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Microbial Toxins and Related Contamination in the Food Industry

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Contents

Chapter 1
Histamine in Fish and Fishery Products

Salvatore Parisi, Caterina Barone, Giorgia Caruso, Antonino Santi Delia, Gabriella Caruso and Pasqualina Laganà

Abstract The consumption of certain fish products containing high levels of histamine (and other biogenic amines) can result in an acute illness with allergy-like symptoms called scombroid syndrome. Fish accumulate toxic levels of histamine when their high level of histidine in muscle tissues is coupled with a proliferation of bacteria rich in the enzyme histidine decarboxylase. Other vasoactive amines—cadaverine, putrescine, etc.—may inhibit detoxification mechanisms that reduce the intestinal absorption of histamine. Moreover, histidine can be transformed by means of another metabolic pathway leading to accumulation in fish muscle of urocanic acid. Recently, interest has been extended to mesophilic and psychrotolerant bacteria. Histamine accumulation is traditionally correlated to microbially contaminated fish and poor storage conditions. In addition, the high thermal stability has to be considered. At present, different methods are available for the analytical determination of histamine ranging from the AOAC fluorometric method to HPLC, ELISA and rapid stick methods.

Keywords ELISA · Histamine · Histidine decarboxylase · HPLC · Mesophilic microorganism · Psychrotolerant bacterium · Refrigeration · Scombroid syndrome

Abbreviations

ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
HACCP	Hazard Analysis and Critical Control Points
HPLC	High-Performance Liquid Chromatography
WHO	World Health Organization

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1.1 Histamine in Fish and Fishery Products: An Introduction

Scombroid fish poisoning, also named histamine poisoning, is one of the most challenging food safety problems in the seafood industry (Hungerford 2010). The consumption of mishandled fish belonging to the families of *Scombridae*, namely tuna and mackerel, *Clupeidae (*sardines and herrings) and *Engraulidae* (anchovies), may result in an acute illness with allergy-like or *Salmonella-like* infection symptoms (Lehane and Olley 2000). This 'scombroid syndrome' occurs if these foods contain high levels of histamine—2-(4-imidazolyl) ethylamine or 4-(2-aminoethyl) imidazole—and other vasoactive (biogenic) amines.

Normally, histamine can be important when related amounts are ≥50 mg/100 g of microbially contaminated fish. In detail, unsatisfactory raw fish usually show more than 50 ppm of histamine (toxic values should be between 100 and 500 ppm), while normal levels should not exceed 10–50 ppm (Chamberlain 2001). On the other side, urocanic acid—an imidazole compound derived from histidine in contaminated products—may be also considered when speaking of scombroid poisoning effects (Lehane and Olley 2000).

Basically, the accumulation of histamine and other decomposition compounds is observed in many fish types, including *Scombridae*. From the biochemical viewpoint, histamine is obtained in scombroid fish (*albacore, bonito*, skipjack, Spanish mackerel, saury, etc.) by means of the enzymatic conversion of free and abundant histidine in muscle tissues (Cattaneo 2011; Lehane and Olley 2000; Rawles et al. 1996; Ruiz-Capillas and Moral 2004; Taylor 1986; Tortorella et al. 2014). However, the abundant presence of free histidine is reported in many fish products, including also (Antoine et al. 1999; Chang et al. 2008; Hungerford 2010; Taylor 1986):

- Anchovies (Engraulis spp.)
- Herring (Clupea spp.)
- Pilchards (Sardina pilchardus)
- Mahi-mahi (Coryphaena spp.)
- Sardines (Sardinella spp.)
- Swordfish (Xiphias gladius).

Actually, histamine poisoning has been reported in relation to non-fish products such as Gouda, Swiss, Gruyere, Cheddar and Cheshire cheeses (Chambers and Staruszkiewicz 1978; Doeglas et al. 1967; Kahana and Todd 1981; Taylor 1986). However, these situations appear circumscribed to a few situations: apparently, cheeses might be considered as a potential problem when speaking of unusual ageing (Taylor 1986). Moreover, other fermented products—*Sauerkraut*, wines—or partially demolished foods (in relation to the protein fraction) such as Italian *pepperoni* and *salami* may contain occasionally high histamine levels (Dierick et al. 1974; Mayer and Pause 1972; Ough 1971; Taylor 1986; Taylor et al. 1978). Anyway, the most part of observed and reported histamine poisoning episodes is

correlated with the consumption of raw fish and finished seafood products (Hungerford 2010).

It has to be considered that the bacterial and enzymatic production of histamine from histidine is strictly correlated with storage temperatures: normally, thermal values should always remain below 4 °C (Hastein et al. 2006; Tsironi et al. 2008). On the other hand, this process may occur in all stages of the food chain (Cattaneo 2014; Kanki et al. 2004). In detail, the production of histamine can be easily be observed in skipjack and big-eye tuna fish at 22 °C after 24–48 h, while this phenomenon may be remarkably delayed at 10 and 4 °C. However, the amount of detectable histamine may be notable after 3 days under refrigerated storage (Silva et al. 1998).

The Food and Drug Administration (FDA) has published a detailed guideline in relation to retail food establishments (scombroid products). According to this document (FDA 2011), raw fish should have internal temperatures below or equal to 10 °C (if fish has been delivered 12 or more hours after death) or 4.4 °C (if fish has been delivered 24 or more hours after death). Anyway, temperatures should be evaluated after receipt (FDA 2011). Certainly, storage at 0 °C can determine the end of histamine production (Chamberlain 2001) but existing levels are not eliminated.

Recently, it has been recognised that the production of histamine from high levels-histamine fish can be assessed when temperatures are higher than 25 °C for 6 h or more (FAO/WHO 2012). On these bases, the recommended amount of histamine in fish products has been defined to be lower than 15 mg/kg on the condition that good hygienic practices and 'Hazard analysis and critical control points' (HACCP)—based strategies have been implemented. The Codex Alimentarius Commission has defined two different levels (100 and 200 mg/kg) in relation to the commercial acceptability and possible food safety problems of fish products respectively (Cattaneo 2014).

These values have a slightly different meaning in the European Union in relation to the Regulation (EC) No. 2073/2005 and subsequent amendments. In detail, nine samples have to be considered and four conditions are possible when speaking of normal fish products:

(a) All results have to be lower than 100 mg/kg. Fish products are fully acceptable
(b) One or two results only are found between 100 and 200 mg/kg while remaining samples are below 100 mg/kg. Fish products are fully acceptable
(c) One or more samples exceed 200 mg/kg. Fish products have to be recalled or withdrawn from the market
(d) More than two samples are found between 100 and 200 mg/kg. Fish products have to be recalled or withdrawn from the market.

These norms are valuable for unsalted fish products. Should salted fish be sampled (nine products), four conditions are possible when speaking of normal fish products (FAO/WHO 2012):

(e) All results have to be 200 mg/kg. Fish products are fully acceptable
(f) One or two results only are found between 200 and 400 mg/kg while remaining samples are below 200 mg/kg. Fish products are fully acceptable

(g) One or more samples exceed 400 mg/kg. Fish products have to be recalled or withdrawn from the market
(h) More than two samples are found between 200 and 400 mg/kg. Fish products have to be recalled or withdrawn from the market.

Interestingly, histamine levels in foods do not appear to be influenced by normal processing treatments such as cooking and smoking. Actually, these processes kill histamine producers but the existing amounts of histamine remain unchanged (Cattaneo 2014). In addition, cold storage cannot reduce the real incidence or diminish possible poisoning episodes when raw fish is partially compromised (FDA 2011).

1.2 Chemistry and Production of Histamine: Importance of Other Biogenic Amines

Actually, histamine is not produced in degraded fish only. In fact, a low amount of histamine is also naturally produced by human beings because of the decarboxylation of histidine (FAO/WHO 2012).

With exclusive reference to fish products, toxic levels of histamine are accumulated when their high level of histidine in muscle tissues is coupled with a proliferation of bacteria rich in the enzyme histidine decarboxylase (Alini et al. 2006). Histamine exerts its negative effects on specific receptors known as H_1 and H_2 receptors located on human cell membranes. H_1 receptors are implicated in allergic reactions with dilation of peripheral blood vessels (rash, namely of the lips and surrounding area, urticaria, headache), while H_2 receptors are responsible for gut motility (diarrhoea, cramps, vomiting). The onset of symptoms either without or with small amounts of histamine implicates other causes or contributory causes of intoxication.

Other vasoactive amines such as cadaverine and putrescine may inhibit detoxification mechanisms that reduce intestinal absorption of histamine by means of catabolic enzymes like histaminase (FAO/WHO 2012). These biogenic amines are produced by means of microbial spoilage and fermentation from amino acids; the precursor is ornithine (FAO/WHO 2012). The role of these molecules is not clear at present: potentially, putrescine and cadaverine might be considered as histamine potentiators (Taylor and Lieber 1979). On the other hand, the real role of these biogenic amines in scombroid poisoning episodes is not clear (FAO/WHO 2012). The same thing can be affirmed when speaking of tyramine, a monoamine molecule formed from tyrosine (Leuschner and Hammes 1999; Prester 2011; Taylor and Lieber 1979). Other notable biogenic amines with some food safety importance are tryptamine, spermine, spermidine and β-phenylethylamine (Shalaby 1996). In relation to the present section, the importance of these amines is reduced because they can be found in many foods including also dairy and meat products, nuts, chocolate, etc. (Emborg 2007).

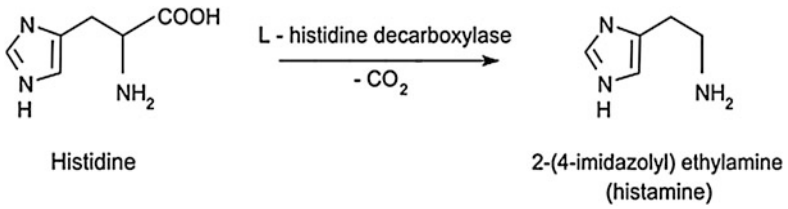

Fig. 1.1 Production of histamine by enzymatic decarboxylation of histidine. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

It has been reported that the presence of histamine may not cause toxic effects at low levels. On the other hand, the contemporary presence of molecules, such as cadaverine and putrescine, can enhance histamine-related toxic effects when the ratio between histamine and remaining biogenic amines is 1:5 (Emborg and Dalgaard 2006; Naila et al. 2010).

After decarboxylation of histidine (Fig. 1.1), the produced histamine can be subsequently converted (Alini et al. 2006) into:

(1) Imidazole acetaldehyde and imidazole acetic acid by the diamine oxidase enzyme, or
(2) Methyl histamine by the methyl transferase enzyme.

In addition to its decarboxylation mechanism, histidine can be transformed by another metabolic pathway leading to accumulation in fish muscle of urocanic acid (Alini et al. 2006), caused by histidine lyase (histidase). The above-mentioned urocanic acid is an imidazole compound whose effects include activation and degranulation of connective tissue mast cells in the human body, releasing histamine from their metachromatic granules. The production of large amounts of histamine in fish is largely due to Gram-negative bacteria, whereas scombroid syndrome is seldom associated with Gram-positive bacteria (FAO/WHO 2012). For a long time, the production of toxic quantities of histamine has been correlated with the excessive growth of mesophilic bacteria belonging mainly to *Morganella morganii*, *Hafnia* and *Raoultella* (*planticola*) species under conditions of thermal abuse at temperatures between 20 and 40 °C. More recently, the interest has concerned also psychrotolerant bacteria belonging to *Morganella psychrotolerans* and *Phosphobacterium phosphoreum* species because they are able to grow below 0 °C (Emborg 2007). On the one side, mesophilic bacteria cause problems when produce suffers even short-term temperature abuse; as a result, bacterial growth is readily controlled by adopting low storage temperatures. On the other hand, psychrotolerant bacteria require short storage times even under low temperature conditions. Histamine accumulation occurs normally in the early stages of the fish supply chain (fishing procedures, storage conditions on board fishing vessels, distribution procedures) and this is certainly the case for fresh fish (FDA 2011). Histamine is a highly heat-stable compound so that once it has formed the produce cannot be

detoxified either by domestic cooking or by transformation treatments including autoclave stabilisation in the canning process. In addition, newly formed histidine may be found in canned fish when production problems expose the produce to unsuitable storage/temperature conditions. This produce is intended to be cooked before sterilisation or already cooked and ready for autoclave. As a consequence, possible post-contamination episodes can occur before the autoclave step or when the canned produce is used for multiple purposes—dressings, sandwiches, pizzas—without adequate hygiene measures (clean utensils, suitable storage temperature between subsequent uses).

Generally, the production of histamine is negligible in certain fish species in the early days after capture on condition that storage temperatures are below 4 °C (Taylor 1986). Substantially, psychrotrophs can prevail in refrigerated tuna and determine the rise of histamine amounts at 10 °C. In addition, the histidine decarboxylase activity is apparently detectable and remarkable at 4 °C (Silva et al. 1998). On the other hand, raw tuna and other fish species such as anchovies can reach notable and dangerous histamine amounts after 24 h only when stored at 22 °C (Behling and Taylor 1982; FDA 1982, 2005; Guizani et al. 2005; Rossano et al. 2006; Silva et al. 1998).

Another important reflection has to be considered when speaking of halotolerant and halophilic histamine-producing bacteria (Hernández-Herrero et al. 1999). Substantially, the production of histamine in fish has also been reported in salted products because of the remarkable activity of halotolerant microorganisms such as *Staphylococcus aureus*. Anyway, *Staphylococcus* spp. is considered the most reported histamine producer species when speaking of fermented salted fish such as anchovies. Other important histamine formers belong to *Vibrio* and *Pseudomonas* species (Hernández-Herrero et al. 1999). The selection of similar halotolerant microorganisms is caused by peculiar pH, water activity and sodium chloride values in salted anchovies.

Interestingly, the presence of these microorganisms has been considered (Hernández-Herrero et al. 1999) in relation to the contamination of fish by human activities (capture and unhygienic handling). When speaking of salted fish products, the production of histamine can only be contrasted (Hernández-Herrero et al. 1999) with adequate refrigeration temperatures and high sodium chloride levels (ideally >20 %).

1.3 Analytical Detection of Histamine

At present, different methods are available for the analytical determination of histamine ranging from the AOAC fluorometric procedure (the most important standardised analysis in the United States of America) to high-performance liquid chromatography (HPLC), considered the ‘gold’ reference test in the European Union (EU). Other remarkable methods include enzyme-linked immunosorbent assay (ELISA) tests—with the status of ‘AOAC Research Institute Performance

Tested Methods (SM) procedure'—and the latest rapid stick methods (lateral flow assay and similar tests) for the semi-quantitative determination of histamine in a matter of minutes.

In detail, the following list shows most used testing methods for the determination of histamine (and other biogenic amines, where possible) in seafood (Emborg 2007; FAO/WHO 2012):

(a) Sensorial evaluation. In other words, several controls may be preliminarily carried out by experienced panelists on seafood products. Naturally, this type of control is based on the perception of degradation and quality losses of sampled fish such as canned tuna. It has to be considered that inexperienced consumers may easily be unable to detect even high concentrations in histamine seafood if sensorial evaluation is preferred (Du et al. 2002; Őzogul et al. 2002)
(b) HPLC procedures (Duflos et al. 1999; Malle et al. 1996). These analytical methods (needed time: 1–2 h; limit of quantification: 1.5–5 ppb) are very useful for confirmation and quantification of histamine, in accordance with Reg. (EC) No. 2073/2005 and subsequent amendments (Emborg 2007; FAO/WHO 2012; Onal 2007). In detail, the European reference method is designed for the detection of histamine, putrescine, cadaverine, spermine and spermidine in all fish products. Detectable amines are extracted with perchloric acid and separated by HPLC after reaction with dansyl chloride (Emborg 2007). On the other side of the Atlantic Ocean, the official AOAC 977.13 fluorometric method recommends the extraction of histamine with methanol (Emborg 2007). Extracted substances have to be eluted through an ion exchange column with hydrochloric acid. Finally, a fluorometric detection is carried out with the addition of *o*-phthalaldehyde to eluted solutions
(c) Spectrofluorometric methods (FAO/WHO 2012). These protocols (needed time: 1 h) can reach 1.5 ppb as the limit of quantification, while analytical ranges are between 1.5 ppb and 100 ppm. Probably, the best advantage of spectrofluorometric methods is strictly correlated with low analytical costs
(d) ELISA systems. These methods are carried out with spectrophotometers. The protocol is fast, simple and is designed to manage multiple samples at the same time
(e) Colorimetry. Similarly to ELISA methods, they are carried out with spectrophotometers. The protocol is fast, simple and is designed to manage multiple samples at the same time. Colorimetric systems offer the same advantages of ELISA testing methods. In addition, a sort of semi-quantitative evaluation can be obtained by means of visual colorimetric protocols.

Other controls may be naturally made on fish and seafood products; however, the evaluation of microbial spoilage and the determination of bacterial species are not the aims of this book. However, it can be highlighted that new strategies also comprehend mathematical modelling programs when speaking of microbial spoilage. In particular, histamine production can be correlated with the growth of microorganisms such as *M. morganii* and *M. psychrotolerans* in certain fish and

fishery products. As a result, the prediction of histamine production may be carried out by means of predictive modelling systems such as the Seafood Spoilage and Safety Predictor (Dalgaard 2009; FAO/WHO 2012; Naila et al. 2010). From the practical viewpoint, several molecular methods for the detection of histamine-producing bacteria are essentially based on the detection of the gene encoding histidine decarboxylase. However, these testing methods need to be improved at present (Emborg 2007).

