestive tracts of fish, shellfish, and shellfish waste, squid liver, octopus saliva	Less harsh than HCl and results in more consistent products
Transglutaminase	Creates protein cross-links to improve rheological properties of gels, i. e., surimi, gelatin	Various fishery items[4]	Strengthens gels with protein cross-linkages

[1] Atlantic cod, carp, harp seals, American smelt, sardine, capelin, salmon, mackerel, orange roughy, tuna; [2] sardine, capelin, cod, cunner, salmon, anchovy. Atlantic white croaker, carp, hybrid tilapia, herring, spiny dogfish, rainbow trout, crustaceans, mollusks, short-finned squid; [3] Atlantic cod, seal, salmon, sardine, Indian mackerel, red sea bream, and others; [4] Red sea bream, rainbow trout, mackerel, walleye, pollock liver, scallop muscles, shrimp, squid

prawns, blue mussels, hoki, tuna, cod, capelin, scads, mackerels, herrings, yellow fin tuna, Alaska pollock, sea cucumbers, and other species. A heptapeptide having a molecular weight of 962 kDa purified from fermented marine blue mussel could scavenge superoxide, hydroxyl, carbon-centered, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Its ability to inhibit lipid peroxidation was higher than α-tocopherol [65.52].

Peptides with antimicrobial activities have been isolated from marine invertebrates, including spider crabs, oysters, American lobsters, shrimp, and green sea urchins [65.28, 30, 51, 54, 56]. Peptides exhibiting high affinity for calcium isolated from pepsin hydrolyzates of Alaska pollack and hoki are able to reduce the risk of osteoporosis. Calcitonin is a hormone (32 amino acid peptide containing a single disulfide bond) known to participate in calcium and phosphorus metabolism. The major source of calcitonin is the thyroid gland, but it is also synthesized in a wide variety of other tissues, including the lung and intestinal tract. The hormone prevents the loss of calcium and phosphorus in urine by reabsorption in the kidney tubules. Calcitonin from salmon can decrease osteoporosis and, hence, is a bone density conservation agent. Salmon calcitonin is about 30 times more potent than that secreted by the human thyroid gland. Nowadays, fish calcitonin is also being made synthetically [65.57]. In addition, the wound healing potential of administering marine collagen peptides (MCP) from chum salmon skin has been demonstrated [65.58]. Some peptides can function as protectants against freezing injury, thereby providing cryostabilization of myofibrils in meat products. Gelatin peptides have been reported to cure osteoporosis. Table 65.5 summarizes various bioactivities of peptides from diverse fishery products.

Marine Enzymes

Seafood processing waste is a rich source of digestive proteolytic enzymes such as gastric, serine, cysteine or thiol proteases, lipases, polyphenol oxidases (PPOs), chitinolytic enzymes, muscle proteases, transglutaminase, and collagenases. Pepsins and gas-

tricins have been isolated from fish gastric mucosa, trypsins and chymotrypsins from pyloric ceca, and trypsin like enzymes from hepatopancreas of marine fish, including cod, mackerel, and salmon, among others. Alkaline phosphatase, hyaluronidase, β-*N*-acetylglucosaminidase, and chitinase have been recovered from shrimp shell waste in good yield. Arctic scallop and clam wastes are good sources of lysozyme, a potential preservative for refrigerated foods [65.8, 59, 60]. A large-scale process for the recovery of enzymes from wastewater of the shrimp processing industry involves flocculation of the water by ferric chloride, concentration by cross-flow ultrafiltration, and then freeze-drying [65.61]. The characteristic properties of marine fish proteinases are a higher catalytic efficiency at low temperatures, lower sensitivity to substrate concentrations, and greater stability at a broader pH range, which are highly useful for their varied applications [65.62].

Collagenase prepared from crab hepatopancreas has been used for skinning of squid (*Loligo* spp.). Protease from mackerel intestines was used for recovery of fish bone from hoki [65.30]. Other applications of endogenous proteases are roe processing, fish sauce, silage, hydrolyzates, and caviar production [65.62, 63]. Because some marine fish and shellfish are inhabitants of extreme low temperatures, their enzymes have significant activities at low temperatures. Further, they may also possess other interesting properties such as salt tolerance and stability to high pressure. Cold-adapted enzymes display a high specific activity associated with relatively high thermosensitivity and lower free energies of activation. Marine enzyme biotechnology can offer novel biocatalysts with properties like high salt tolerance, hyperthermostability, barophilicity, cold adaptivity, and ease in large-scale cultivation. Genes encoding chitinases, proteases, and carbohydrases from microbial and animal sources have been cloned and characterized. Their commercial applications include food processing, biomass conversion, molecular biology, environmental biosensors, bioremediation, and several other processes. Proteases and peroxidases have found industrial applications [65.22, 63–65]. Table 65.6 depicts various enzymes from seafood wastes.

65.5 Lipid-Based Nutraceuticals

65.5.1 Omega-3 Fatty acids

Marine fish generally contain high (> 7%) amounts of lipids, composed of neutral lipids comprised of triacyl glycerols, phospholipids, sterols, wax esters, and some unusual lipids, such as glyceryl esters, glycolipids, sulfolipids, and hydrocarbons. Commercial fish oils are characterized by fatty acids with 12–26 carbon atoms with 0–6 double bonds. The fatty acids are made up of saturated (15–25%), monounsaturated (35–60%) and polyunsatured (25–40%). Marine lipids are rich in long-chain polyunsaturated fatty acids (PUFA) (with more than 14 carbon atoms), particularly, omega-3 (also referred to as ω-3 and or *n*-3) fatty acids (3 indicating the position of the first double bonds at the third carbon from the methyl end of the fatty acid structure). Other dietary fatty acids include *n*-6 PUFA, namely, linoleic, $C_{18:2w6}$, γ-linolenic, $C_{18:3w6}$, and arachidonic, $C_{20:4w6}$ acids. The popular ω-3 fatty acids are eicosapentaenoic acid containing five double bonds ($C_{20:5w3}$, cis-5,8,11,14,17-eicosapentaenoic acid, designated as EPA), and the 22-carbon docosahexaenoic acid, containing six double bonds ($C_{22:6w3}$, cis-4,7,10,13,16,19-docosahexaenoic acid, DHA). In contrast with other fats and oils, marine fish oils contain large amounts of EPA and DHA, in the range of 14–19 and 5–8%, respectively. Fish oils such as those from tuna, salmon, etc., may contain more DHA than EPA. Commercial cod liver oil is a complex mixture of more than 50 different fatty acids, forming triacyl glycerols, of which there is usually 8–9% each of EPA and DHA. Marine oils also contain significant amounts of fat-soluble vitamins A and vitamin D. Halibut, shark and cod liver oils are rich sources of vitamin A and D. A 3.5-oz portion of salmon can provide 90% of daily human need of vitamin D. The content of vitamin E (α-tocopherol) is a powerful antioxidant related to its availability from feed. Vitamin E can help protect skin cells and tissues because of its antioxidant activity. The fat soluble vitamin A and carotenes are relatively stable at normal cooking temperatures. Oil from Antarctic krill (*Euphausia suberba*) is rich in omega-3 fatty acids, phospholipids, and also natural pigments and vitamins [65.66, 67].

The beneficial health effects of marine fish oils, because of the rich presence of omega-3 PUFA, have been well documented. A significant body of evidence

indicates that intake of the long-chain *n*-3 polyunsaturated fatty acids (omega-3 fatty acids) found in fish is cardio-protective [65.68]. Besides, they can reduce hypertension, lower autoimmune and inflammatory diseases, depression, attention-deficit hyperactivity disorder (ADHD) in children and muscle degeneration in the elderly, arthritis and some types of dermatitis. These fatty acids are also involved in the structure of cell membranes, the development of the nervous system and influence the synthesis of cell mediators (prostaglandins and leukotrienes), which play important roles in coagulation, inflammation and proliferation of certain cells. DHA and arachidonic acid (*n*-6) are also important in visual function. Deficiency of these compounds causes disorders such as restrictive growth, abnormal skin and hair, damage of reproductive system, among others [65.69, 70]. Many psychiatric disorders, particularly schizophrenia and major depressive disorder (MDD), have shown positive results when supplementation has been used as an adjunct to standard pharmacotherapy [65.71, 72]. Cod liver oil has been shown to help slow the destruction of joint cartilage in patients with osteoarthritis [65.73]. The health benefits of PUFA have encouraged regulatory agencies to recommend regular public consumption of fatty fish such as herring, sardine, and mackerel. The WHO recommends consumption of 1–2 servings of fish, containing 200–500 mg of EPA and DHA, per week. The American Heart Association recommends that persons diagnosed with cardiovascular diseases consume 1 g each of the fatty acids per day [65.72, 74]. Table 65.7 summarizes potential health benefits of PUFAs.

Whole or processing wastes (particularly liver) of fish, including anchovies, capelin, Atlantic cod, Atlantic herring, Atlantic mackerel, Atlantic menhaden, cod, saithe, haddock, salmonids, and sardines are rich sources of marine lipids rich in PUFA, particularly EPA and DHA. However, lipid composition in these fish varies with season and also depends upon the tissues being analyzed. The oil contents can be as high as 21% in herring, 22% in tuna, and 18% in sardines in winter. Fish livers are the ideal source of these lipids. Enzymatic methods using proteases are useful to release the bound oil from fish tissues. Depending on the proteolytic enzyme used, oil yield from red salmon heads varied between 4.9–10.6% [65.37]. Figure 65.3 depicts a typical alkaline refining process for fish oil extraction. PUFA contents in different marine fishes and methods for their extraction and fractionation, in terms of fatty acid constituents in the form of methyl esters have also been provided. Methods of isolation consist of molecular and fractional distillation, solvent, and supercritical extraction [65.75, 76]. Because omega-3 fatty acids in oil are highly unsaturated, they are sensitive to oxidation with the formation of hydroperoxides, which depends on exposure to heat, light, and moisture, and the presence of metal ions. Therefore, during their isolation PUFAs must be protected against oxidation by incorporating small amounts of antioxidants such as tertiary butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and octyl gallate, and the oil must be stored in ampoules with minimum headspace [65.77]. Supercritical fluid extraction (SFE) is a popular technology due to its nontoxic and nonflammable nature. The carbon dioxide atmosphere employed during extraction also protects PUFAs from oxygen-induced oxidation. In the process, PUFAs are usually extracted at an elevated pressure of 10–30 MPa and at 40–80 °C [65.76].

Table 65.7 Potential health benefits of omega-3 fatty acids

Prevention of atherosclerosis
Protection against arrhythmia
Reduction of blood pressure
Beneficial for diabetic patients
Good against manic-depressive illness
Reduction of symptoms in asthma patients
Protection against chronic obstructive pulmonary diseases
Alleviation of symptoms of cystic fibrosis
Prevention of relapse in patients with Crohn's disease
Prevention of various cancers
Good for bone health
Improvement of brain functions in children

Marine oils have been isolated commercially from several gadoid finfish species [65.3, 77–81]. Tuna fish has a total lipid content of about 22% with up to 20 and 4% of DHA and EPA, respectively Monounsaturated and *n*-6 fatty acids are also present at 23.3 and 3.8%, respectively. The huge discards from the global tuna canning industry, estimated at 450 000 t annually, could be a rich source of oil [65.78]. Total lipid contents in the head, meat, and waste of three commercial varieties of Indian marine fishes, namely, pink perch, Indian mackerel, and Indian oil sardine are in the range of 4.3–13.6, 2.53–10.97, and 2.7–15.1% (wet weight basis), respectively. Neutral lipids were higher in the head (83.2–89.2%). The saturated fatty acid, palmitic acid, was present in all the fishes, irrespective of the body components. EPA and DHA, however, were found in higher concentrations [65.82]. Shark liver is 22–30% of the body weight, with an oil content as high as 90%. Liver oils of some sharks found under a depth