1.4 New Possible Strategies Against Histamine in Fish Products

Finally, the control of biogenic amines and histamine in the food industry (and correlated sectors) can be briefly described. At present, main strategies are (Brunazzi et al. 2014; Emborg 2007; López-Sabater et al. 1994; Naila et al. 2010; FAO/WHO 2012; Parisi 2009):

(a) The addition of starter cultures with the aim of degrading produced histamine
(b) The application of hydrostatic pressures
(c) Irradiation methods
(d) The addition of chemicals (citric acid, D-sorbitol, sodium and potassium sorbate, etc.) with different functions, including preservatives
(e) The modification of the microbial ecology in certain products (in accordance with mathematical predictive models)
(f) The use of innovative packaging solutions: modified atmosphere packaging techniques and active (smart) packages.

Many of these procedures can be surely defined 'risk management options' when speaking of safety and risk assessment (FAO/WHO 2012). The remaining part of possible strategies can be briefly listed as follows:

- Chilling
- Freezing
- Gutting and gilling of highly-perishable fish products
- Heating processes.

Naturally, the use of one or more of these strategies has to be considered in the ambit of HACCP plans. The use of a reasonable risk management approach can be very useful with reference to food safety and the reduction of rejection costs, although the decrease of histamine levels cannot be always correlated with economic advantages in specific business areas (FAO/WHO 2012).

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Chapter 2
Biological Toxins from Marine and Freshwater Microalgae

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Abstract In the last decades the increased occurrence of intoxications caused by biological toxins produced from marine and freshwater microalgae has underlined their relevance as emerging risks for food safety. Biological toxins from algae (i.e. saxitoxin, brevetoxin, okadaic acid, domoic acid) are recognised as a major threat for human and animal health, especially where Harmful Algal Blooms phenomena develop. Many of these toxins are responsible for severe illness or death, mostly related to consumption of seafood contaminated by toxic algae. The present book summarises current knowledge and perspectives for future research on marine and freshwater algal toxins. Specific topics are: overview of the different species producing toxins, their survival strategies in the environment; typologies of toxins, their chemical structure and mechanisms of actions; methods currently in use for their monitoring; emerging issues and future outlooks for their control. The importance of biotoxin monitoring in the framework of the European Marine Strategy Framework Directive is also discussed.

Keywords Brevetoxin · Ciguatoxin · Cylindrospermopsin · Domoic acid · Harmful algal bloom · Microcystin · Okadaic acid · Poisoning · Saxitoxin · Yessotoxin

Abbreviations

ASP	Amnesic Shellfish Poisoning
AOAC	Association of Official Analytical Chemists
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BMAA	β-Methylamino-L-Alanine
PbTx	Brevetoxin
CYN	Cylindrospermopsin
BIOTOX	Development of Cost-Effective Tools for Risk Management and Traceability Systems for Marine Biotoxins in Seafood
DSP	Diarrhetic Shellfish Poisoning
DST	Diarrhetic Shellfish Toxin
DiCANN	Dinoflagellate Categorisation by Artificial Neural Network
DTX	Dinophysistoxin

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DA	Domoic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EFSA	European Food Safety Authority
EU	European Union
GEOHAB	Global Ecology and Oceanography of Harmful Algal Blooms
GES	Good Environmental Status
HAB	Harmful Algal Bloom
HPLC	High-Performance Liquid Chromatography
LD_{50}	Median Lethal Dose (50 % of the population)
LOQ	Limit of Quantification
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
MSFD	Marine Strategy Framework Directive
MALDI-TOF	Matrix-assisted Laser Desorption/Ionisation Time-of-Flight
MCY	Microcystin
MCY-RR	Microcystin-RR
MW	Molecular Weight
NSP	Neurotoxic Shellfish Poisoning
N	Nitrogen
OA	Okadaic Acid
PLTX	Palitoxin
PSP	Paralytic Shellfish Poisoning
PTX	Pectenotoxin
P	Phosphorus
PCR	Polymerase Chain Reaction
PSU	Practical Salinity Unit
Q-TOF	Quadrupole-Time-of-Flight
STX	Saxitoxin
SPX	Spirolides
USA	United States of America
YTX	Yessotoxin

2.1 Toxin-Producing Microorganisms

2.1.1 Generalities on Phytoplankton and Toxic Species: Spatial and Temporal Distribution—Environmental Drivers

Phytoplankton comprises unicellular or colonial, microscopic organisms (microalgae) that inhabit many aquatic ecosystems. Algae are autotrophic organisms, which are able through the photosynthetic process to convert carbon dioxide and water

into sugars for the cell metabolism, using the energy from sunlight. They are subdivided into 10 groups (Bold and Wynne 1985): *Cyanophyta, Prochlorophyta, Chlorophyta, Charophyta, Euglenophyta, Phaeophyta, Chrysophyta, Pyrrophyta, Rhodophyta, Cryptophyta* and another group including other species.

Within the planktonic food web, phytoplankton occupies the first trophic level with a key role as primary producer of organic matter. The phytoplankton community inhabiting aquatic environments undergoes seasonal changes characterised by the succession of different unicellular or colonial taxa (Bruno 2000).

The temporal variability in the composition of the phytoplankton community is associated with spatial variability. Along the water column, phytoplankton species are differently distributed depending on the different tolerance of their photosynthetic pigments to light wavelength spectra and on the ability to move towards zones more enriched in nutrients. *Cyanophyceae* are more frequent on surface layers as their coloured pigments protect chlorophyll from ultraviolet (UV) denaturation, while Dinoflagellates inhabit shallow, weakly lighted, environments. At intermediate depths, *Chlorophyceae* are dominant with the exception of lakes in spring and autumn, and spring waters where *Crysophyceae* are prevalent. Close to the thermocline layer, Diatoms predominate.

Of the approximately 5,000 species of identified microalgae, about 300 are able to develop under massive growth, producing 'red-water' phenomena, whose occurrence was reported thousands of years ago. In some cases, the proliferation of planktonic algae (the so-called 'algal bloom') is a real benefit for aquaculture. Many species can create extended marine algal blooms named 'red tides': sometimes, they do not constitute a hazard to human health. Red tides are produced when layers of deep water, rich in nutrients, overlap layers of warmer surface water, due to solar heating or due to surface freshwater supplies. In eutrophic environments, algal blooms involve one or two phytoplanktonic species, which represent 80–90 % of the total biomass. In oligotrophic environments, seasonal blooms also occur but they are never mono-specific. These phenomena occur when warmer surface water overlaps deeper water rich in nutrients; under these conditions, rapidly growing algae consume nutrients from the surface waters, leaving those present under the pycnocline. Motile algae arrive to this layer, where they produce blooms that move towards the surface during the day to capture light and heat (Bruno 2000). Algal species quickly exhaust the nutrients of the surface layers with a rapid growth, leaving those in the colder layer below the pycnocline.

Some algae species such as Dinoflagellates, able to migrate vertically even with higher speeds of 10 m/day, can reach this layer where they find optimal conditions of temperature and nutrients for their growth. Those algal species that can compete successfully for available growth-limiting nutrients have the potential to become dominant and produce blooms (Granéli et al. 2008). In some cases, however, algal blooms are recognised dangerous agents because of the ability of modifying the visual appearance of water. In addition, they can be considered foam-producing organisms and able to cause toxic effects, with possibility of death, on the human population and fish (ICES 1984). For these reasons, these life forms are considered Harmful Algal Blooms (HAB). An approximate number of 75 species, mostly

represented by dinoflagellates and diatoms, inhabit both marine and freshwater ecosystems. They are recognised as toxic substance producers since they produce biotoxins (phycotoxins) that include the most powerful non-protein toxins known to date. In fact, the blooms of toxic microalgae occurring on the coasts have been responsible for die-offs of wild animals, livestock and pets. The consumption of shellfish, fish or water contaminated by algal toxins has been associated with very serious cases of poisoning in humans and negative effects on aquatic environments.

According to the produced effects, the species involved in outbreaks of toxic algal blooms can be distinguished in three main groups (Bruno 2000):

- 1st group. Species that cause water colouration only, resulting in a decrease of the water transparency, and which may exceptionally grow causing some episodes of fish and invertebrate mortality, related to oxygen consumption during their decomposition. Species of dinoflagellates and diatoms belong to this group
- 2nd group. Species which produce powerful toxins that accumulate along the trophic web and can cause effects in upper consumers (animals and humans); dinoflagellates belonging to *Alexandrium, Gymnodinium, Dinophysis, Prorocentrum* and diatoms belonging to the genus *Pseudo-nitzschia* are included in this group
- 3rd group. Species that are not toxic to humans but are noxious to fish and invertebrates (i.e. *Gyrodinium aureolum*, *Chaetoceros convolutus, Nodularia spumigena, Chattonella* spp). In addition, some toxic species spread their toxins through the production of aerosols reaching the coasts (i.e. *Gymnodinium breve* and *Ostreopsis* spp).

Phytoplankton life forms can cause many problems in Europe and worldwide (Anderson 1989). This bloom results in severe economic and sanitary consequences when waters are used for recreational and productive purposes (tourism, fisheries and aquaculture). Algal toxins have negative impacts on the health of marine organisms, such as fish, shellfish and crustaceans; moreover, they are concentrated in seafood products through water filtration mechanisms and become dangerous to human health when contaminated seafood are consumed. Negative impacts and correlated mechanisms are extremely different. Many of these impacts are characterised by chemical–ecological interactions mediated by secondary metabolites of various bioactivity, as shown by their diverse structural classification and the range of receptors and metabolic processes affected (Cembella 2003). Many biologically-active molecules, including dangerous toxins for animal life, can be chosen as good indicators (Codd 2000). Toxic microalgae are common only among the dinoflagellates, diatoms and cyanobacteria (Katircioğlu et al. 2004).

In marine environments, the division of *Pirrhophyta* includes the greatest number of algal species currently known as producers of toxins or harmful substances: two classes of *Dinophyceae* and *Desmophyceae* are considered. The toxic species such as *Dinophysis, Gymnodinium, Peridinium* and *Gonyaulax* belong to the class of *Dinophyceae*, while *Prorocentrum* belongs to the class of *Desmophyceae*. Many toxins are produced by dinoflagellates, but also some diatoms are also toxic.

In freshwater environments, toxic algal species mostly belong to *Cyanophyceae*. They are a well-defined group of prokaryotic organisms, concerning about 150 genera and over 2000 species (van den Hoek et al. 1995). These algae have a photosynthetic apparatus similar in structure and function to that of chloroplasts of eukaryotes, thanks to the presence of chlorophyll *a* responsible for oxygenic photosynthesis. *Cyanophyceae* show a great diversity in morphology, structure and functions, and phenotypes; these species are represented by complex populations (ecotypes) which express particular genotypes. The toxic species are about 40; the ability to produce toxins has an important taxonomic significance (Skulberg and Skulberg 1985).

Cyanophyceae are ubiquitous organisms present in aquatic environments with wide salinity ranges and temperature up to 73–74 °C, in soil, in rocks; some genera are able to fix atmospheric nitrogen through heterocysts and members of symbiotic relationships. Many filamentous and unicellular species are motile, through mucilage or filaments. Cytoplasm shows some gas vacuoles that regulate their floating over water surface. There are three orders: *Chroococcales, Chamaesiphonales, Oscillatoriales*; the first and the third include some toxin-producing species.

Many species belonging to Cyanobacteria are responsible (O'Neil et al. 2012) for HAB in freshwater, estuarine and marine environments. The incidence of cyanobacterial blooms has been observed with high results in different aqueous environments (Carmichael 2008; Paerl 2008; Paerl and Huisman 2008; Paul 2008). Some new cyanobacterial toxins, such as β-methylamino-L-alanine (BMAA), have been isolated, as well as new genera of toxin-producing cyanobacteria (Brand 2009; Cox et al. 2005, 2009; Kerbrat et al. 2011).

2.1.2 Major Toxic Algal Species: An Overview

2.1.2.1 Diatoms—*Pseudo-nitzschia* spp

Pseudo-nitzschia was observed with other diatom species in certain Italian and Spanish areas (Quijano-Scheggia et al. 2005; Totti et al. 2000). The production of toxin (domoic acid) was found in different Mediterranean areas (Azmil et al. 2001; Kaniou-Grigoriadou et al. 2005), possibly in association with two *Pseudo-nitzchia* microrganims (Cerino et al. 2005; Orsini et al. 2002).

The abundance and distribution of toxic *Pseudo-nitzschia* species (particularly *P. calliantha* and *P. delicatissima*, two potential 'Amnesic Shellfish Poisoning' toxin producers) was studied in Italian waters (Caroppo et al. 2005). *P. calliantha* showed a stronger seasonal distribution and was correlated with winter water conditions than *P. delicatissima*, which in turn exhibited a broader temporal distribution and appeared independent from major environmental constraints. *Pseudo-nitzschia* spp have been detected in diverse environments such as high-nitrate and low-chlorophyll regions (open ocean), but also in fjords, gulfs and bays; the same thing may be observed when speaking of produced toxins because of the well known stability.

2.1.2.2 Dinoflagellates

Alexandrium spp

The genus *Alexandrium*, including the species *A. minutum, A. catenella, A. tamarense* and *A. taylori*, is recognised to be responsible for different toxic episodes in many Mediterranean areas (Giacobbe et al. 2007; Penna et al. 2005; Vila et al. 2001, 2005).

Basically, *Alexandrium* is well known because of repeated observations in many different ecosystems (Anderson et al. 2012a). In addition, the specificity of *Alexandrium* species is correlated with the production of three different toxin groups, in spite of the multiplicity of nutritional exigencies. By the hygienic viewpoint, *Alexandrium* blooms have been considered as one of the most important topics when speaking of HAB-related toxin episodes. As a result, a notable amount of scientific literature is available at present with relation to different aspects, including also effects on the environment.

Dinophysis spp

Being a cosmopolitan genus, *Dinophysis* spp has been considered as one of the main problems for shellfish aquaculture in many European Countries and other regions because of the production of okadaic adic and pectenotoxins, powerful lipophilic toxins. The first report on *Dinophysis* species concerned (Caroppo et al. 2001) the composition and spatio-temporal distribution in the oligotrophic waters of the southern Adriatic coasts (Apulia, Italy).

Dinophysis sacculus, D. fortii, D. caudata, Phalacroma rotundatum and *P. mitra* are potentially producers of diarrhetic shellfish poisoning; these dinoflagellates were associated with mixing conditions, low water temperatures and high nutrient inputs; significant correlations of these *Dinophysis* species with chlorophyll *a* were found (Caroppo et al. 2001). These and other important results have been obtained recently (Reguera et al. 2012) in spite of known culture difficulties.

Pyrodinium bahamense

Pyrodinium bahamense, belonging to the family *Gonyaulacaceae*, is a tropical euryhaline dinoflagellate found mainly in the Atlantic ocean, particulary in marine waters that have more than 20 Practical Salinity Units (PSU) of salinity—the amount of dissolved salts in water—and are warmer than 20 °C. The optimal salinity is considered to be around 35 PSU. The cultivation of *P. bahamense* is explained by its specific nutrition needs. It shows optimal growth and chlorophyll levels when nitrogen levels in its environment are greater than 100 μM. Nitrogen is considered an important factor for the synthesis of toxins in *P. bahamense*.

This organism displays bioluminescence when agitated, glowing red due to its pigments. *Pyrodinium*—a mono-specific species with two varieties—was first discovered in 1906 in waters around New Providence Island in the Bahamas. These organisms are a major cause of seafood toxicity and cause of paralytic shellfish poisoning, especially in Southeast Asia and in Central America.

Karenia brevis

Karenia brevis, a microscopic and unicellular marine dinoflagellate common in the Gulf of Mexico, is considered responsible for red tides in Florida and Texas. This life form is correlated with the detection of brevetoxins, powerful compounds that can cause gastrointestinal and neurological problems in other organisms and are responsible for large die-offs of marine organisms and seabirds. *K. brevis* is unarmored and does not contain *peridinin*. The region around southwest Florida is one of the major hotspots for red tide blooms where *K. brevis* grow to very high concentrations and the water can take on a reddish or pinkish colouration.

Gambierdiscus toxicus

Taxonomy, geography, ecophysiology and toxicology of *Gambierdiscus* have been recently reviewed (Parsons et al. 2012). This life form is normally correlated with the production of certain gambiertoxins. *Gambierdiscus toxicus* was originally described from the French Polynesia, while *G. beliseanus* is described from coastal waters of Belize. Three additional *Gambierdiscus* species have been isolated from French Polynesia: *G. polynesiensis*, *G. pacificus* and *G. australes*.

The global distribution of 10 *Gambierdiscus* species has been documented recently (Litaker et al. 2010), with most species found in the Atlantic being distinct from those in the Pacific region. New species of *Gambierdiscus* have been detected in European Atlantic waters and in the Mediterranean Sea.

Gambierdiscus is likely to grow in shallow water habitats (<50 m) where annual temperatures range between 21 and 31 °C (optimum between 25 and 29 °C), with high, stable salinities, light levels 10 % of incident light and adequate substrate (algae, biofilms). *Gambierdiscus* prefers low light intensities. Many researchers found a positive correlation between *Gambierdiscus* spp cell abundance and water temperature. Toxin production may also be affected by increasing temperatures. On the other hand, *Gambierdiscus* cells appear to grow poorly in low salinity waters preferring a salinity range of 28–35 °C.