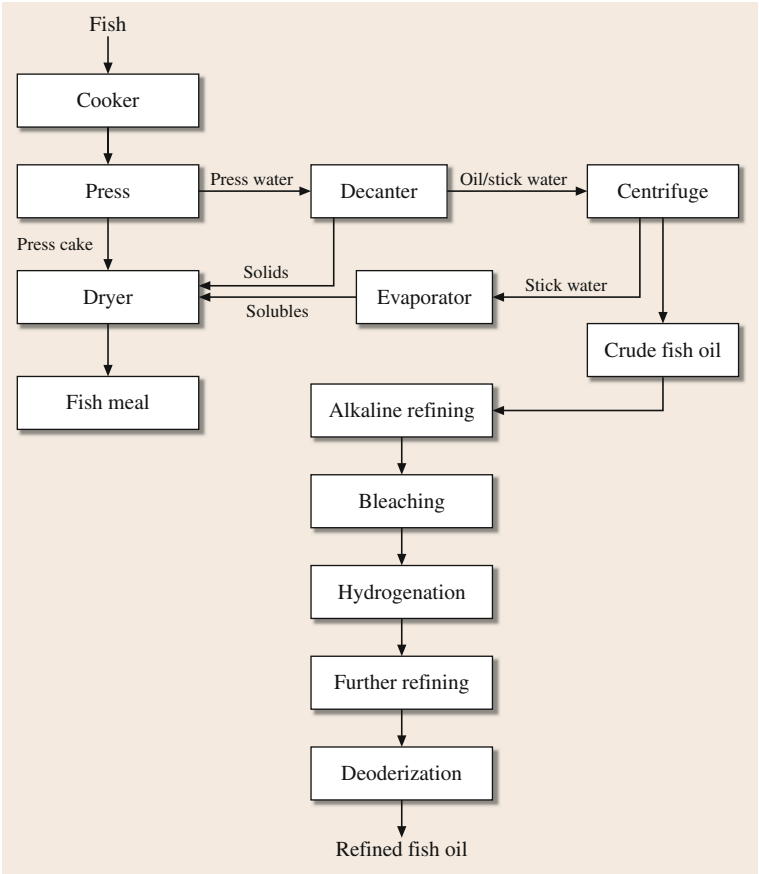

Fig. 65.3 Process for recovery of fish oil

of 300–3000 m in the Pacific, North Atlantic, and Indian Ocean contain ≈ 85–90% unsaponifiable matter, mainly the hydrocarbon, squalene. Shovelnose dogfish liver oil contains 60% hydrocarbons, consisting mainly of squalene and pristane, and 25% diacyl glyceryl ether. Squalene, $C_{30}H_{50}$ is 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) and squalamine, an amino sterol antibiotic, are found in shark liver. The recovery of oil from shark liver consists of natural decomposition, acid ensilage in presence of formic acid, alkali digestion, and steam rendering. The oil recovered is degummed, bleached, and deodorized. The process for isolation of squalene from shark oil consists of heating chopped liver in 2% caustic soda solution for 30–40 min to separate the oil. After removing water by anhydrous sodium sulfate, the oil was subjected to vacuum distillation at 240 °C [65.79]. Herring oil has been extracted from dried fish, giving 41% oil rich in PUFA [65.3]. Enrichment of marine oil to increase the contents of PUFA has been attempted by making use of lipolysis [65.83, 84]. Several industries specialize in fish oil production and purification [65.84–86]. Table 65.8 shows the contents of omega-3 fatty acids in as a percentage of total fatty acids in various commercial fish oils.

65.5.2 Carotenoids

Carotenoids are a family of fat-binding compounds responsible for the red and yellow color of crustaceans and also many plants, algae, and cyanobacteria. The carotenoids found in nature can be classified into two groups, namely, hydrocarbons, such as β-carotene and xanthophylls, and the oxygenated derivatives such as astacene, astaxanthin, canthaxanthin, cryptoxanthin, neoxanthin, violaxanthin, and zeaxanthin, lutein, etc. The color of carotenoids is due to chromophores containing conjugated double bonds. The naturally abundant β-carotene ($C_{40}H_{56}$) is a polyunsaturated hydrocarbon made up of two retinal molecules.

Table 65.8 Contents of omega-3 fatty acids in commercial fish oils (values give the percentage of fatty acids in total fatty acids; after [65.87], courtesy of Wiley-VCH)

Species	18 : 3	18 : 4	20 : 5	22 : 5	22 : 6	EPA + DHA
Anchovy	1	2	22	2	9	31
Atlantic menhaden	1	3	14	2	12	26
Sardine/ pilchard	1	3	16	2	9	25
Gulf menhaden	2	3	13	3	8	21
Pollock		2	15		4	20
Capelin	1	3	8		6	14
Sand eel	1	5	11	1	11	22
Mackerel	1	4	7	1	8	15
Blue whiting	1	3	7	1	8	15
Herring	2	3	6	1	6	12
Tuna	1	1	6	2	22	28
Norway pout	1	3	9	1	14	23
Whitefish spp.	1	2	9	2	13	22
Salmon, wild	2	1	8	4	11	19
Salmon, farmed	1	3	9	2	11	18
Sprat	2		6	1	9	15
Tilapia, farmed	2			3	5	5
Catfish, farmed	1		1	1	3	4

Approximately 60 carotenoids possess varying levels of provitamin A activity, β-carotene possessing maximal provitamin A activity. Carotenoids possess remarkable antioxidant properties, which is their primary beneficial role in the diet of humans and animals. Astaxanthin is about ten times stronger in antioxidant activity than other carotenoids (including β-carotene, canthaxanthin, and lutein). Animals, including humans, do not synthesize carotenoids de novo and rely upon diet for these compounds. Oral supplementation of carotenoids appears to increase the photo-protective properties of the epidermis and dermis against environmental stress (e.g., UV radiation, pollution, smoke) by quenching free radicals generated by oxidative stress. Studies have also shown the ability of carotenoids to reduce DNA damage and protect against depletion of Langerhans cells, a key component of immune function. Because of their potent antioxidant properties, carotenoids have been suggested to have a protective role against cancer, aging, ulcers, heart attack, and coronary artery disease [65.88].

Crustacean wastes are abundant sources of β-carotene and astaxanthin (3,3′-dihydroxy-β, β-carotene-4 and 40-dione), the oxidized form of β-carotene. Astaxanthin is responsible for the pink-to-red pigmentation of crustaceans and wild salmonids [65.89–91]. The process of extraction of carotenoids from shell waste consists of initial treatment with proteases to detach the pigments from the bound proteins, followed by extraction in organic solvents. Upon treatment with trypsin at the optimum temperature 45–55 °C for 2 h the carotenoid-rich head wastes of commercially important Indian marine shrimp species yielded significant quantities of astaxanthin, and also minor amounts of β-carotene, canthaxanthin, lutein, zeaxanthin, and crustacyanin [65.74]. Astaxanthin in the form of carotenoprotein can be extracted with a yield of 49% by treating shrimp waste with proteolytic enzymes such as trypsin in the presence of ethylenediamine tetraacetic acid (EDTA) at a pH of 7.7 and 4 °C [65.56].

65.6 Polysaccharide-Derived Nutraceuticals

Chitin is a cationic polysaccharide formed by units of *N*-acetyl-D-glucosamine, joined by (1–4) β-bonds, viz., β-(1-4)-*N*-acetyl-D-glucosamine, which is β-(1-4)-*N*-acetyl-2-amino-2-deoxy-D-glucose.

65.6.1 Chitin and Chitosan

Marine crustaceans, which include shrimp, crabs, squid, cuttlefish, krill, and oysters, are rich in chitin. Chitin forms the outer protective coatings of crustacean shells in a covalently bound network with proteins and dihydroxy phenylalanine, together with some metals and carotenoids. Chitin occurs in three polymorphic forms, α, β, and γ, which differ in the three-dimensional arrangements of the molecular chains. Its most common form is α-chitin, where two units of *N*-acetyl-D-glucosamine are in an anti-parallel arrangement. Chitin is a very light, white or yellowish, powdery/flaky prod-

uct. It is insoluble in water, almost all common organic solvents, and in acidic and basic aqueous solutions. Chitin may be solubilized in carbon disulfide after treatment with caustic soda and then re-precipitated as a filament or film in the manner of viscose rayon. Chitin swells in cold alkali when deacetylation takes place [65.8, 15, 44, 92–96].

Shellfish waste consisting of crustacean exoskeletons is currently the main source of biomass for chitin production. The isolation process for chitin consists of three-step treatments of shell wastes, and includes demineralization, deproteinization, and bleaching, followed by extraction of chitin using acetone [65.97]. Demineralization is usually carried out by an extraction of 1−3 h with diluted hydrochloric acid, but harsher conditions such as 90% formic acid, 22% HCl, 6 N HCl, or 37% HCl have also been applied. Deproteinization is performed by treating the raw material with either sodium hydroxide or potassium hydroxide at concentrations between 1−10%(w/v) and at temperatures of 65–100 °C for a duration ranging from 0.5–6 h. Since the harsh conditions of alkaline digestion may cause depolymerization and deacetylation of chitin, enzymatic deproteinization by digestion with proteolytic enzymes such as papain, pepsin, trypsin, or pronase has been used. The following step of solvent extraction (e.g., acetone, chloroform, ethanol) helps remove pigments like melanins and carotenoids [65.15, 93–95].

Chitosan is a collective name representing chitin deacetylated to different degrees. Structurally, chitosan is poly-(1-4)-linked-2-amino-2-deoxy-D-glucose. Commercially chitosan is produced by deacetylation of crustacean chitin using 30−60% (w/v) sodium or potassium hydroxide at 80−140 °C. The preparation is purified by dissolving it in dilute acetic acid, reprecipitation with alkali, followed by washing and drying to obtain flakes of chitosan. The resulting molecular weight distribution of deacetylated units along the polysaccharide chain depends on the alkali concentration applied, and the temperature and time of the process. The degree of deacetylation of the polysaccharide can be increased by using high temperatures in the process, but such harsh conditions also cause depolymerization and lead to a reduction in the size of the molecules. To obtain more defined chitosans and to avoid polysaccharide degradation by oxygen it is also recommended to carry out the deacetylation process under nitrogen or to employ thiophenol or sodium borohydride addition as scavengers of oxygen. Alternately, chitin deacetylases derived from different organism sources can be used [65.94]. Shell waste can also be used for fermentation to produce chitinase [65.98]. Figure 65.4 shows a typical process for chitin extraction and its subsequent processing to yield chitosan.