Prymnesiophytes—*Prymnesium*

Prymnesium organisms have been studied because of two main features at least (Granéli et al. 2012):

(a) These life forms, in particular *P. parvum*, are able to create remarkable blooms in many water areas
(b) The presence of *Prymnesium* organisms can influence the survival and the reproductive cycle of other life forms such as other algae, fish and plankton because of the production of peculiar haemolytic toxins
(c) Finally, life forms such as *P. parvum* are reported to use water-soluble organic compounds when available. This possibility gives these microrganisms higher survival chances.

Dinoflagellates—*Coolia monotis*

Coolia monotis is a benthic Dinoflagellate belonging to the family *Ostreopidaceae*. It is a euryhaline species, growing at a salinity range of 20–50 % and surviving in a range of 15–60 %, with mean values of around 35 % (Aubert and Aubert 1986). Optimal temperatures for growth is 25 °C and irradiance values are 30–100 μmol photons m^{-2} s^{-1}; in these conditions, the duplication time of *Coolia* is 3–4 days. *Coolia* produces aggregates due to the release of mucous substances; these compounds could have antifungal properties (Pearce et al. 2000). This species is of particular interest since, although it is a cosmopolite organism (Bruno et al. 1997), it has been recently included within the five genera of benthic dinoflagellates which are involved in *ciguatera* episodes in tropical and sub-tropical areas. Although no direct relationships of *Coolia monotis* and toxic phenomena have not been fully demonstrated, some studies have shown the production of a toxin, derived from yessotoxin, called cooliatoxin (Holmes et al. 1995).

This species is reported to be toxic on *Dunaliella salina* (Donner et al. 2000). It may cause effects similar to those observed with maitotoxin; the toxic effect, however, is pH- and temperature-dependent; the pH should be between 8 and 9. In addition the organism is inhibited by a temperature of 40 °C.

Dinoflagellates—*Ostreopsis* spp

The genus *Ostreopsis* consists of benthic/epiphytic dinoflagellates living in tropical and sub-tropical waters. Salinity is reported to have an optimum of 32 % and pH between 8 and 8.15. Nine species of *Ostreopsis—O. siamenses, lenticularis, heptagona, mascarenensis, ovata, labens, marinus, beliseanus* and *caribbeanus*—at least have been reviewed for their morphometric characteristics and habitats (Faust 1999; Faust et al. 1996).

Ostreopsis spp have been the object of several studies during the last decades due to its relationships with *Ciguatera* (Holmes et al. 1995). Although the dinoflagellate *Gambierdiscus toxicus* is the only species recognised to be responsible for this phenomenon, some studies performed along the Puerto Rico coast have shown the production of toxic compounds from some dinoflagellates, mostly *O. lenticularis* (Tosteson et al. 1998).

The production palytoxin has been reported in the Mediterranean Sea by *O. ovata* which usually lives on the surface of red and brown macroalgae (Ciminiello et al. 2009). Blooms of this alga have sometimes been associated with mortalities of benthic organisms and respiratory problems in swimmers or people who were close to the affected area. It is not known if the toxins produced by *O. ovata* or other organisms associated to it may accumulate along the food chain, and whether its accumulation can result in a significant health risk.

Since 1998, summer algal blooms of *O. ovata* were recognised to be an emerging problem in the Apuan benthic seawaters (Tuscany, Italy), inducing heavy consequences on benthic communities- like molluscs, coelenterates and echinoderms—and evident alterations of water quality (Sansoni et al. 2003). Other health problems—respiratory illness and fever in about 200 hospitalised people following inhalation of marine aerosols—were reported in northern Italy. As the exposure to produced toxins—palytoxins (PLTX) compounds (Botana et al. 2013)—occurs through respiration, the banning of coastal areas to recreational activities is not a good tool to reduce toxic risks.

The importance of *Ostreopsis* spp is strictly correlated with the geographical distribution. Originally, these life forms were reported in the Mediterranean Sea and in selected areas only (Durando et al. 2007). However, climatic modifications—thermal values above all—and remarkable salinity values have been considered the main reasons for the broadened spreading of *Ostreopsis* spp in other extra-European areas. The diffusion of PLTX is the natural consequence.

Significant abundances of *O. ovata* bloom have been reported (Accoroni et al. 2011, 2012) in the northern Adriatic Sea in September (1.3×10^6 cells/g corresponding to 63.8×10^3 cells/cm^2) and on hard substrata (rocks) than on seaweeds. Hydrodynamism played a major role in *Ostreopsis* blooms, as higher abundances were observed in sheltered sites compared with exposed ones (Totti et al. 2010). Temperature and nutrients did not seem to cause an important effect on *O. ovata* blooms, as this species peaked when temperature values were decreasing. High levels of toxins were recorded in natural samples by high resolution liquid chromatography-mass spectrometry. High total toxin contents were demonstrated (up to 75 pg/cell) including putative palytoxin and ovatoxins; episodes of death of both benthic invertebrates (limpets, sea urchins and mussels) and macroalgae were commonly observed during algal blooms.

Gymnodinium sanguineum

G. sanguineum is a toxic Dinoflagellate which may give red tides, causing sometimes a decrease in the abundance of herbivorous zooplankton and of its filtration rate (Fiedler 1982).

Glenodinium cf. *foliaceum*

G. foliaceum is a toxic Dinoflagellate species known since 1960. It caused frequent intoxications following the ingestion of the mollusc *Cardium edule* in the lagoon of *Obidos* (Portugal), where a bloom of this dinoflagellate produced red-brown tides. The same species was also found in high numbers in British brackish waters, including also other areas such as: the Baltic Sea, United States of America (USA) and the Mediterranean Sea (Dodge 1982). It can be distinguished from *Protoperidinium* spp due to its preference for brackish habitats and the presence of green-brown chromatophores inside the cell.

Prorocentrum minimum

P. minimum is a tecate dinoflagellate sometimes associated with fish mortalities (Rabbani et al. 1990); the related toxicity has been demonstrated for *P. minimum* var. *maria lebouriae* (Okaichi and Imatomi 1979).

This microalgal species is common in many coastal and estuarine areas worldwide (Hajdu et al. 2005). The origin of *Baltic P. minimum* is unclear; it could have been transported by ballast water or spread successively by currents from the Skagerrak into the brackish Baltic Sea.

Since the early 1980s, blooms of *P. minimum* have been reported from many eutrophic coastal areas of the Baltic Sea. A laboratory experiment (Hajdu et al. 2000) revealed that the species had optimum growth at 15 PSU, but it could also grow well at salinity below 5 PSU. The growth rate ranged from 0.13 to 0.6 m per day below 10 PSU. Another species belonging to the genus *Prorocentrum* is *P. lima*, a cosmopolitan species that is toxic since related to diarrhoeic shellfish poisoning (DSP) phenomena (Sechet et al. 1998); it is epiphytic-benthonic and is frequently associated with macroalgae.

Cyanobacteria

Cyanobacteria—Microcystis

Microcystis is reported to be a powerful bloom-producing microorganism with a worldwide distribution in freshwaters, with the exception of some inhospitable areas (Fristachi et al. 2008). Very frequent in China lakes (*Taihu*), it is present as aggregated single-cell coccoid genera (O'Neil et al. 2012; Paerl and Otten 2013).

The importance of these life forms is strictly correlated (Fristachi et al. 2008) with:

(a) Survival and growth rates when certain environmental conditions are assures. In detail, *Microcystis* can survive well in warm waters, with abundance of bioavailable substances and the concomitant presence of carbon dioxide. This substance is abundant in warm ecosystems
(b) The diversification of toxins. These organisms can synthesise a group of powerful molecules, including microcystins (MCY), anatoxin-a and BMAA. Actually, certain *Microcystis* cells are not able to synthesise MCY.

Cyanobacteria—Planktothrix rubescens

Formerly known as *Oscillatoria rubescens*, *P. rubescens* is a filamentous cyanobacterium. In lakes, the presence of these algae is constant throughout the year; in summer, however, this species—due to its photosynthetic pigments and its needs of low temperature—is distributed preferentially into the deepest part of the lake, while in winter—under conditions of low temperature and light radiation- moves to the surface resulting in conditions favourable for toxic blooms. The related growth depends mostly on the availability of nitrogen compounds and to a lesser extent of phosphate, water temperature and light. Usually, this alga is transported from already contaminated sites by water birds that transport it as sporae. It is able to produce several toxins called microcystins which yield hepatotoxic, carcinogenic and gastrointestinal effects.

Cyanobacteria—Anabaena

Anabaena is a filamentous, heterocystous *Cyanobacteria* genus. Generally, this type of organism is considered ubiquitous (freshwater environments) and able to grow with low nitrogen and carbon dioxide sources (O'Neil et al. 2012). The importance of *Anabaena* is correlated with the production of MCY, anatoxins, cylindrospermopsin (CYN) and a saxitoxin (STX) where possible.

Cyanobacteria—Cylindrospermopsis

Cylindrospermopsis is a solitary, filamentous diazotroph cyanobacterium, which in the last decade has expanded its geographical range across every continent, except Antarctica (O'Neil et al. 2012). The structure of its cylindrospermopsin was determined in 1992. Other cyanobacteria, including U*mezakia natans, Aphanizomenon ovalisporum* (Carmichael 2001), *Lyngbya wollei, Raphidiopsis mediterranea*, and *Anabaena lapponica* were found to be capable of producing CYN (O'Neil et al. 2012).

Cyanobacteria—Nodularia

Nodularia is a filamentous, heterocystous *Cyanobacteria* genus, causing blooms in brackish waters worldwide (O'Neil et al. 2012) especially in the Baltic Sea

N. spumigena was the species responsible for the first bloom of a toxic cyanobacterial species reported in the world. Morphological features—like the presence of gas vesicles, the dimensions and shapes of vegetative cells, heterocytes, akinetes, the size and shape of trichomes—did not accurately differentiate *Nodularia* strains in the Baltic Sea. Historically, the first bloom episode correlated to a cyanobacterial species has been ascribed to a *Nodularia* organism. This life form, *N. spumigena*, produces nodularin which can promote liver tumours and act directly as a liver carcinogen, due to inhibition of protein phosphatases.

Cyanobacteria—Lyngbya

Lyngbya sp is a filamentous, non-heterocystous *Cyanobacteria* genus (O'Neil et al. 2012). Actually, different organisms belonging to this species are reported at present: they can produce different toxins and show very dissimilar attitudes when speaking of environmental adaptability. The freshwater species *L. wollei* is capable of producing saxitoxin as well as cylindrospermopsin.

One of the most known life forms of this species, *L. majuscula*, was first reported in Hawaii, USA, during the 1950s to the 1970s, but also during the late 1990s in Australia, in Moreton Bay, Queensland as well as near Perth and Broome. From the health viewpoint, severe illnesses have correlated with the action of *L. majuscula* on professional fishermen (O'Neil et al. 2012). Blooms have also been reported in Florida as well as active spots in the Caribbean and the South Pacific.

This cyanobacterium produces several demotoxic alkaloids, neurotoxins, and bioactive compounds. Toxins associated with *L. majuscula* include lyngbyatoxin-A and debromoaplysiatoxin, causing asthma-like symptoms and severe dermatitis in humans.

In addition to previously reported bioactive compounds toxic for fish and invertebrates, new microcolins, lyngbyamides and barbamides have been identified (Liu et al. 2011).

Cyanobacteria—Oscillatoria

Oscillatoria sp is a filamentous, non-heterocystous cyanobacterial genus commonly found in watering-troughs waters, and is mainly blue-green or brown-green. This organism uses photosynthesis to survive and reproduce. Each filament of *Oscillatoria* consists of trichome which is made up of rows of cells.

Cyanobacteria—Trichodesmium

Trichodesmium spp is a group of colonial non-heterocystous filamentous cyanobacterium species belonging to *Oscillatoriales*. Basically, these organisms are

reported to be strong bloom-producing agents. Moreover, they are able to grow in many environmental marine ecosystems including tropical waters. In addition, *Trichodesmium* spp need low available nutrient substances but waters have to be clear enough to allow light penetration. These life forms synthesise water-soluble toxins with consequent aggressive action on other marine competitors (O'Neil et al. 2012).

Interestingly, different substances have been correlated with these organisms, including palytoxin, microcystin (MCY)-LR and a MCY-like cyclic peptide (Kerbrat et al. 2011).

Synechococcus is a cosmopolitan open ocean cyanobacterium, but it also forms harmful blooms covering vast areas in Florida Bay, USA. *Synechococcus* blooms are also known to inhibit zooplankton grazing (O'Neil et al. 2012).

2.1.3 Harmful Algal Blooms: Occurrence and Causes

HAB—well known because of the adverse action on human and wildlife health—are increasing in frequency and intensity worldwide (Hallegraeff 1993; O'Neil et al. 2012; Van Dolah 2000). These phenomena afflict most temperate and tropical coastal nations; their frequency and negative impacts on fisheries have increased markedly in the last decades since 1970 (Van Dolah 2000). Blooms of *Cyanophyceae*—mostly due to *Microcystis aeruginosa, Oscillatoria rubescens* and *Anabaena flos-aquae*—have recently been recorded in freshwaters: Australia, Japan and South Africa (O'Neil et al. 2012).

The widespread occurrence of HAB both in marine and freshwaters has resulted in a growing public interest for their negative interferences on economic activities related to the use of marine resources (fishing, mariculture and tourism) and for their negative implications on biodiversity and ecosystem health (Botana 2014; Cabado and Vieites 2012; Evangelista et al. 2008; Rossini 2014). Simultaneous advances in the monitoring and surveillance have also contributed to increasing records of HAB (Pitcher 2012).

Since selected groups of phytoplankton species occur regularly in the presence of precise chemical and physical conditions, several different models have been suggested to explain species occurrence and abundance (Margalef 1978; Reynolds 1988; Reynolds and Smayda 1998). On the other hand, the role of endogenous regulation in species timing has been considered only marginally (Eilertsen and Wyatt 2000; Garrison 1981). Actually, many other conditions and factors should be taken into account (Garcés et al. 2002; Zingone and Wyatt 2005; Zingone et al. 2001).

In addition, it should be noted that bacteria may play a role in the population dynamics and toxicity of harmful algae (Doucette et al. 1998). Bacteria have been reported to be involved—directly or indirectly—in the production of biotoxins, the promotion or inhibition of the growth of HAB species and the stimulation or inhibition of phytoplankton.

Although natural transport and dispersal mechanisms and natural habitat extensions, driven by environmental change, may contribute to increased HAB observations, anthropogenic activities—including the eutrophication of coastal systems and the translocation of some species—are considered to be main drivers of HAB events. Climate changes caused by global warming consequent to the greenhouse effect and to the hole in the ozone layer, as well as the increased anthropic influence on aquatic ecosystems have been recognised as the main factors involved in the outbreaks of HAB. Proliferations of toxic microalgae have increased with the rise of surface water temperature up to 2° above the seasonal average values. Increasing outbreaks of HAB along Mediterranean coasts suggest that this ecosystem is changing towards conditions typical of tropical and sub-tropical regions (Giacobbe 2008).

Among the causes invoked to explain the expansion of HAB (GEOHAB 2001), several factors have to be highlighted, including also increased nutrient inputs and surface water temperatures due to changing global climate. The general increase in water eutrophication and phytoplankton blooms is usually associated with the massive input of nitrogen and phosphorus (Granéli et al. 2008). The nutrient enrichment of seawater caused by organic wastes released from anthropic activities (i.e. sewages, agriculture, fertilisers) stimulates the algal proliferation. On the other hand, the spreading of toxic species may be explained by the occurrence of natural mechanisms, increased aquaculture activity, improved analytical methods which determine the discovery of new toxins and toxic events (Hallegraeff 1993). Blooms of algal exotic species have also increased due to the spreading of resting/dormant stages in ballast waters, as well as due to transfers of shellfish stocks (Garcés et al. 2001). Ballast waters and the progressive tropicalisation of Mediterranean waters have favoured the dispersion of toxic species from a geographic area to others, leading to the increase of the episodes of HAB.

The frequency and magnitude of processes (i.e. eutrophication and climate changes) that may promote the proliferation of cyanobacterial HAB are expected to increase in next future (Anderson et al. 2002; O'Neil et al. 2012). While some cyanobacterial blooms are associated with eutrophication—being cyanobacteria highly competitive for inorganic phosphorus (P) and able to acquire organic P compounds—other genera appear dependent on the reduced abundance of P and inorganic nitrogen (N). The role of eutrophication in HAB is controversial. The importance of life cycles in this context should be considered (Zingone et al. 2001).

Globally, climate changes can modify all the aquatic ecosystems. Several examples concern the augment of thermal values, the modification of ocean flows, the increased rate of photosynthetic processes, etc. (Hallegraeff 2010). On the other hand, more research is needed when speaking of the correlation between cyanobacteria and the amount of carbon dioxide (Katırcıoğlu et al. 2004).

However, the increasing uncertainty in progressing from climate change to ocean response to biological impact leads to considerable doubt on the ecosystem scenario development (Hallegraeff 2010). Climate simulation scenarios are affected by uncertainties; the comprehension and predictions of how climate may select for HAB are still limited by the scarcity of long-term records of algal blooms required

for assessment of past climate variability on HAB at least. The prediction of phytoplankton community responses and more specifically the response of HAB to climate change, requires accurate forecasts of these environmental and ecological parameters (Garcés et al. 2001).