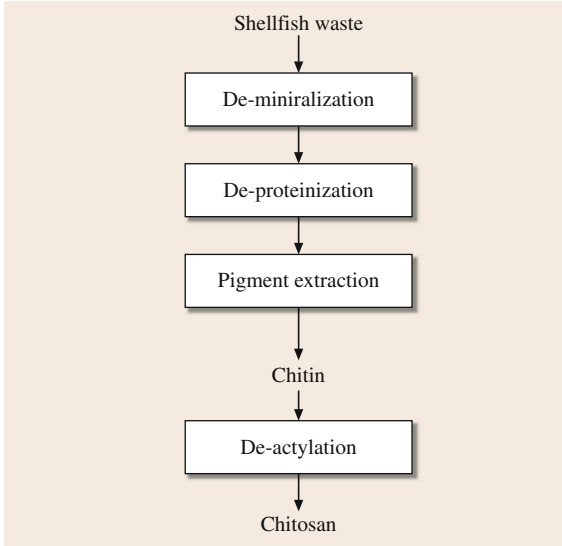

Fig. 65.4 Recovery of chitin and chitosan from shellfish waste

The molecular weight of natural chitosan is generally higher than 10^6 Da. Chitosan is a polycationic, long-chain biopolymer because of the presence of one free amino group and two free hydroxyl groups for each glucose ring. Because of this chemical nature, it has a natural affinity for negatively charged compounds, including biological membranes. Chitosan is insoluble in pure water, but unlike chitin it is soluble in weakly acidic aqueous media. A minimum deacetylation of 70% is required for chitosan to be acceptable for various purposes. The pK_a value for the positively charged ammonium group is ≈ 6.2. When the pH is raised to ≈ 6.5, chitosan precipitates in a gel form. The multidimensional utilization of chitin derivatives, including chitosan, is due to a number of characteristics, including their polyelectrolyte and cationic nature, the presence of reactive groups, the high adsorption capacities, and bacteriostatic and fungistatic influences, which make them very versatile biomolecules [65.99]. Because of its cationic nature, chitosan is incompatible in solution with most anionic water-soluble gums such as alginates, pectate, sulfated carrageenan, as well as carboxymethyl cellulose. On the other hand, acidic solution of chitosan is compatible with nonionic water soluble gums such as starch, dextrins, glucose, polyhydric alcohols, oils, fats, and nonionic emulsifiers.

Chitosan can form films, which are tough, flexible, and transparent. Chitosan is biodegradable by the specific enzyme, chitosanase. By enzymatic treatment, soluble chitosan can also be obtained in oligosaccharide form. Chitosan derivatives in the form of acetate, ascorbate, lactate, malate, and others are water-soluble and have varying functional properties. The role of chitosan as a fiber, however, is challenged by popular fiber products such as oats, soy, and bran. Chitosan film can be extruded from its acidic solution into a 70 °C coagulating bath containing caustic soda and sulfonic acid esters of high molecular weight alcohols. Chitosan in microcrystalline form has several advantages when compared to standard chitosan [65.8, 95, 100, 101].

Polysaccharide-based biomaterials, particularly chitosan, are emerging for applications in several biomedical fields, such as tissue regeneration, particularly for cartilage, drug delivery devices, and gel entrapment systems for the immobilization of cells. Their salient beneficial properties are controllable biological activities, biodegradability and the ability to form hydrogels [65.101]. Recent years have witnessed a marked growth in chitosan and its derivatives, the applications encompassing food and nutrition, biotechnology, material science, drugs and pharmaceuticals, cosmetics, water treatment, cosmetics, agrochemicals, biotechnology, environmental protection, and gene therapy. The medical applications of chitosan cover such diverse fields as hemodialysis membranes, artificial skin, hemostatic agents, hemoperfusion columns, and drug delivery systems. Oral administration of chitosan suppresses the serum cholesterol level and hypertension. The bacteriostatic and fungistatic properties of chitosan mean that it can be used as an ingredient in tropical skin ointments as a wound healing agent [65.43]. The property of chitosan to form gel at slightly acid pH provides antacid and anti-ulcer activities. The antimicrobial activity and film-forming property of chitosan make it a potential source of food preservatives or a coating material of natural origin. Chitosan is also a popular drug carrier. It enhances dissolution properties of poorly soluble drugs and also helps transdermal delivery of drugs [65.43, 95, 102–105]. When intravenously injected, chitin/chitosan oligosaccharides enhance antitumor activity by activating macrophages [65.106]. Chitosan-based scaffolds are useful for tissue regeneration [65.107]. The use of chitin as a source of dietary fiber in chicken feed enhances the growth of bifidobacteria in the guts, which reduce other microorganisms and produce the β-galactosidase necessary for the digestion of feed supplemented by whey or other dairy by-products. The effect has also been noticed in the case of chitosan feed meant for pigs and fish [65.43].

65.6.2 Glucosamine

Glucosamine is natural amino sugar found in large concentrations in certain foods such as milk, eggs, liver, yeast, molasses, and yeast. Glucosamine is obtained by extensive hydrolysis of chitosan with mineral acid, such as hydrochloric acid. If it is produced from chitin, it must be deacetylated. Glucosamine can easily be absorbed into the human intestine [65.93].

65.6.3 Glycosaminoglycans (GAGs)

Glycosaminoglycans are multifunctional polysaccharides composed of repeating disaccharide units. Based on the disaccharide composition, linkage type, and the presence of sulfate groups, GAGs include chondroitin sulfate, dermatan sulfate, heparin sulfate, keratin sulfate, and heparin, and are present in the connective tissues of living systems together with collagen, elastin, fibronectin, and laminin, forming a complex extracellular

Table 65.9 Useful features of chitosan in food applications

Properties	Applications
Renewable resource	Abundantly available from marine sources and hence renewable
Bioactivity	Antimicrobial activity and its use in food packaging as antimicrobial additive, stimulation of immune system, anti-cholesterolemic activity, use as fiber in foods, obesity control
Biodegradability	Substrate for single cell production, biodegradable packaging material, controlled release of drugs, nutrients, etc.
Reactivity of deacetylated amino groups	Moisture control, thickening action
Chelating capacity	Removal of metals, water treatment, antioxidant activity
Complex formation with other macromolecules	Complexes with proteins (useful to remove hypoallergenic β-lactoglobulin from whey, clarification of wines). Chitosan-alginate removes protein from seafood industry waste water
Biocompatibility	Nontoxic and biological tolerance
Film-forming properties	Useful as edible packaging, encapsulation materials, and delivery of nutraceuticals

Table 65.10 Applications of chitin, chitosan, and some of their derivatives in healthcare (after [65.43, 101, 102, 105, 108])

Compound	Applications
Chitin	Wound dressing In vivo absorbable sutures Drug delivery Dialysis membrane
O-Carboxymethyl chitin and *O*-hydroxypropyl chitin	Cosmetic ingredient
N-Actylchitohexasacharide	Antitumor agent
Chitosan and its various derivatives	Artificial skin Blood anticoagulant and hemostatic materials Chitin and chitosan hydrogels for delivery of nutraceuticals Protein absorbents Drug delivery systems Haemodialysis membranes Immunostimulation, molecular recognition, and entrapment of growth factor, etc. Nanofiber scaffold for nerve tissue regeneration Hypocholesterolemic agents Wound-healing materials Nutrition (dietary fiber, weight reduction, hypocholesterolemic agent, etc.) Water treatment (removal of metals, radioisotopes, pesticides, etc.) Cosmetics (shampoo, skin products)
N-Hexanoylchitosan and *N*-octanoylchitosan	Antithrombogenic material for artificial blood vessels Contact lenses Blood dialysis membranes Artificial organs
N-Carboxybutylchitosan	Wound dressing
5′-Methylpyrrolidinone chitosan	Dentistry
Sulfates of chitin and chitosan	Anticoagulant and lipolytic agents

matrix providing a cushion between bones and joints. Most sulfated GAGs are covalently linked to proteins to form proteoglycans. Chondroitin is composed of a chain of *N*-acetylgalactosamine and glucuronic acid residues linked through alternating β (1–3) and β (1–4) bonds. Chondroitin sulfate (CS) has anti-inflammatory properties, as well as anticancer properties. While chondroitin provides cartilage with strength and resilience, glucosamine inhibits inflammation and stimulates cartilage cell growth [65.109]. Table 65.9 summarizes food applications of chitosan and Table 65.10 shows applications of chitin, chitosan, and some of their derivatives in healthcare.

GAGs in terrestrial vertebrates have been well studied. Cartilage of marine animals such as shark, skate, mussels, and squid can be good sources of these polysaccharides. Shark cartilage, which is rich in chondroitin sulfate, has been found to have several therapeutic effects against diseases such as arthritis and tumors. Shark cartilage along with glucosamine is a highly effective treatment for arthritis and osteoporosis. A process for the preparation of chondroitin sulfate from skate cartilage has been reported [65.84]. Another compound, hyaluronic acid (HA), is a natu-

Table 65.11 Some applications of glycosaminoglycans in medicine (after [65.44, 101, 105])

Glycosaminoglycans	Applications
Chondroitin sulfate and other glycosaminoglycans	Helps rebuild cartilage and joints. Supports body's ability to joint health integrity
Hyaluronic acid	Eye surgery, osteoporosis, tumor marker etc.
Dermatan sulphate	Stimulates cell growth. Role in wound healing, cardiovascular disease, etc.
Heparin	Anticoagulant
Keratan sulfate	Eye treatment (corneal transparency)
Chitin	Chitin and chitosan have wide applications
Chondroitin	Helps rebuild cartilage and joints. Supports body's ability keep joints healthy

rally occurring polysaccharide consisting of glucuronic acid and *N*-acetyl-Dglucosamine units. HA functions as an effective moisturizer due to its strong water holding capacity. With age, the levels of HA in the body decrease, reducing the moisture binding capacity of the skin, resulting in dry skin and wrinkles. Oral consumption of HA can help increase moisture on the surface and within the skin. A combination of gelatin, hyaluronic acid, and chondroitin can have potential in wound healing [65.53]. Table 65.11 shows some medical applications of glycosaminoglycans. The global market for marine-derived drugs was US$ 4.8 billion in 2011 and is expected to reach US$ 8.6 billion in 2016 [65.110].

65.7 Mineral-Based Nutraceuticals

Bone comprises a significant part of seafood processing wastes. Filleting of fish generates a large amount of fish frames. For instance, backbone wastes from the processing of Atlantic cod account for approximately 15% of the wet weight of the fish. Fish bone contains about 40% crude protein and 6% collagen, on a dry weight basis. Inorganic minerals constitute approximately 60% of bones, the major ash components being calcium and phosphorus [65.11, 23, 111]. The mineral constituents can be classified as into three groups, composed mainly of either hydroxyapatite (HAP), tricalcium phosphate, (TCP), or a mixture of HAP and TCP. Sea bream, horse mackerel, carp, and shark have hydroxyapatite type phosphate (as in the case of cattle, swine, and fowl), while Japanese anchovy has TCP type phosphate [65.112].

Fish bone is a potential source of calcium. To isolate the mineral in a bioavailable form, the bone is initially softened by either hot water or hot acetic acid. Superheated steam can reduce the loss of soluble components from fish tissue, which enables better recovery of bone within a shorter period. The treated bones are subjected to saponification, degreasing, and degumming. The preparation is a source of dietary calcium, in addition to some phosphorus [65.113]. Peak bone stone is the bony structure situated near the vertebral column of the dorsal fin base obtained from large fishes like ghol (*Protonibea diacanthus*), koth (*Otolithes biauratusa*), and dara (*Filamanus heptadactyla*). There is potential to use the product as raw material for calcium powder [65.8]. The skeleton discarded from the industrial processing of hoki was digested by a heterogeneous enzyme extracted from the intestine of bluefin tuna, which yielded a fish bone oligophosphopeptide containing 23.6% phosphorus. The peptide (molecular weight of 3.5 kDa) could be a nutraceutical with a potential calcium-binding activity [65.114]. Fish and shellfish are also rich sources of selenium [65.66, 67].