Many research programs have focused on the monitoring and prevention of HAB phenomena. First, a recent international project has studied the correlation between the abundance of certain HAB species and specific factors such as interactions between different organisms, the availability of nutrients and other ecological agents (GEOHAB 2001). In more recent years, many researches have focused on HAB with the specific objective of developing new assays for rapid detection of biotoxins and of advanced early warning tools for the detection of shellfish toxins as well as of decontamination methods for toxic shellfish.

The recent episodes of blooms due to tropical species typically have highlighted the need to undertake more scientific, organisational and institutional initiatives when speaking of management and protection of aquatic resources.

Different coastal monitoring programmes have been performed in Italy, for the Adriatic Sea and then for all other Regions having shorelines. Lines and protocols of intervention and monitoring have been set up in cooperation among the Italian Ministry of Environment and Health, Local Regional Agencies for Environmental Protection and Research institutes (University, National Research Council) involved in the field of environmental and human protection.

The extensive monitoring for *Alexandrium* spp in Mediterranean coastal waters —including the Catalonia and Balearic Islands (Spain), Aegean regions (Greece), Sardinia and Sicily (Italy)—indicated that major HAB emergencies are in the Catalan area which is characterised by the highest nutrient load, especially nitrogen, and marked human activities along the coast (Giacobbe et al. 2006). The same thing was observed in the Catalonia region and other areas (Luglié et al. 2004).

2.2 Typologies of Toxins, Mechanisms of Action and Syndromes

2.2.1 Typologies of Toxins: Chemical Structure

Damages caused to humans by algal blooms are primarily derived from the production of toxins and to a lesser extent from the effects of dermal sensitisation in relation to cell membranes or volatile substances present in aerosols on the surface of the blooms.

Basically, shellfish toxins are subdivided in two groups depending on the affinity to water or lipids. Table 2.1 shows most important molecules with relation to water affinity or lipophilic attitude. In addition, these toxins are associated with six different syndromes.

Table 2.1 Groups of marine toxins and correlated syndromes (Botana 2014; Gerssen et al. 2010; van Dolah 2000)

Toxin group	Toxin sub-group	Syndrome
Hydrophilic toxins	Domoic acid	Amnesic shellfish poisoning (ASP)
	Saxitoxins	Paralytic shellfish poisoning (PSP)
Lipophilic toxins	Brevetoxins	Neurotoxic shellfish poisoning (NSP)
	Okadaic acid	Diarrhetic shellfish poisoning (DSP)
	Dinophysistoxins	
	Pectenotoxins	
	Yessotoxins	
	Azaspiracids	Azaspiracid shellfish poisoning (AZP)
Non-regulated lipophilic toxins	Spirolides	–
	Gymnodimines	–
	Pinnatoxins	–
	Ciguatoxins	Ciguatera fish poisoning

The severity of effects produced by toxins depends on the different solubility of these compounds (in water or lipids); modality of exposure, mode of action and the different susceptibility of species are important. Consequently, concentrations below a threshold may result only in a weak physiological or behavioural response if organisms are able to cope with high toxin levels. On the other hand, high concentration of toxins may have lethal effects.

A group of different experts has formulated an indication that toxins are classified according to their chemical characteristics into: saxitoxins (STX), okadaic acid (OA) and dinophysistoxins (DTX); pectenotoxins (PTX); yessotoxins (YTX); brevetoxins (PbTx); domoic acid (DA), azaspiracids (AZA), spirolides (FAO/IOC/WHO 2005).

A synthesis of main syndromes associated with marine toxic algae is reported in Table 2.1.

2.2.1.1 Saxitoxins

STX (Fig. 2.1) are the most common toxins. These are a group of around 30 water-soluble, hydrophilic, tetrahydropurine neurotoxins produced by dinoflagellate species belonging to *Alexandrium* spp., *Gymnodinium catenatum* and *Pyrodinium bahamense* (Halstead 2002; Mitrovic et al. 2004). The STX family includes different chemical compounds, divided into subgroups: saxitoxin, neosaxitoxin, tetrodotoxin and gonyautoxins (from 1 to 4, B1 and B2, C1 to C4). Most of these molecules are metabolites of saxitoxin and neosaxitoxin. In general, they are responsible for PSP. Saxitoxins (carbamate alkaloid neurotoxins), a variegated group of chemical compounds, are produced by different freshwater cyanobacteria (Codd 2000; Codd et al. 1999).

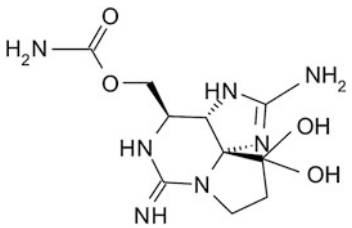

Fig. 2.1 Chemical structure of saxitoxin (STX). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

These heterocyclic guanidine compounds interfere with the production of action potentials of some types of excitable cells, causing inhibition in nerve conduction. The block of the influx of sodium ions into the cells (and therefore of the conduction of neuronal axons) occurs through the bond on the receptor exterior to the sodium channel by negatively charged amino acids on the alpha subunit II. The biosynthesis of the toxin occurs through the pathway of methylenetetrahydrofolate glycine-serine—and of the ornithine-arginine—with closure of the imidazole ring.

PSP-producing species have been found in the Adriatic Sea (Honsell et al. 1992); Tyrrhenian Sea (Carrada et al. 1991) and in Cape Peloro Lakes (Giacobbe and Maimone 1994), although the detection of saxitoxins has been also reported in extra-european Countries also (Gerssen et al. 2010). The median lethal dose at which 50 % of the population responds (LD_{50}) for adult men is 1–2 mg, orally. Saxitoxin persists in the environment and should be subject to biological magnification. STX has been found in bottom sediments, within resting cysts and can accumulate in the hepatopancreas, adductor muscle and viscera of marine molluscs (like *Mytilus galloprovincialis*, *M. californianus* and *Patinopecten yessoensis*), but also of freshwater molluscs (*Corbicula sandai*). The toxin can accumulate in *Tindinnids*, crustaceans like *Tigriopus californicus* and *Cancer anthonyi larvae* (Bruno 2000). Subsequently, the accumulated toxin is metabolised and the shellfish are decontaminated, but the metabolites retain their toxic properties. The mantle or the kidney remain toxic for up to six months after intoxication while the hepatopancreas detoxifies itself much earlier. Toxins cause a species-specific toxic effect in the same shellfish: it is manifested by altered shell activity, decreased or increased oxygen consumption, altered heart rate, reduced production of fine linen, altered filtration and nutrition rates.

At present, different measures have been taken into account with the aim of protecting human health from PSP within the EU (EFSA 2009a).

2.2.1.2 Diarrhetic Shellfish Toxins

Diarrhetic Shellfish Toxins (DST) are a family of lipid-soluble toxins which includes okadaic acid (OA, Fig. 2.2) and its congeners, dinophysistoxins (DTX 1–4); DTX are polyethers derived from a fatty acid with 38 carbon atoms. These compounds are

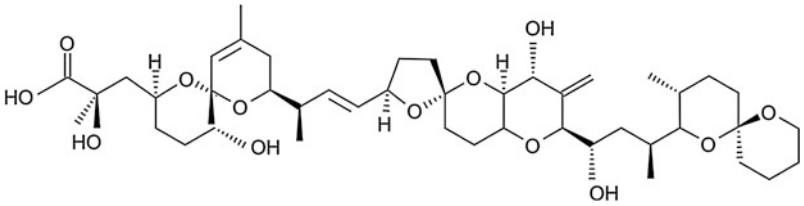

Fig. 2.2 Chemical structures of okadaic acid (OA). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

isolated from algae of genera *Dinophysis* and *Prorocentrum*, and are responsible for DSP (Landsberg et al. 2005; Lee et al. 1989; Yasumoto et al. 1978). At present, it has to be highlighted that OA toxins are repeatedly found in many organisms and various geographical areas with notable amounts (Gerssen et al. 2010). DSP-producing algae have been found in all the world. In Italy, these have been found in the Adriatic Sea (Tubaro et al. 1992) and in brackish waters of Sicily (Giacobbe et al. 1995).

DST include also YTX and 45-hydroyessotoxin, produced by the dinoflagellate *Protoceratium reticulatum*. These toxins may accumulate in shellfish which may be contaminated even with a few hundreds of cells per liter.

The so-called pectenotoxin group, including PTX from 1 to 7, is also considered in the DST category.

YTX and PTX, included into the group of DSP toxins due to their frequent co-occurrence with OA and DTX analogues, do not cause diarrhoea; they should be removed by DSP group (Gerssen et al. 2010). With relation to YTX, a remarkable differentiation has to be considered from the chemical viewpoint. However, four toxins only are been found repeatedly with respect to the total number of similar YTX; some of these molecules have been reported in Europe only (Gerssen et al. 2010). Yessotoxin is a polyether disulphate that exerts its toxic effects on cells of the cardiac muscle, causing a marked cytoplasmic oedema. It has a high toxicity, with a LD_{50} of 100 μg/kg of body weight in mouse. For this reason, the current EU limit is 1 mg/kg; however, YTX have not been recognised responsible for human illnesses at present (Gerssen et al. 2010).

On the other side, 15 different PTX toxins have been studied. Apparently, these toxic molecules are generally found in Europe. For this reason, PTX should be considered in the OA group with relation to European legislation. However, the EFSA has recently recommended a different classification (and different safety limits) for PTX (EFSA 2008a, b, c, 2009b; Gerssen et al. 2010).

2.2.1.3 Brevetoxins

Brevetoxins (PbTx) are lipophilic, complex, neurotoxic polycyclic ethers, produced by the dinoflagellate *Karenia* (*Ptychodiscus*) *brevis* (previously *Gymnodinium breve*) which may be differentiated in two main types (Baden 1989). They cause NSP (Gerssen et al. 2010).

Because of the localisation of NSP in extra-European Countries, this danger has been considered with the determination of safe limits by the Food and Drug Administration, while a scientific opinion is available in Europe (EFSA 2010c).

PbTx may spread through release into the water after cell lysis, or stored in animal tissues or inhaled by the simple breath of aerosol during the algal blooms. They are eliminated through the urine and faeces. Brevetoxins can cause widespread death of fish and even of marine mammals (*Tursiops truncatus*) due to biological magnification.

2.2.1.4 Domoic Acid

Domoic acid (DA) is produced by certain diatom species (Sect. 2.1.2.1). This molecule is a cyclic amino acid (Fig. 2.3): a specific, high affinity- glutamate antagonist at the neuronal level. LD_{50} is ranged between 35 and 70 mg/kg in rats (Iverson et al. 1989). However, human toxicity was observed in Canada after the consumption of molluscs (*Mytilus edulis*) containing an amount of 1 mg/kg of DA; most people died, whereas those surviving had permanent neurological damages (Perl et al. 1990). The depuration time of molluscs is more than 18 days in uncontaminated waters. At present, ASP has been studied in Europe also (Gerssen et al. 2010). For this reason at least, a permitted level of 20 mg DA/kg shellfish has been defined by the European Legislation (EFSA 2009c). However, new norms may be expected in future.

2.2.1.5 Ciguatoxins

Nonprotein- ciguatoxins are produced by *Gambierdiscus toxicus* and *Ostreopsis lenticularis*, tropical benthic dinoflagellates (Bagnis 1968). Other species which contribute to this toxin are *Coolia, Prorocentrum* and *Amphidinium*.

Chemically, they are cyclic polyethers which are soluble in lipids (ciguatoxins) or in water (maitotoxins); they act as exciting agents causing repeated activation of nerve axons (Holmes et al. 1991). The binding site is the same as that of brevetoxins. Ciguatoxins have a LD_{50} of 0.45 mg/kg of body weight in mouse. Ciguatoxins

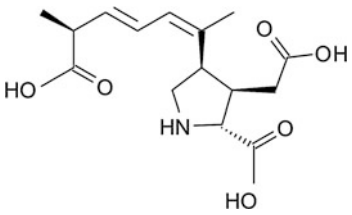

Fig. 2.3 Chemical structure of domoic acid (DA). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

accumulate through the trophic web, whereas maitotoxins remain located in the digestive tract of plankton-feeding fish (Halstead 2002). An opinion on ciguatoxins has been reported by EFSA (EFSA 2010a).

2.2.1.6 Azaspiracid Shellfish Poisoning (AZP)

AZP is correlated with 24 different toxins but only three molecules—azaspiracid-1, -2 and -3—can be defined the most important menaces for human and animal health (Gerssen et al. 2010). Generally, AZP is reported in Europe and in American Countries.

At present, European legislation has modified the previous safety limit for azaspiracid-1 and other toxins: 30 μg azaspiracid-1 equivalents/kg shellfish instead of the previous amount

2.2.1.7 Spirolides and Gymnodimines (Cyclic Imines)

Spirolides (SPX) and gymnodimines are considered powerful toxins because of rapid lethal effects on test animals. Chemically, it can be affirmed that these molecules are cyclic imines. In addition, the detection of SPX is not circumscribed to European Countries only: however, the importance of these substances in the EU legislation (EFSA 2010b) has to be noted. On the other side, gymnodimines appear to be reported in Oceania only and shellfish from New Zealand.

2.2.1.8 Palytoxin

PLTX toxins appear similar because of the general structure: an extended compound ranging from 2,659 to 2,680 Da. Interestingly, these molecules have hydrophilic attitudes and lipidic affinity at the same time (Botana et al. 2013). A Scientific opinion on PLTX has been recently reported by the EFSA (EFSA 2009d).

2.2.1.9 Aplysiatoxins

Aplysiatoxins are bis-lactones of organic acids produced by tropical marine *Oscillatoriaceae* belonging to *Lynbya majuscula, L. gracilis, Schizothrix calcicola* and *Oscillatoria nigroviridis*.

These species can grow as epyphites on macroalgae like *Acanthophora spicifora, Laurencia intermedia* and *L. okamurai*. Aplysiatoxins may reach humans through the biological magnification performed by herbivorous fish and molluscs

(*Siganus fuscescens, Aplysia kurodai*). There are at least 12 compounds belonging to aplysiatoxins, including indole alkaloids (such as lyngbyatoxin a) and polyacetates (such as aplysiatoxin), which may cause epithelial and gastrointestinal cancers.

2.2.1.10 Cyanotoxins

Cyanotoxins are composed of different molecules—anatoxins, microcystins and saxitoxins—produced by cyanobacteria (Sivonen and Jones 1999).

MCY and cylindrospermopsin (CYN) are natural hepatotoxins toxins produced by cyanobacteria (blue-green algae) that grow worldwide in eutrophic freshwaters and cause animal and human water-based toxicoses (Sivonen and Jones 1999). Cyanotoxins fall into three broad groups of different chemical structure (Katırcıoğlu et al. 2004): cyclic peptides, alkaloids and lipopolysaccharides. On the other side, potent neurotoxins are anatoxin-a ($C_{10}H_{15}NO$), molecular weight (MW): 165 Da, (2) anatoxin-a(s) (CHN_4O_4P), MW = 252 Da, and (3) saxitoxins.

Microcystins, produced by genera *Anabaena, Microcystis, Nostoc* and *Oscillatoria*, are monocyclic hepta-peptides with MW of about 800–1,000 Da. These molecules are constituted by a blocking carbohydrate, seven amino acidic residues and a methylamine. They differ according to two L- amino acids, and more than 60 variants of the first isolated toxin (microcystin-LR) are known (Codd 2000). They show potent hepatotoxicity and activity as tumour promoters.

Nodularin, produced by *Nodularia spumigen*, a brackish water cyanobacterium, is a cyclic pentapeptide structurally very similar to microcystins.

With concern to the health importance of CYN, the chemical compound—a cyclic guanidine alkaloid—has been associated with outbreaks of human sickness and cattle mortality: a certain carcinogenic activity has been reported (Katırcıoğlu et al. 2004).

In relation to neurotoxins, anatoxins are non-sulphated alkaloid toxins of freshwater cyanobacteria and act as neurotoxins. Anatoxin-a (Fig. 2.4) is an alkaloid with a median lethal dose (at which 90 % of the mouse population responds) of 0.3 mg/kg (Metting and Pyne 1986; Mitrovic et al. 2004). Other important neurotoxins are anatoxin-c and Homoanatoxin-a (Skulberg 1999).

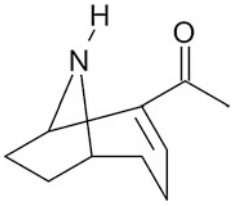

Fig. 2.4 Chemical structure of a neurotoxic alkaloid: anatoxin-a. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

Prymnesin is an icthyotoxin produced by *Prymnesium parvum*, a brackish-water golden-brown phytoflagellate (Metting and Pyne 1986).

The presence and correlated health risks of the neurotoxic compound, BMAA, has been recently described (Cox et al. 2003, 2005, 2009). BMAA has been found in remarkable amounts (fish and shellfish tissues) in Northern European waters (Jonasson et al. 2010).

2.2.2 Mechanisms of Action of Algal Toxins

The most part of marine algal toxins cause changes in the concentration of sodium and calcium ions. As a result, they interfere with electrical transmission.