65.8 Novel Marine Organisms and Compounds

Marine invertebrates have been recognized as rich sources of more than 400 bioactive compounds, including hypotensive agents, cardio active substances, muscle relaxants, antibiotics, and antiviral and antitumor agents [65.115]. Sea cucumbers or holothurians are spiky-skinned animals of the phylum Echinodermata (class Holothuroidea). They are commonly found in shallow water areas of the sea to deep ocean floors. Related species are the sea lily, sea urchin, star fish, and sand dollars. The cell wall of the sea cucumber contains large amounts of sulfated glycans. The cell wall polysaccharide is comparable in backbone structure to mammalian chondroitin sulfate, but some of the glucuronic acid residues display sulfated fucose branches. The specific spatial array of the sulfated fucose branches in the fucosylated chondroitin sulfate not only confers high anticoagulant activity to the polysaccharide but also antithrombin activity [65.116].

These glycans also exhibit a wide range of other biological activities, which include recombinant HIV reverse transcriptase activity, anti-inflammatory, antiangiogenic, and antiadhesive properties [65.117]. Green fluorescent protein (GFP) is a novel photoactivatable fluorescent protein (PAFP) compound that was first isolated from jellyfish in 1962. The green color of the protein turns red when exposed to an intense pulse of visible blue light. GFP is an invaluable tool that is useful for in vivo bioprocess monitoring such as protein interactions and drug interaction [65.118].

The ink of squid and cuttlefish has been found to possess some therapeutic activity. Mollusks are another important species that have a wide range of uses in pharmacology. Guanine, (2-amino-6-oxypurin) found as a constituent in scale membranes is responsible for the gleaming effect of fish. Guanine combines with collagen and calcium phosphate, yielding a silvery white thinning substance. The lustrous material can be extracted as crystals from fish scales like those of sardine, herring, ribbonfish, carp, etc. The yield of the recovered material, often called pearl essence because of its luster, is $\approx 0.3\%$ of the body weight in the case of ribbon fish.

65.9 Commercial Aspects

In recent years marine compounds, including those derived from seafood, have been entering commercial markets. Some marine nutraceuticals currently marketed in the US include fish and algal oils that are rich in omega-3 fatty acids, chitin and chitosan, fish and shark liver oil, and marine enzymes and chondroitin from shark cartilage, sea cucumbers, and mussels. Commercially important nitrogen-based seafood nutraceuticals include peptides, fish bone phosphopeptide with calcium binding activity, salmon calcitonin, and squalamine. Salmon calcitonin (CAS No. 47931-85-1) is approved for postmenopausal women who cannot tolerate estrogen.

In Japan foods fortified with nutraceuticals are approved as food for specified health uses (FOSHU). Several FOSHU products with a fish (e.g., sardine) peptide as the functional ingredient have been approved for the control of mild hypertension [65.119]. Enzymes such as trypsin and chymotrypsin, purified from cod viscera, are available commercially. The global market for industrial enzymes was valued at US$ 3.9 billion in 2011 and is supposed to grow to reach $ 6 billion in 2016 [65.110]. Sea cucumber, processed after boiling and sun-drying, marketed as *beche-de-mer* (meaning processed sea slug or sea cucumber) is a highly priced product on the international market. It is said to cure low blood pressure, kidney disorders, and impotence and to prevent ageing [65.120]. The global market for marine-derived drugs was US$ 4.8 billion in 2011 and is expected to reach US$ 8.6 billion in 2016 [65.110].

Omega-3 fatty acids remain one of the most successful and promising functional ingredients in the food and beverage industry. The global market for the product is about 49 000 t annually, worth around US$ 700 million. Marine-based oils used in the food industry include oils from cod, sardine, tuna, and salmon, and also algal oil, many of these oils finding use in margarine and butter. Capsules containing PUFA-rich oils of fish such as cod and salmon, rich in DHA and EPA, as well as vitamins A and D, are being prescribed to reduce blood pressure and to and improve joint mobility. Foods fortified with omega-3 fatty acids are marketed particularly in the US, Europe, Japan, and Southeast Asia. Incorporation of DHA into baby foods is being practiced to enhance memory development. Other fortified products include bakery items, bread spreads, salad dressing, and others [65.85]. The market for these products was propelled when the US Food and Drug Administration (FDA) in 2004 approved a qualified health claim for omega-3 fatty acids (EPA and DHA). About 75% of fortified products on the European market include milk, cake, pasta, cheese, yogurt and chocolate milk, and infant formulae, which contain omega-3 from marine fish and the rest from algae and flax seed oils [65.7]. The demand for omega-3-enriched consumer packaged goods (CPGs) is expected to exceed a value of US$ 13 billion in 2015 [65.49]. Marine oils are also being used to fortify livestock and aquaculture feed to produce omega-3-rich farmed fish, eggs, and milk. The current interests in Japan encompass the potential anticancer properties of deep.sea shark liver oil and tuna body oil as a less expensive source of DHA [65.119].

The worldwide carotenoid demand is growing at an estimated average annual rate of 2.9%. Currently, β-carotene from the alga *Dunaliella salina* is a commercialized high-value product, its major producers being Australia, US, and Israel. β-Carotene is sold mainly as an extract or suspension in vegetable oil or as β-carotene-rich algal powder. Astaxanthin is widely used to pigment salmon and trout, although in recent times, canthaxanthin has become popular in fish pigmentation and also in poultry to give a red color to egg yolks.