Saxitoxins are responsible for the Paralytic Shellfish Poisoning (PSP). In other words, neurotransmission is blocked (Gerssen et al. 2010). The primary route of clearance is via the urinary tract, both in humans and in animals.

DST are inhibitors of serine and threonine phosphatases PP1 and PP2A that are key components of cellular processes related to metabolism, ion balance, and neurotransmission. Diarrhoea originates mostly from the hyperphosphorylation of proteins in the intestinal epithelia.

The tolerance limit set for OA is 40 μg/100 g of shellfish, while the analogous limit for dinophysistoxin-1 is 36 μg/100 g of mollusc.

Brevetoxins, which cause NSP, bind to the sodium channel but in a site different from saxitoxin. In brief, by this mechanism, PbTx alter membrane properties of excitable cells, leading to membrane depolarisation and subsequent disruption of cardiac functions (Baden 1989). PbTx are potent neurotoxins and hemolysins: they can cause depolarisation of smooth muscles in bronchial and tracheal tissues.

DA, which causes ASP, is a neurotoxin. It can produce neurological lesions with symptoms such as memory loss, neuro-excitatory and neuro-degenerative effects in mammals (Bruno 2000).

Spirolides—cyclic imines produced by *Alexandrium ostenfeldii* and *A. peruvianum*—are 'fast-acting' algal neurotoxins isolated from shellfish and plankton samples off the eastern shore of Nova Scotia, Canada (Landsberg et al. 2005). The molecular basis for these effects may, at least in part, be due to the recently reported antagonist actions of spirolides at nicotinic-type and muscarinic-type acetylcholine receptors (Munday et al. 2012).

The known activity of PLTX causes different symptoms in animals and humans. This type of human poisoning is called palytoxicosis or clupeotoxicosis. Debromoaplysiatoxins bind stably to the membrane receptor of protein kinase C. These toxic compounds have also been isolated from other *Oscillatoriaceae* (Chorus and Bartman 1999). Finally, anatoxin-a, homoanatoxin-a and anatoxin-a(s) are powerful neurotoxins.

2.2.3 Syndromes Caused by Algal Toxins

The main human poisoning syndromes associated with the consumption of shellfish have been reported, depending on the type of symptoms, such as paralytic (PSP), diarrheic (DSP), neurotoxic (NSP), and amnesic (ASP) poisoning, respectively (Botana 2008; van Dolah 2000). AZP is also reported.

The ingestion of edible molluscs containing toxins can cause the onset of very serious symptoms in humans, sometimes with fatal outcome. Algal toxins for the major part are thermally stable and are known for their effects on the nervous system, which often happen within 1 h from food consumption.

Symptoms due to algal toxins can be summarised as follows (Bruno 2000):

(1) PSP caused by Saxitoxins. PSP is a life-threatening syndrome; the symptoms are purely neurological with rapid onset. Acute intoxication with early symptoms includes tingling of the extremities and perioral area, loss of motor control, incoherence and death by respiratory paralysis
(2) DSP caused by *Dinophysis* toxins diarrhoea, vomiting, etc. However, no human intoxications by PTX have been reported yet
(3) NSP caused by brevetoxins. NSP is responsible for an intoxication syndrome with both neurological and gastrointestinal symptoms. NSP toxins can cause symptoms such as dry cough, wheezing, watery eyes, paresthesias, myalgia, vertigo, headache, nausea, diarrhoea, abdominal pain, bradycardia and dilated pupils (Baden 1983), asthma, etc. Exposure to brevetoxin aerosols can result in conjunctival irritation, rhinorrhea and broncho-constriction; asthmatic people may have wheezing (Baden et al. 1982). After exposure to brevetoxin aerosols during a *K. brevis* bloom, inflammatory responses with upper and lower respiratory symptoms have been reported (Backer et al. 2003). NSP syndromes are endemic in Florida and in the Gulf of Mexico
(4) ASP caused by DA. ASP can be a life-threatening syndrome characterised by a variety of acute symptoms—vomiting, nausea, diarrhoea, hallucinations, confusion, disorientation, breath difficulties, short-term memory loss and in severe cases, death—which may be both neurological and gastrointestinal, and take place within 24 or 48 h of ingestion
(5) *Ciguatera* fish poisoning by Ciguatoxins. This syndrome is generally confined to tropical and subtropical seas. Since the ciguatoxins include multiple toxic components in fish, several and different symptoms have been identified in humans (Backer et al. 2003). They occur 8–48 h after consumption of the contaminated food (neurological symptoms: paresthesias, myalgia, arthralgia and inversion of sensitivity to heat; gastrointestinal disorders; hearth disorders like bradycardia and ipotension; weakness and skin reactions; psychiatric disorders). Usually, the consumption of toxic herbivorous fish is reported to be linked with gastrointestinal or neurological problems, while toxic carnivorous fish may produce cardiovascular and neurological symptoms (Bagnis 1968). Interestingly, different cooking of freezing methods do not lead to toxin inactivaction

(6) Symptoms caused by AZA. Toxic effects observed during AZP intoxication are gastrointestinal disorder, diarrhoea and abdominal cramps
(7) Symptoms caused by spirolides. Exposure to spirolides in mice induces a number of symptoms that vary with survival time, such as piloerection, ataxia, ophthalmia, abdominal muscle spasms, hyperextension of back, and tail whipping. In terms of toxicity, the lethal dose of desmethyl-spirolide C delivered intraperitoneally to mice was reported to be 40 μg/kg
(8) Symptoms caused by PLTX. These toxins, inhaled by aerosol formed in the presence of high cell concentrations and waves, may cause respiratory infections and conjunctivitis; in other cases, dermatitis and alterations of body temperature were reported. Another disorder is leukocytosis with additional symptoms with nausea and headaches
(9) Symptoms by aplysiatoxins. These toxins cause an acute toxic effect which consists of strong itching following contact with algae, that evolves within 3–8 h into a strong erosive dermatitis with erythema on the skin exposed to the toxins action
(10) Symptoms caused by microcystins and nodularins: nausea, vomiting, intestinal pains within 3–4 h of water ingestion, later fever, acute headache, muscular pains, hepatic damages, death in some immunosuppressed individuals. Microcystins produced by *Cyanophyceae* have a direct effect on humans or animals, who have a contact with lake water during algal blooms
(11) Symptoms by anatoxins: diarrhoea, vomiting, nausea, no sensitivity; paralysis, breath difficulties, death.

2.3 Brief Notes on Detection Methods

2.3.1 Analytical Methods for the Determination of Algal Toxins

The Regulation (EC) No. 853/2004 prescribes—Annex III, Section VII, Chapter V (2)(c) and (e)—that live bivalve molluscs commercialised for food purposes have to comply with strict toxin levels (maximum amounts) such as 800 μg/kg for PSP-associated toxins.

The main analytical procedures used for the examination of poisoning syndromes are examined in this section. When speaking of PSP, the following methods are used:

(a) Biological Test
(b) Liquid chromatography/mass spectrometry
(c) Test of sodium channels for cell viability.

For DSP, the following methods are used:

(a) Cytotoxicity test
(b) Bioassay
(c) Liquid chromatography high resolution
(d) Immunoassay
(e) 'Ion-spray Liquid Chromatography-Mass Spectrometry'
(f) Test of phosphatase inhibition
(g) Thin-layer chromatography.

For NSP the following methods are mentioned:

(a) Biological Test on mouse
(b) Test radioimmunoassay
(c) Test of sodium channels for cell viability
(d) Competitive bound to sodium channels
(e) Enzyme-Linked ImmunoSorbent Assay (ELISA) with immunoelectrochemical biosensors
(f) High-performance liquid chromatography (HPLC).

When speaking of ASP, the following methods are used:

(a) Bioassay
(b) Capillary Electrophoresis
(c) Liquid chromatography high resolution
(d) Immunochemical tests
(e) Mass spectrometry
(f) Bound to competitive receptors
(g) Thin-layer chromatography.

For the *Ciguatera* syndrome, the following methods are mentioned:

(a) Biological Test on mouse
(b) Liquid chromatography
(c) Test of sodium channels for the cell viability
(d) Competitive binding of sodium channels
(e) ELISA
(f) Latex agglutination test.

In relation to EU official testing methods for the detection of toxins in shellfish, a recent overview has been made available; interestingly, some alter alternative methods have been also described (Gerssen et al. 2010). The Reader is invited to consult this and other documents with concern to the detection of above mentioned toxins in these products. With concern to alternative methods, polymerase chain reaction (PCR) is one of the last research lines (Penna et al. 2007; Perini et al. 2011).

In brief, EU protocols often consider mouse bioassays or rat bioassays (RBA) when speaking of the detection of common toxins according to the Commission Regulation (EC) No. 2074/2005 (Gerssen et al. 2010).

With the exclusion of bioassays and competitive microplate receptor-binding assays, other commonly used analytical methods are based on biomolecular techniques (EFSA 2010a), immunoassays such as ELISA tests (Gerssen et al. 2010) and chemical approaches. The last group of systems—HPLC, liquid chromatography-mass spectrometry (LC-MS) methods, capillary and high-performance capillary electrophoresis—is very interesting. Some examples of of chromatographic methods are reported below.

Total MCY levels released by the cyanobacterium *Planktothrix rubescens* were measured (Messineo et al. 2006) in lake Albano (central Italy) by liquid chromatography-tandem mass spectrometry (LC-MS). MC levels up to 14.2 μg/l were found, with high concentrations in summer at a 20–25 m-depth. The intracellular toxin content varied between 1.5 (surface, January 2004) and 0.21 pg/cell (surface, May 2004). Six different MC were detected, the most abundant being two desmethyl-MC-RR isomers.

A 'liquid chromatography/electrospray ionisation- quadruple time-of-flight' method was firstly described (Ferranti et al. 2009) to analyse MCY in freshwaters and allowed the characterisation of a novel variant of microcystin-RR (MCY-RR).

In a water reservoir in southern Italy where a bloom of Planktothrix rubescens occurred (Ferranti et al. 2013), cyanobacterial toxins were used using 'matrix-assisted laser desorption/ionisation time-of-flight' (MALDI-TOF) mass spectrometry and liquid chromatography coupled to quadrupole-time-of-flight (Q-TOF) tandem mass spectrometry. MALDI-TOF mass spectrometry was employed for rapid screening over a wide mass range; liquid chromatography coupled to Q/TOF tandem mass spectrometry allowed for molecular structure confirmation of each single cyanotoxin. Microcystins, anabaenopeptins and aeruginosins were the main cyanotoxins identified by liquid chromatography coupled to Q/TOF tandem mass spectrometry.

A LC-MS method was developed in the multireaction monitoring mode (Bogialli et al. 2006a): microcystin extraction was performed with a sorbent (Carbograph 4) cartridge. As Cylindrospermopsin is a highly polar compound that is scarcely retained by any sorbent material, 0.5 mL of filtered lake water was directly injected into the LC column. Limit of quantification (LOQ) of the five microcystins were within the 2–9 ng/L range, whereas the LOQ of cylindrospermopsin was 300 ng/L. Two demethylated forms of MCY-RR and one demethylated variety of microcystin-LR were found. Demethylated MCY-RR is known to be even more toxic than MCY-RR towards zooplanktonic grazers.

LC/MS/MS toxin analyses were performed on surface water samples collected from 28 Italian lakes (Messineo et al. 2009). The most widespread species associated with toxin production belonged to the genera *Microcystis, Planktothrix* and *Anabaena*. Values up to 226.16 ng/mL were recorded for microcystins (sum of all variants), upto 126 ng/mL for total cylindrospermopsin and to 100 μg/g (dry weight) for anatoxin-a.

With concern to the detection of toxins in fish fillets, a simple, specific, and sensitive procedure for determining six cyanotoxins (microcystins-RR, -LR, -YR, -LA, -LW and nodularin) in fish muscle tissue was reported (Bogialli et al. 2005).

This method was based on the matrix solid-phase dispersion technique with heated water as extractant followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) equipped with an electrospray ion source. The limits of quantification were estimated to range between 1.6 and 4.0 ng/g.

The identification of anatoxin-a in water and fish fillets was reported by LC-MS/MS with electrospray ionisation (Bogialli et al. 2006b), with limits of quantification estimated to be 13 ng/l and 0.5 ng/g in water and fish fillet, respectively.

Liquid chromatography coupled to electrospray ion trap mass spectrometry was described for determination of CYN in freshwater and fish muscle (Gallo et al. 2009). This method bacame highly selective and reliable in unambiguous identification of CYN in water and cyanobacteria extracts from Lake Averno, near Naples (Italy), with limits of quantification which were 0.10 ng/mL in freshwaters and 1.0 ng/g in fish muscle, respectively.

CYN produced by the cyanobacteria *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* was detected by Messineo et al. (2010) in lake Albano (Italy) by LC-MS/MS and ELISA immunoassay, showing extracellular superficial values ranging from 2.6 to 126 µg/L, and water column values ranging from 0.41 to 18.4 µg/L. Moreover, CYN was detected in tissues from two *Salmo trutta* trouts (up to 2.7 ng/g).

Microcystin detection was reviewed in contaminated fish from Italian lakes using ELISA and LC-MS/MS analysis (Bruno et al. 2009). As a result, 87 % of the analysed extracts of tissues (muscle, viscera and ovary) were positive for the presence of microcystins. ELISA test results were from 3 to eightfold higher than calculated concentration by LC-MS/MS analyses. The rapid screening and accurate mass-based identification of cyanobacteria biotoxins were easily afforded by MALDI-TOF/MS, spanning over wide molecular mass ranges. Nevertheless, accurate structure characterisation of all compounds was attained only studying their own fragmentation patterns by LC-Q-TOF-MS/MS. This hybrid mass spectrometry detector was highly sensitive, selective and repeatable in measuring the characteristic ions from each cyanotoxin studied; this technique was successfully employed in confirming known toxins, as well as in elucidating the molecular structure of several new compounds never described previously. On the other hand, ion trap and triple quadrupole LC-MS/MS offered high repeatability and sensitivity for identifying targeted known compounds, such as some microcystins, but could fail in detecting the presence of structural modified derivatives, or less abundant molecules. As a result, hybrid MS/MS detectors giving full details about the molecular structure of many different biotoxins represent the most modern approach for ‘profiling’ contamination levels and assessing the risk deriving to the consumers, both through freshwaters and foods. More recently, it was highlighted (Bruno 2013) that there is a strong need to homogenise the procedures for risk assessment evaluation and to define threshold limits based on cyanobacterial abundance and/or cyanotoxin concentrations, studying implications deriving from the presence of risk cofactors like heavy metals or pesticides, too. These aspects are needed for the preparation of transnational guidelines for risk assessment and management.

2.3.2 *Automatised Methods for the Detection of HAB Species*

A new method for the continuous monitoring and control of HAB species—the 'Dinoflagellate Categorisation by Artificial Neural Network' (DiCANN) system—has been developed (Culverhouse et al. 2001). This software is able to detect and identify automatically all species belonging to *Dinophysis*-type DSP.

The software is also able to identify other dinoflagellates like *Prorocentrum lima, Ceratium* and *Protoperidinium* (Culverhouse et al. 2001); it may be implemented in future for the identification of other planktonic taxa.

Another system, the HAB Buoy, has also been released (Cabrini et al. 2010); this, automatised system, very innovative, allows the in situ detection of toxic algae in 5 s only. HAB Buoy is composed by a near-infrared light source, a flow cell, an objective and a digital camera connected to a dual-processor computer equipped with 2 GB of random access memory (RAM). The HAB Buoy can be remote controlled through a Wireless Local Area Network. At present, this system is available as a prototype which needs further tests before its commercialisation.

2.4 Emerging Issues and Perspectives for Future Research

The understanding of toxic water blooms and extensive intoxications needs a new synthesis of the relevant knowledge in several fields (biology, ecology, chemistry and epidemiology) in order to address issues related to water quality preservation, nature conservation, planning and improvement of the physical environment. There is an emerging evidence that several biotoxins produced from a variety of microalgae—including blue-green algae—are chemically similar and cause analogous physiology and toxicological effect (Katırcıoğlu et al. 2004).

As future perspectives in the field of algal toxins are concerned, monitoring and control of HAB phenomena requires interdisciplinary research approaches. This strategy is strongly recommended as a challenge for future research. Priority to the advancement in both control and prevention strategies should be given for the safeguard of the coastal marine environment and public health.

The transfer of phycotoxins through aquatic food webs is an important aspect of HAB dynamics, which affects multiple trophic levels (Doucette et al. 2006). In addition to direct effects of toxic algal blooms on humans (which are ascribable to toxins that are transmitted to humans through the food chain), indirect effects on the environment also occur in association with some toxic algal blooms. These effects mainly relate to the both sessile (barnacles, bivalves, gastropods) and mobile epibenthos (echinoderms, cephalopods, small fish).

Trophic linkages between HAB and their ecosystems, and the relevant effects of HAB on aquatic organisms have been recently (Landsberg 2002). Ecological and hygienic-sanitary impact of HAB on the trophic web concerns several levels:

- Copepods, for example, show physiological reactions to toxic species, among which disequilibria in the process of grazing and emission of faecal pellets, with severe consequences in the trophic fluxes and in the interactions between water column and sediments (Sykes and Huntley 1987)
- Molluscs, like bivalves, which feed on phytoplankton through filtration, may accumulate toxins. Different species have different susceptibility to these toxins. In the Adriatic Sea, *Mytilus galloprovincialis* was more able to accumulate DPS and PSP toxins compared to other bivalves (Poletti et al. 1995).

In situ and laboratory observations have shown that many invertebrates (molluscs and worms) apparently are unaffected by algal toxicity even at high cell concentrations (10^5–10^7 cells/l). However, death may be caused by oxygen depletion due to the high algal concentration rather than due to direct neurotoxic effects (Steidinger et al. 1973). Conversely, toxic algae may exert both direct and indirect neurotoxic effects on *Teleostea*; mortalities of fish feeding on plankton have been recorded during blooms of some Dinoflagellates like *Alexandium* spp. and *Pyrodiscus breve*, following the ingestion of toxins causing neuromuscular lesions (Steidinger et al. 1973). Direct effects have also been recorded on fish of commercial interest (anchovies, herrings, salmons, etc.) which are susceptible to PSP-like toxins that accumulate into their tissues reaching concentrations dangerous for human consumption (White 1984). Significant decreases of fish larvae and juveniles have been found in association with blooms of the dinoflagellate *Gyrodinium aureolum* (Potts and Edwards 1987).

From a publih health point of view, damages produced by Dinoflagellates toxic to fish have been recorded in many Countries worldwide. Food intoxications following consumption of seafood contaminated by toxic algae have been reported since the 1970s, even in Italy since 1968 (Viviani 1992).

In the context of seafood safety and health preservation, some aspects that deserve particular attention are those related to the mechanisms toxins uptake, accumulation and detoxification performed by molluscs (Asakawa et al. 2006; Blanco et al. 2003; Chen and Chou 2002; Choi et al. 2003; Ichimi et al. 2001). Several examples can be reported here.

Comparing the detoxification mechanisms of toxic scallop (*Patinopecten yessoensis*) and clam (*Saxidomus purpuratus*), it was shown (Li et al. 2012) that the biotransformation of toxins was species-specific. The reductive enzyme was more active in clams than in scallops and that an enzyme in scallops is more apt to catalyse hydrolysis of both the sulphonate moiety at the *N*-sulphocarbamoyl of C toxins and the 11-hydroxysulphate of C and GTX toxins to produce metabolites.

With reference to domoic acid, little knowledge is available about the accumulation of this toxin in these molluscs. Domoic acid was found in the digestive gland of common cuttlefish with the highest values during spring and summer months (Costa et al. 2004), periods when *Pseudo-nitzschia* occur in the plankton; domoic acid was also found in branchial hearts of the same species during *Pseudo-nitzschia* blooms (Costa et al. 2005), suggesting the degradation and biotransformation of the

toxin. PSP toxins were also found in the digestive gland of common octopus during blooms of *Gymnodinium catenatum* (Costa et al. 2009).

In order to explore adverse effects on benthic invertebrates produced by algal toxins, Gorbi and coworkers have recently investigated in mussels *Mytilus galloprovincialis* the effects produced on immunological, histological and oxidative parameters by exposure to *Ostreopsis* cf. *ovata* (Gorbi et al. 2013). A clear involvement of the immune system of mussels was observed with a significant decrease of granulocytes, phagocytosis activity and lysosomal membrane stability in haemocytes, after both 7 and 14 days of exposure to *O*. cf. *ovata*. A decrease of the digestive gland wall thickness and of neutral lipid levels, the dilatation of tubules and haemocytes infiltration into the digestive gland were found in exposed mussels; a possible inhibition of the feeding activity, with a consequent induction of autophagic phenomena and utilization of stored reserve products such as neutral lipids was suggested. Antioxidant parameters revealed a limited role of *O*. cf. *ovata* to induce oxidative stress, except for a slight increase of catalase, glutathione reductase and glutathione peroxidases activities, and a significantly higher capability to neutralise peroxyl radicals in mussels exposed for 14 days. The observed effects on the general health status of exposed mussels should be adequately considered when assessing the ecological relevance of algal blooms.

As an output of the 'Development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood' (BIOTOX) Project (2005–2008), a review of 'European Shellfish producing countries and EU Food Safety legislation on monitoring and control of Bivalves' was produced, including an overview of industry practice on shellfish toxin control by industry case studies. The detection and depuration methods developed during BIOTOX will be incorporated into 'Hazard Analysis and Critical Control Points' verification procedures in Europe for the standardised monitoring, depuration and traceability of biotoxins in shellfish. The risk management practices of European Member States were harmonised to identify potential trade barriers and assist the industry in reaching its full commercial potential.

Within the recent Marine Strategy Framework Directive (MSFD, European Directive 2008/56/EC), some criteria are indicated in the Decision 2010/477/EU for the achievement of Good Environmental Status (GES) inherent to the descriptors listed in Annex I to the Directive. With respect to the Descriptor 5 'Eutrophication', the EU Commission Decision 2010/477/EU on criteria and methodological standards on GES of marine waters requires that: '*it is minimised eutrophication of human origin, in particular its negative effects, such as loss of biodiversity, ecosystem degradation, harmful algal blooms and oxygen deficiency in bottom waters*'.

The Decision indicates that the assessment of eutrophication in marine waters must take into account the assessment of coastal and transitional waters under the Directive 2000/60/EC (Annex V, points 1.2.3 and 1.2.4) and related guidelines, so as to ensure comparability, taking into consideration the information and knowledge gathered and approaches developed in the framework of regional sea conventions.

The assessment must combine the information on the level of nutrients and those relating to a number of primary and secondary effects which are relevant from an ecological perspective.

For each criterion, the Decision identifies a number of indicators that can be used for the purpose of description and subsequent assessment of GES, as follows (Criterion 5.2. Direct effects of nutrient enrichment):

- Indicator 5.2.1. Chlorophyll concentration in the water column
- Indicator 5.2.2. Water transparency related to increase in suspended algae, where relevant
- Indicator 5.2.3. Abundance of opportunistic macroalgae
- Indicator 5.2.4. Species shift in floristic composition such as diatom to flagellate ratio, benthic to pelagic shifts, as well as events of toxic algal blooms, due, for example, to cyanobacteria, caused by human activities.

Kalogerakis and coworkers suggest that the link between GES and seafood safety is explicit in MSFD Descriptor 9 in the future monitoring of HAB: contaminants in seafood must not exceed relevant standards (Kalogerakis et al. 2014). According to these Authors, real-time status information of the marine environment and seafood safety is urgently needed by stakeholders (seafood and aquaculture industries, policy makers) to respond timely to rapidly occurring phenomena such as harmful algal blooms (HAB). Thus, it is important to develop new innovative sensors for in situ detection of known and newly identified HAB species and the detection of marine biotoxins. The development of better operational model systems will allow the prediction of early warning proxies/indicators to assess the development and spread of HAB. The development of efficient end-user interfaces for governance, farmers, fishermen, and the general public for access to, for example, simulation and forecast results, toxicity information and advice regarding HAB is important. Finally, the development of new methods and technologies for prevention and controlling of HAB, like nanotechnology, marine ecosystem models, natural and artificial upwelling, are important elements of an integrated management strategy for HAB.

The assessment of phytoplanktonic abundance and composition will be performed within the Proposal of guidelines for 'Marine Strategy Framework Directive' monitoring plans'. Particular interest has also been given to the detection of toxic algae and related biotoxins (e.g. PSP, ASP, DSP, YTX, AZA) already considered within Regulations (EC) Nos. 853/2004 and 854/2004, for the safety of shellfish production areas.

The standard monitoring of shellfish farms for the presence of harmful algae and related toxins usually requires the microscopic examination of phytoplankton, bioassays and toxin determination by HPLC. Microscopy procedures are time-consuming and require taxonomic expertise, thus limiting the number of specimens that can be analysed. Molecular biology techniques have great potential in the detection of target organisms in field samples.

Toxins that have affected mainly the Mediterranean Sea over the last two decades are OA and some of its derivatives, yessotoxins and saxitoxins; the presence of DA has been sporadically detected, but at a lower concentration compared to the tolerance limits prescribed by the current law (Ciminiello et al. 2009).

In the northern Adriatic Sea, a general oligotrophication has been reported (Mozetič et al. 2010); nevertheless, some HAB phenomena have been reported in the last 6 years, probably because of nutrient inputs from the *Po* river, as shown by the Report for Year 2011 on the Environmental quality of seawaters in Emilia Romagna (ARPA Struttura Oceanografica Daphne—Emilia Romagna Region 2012).

The biological complexity of toxic algae needs to be matched with appropriate complexity in the representation of environmental and ecological parameters to allow either short-term regional predictions or long-term predictions of their response to global climate changes (Pitcher 2012).

The prediction of HAB is highly desirable for the management of their impacts; nevertheless, only a few HAB species can be predicted with any success to date, although many of them are regular components in the seasonal succession of phytoplankton. Model development is fundamental to achieve HAB prediction. Through retrospective and predictive calculations, models may be used to analyse, synthesise and test understanding of the dynamics of HAB in complex systems (Pitcher 2012).

Attention should also be paid to the development of advanced technologies for the mitigation of HAB events. It has been suggested (Paerl and Otten 2013) that the proliferation of cyanobacterial HAB in a wide range of aquatic ecosystems is favoured by multiple factors such as anthropogenic nutrient loading, rising temperatures, enhanced vertical stratification, increased residence time and salination, etc. The reduction of nutrient inputs is the most obvious target expected to play a key role in any cyanobacterial HAB mitigation strategies in both freshwater and marine environments.

The reduction of phosphorus is an effective means of reducing cyanobacterial dominance in aquatic, and especially freshwater, ecosystems. However, nitrogen reductions are also needed, especially in eutrophic lakes, rivers, estuaries and coastal waters. A management priority is to establish N and P input thresholds, below which cyanobacterial HAB can be controlled in terms of magnitude, temporal and spatial coverage. For this reason, total nutrient loads and concentrations need to be considered in the management of cyanobacterial HAB. The ratios of N to P inputs should be considered when developing these thresholds. For example, total molar N:P ratios above 15 do not favour cyanobacterial HAB dominance. Nutrient inputs may be point or non-point source. Point sources (i.e. wastewater, industrial effluents) are often associated with well-defined discharge sites; therefore, they are relatively easy to control. The remaining major challenge concerns the control of non-point sources, which frequently are the largest sources of nutrients; therefore, their controls are likely to play a critical role in mitigating cyanobacterial HAB. Nutrient management strategies may also include the removal of nutrients from receiving waters after their discharge, by dredging sediments, harvesting

macrophytes that have assimilated nutrients, and in some cases stocking and then removing higher trophic level consumers (finfish and shellfish) to eliminate nutrient-containing biomass. Some of these treatments, however, can have negative effects: sediment dredging can disrupt important bio-geochemical processes in surface sediments and benthos and lead to enhanced mobilisation of previously retained nutrients. Also, disturbance of the sediment meso- and micro-fauna, as well as microbial communities, can disrupt nutrient, oxygen and carbon cycling to the detriment of ecosystems undergoing mitigation and restoration.

Beneficial effects are expected from manipulating physical factors that are known to play key roles in cyanobacterial HAB competition versus other eukaryotic phytoplankton. For example, devices favouring vertical mixing or breaking down stratification have proved to be effective in controlling outbreaks and persistence of cyanobacterial HAB. The increase of flushing rates with nutrient-low waters, and thereby the decrease of water residence times, can be effective in reducing or controlling cyanobacterial HAB. However, these devices may exert some applicability in small lakes or reservoirs, while they are not suitable over large areas and volumes. In most of the cases, nutrient input reductions, combined with physical controls, are the most effective, simple and economically feasible management strategy.

As nutrient controls can be expensive, alternative nutrient removal strategies may be required, such as cultivation and stimulation of macrophytes, stocking of herbivorous (and specifically cyanobacteria consuming) fish and shellfish species.

Anderson and coworkers have reviewed different approaches adopted by countries and commercial enterprises worldwide to monitor and manage HAB in coastal waters (Anderson et al. 2012b). This result is typically achieved through the establishment of programmes for toxin and cell detection (and quantitation) in water, aerosols, shellfish, fish, etc.; the development of bloom forecasting and early-warning capabilities as well as medical intervention and therapeutic strategies; and the development of bloom prevention and mitigation strategies. Owing to the complexity and diversity of HAB phenomena, many challenges are associated with these activities.

In conclusion, despite significant advances in research, commercial biotechnological applications for in situ monitoring of marine ecosystems are still not available for most groups of toxins. Even if biosensors or technologies are sufficiently sensitive to comply with the regulatory limits, few of these methods have been validated and/or accepted as alternative to the mouse bioassays. Biosensor technologies offers several advantages over analytical methods and animal bioassays, including speed, ease of use and low cost, no ethical issues related to the use of laboratory animals. In most cases, these techniques would be good tools to be used as screening methods in order to reduce the number of animal bioassays. This is reported as an important challenge for future development in Marine Biotechnology for protection and management of marine ecosystems (Marine Board-European Science Foundation 2010).

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Chapter 3
Brief Notes About Biofilms

Pasqualina Laganà, Gabriella Caruso, Francesco Mazzù, Giorgia Caruso, Salvatore Parisi and Antonino Santi Delia

> ….There are several steps that we must take to optimize our lives in a city. The first is to choose the city in which we will live, then we must select the neighborhood in the city that best suits our needs, and finally we must make our home amongst the homes of many others. Occasionally, when life in the city sours, we leave….
>
> P. Watnick, R. Kolter (2000) Biofilm, City of Microbes

Abstract The biofilm is a microbial community characterised by sessile bacterial cells strongly adherent to a substrate and/or an interface and incorporated in a polymeric matrix of microbial origin. In this condition, microbial cells exhibit an altered phenotype in comparison to corresponding free or planktonic forms. Life in a biofilm represents probably the prevailing mode of growth for microbes in several environments. After the development of new observation methods and the modification of different procedures, biofilms may be identified on known substrates and on new sites. The concept of biofilm has acquired the importance that still plays in the health sector, especially in areas where the use of invasive instruments and the continuous temporal localisation is expected. An important role is also played in the food industry because of the direct presence on food surfaces.

Keywords Alginate · Biofilm · Cellulose · Exopolysaccharide · Extracellular polymeric substance · *N*-acetylglucosamine · Succinoglycan

Abbreviations

CLSM	Confocal laser scanning microscopy
DNA	Deoxyribonucleic acid
ES	Exopolysaccharide
EPS	Extracellular polymeric substance

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3.1 Biofilm: An Introduction

The biofilm is a microbial community characterised by sessile bacterial cells strongly adherent to a substrate and/or an interface and incorporated in a polymeric matrix of microbial origin. In this condition, microbial cells exhibit an altered phenotype with respect to growth rates and the gene transcription in comparison to the corresponding free or planktonic forms.

It has been reported that the bacterial adhesion triggers gene control mechanisms for the production of required molecules in the formation of biofilms (Donlan and Costerton 2002). The definition of biofilm involves not only a description of the morphostructural type but also biomolecular factors (Donlan 2002).

After the development of new observation methods such as scanning electron microscopy and the modification of different procedures, biofilms may be identified on known substrates and on new sites. Consequently, persistent and antibiotic-resistant infections have been explained in this way. In recent years, the 'biofilm phenomenon' has been analysed in different ways and situations. Many microorganisms have been the object of study, including:

- *Candida albicans* (Hawser and Douglas 1994; Sherry et al. 2014)
- *Escherichia coli* (Pratt and Kolter 1998)
- *Klebsiella pneumoniae* (di Martino et al. 2003)
- *Legionella pneumophila* (Atlas 1999; Declerck 2010; Declerck et al. 2007; Hindré et al. 2008; Murga et al. 2001; Storey et al. 2004)
- *Proteus mirabilis* (Holling et al. 2014; Jones et al. 2007; Moryl et al. 2014)
- *Pseudomonas aeruginosa* (Klausen et al. 2003; O'Toole and Kolter 1998; Ramsey and Whiteley 2004; Savoia 2014)
- *Staphylococcus aureus* (Kiedrowski et al. 2014; Islam et al. 2014; Wojtyczka et al. 2014).

3.1.1 Extracellular Polymeric Substances

As mentioned above, biofilm is a complex structure which is made of aggregates of microbial cells within a matrix of extracellular polymeric substances (EPS). EPS are high-molecular-weight compounds secreted by microorganisms into their environment.

The matrix structure constitutes the elastic part of the biofilm. Interstitial voids and channels separating micro-colonies contain a liquid phase, mainly constituted by water. The liquid phase is the viscous part of biofilms. The EPS matrix provides the biofilm with mechanical stability through these viscoelastic properties (Shaw et al. 2004) and the consequent functional and structural integrity. Physiochemical properties of biofilms are mainly determined by the qualitative and quantitative composition of EPS matrices (Flemming and Wingender 2010)

All major classes of macromolecules—polysaccharides, proteins, nucleic acids, peptidoglycan and lipids—can be present in a biofilm. Although extracellular polysaccharides are considered as the major structural components of the biofilm matrix, extracellular deoxyribonucleic acid (DNA) plays an important role in the establishment of biofilm structures (Whitchurch et al. 2002)

EPS are the construction material of bacterial settlements and either remain attached to outer cell surfaces or are secreted into its growth medium. These compounds are important in biofilm formation and cells' attachment to surfaces. EPS constitutes 50–90 % of the total organic matter in biofilms (Donlan 2002; Donlan and Costerton 2002; Flemming et al. 2000).