Japan is the major producer of chitin and chitosan from the shells of crabs and shrimp (FAO, 2012). The market for chitosan in the US is about US$ 20 million [65.13]. Chitosan is essentially used to assist

weight loss. Chitosan-fortified fruit juices and chocolates are marketed in the US. In Europe chitosan is sold as dietary capsules to assist weight loss. Some of the products include *Fat Absorb*, a US product containing 250 mg of chitosan per capsule, the *Seaborne* range of products such as *Essential Sea* and *EssentialSeaPlus*, which contain chitosan together with lecithin, vitamins C and E, garlic, and β-carotene, and the fat trimmer, *Minfat*. In Japan, chitosan is added to noodles, potato crisps, and biscuits. There is potential for supplementing beverages with glucosamine in hydrochloride or sulfate forms prepared from chitosan. It is expected that current clinical trials in Japan and many other countries will result in identifying new functional ingredients beyond the fish oils, chitosan and peptides, that can throw more light on the health protection ability of seafood and other marine products [65.119]. As was pointed out recently [65.121], the refinement of existing technologies with inputs from advances in biotechnology can further help with the isolation of seafood components that are safe and can retain their potential bioactivities for diverse applications.

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Acknowledgements

A.2 Marine Fungal Diversity and Bioprospecting

by Kalaiselvam Murugaiyan

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A.8 Marine Sponges – Molecular Biology and Biotechnology

by Stephen A. Jackson, Jonathan Kennedy, Lekha Menon Margassery, Burkhardt Flemer, Niall O'Leary, John P. Morrissey, Fergal O'Gara, Alan D. W. Dobson

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B.11 Microbial Bioprospecting in Marine Environments

by Mariana Lozada, Hebe M. Dionisi

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B.12 Novel Bioreactors for Culturing Marine Organisms

by Debashis Roy

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B.13 Transgenic Technology in Marine Organisms

by Thomas T. Chen, Chun-Mean Lin, Maria J. Chen, Jay H. Lo, Pinwen P. Chiou, Hong-Yi Gong, Jen-Leih Wu, Mark H.-C. Chen, Charles Yarish

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B.15 Biofouling Control by Quorum Quenching

by Vipin C. Kalia, Prasun Kumar, Shunmughiah K. T. Pandian, Prince Sharma

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B.16 Detection of Invasive Species

by Nathan J. Bott

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D.22 Cell Wall Polysaccharides of Marine Algae

by Andriy Synytsya, Jana Čopíková, Woo J. Kim, Yong Il Park

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D.23 Iodine in Seaweeds: Two Centuries of Research

by Frithjof C. Küpper

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E.27 Marine Actinomycetes in Biodiscovery

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F.32 Marine-Derived Fungal Metabolites

by Sherif S. Ebada, Peter Proksch

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F.33 Marine Dinoflagellates-Associated Human Poisoning

by Samanta S. Khora

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F.36 Fatty Acids of Marine Sponges

by Pravat Manjari Mishra, Ayinampudi Sree, Prasanna K. Panda

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G.42 Marine Functional Foods

by Ana C. Freitas, Dina Rodrigues, Ana P. Carvalho, Leonel Pereira, Teresa Panteleitchouk, Ana M. Gomes, Armando C. Duarte

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G.44 Cosmetics from Marine Sources

by Elena M. Balboa, Enma Conde, M. Luisa Soto, Lorena Pérez-Armada, Herminia Domínguez

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G.45 Omega-3 Fatty Acids Produced from Microalgae

by Munish Puri, Tamilselvi Thyagarajan, Adarsha Gupta, Colin J. Barrow

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G.48 Vector and Agricultural Pest Control

by Venkateswara Rao Janapala

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H.49 Nanotechnology – from a Marine Discovery Perspective

by Ramachandran S. Santhosh, Visamsetti Amarendra

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H.51 Biofuel Innovation by Microbial Diversity

by Thiago Bruce, Astria D. Ferrão-Gonzales, Yutaka Nakashimada, Yuta Matsumura, Fabiano Thompson, Tomoo Sawabe

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H.52 Marine Biomaterials as Antifouling Agent

by Parappurath Narayanan Sudha, Thandapani Gomathi, Jayachandran Venkatsan, Se-Kwon Kim

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I.54 Marine Materials: Gene Delivery

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I.55 Marine Organisms in Nanoparticle Synthesis

by Pallavi Mohite, Mugdha Apte, Ameeta R. Kumar, Smita Zinjarde

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I.56 Marine Biomaterials in Therapeutics and Diagnostic

by Ashutosh Srivastava, Arti Srivastava, Ananya Srivastava, Pranjal Chandra

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I.57 Enzymatically Synthesized Biosilica

by Xiaohong Wang, Heinz C. Schröder, Werner E.G. Müller

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I.58 Biomineralization in Marine Organisms

by Ille C. Gebeshuber

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J.59 Functional Feeds in Aquaculture

by Jorge Olmos Soto, José de Jesús Paniagua-Michel, Lus Lopez, Leonel Ochoa

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J.63 Marine Actinobacterial Metabolites and their Pharmaceutical Potential

by Panchanathan Manivasagan, Jayachandran Venkatesan, Kannan Sivakumar, Se-Kwon Kim

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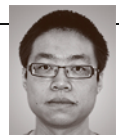

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Detailed Contents

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Erratum to: Springer Handbook of Marine Biotechnology

Se-Kwon Kim

Erratum to:

S.-K. Kim (Ed.), Springer Handbook of Marine Biotechnology, DOI 10.1007/978-3-642-53971-8,

Dear Reader,

thank you for your interest in this Springer Handbook. In spite of careful checking by the editorial und production team, a mistake has unfortunately slipped in, which we only noticed after printing.

The name of the contributor Christine Delbarre-Ladrat was misspelled. We corrected this error in the electronic version. We apologize to the reader and Christine Delbarre-Ladrat.[1]

[1] The online version of the original book can be found under DOI 10.1007/978-3-642-53971-8.