The development of microbial biofilms is observed virtually on all submerged surfaces in natural and industrial environments. Biofilms are also observed at interfaces as pellicles, or in the bulk of aquatic environments as flocs or granules (Marti et al. 2011).

Microorganisms synthesise a wide spectrum of multifunctional polysaccharides including intracellular, structural and extracellular polysaccharides or exopolysaccharides (ES). ES are high-molecular-weight polymers, the composition includes sugar residues which are secreted by a microorganism into the surrounding environment.

ES generally consist of monosaccharides and some non-carbohydrate substituents such as acetate, pyruvate, succinate and phosphate groups. Owing to the wide diversity in composition, ES have found multifarious applications in medical, pharmaceutical and food industries.

ES of some strains of lactic acid bacteria, including *Lactococcus lactis* subsp. *cremoris*, contribute a gelatinous texture to fermented milk products such as *Viili* (a yoghurt-like mesophilic fermented milk); these polysaccharides are also digestible (Ljungh and Wadstrom 2009; Welman 2009). An example for the industrial use of ES is the application of dextran in the Italian *panettone* and other breads in the bakery industry (Ullrich 2009).

Lembre and coworkers have reported various examples of ES and carbohydrates in bacterial biofilm (Lembre et al. 2012). The composition of ES can vary according to producing bacterial species (Sects. 3.1.1.1–3.1.1.3).

3.1.1.1 Alginate and N-Acetylglucosamine

Alginate, a polysaccharide extracted by brown algae and different bacteria such as *Azotobacter vinelandii* (Gorin and Spencer 1966) and *P. aeruginosa* (Davies et al. 1993; Davies and Geesey 1995; Linker and Jones 1964), has been extensively studied (Fig. 3.1). In detail, alginate is an ES with a relatively high molecular mass (10^4–10^6 g/ml). This compound consists (Gacesa 1998) of two uronic acid residues: β-D-mannuronate, also named M, and its C-5 epimer, α-L-guluronate, also named G (Fig. 3.1).

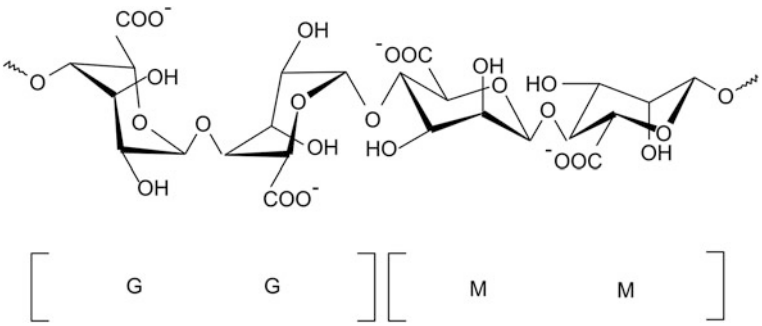

Fig. 3.1 Exopolysaccharides in biofilm matrices: structure of G- and M-alginates. G-structures mean 'poly-α-L-guluronate' while M-blocks are 'poly-β-D-mannuronate'. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

Another interesting ES, *N*-acetylglucosamine (Fig. 3.2), is produced by *E. coli*, *S. aureus* and *S. epidermidis* (Cerca and Jefferson 2008; Kaplan et al. 2004; Izano et al. 2008).

3.1.1.2 Succinoglycan

Succinoglycan is produced by *Alcaligenes faecalis* var. *myxogenes* 10C3, a microorganism our research group has isolated from soils. This microorganism produces a water-soluble and an insoluble extracellular polysaccharide. The former compound, succinoglycan, is composed of glucose, galactose, pyruvic acid and succinic acid (molar proportions are 7:1:1:1), with (β1-3)-, (β1-4)- and (β1-6)-glucosidic linkages. These polymers or oligomers are also produced by many *Agrobacterium* and *Rhizobium* strains (Harada 1983; Harada and Amemura 1979; Hisamatsu et al. 1982; Tomlinson et al. 2010).

3.1.1.3 Cellulose

Cellulose (Fig. 3.3) is the most abundant sugar polymer found on the surface of the planet Earth. It is found throughout the living world: in plants, animals, fungi and in bacteria such as *Salmonella* spp, *E. coli, Acetobacter, Agrobacterium* and *Rhizobium* (Matthysse et al. 2005; Solomon et al. 2005; Spiers and Rainey 2005).

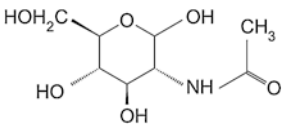

Fig. 3.2 Structure of *N*-acetylglucosamine. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

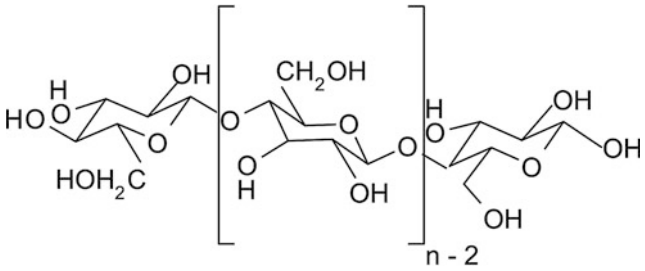

Fig. 3.3 Structure of cellulose. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

3.2 The Biofilm and Its Creation

As mentioned earlier, biofilms are an aggregation of microorganisms attached to and growing on a surface (Costerton and Stewart 2001). The formation and the subsequent development of biofilms are affected by many factors, including the specific bacteria strain (Borucki et al. 2003; Chae and Schraft 2000), material surface properties and environmental parameters such as pH, available nutrient levels and temperature (Donlan 2002).

The biofilm formation is a dynamical process consisting of five steps: (1) initial attachment, (2) irreversible attachment, (3) early development of biofilm architecture, (4) maturation and (5) final dispersion (Fig. 3.4).

3.2.1 Initial Attachment

The initial attachment of microorganisms can be active or passive, depending on their motility, the gravitational transportation of their planktonic species (free floating) or the diffusion of the surrounding fluid phase (Kumar and Anand 1998). Physical properties of the environment are essential for microorganism's attachment to the substratum, biofilm formation and microbial processes. The cell adhesion during this process strongly depends on the physiochemical properties of the

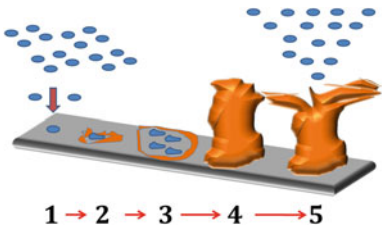

Fig. 3.4 Steps of biofilm development: (**a**) reversible attachment; (**b**) irreversible attachment; (**c**) micro-colony formation; (**d**) maturation; (**e**) final dispersion

bacterial cell surface (Begoude et al. 2007). At first, adherent cells possess only a small quantity of EPS; many of these life forms are capable of independent movement (O'Toole and Kolter 1998) by pilus-mediated twitching or gliding motility. The adhesion is reversible in this stage (Fig. 3.4).

3.2.2 Irreversible Attachment

The switch of the biofilm from the reversible to the irreversible state is related to the interaction between the presence of bacteria, EPS production and the surface (Fig. 3.4). In this step, cells lose their flagella-driven motility (Stoodley et al. 2002). After this irreversible attachment, a very strong force is required to remove the biofilm. For this reason, there is the need of chemicals such as enzymes, detergents or surfactants. McCoy and coworkers have shown that microbial adhesion strongly depends on hydrophobic–hydrophilic properties of interacting surfaces (McCoy and Brown 1998).

3.2.3 Micro-Colony Formation

Micro-colony formation results from the simultaneous aggregation and the increase of microorganisms (Fig. 3.4). As a result, the formation of biofilms is associated with the production of EPS, which helps to reinforce links between bacteria and the substratum. In addition, the colony is protected from any environmental stress (Donlan 2002).

Micro-colonies are discrete matrix-enclosed communities that may include cells of one or many species. Depending on the involved species, the micro-colony may be composed of 10–29 % cells and 75–90 % EPS- matrix (Costerton et al. 1987). Bacterial cells within the matrix are characterised by their lack of Brownian motion; the structural analysis of many micro-colonies often reveals a mushroom-like shape (Costerton et al. 1995).

Studies of bacterial species in natural systems have shown (McLean et al. 1997; Swift et al. 2001) that planktonic cells from the neighbouring medium can be included in this stage into the biofilm using the cell–cell communication (also named *quorum sensing*).

3.2.4 Maturation

After attachment to a surface, bacteria undergo further adaption to life in a biofilm. Two significant properties are often associated with this step: the increased EPS synthesis and the development of antibiotic resistance. In this step, bacteria develop other properties such as ultraviolet light—resistance, increased rates of genetic

exchange, altered biodegradative capabilities and the increased production of secondary metabolites (O'Toole et al. 2000). For example, the presence of protein from milk or meat can alter physicochemical properties of surfaces (charge, free energy, hydrophobicity, etc.), which further results in a greater bacterial attachment (Kumar and Anand 1998).

The biofilm maturation (Fig. 3.4) is the step where the living matrix develops into an organised structure which can be flat or mushroom-shaped (Fig. 3.4), depending on available nutrient sources (Klausen et al. 2003).

In order to reach structural maturity, periods of 10 days or more are required (Stoodley et al. 2002). Bacteria grow under sessile form in motley complex-enclosed micro-colonies scattered with open water channels (Davey and O' Toole 2000). Mature biofilms have also been evaluated and compared with chemostat cultures of *P. aeruginosa* by the DNA microarray technology. Results have shown that over 70 genes were altered, including genes encoding proteins involved in translation, metabolism, membrane transport and/or secretion, and gene regulation.

3.2.5 Dispersion

After the fourth stage, several attacked bacteria can leave the 'aged' biofilm individually or in groups and disperse in the environment. Results are the survival of these life forms and the colonisation of new niches. This detachment is a discontinuous and occasional process; it depends on various factors such as:

(a) Flow variations of the surrounding liquid
(b) The presence of chemicals or the modification of surface properties of cells or the colonised substrate
(c) Internal biofilm processes, such as endogenous enzymatic degradation
(d) The release of EPS or surface-binding protein.

These causes can occur at the same time; in addition, starvation is considered as a reason for the detachment and forces bacteria to search for a nutrient-rich environment (Srey et al. 2013). Every microorganism that breaks off can be transported in the environment and start again the process of biofilm formation (Fig. 3.4).

3.3 Some Environmental Factors Affecting Biofilm Formation

3.3.1 pH and Temperature

Bacteria respond to internal and external pH and temperature modifications with the variation of the activity and synthesis of proteins associated with many different cellular processes (Olsen 1993). A gradual increase in acidity determines good

chances of cell survival if compared to a sudden increase by the rapid addition of hydrochloric acid (Li 2001). This result suggests that bacteria contain mechanisms in place which allow the microbial population to adapt to small pH changes (Garrett et al. 2008). Bacteria have membrane-bound proton pumps which extrude protons from the cytoplasm to generate a trans-membrane electrochemical gradient, i.e. the proton motor force. The passive influx of protons in response to the proton motive force can be a problem for cells attempting to regulate their cytoplasmic pH (Booth 1985). Large variations of external pH values can overwhelm such mechanisms and have a biocidal effect on microorganisms. The optimum pH for polysaccharide production depends on the individual species; however, it has been reported that approximate pH values of around 7 are good for most bacteria (Oliveira et al. 1994).

3.3.2 Surface Topography

The roughness of substrates is known to be one of the key factors in determining the extent of bacterial colonisation (Crawford et al. 2012; Oh et al. 2009). Also, it plays a significant role in the attachment process, particularly when superficial irregularities are comparable to microbial sizes and can provide shelter from unfavourable environmental factors (Mitik-Dineva et al. 2008). Moreover, levels of bacterial adhesion are determined by the surface topography. The relationship between surface roughness and the attachment (and growth) of bacteria may vary depending on involved microbial species. Furthermore, once bacteria have colonised a surface, the resistance of biofilms to the removal by simple friction increases in proportion to the superficial roughness (Preedy et al. 2014).

3.3.3 Hydrophobicity and Hydrophilicity

Hydrophobicity plays an important role in the microbial attachment on surfaces: this factor has an impact on the temporal length of cells association with the substratum. The hydrophobicity of cell surfaces is also important in adhesion because the hydrophobic interaction tends to augment when non-polar properties of each surface increase. (Alsteens et al. 2007; Faille et al. 2002; van Oss 1995, 1997)

3.4 Food and Food Industry

The role of biofilms in the food sector is relevant. Many authors have demonstrated the presence of microorganisms organised in biofilms directly on the food as well as on different materials that will be in contact with it. Germs can adhere strongly to glass or stainless steel tools and ceramic and polypropylene surfaces, with the

resulting difficult eradication (Adetunji et al. 2014; Balsanelli et al. 2014; Regina et al. 2014).

In environments of food processing bacteria with other organic and inorganic molecules, such as milk—or meat—proteins, a conditioning film can be formed. This film, composed of many complexes, can be displaced by diffusion or transported by liquid flows. The accumulation of molecules at the solid–liquid interface on the contact surface of the food can lead to a high concentration of nutrients with respect to the fluid phase. The increased level of nutrients deposited on contact surfaces can favour the formation of biofilm, acting as a conditioning film.

The adhesion of bacteria to food or food- contact surfaces represents a serious hygiene problem; it can determine economic losses due to possible food spoilage. In addition, some studies have demonstrated the persistence of some foodborne pathogens on surfaces in contact with foods and some germs has been recently added to the existing list: *Salmonella* (de Oliveira et al. 2014; Lianou and Koutsoumanis 2012, 2013; Patel et al. 2013; Schonewille et al. 2012; Wang et al. 2013); *Listeria monocytogenes* (Barbosa et al. 2013; Borucki et al. 2003; Ferreira et al. 2014; Valderrama and Cutter 2013); *Yersinia enterocolitica* (Zhou and Yang 2011; *Campylobacter jejuni* (Joshua et al. 2006; Reeser et al. 2007; Teh et al. 2010, 2014); *Aeromonas hydrophila* (Elhariry 2011; Jahid et al. 2013); *E. coli* and *E. coli 0157: H7* (Dourou et al. 2011; Gomes et al. 2014; Silagyi et al. 2009; Simpson Beauchamp et al. 2012); *S. aureus* (Gutiérrez et al. 2012). The persistence and the accumulation of germs in biofilms could be a source of subsequent contamination, leading to a reduced shelf life of the product. The transmission of pathogens may also occur by produced aerosols during surface-cleaning processes in food plants (Italian Institute of Packaging 2009; Parisi 2011, 2012; Steinka 2015).

One of the main objectives of the food industry is the production of safe and healthy products with good organoleptic quality. Consequently, the growth of microorganisms has to be monitored in order to minimise the risk of food contamination by germs into food plants (environmental contamination).

Moreover, monitoring activities have to consider methods of production and handling of food products, involving frequent contact of raw materials, semi-finished and finished products with work surfaces and utensils. It can be assumed that the transfer of microbial flora with pathogenic or degrading features to foods may be easily observed when considering work surfaces, utensils, packaging materials and correlated machinery equipment (Parisi 2013).

The presence of nutrient residuals on surfaces can promote the formation of biofilms; on the other hand, the simple presence of organic substance in drinking water may be sufficient, when surfaces are washed.

From the industrial viewpoint, biofilms represent a problem that mainly concerns food industries: breweries, dairy companies, meat industries, etc. (Chen et al. 2007; Frank et al. 2003; Jessen and Lammert 2003; Simões and Vieira 2009; Simões et al. 2010; Somers and Wong 2004).

With reference to food production environments, bacteria and chemical compounds (organic and inorganic molecules such as milk or meat proteins) can adhere to non-food surfaces with the consequent formation of conditioning films. Organic

and inorganic molecules are transported together with microorganisms on surfaces by diffusion or by the turbulent flow of liquids in some cases (Poulsen 1999; Sharma and Anand 2002). In these systems, the amount of residual nutrients with strong adherence to surfaces is notable and can condition the surface itself (Hood and Zottola 1997). Basically, the increase of nutrients promotes biofilm formation (Jeong and Frank 1994).

The creation of biofilm on food-contact materials leads to a number of hygienic and economic problems at the same time: in fact, biologic contamination can cause food deterioration before of the labelled expiration date (Parisi 2002a, b, c).

On the other hand, it is known that surfaces and tooling in contact with foods must be made with easily cleanable and disinfectable materials (Gurnari 2015). The use of steel and hardness plastics has been promoted for this reason, excluding porous materials such as wood.

For example, dirty or unclean equipment are considered as the main sources of contamination (with airborne microflora) of milk and milk products. The accumulation and the persistence of biofilms can cause post-process contamination, shortening shelf-life values of food products (Jin and Zhang 2008; Zottola and Sasahara 1994).

The contamination may occur between packaging and consumption steps at any time. According to recent literature on polyethylene terephthalate (common plastic bottles for drinks are made with this plastic matter), bacteria can adhere tenaciously and constitute biofilms in less than 24 h; on the other hand, polypropylene (containers and plates) is not easily contaminated by moulds and bacteria (Byun et al. 2007).

The modern equipment used in food production processes are made of different materials such as stainless steel, glass, plastic, rubber and polytetrafluoroethylene (Teflon®); the degree of hydrophobicity of these surfaces can influence microbial adhesion (Sinde and Carballo 2000; Teixeira et al. 2005).

Food contamination may appear at different stages of the production chain; in particular, certain perishable products such as milk have to be considered. Milk can be contaminated during the milking stage by faccal contamination or the simple use of contaminated water. With specific reference to milk and its derivatives, saprophytic or pathogens bacteria may creep under the seals of transformation or packaging machinery and contaminate food products and/or the final container (or single parts), thus leading to the formation of biofilm. Should the contamination concern packaging materials, this phenomenon and the consequent biofilm development would occur after pasteurisation. Surfaces such as shelves and walls can be indirectly source of contamination: germs can be transported from the air, as well as by operators' hands.

The design of equipment and the careful selection of materials of work surfaces are of crucial importance in preventing the formation of biofilms (Gurnari 2015). The most suitable material is stainless steel (it can be easily cleaned using common detergents).

The prerequisite for an efficient sanitation programme is correlated with the so-called hygienic design (Gurnari 2015). Corners, cracks, joints, gaskets and

crevices are vulnerable points for the accumulation of biofilm. Should the design of equipment and work surfaces be improper, any sanitation programme would represent possible contamination problems. Therefore, best strategies for the control of food-contact surfaces can be expressed as follows:

(a) The design of equipment should aim towards an efficient hygiene program, perform a proper cleansing and implement a good sanitation
(b) An effective cleaning programme removes undesired materials from food-contact surfaces, including microorganisms, foreign matter and any residues of detergent.

3.5 Strategies of Biofilm Control

3.5.1 Physical Methods

With exclusive reference to the removal of biofilms in food industries, the most innovative methods of physical nature include:

(a) High-frequency ultrasound
(b) High-frequency pulsed electric fields (the concomitant use of organic acids can be considered)
(c) Low-frequency electric fields (with the aim of increasing the effect of biocides).

Recently, the use of electric fields associated with antibiotics has been defined effective when speaking of *Pseudomonas* biofilm control.

3.5.2 Chemical Methods

The effective elimination of microorganisms on surfaces is important before disinfection procedures. Germs become much more sensitive to disinfectants when the biofilm is removed from surfaces. Even the mechanical or chemical break of polysaccharide matrices is important for the control of biofilms. Among the detergents with effective power against biofilms, chelating substances should be mentioned: these compounds include ethylenediaminetetraacetic and aminoethylene glycolethyl tetracetic acids. Some oxidising disinfectants are able to depolymerise ES, including acid peracetic acid, chlorine, iodine and hydrogen peroxide. These substances allow the detachment of biofilms formed by certain types of bacteria from surfaces.

3.5.3 Biological Methods

An innovative strategy for the control of biofilms consists in the adsorption of bioactive molecules such as bacteriocins and enzymes on food-contact surfaces. For example, nisin has been adjudged effective when speaking of adherence reduction of *L. monocytogenes* to surfaces (Saà Ibusquiza et al. 2011). The use of peculiar lactic bacteria cultures and their extracts allows the inhibition of degrading microorganisms and pathogens on food-contact (poultry products). As regards enzymes, they have been defined very efficient as detergents because of the inactivation of biofilm exocellular polymers and the enhancement of biofilm removal.

3.6 Biofilm in Hospital Setting and Antibiotic Resistance

It has to be noted that the chronicity of persistent bacterial infections is due to bacterial biofilm formation, in contrast with planktonic bacteria found in acute infections (Bjarnsholt 2013).

The epidemiologic evidence has clearly shown that biofilms have a role in infectious diseases (cystic fibrosis, periodontitis, bloodstream and urinary tract infections) as a result of indwelling medical devices. The process may be particularly relevant for immunocompromised patients who lack the ability to contrast invading organisms. Beyond the evidence, however, the exact processes by which biofilm-associated organisms elicit disease in the human host are poorly understood. Suggested mechanisms include the following steps:

(1) Detachment of cells or cell aggregates from indwelling medical device biofilms, resulting in bloodstream or urinary tract infections
(2) Production of endotoxins
(3) Resistance to the host immune system
(4) Provision of a niche for the generation of resistant organisms (through resistance plasmid exchange).

One of the most fascinating defence mechanisms of a biofilm is based on a peculiar type of intercellular signalling, the so-called *quorum sensing*. Some bacteria release a molecular signal, also called inductor. These molecules are acylated homoserine lactone for Gram-negative bacteria and peptic substances (peptides, amines, amino acids) for Gram-positive microorganisms.

As the cell density rises, the concentration of these molecules increases. Inductors interact with specific receptors in each cell to activate *quorum sensing* genes and start a cascade of events, thus causing the expression or repression of numerous other genes on the bacterial chromosome.

The structure and physiological attributes of biofilm-producing organisms confirm the inherent resistance to antimicrobial agents such as antibiotics, disinfectants

and germicides. The mechanisms responsible for resistance may be described approximately as follows:

1. Loss of penetration power of antibiotics through the biofilm matrix
2. Percentage of altered growth of germs which constitute the biofilm
3. Other physiological changes due to the mode of growth of the biofilm.

Antibiotic molecules must diffuse into the biofilm matrix to inactivate included cells. Extracellular polymeric substances that constitute the matrix represent a barrier to the diffusion of these molecules so as to hold both the percentage of the transport of molecules to the inside of biofilm and the interaction of the antimicrobial matter with the material from the matrix. A loss of power of penetration of ciprofloxacin against *P. aeruginosa* has been demonstrated (Suci et al. 1994); in other words, the normal sterilisation of surfaces (needed time: 40 s) would require 21 min when surfaces are covered by biofilms. Bacterial cells have been reported to be 15 times more sensitive to tobramycin if compared to cells of an intact biofilm (Hoyle et al. 1992).

Another mechanism has been proposed to explain the resistance to antimicrobial agents. In brief, cells associated with the biofilm may grow significantly more slowly than planktonic cells with the delayed absorption of antimicrobial. Anwar and coworkers have demonstrated that old cells (life: ten days) of *P. aeruginosa* biofilms were much more resistant to tobramycin and piperacillin biofilm cells (Anwar et al. 1989) if compared to younger cells (life: two days).

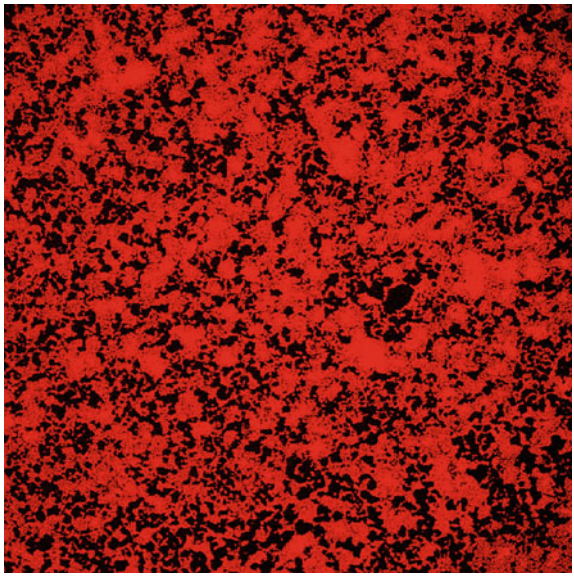

Fig. 3.5 A biofilm formed by *S. aureus*, grown on a glass surface (CLSM image; 2014, Laganà P, Delia S., University of Messina, Italy)

A dosage of 500 mg of piperacillin + tobramycin (5 mg/ml) can inactivate completely planktonic cells and biofilms of young cells. On the other side, older cells have been reduced only by approximately 20 % in the same condition.

3.6.1 Measurement and Observation of Biofilms

Biofilm growth and structure has been measured using diversified methods: fluorescence, transmission electron, scanning electron, atomic force and confocal laser scanning microscopy (CLSM). In relation to the CLSM system, our research group has used a TCS SP2- laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany), equipped with argon-krypton laser and coupled to a Leica DMIRB-inverted fluorescence microscope. The use of CLSM requires the use of fluorescent stains as propidium iodide, a nucleic acid intercalating and fluorescent agent (Sigma). A biofilm produced by *S. aureus*, grown on glass and analysed by CLSM, is shown in Fig. 3.5.

Numerous methods are used with the aim of quantifying biofilm production (Sternberg et al. 2014). Microtiter plates are among the most frequently used biofilm model systems (Djordjevic et al. 2002; Elkhatib et al. 2014; Kwasny and Opperman 2010; Pierce et al. 2010). In these systems, biofilms are either grown on the bottom and the walls of the microtiter plate (most commonly a 96-well plate); results are usually read using a spectrometer (Fig. 3.6) (Table 3.1).

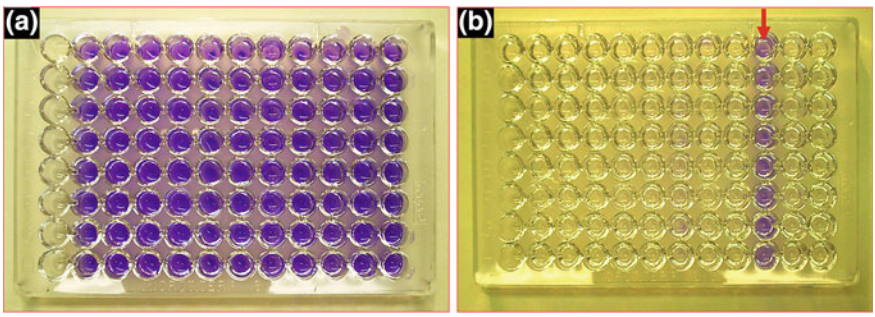

Fig. 3.6 (**a**) A microtiter plate where *S. aureus* and *P. aeruginosa* are incubated after 45 min at room temperature with 1 %-crystal violet. (**b**) A microtiter plate after washing and addition of 95 % ethanol. The arrow shows a strip with a strain of *S. aureus* (Laganà P and Delia S, University of Messina, Italy)

Table 3.1 Summary table of the main strategies for the management and control of biofilms

Classification of operative procedures for biofilm control	Definition of strategies
Physical Methods	High intensity-magnetic fields
	High energy-pulsed electric fields
	Low energy-electric fields
Mechanical	Brushing
Methods	Scraping
Chemical	Chelating agents
Methods	Peracetic acid
	Chlorine
	Iodine
	Hydrogen peroxide monolaurate
	Cetylpyridinium chloride
	Other chemical agents
Biological	Bacteriocins (e.g.,nisin)
Methods	Lactobacillaceae
	Microbial enzymes: protease, alpha-amylase, beta-gluconase, endo-glycosidases (endo-H), exo-polysaccharidase, etc.

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Chapter 4
Microbial Toxins in Foods: The Importance of *Escherichia coli*, a Versatile Enemy

Giorgia Caruso, Antonino Santi Delia, Gabriella Caruso, Salvatore Parisi and Pasqualina Laganà

Abstract When speaking of food microbiology, analysts and hygiene professionals are accustomed to starting their discussions with a relatively less number of food pathogen bacteria, in spite of the great variety of microbiological risks in the food and beverage sector. A few micro-organisms are well known with reference to the possibility of showing diversified strains. In other words, one specific micro-organism can spread into the environment and in selected 'culture media' like foods with the possible 'permutation' of the original strain in several sub-strains with different properties and features. This is the situation of *Escherichia coli*, the most important micro-organism in the group of so-called 'Coliform' bacteria. *E. coli* is a typical commensal of the intestinal tract of animals and humans. For its abundant presence in the colonic microflora, it is used as one of the most important indicators of faecal contamination in food and water. However, some subsets within this species have acquired specific virulence genes. Enteric strains have been divided into different pathotypes, depending on virulence factors and pathogenic features. In the present chapter, various *E. coli* pathotypes are described; particular relevance is given to toxin-producing strains. Additionally, this work intends to provide useful information about related toxins, their known chemical properties and the most used analytical methods in the food sector.

Keywords *Escherichia coli* · Faecal contamination · Food-borne outbreak · Heat-labile toxin · Heat-stable toxin · Shiga toxin

Abbreviations

AAF	Aggregative Adherence Fimbrium
A/E	Attaching-and-Effacing
CT	Cholera Toxin
CF	Colonisation Factor
CFU	Colony Forming Unit
DNA	Deoxyribonucleic Acid
DAEC	Diffusely Adherent *E. coli*
EAEC	Enteroaggregative *E. coli*
EAST-1	Enteroaggregative Heat-Stable Toxin 1

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EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
LT	Heat-Labile
ST	Heat-Stable
STa	Heat-Stable a
STb	Heat-Stable b
IMS	Immunomagnetic Separation
LEE	Locus Enterocyte Effacement
LPS	Lipopolysaccharide
PCR	Polymerase Chain Reaction
RTE	Ready-to-Eat
RKI	Robert Koch Institute
SPATE	Serine Protease Auto Transporter of *Enterobacteriaceae*
Stx	Shiga Toxin
STEC	Shiga Toxin-Producing *E. coli*
ShET-1	*Shigella* Enterotoxin 1
SMAC	Sorbitol-MacConkey
VTEC	Verotoxigenic *E. coli*

4.1 Introduction

Escherichia coli is a member of the family *Enterobacteriaceae*: it is represented mostly by motile Gram-negative bacilli. It occurs in diverse forms in nature, displaying an impressively versatile behaviour, which reflects the immense diversity within the species (Bergthorsson and Ochman 1998). Far from being only an enteric species, *E. coli* has recently been also recognised as a free-living bacterium; environmental reservoirs of this organism are known (Cox et al. 1988). In fact, evidences from a number of studies suggest that *E. coli* may not only persist, but also multiply in natural environments such as warm, sub-tropical soils and waters (Byappanahalli and Fujioka 1998, Solo-Gabriele et al. 2000), even if survivorship in open environments is strain -dependent (Topp et al. 2003). Nevertheless, the intestinal system of humans and other mammals is considered to be more favourable for its growth, as resource availability and the constancy of the abiotic features represent the ideal conditions for *E. coli* (van Elsas et al. 2011). Among the human colonic flora, this microorganism is the predominant facultative anaerobe. *E. coli* can colonise the gastrointestinal tract of infants within hours after birth and afterwards, establishing an enduring relationship with mutual benefits for the rest of the

host's life (Kaper et al. 2004). *E. coli* commensal strains thrive in the mucous layer of the mammalian colon, allowing the host to break down particular carbon compounds. These commensal strains remain confined harmlessly in their niche and are rarely pathogenic, except when gastrointestinal barriers are broken.

However, the versatile behaviour of *E. coli* ranges also to pathogenic forms. In fact, several strains have acquired specific virulence factors that affect a wide array of cellular processes; these strains possess genomic islands (i.e. regions) which are full of genes that encode key traits for adherence, colonisation, invasion and production of toxic compounds (Touchon et al. 2009). These virulence determinants have been acquired by means of bacteriophages or plasmid deoxyribonucleic acid (DNA); these determinants have been frequently spread by horizontal gene transfer, leading to the creation of novel combinations of pathogenic strains. The various commensal and pathogenic forms of *E. coli* are known to have genomes that may diverge by up to 20 % (Ochman and Jones 2000). Generally, infections due to pathogenic *E. coli* result in three general clinic syndromes: urinary tract infections, in which *E. coli* is by far the most common causing agent; sepsis/meningitis; enteric/diarrhoeal diseases. Among the diarrheagenic strains, there are (Kaper et al. 2004) six well-known pathotypes (i.e. group of strains of a single species that cause a disease using a common set of virulence factors): enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC).

Several other potential *E. coli* pathotypes have been reported but have not been as well established as the ones listed above. Among the six pathotypes, some include strains of worldwide public health importance, which are recently emerging. In fact, certain groups can cause life-threatening diarrhoea, which in addition may lead to severe sequelae.

4.2 Diarrhoea-Associated *E. coli*

All the six pathotypes possess specific proteins, which enhance their colonising and adherence ability, overwhelming competition with the indigenous flora. On the other hand, various pathotypes are classified on the basis of virulence factors, as they differ in the pathogenetic strategy, which may consist in:

- Intracellular invasion
- Intimate adherence to epithelial cell membranes
- Production of toxins able to disrupt fundamental eukaryotic processes.

In addition to the so-called virotyping, serology is also used. In fact, *E. coli* strains are classified on the basis of antigenic differences. Serotyping is performed on the primary components of cell surface: the lipopolysaccharide (LPS) which is termed O antigen, the flagella (H antigen) and some strains possess a capsular (K) antigen. Each O antigen defines a serogroup, while the combination of O and H

antigens identifies the serotype (Kaper et al. 2004). More than 180 O, 60 H and 80 K different antigens have been proposed (Stenutz et al. 2006). Nevertheless, there is not always a definite correlation between the serogroup and the pathotypes. One pathotype may comprise several serogroups, which conversely may belong to various pathotypes on the basis of virulence factors. Furthermore, virulence factors usually reside in mobile genetic elements (as plasmids and phages), which thus may be transferred from one strain of *E. coli* to another.

Therefore, even if these categorisations may become useful in approaching classification, they should not be considered absolute.

4.2.1 *Enteropathogenic* E. coli *(EPEC)*

EPEC has been the first identified pathotype. The characteristic of infections due to EPEC is the 'attaching-and-effacing' (A/E) histopathology. Specifically, bacteria intimately attach to the intestinal epithelial cells causing marked cytoskeletal changes, including accumulation of polymerised actin and the effacement of intestinal microvilli. EPEC virulence factors are encoded in a plasmid of 70–100 kb, called EPEC Adherence Factor (EAF) plasmid, and in genes on a 35 kb pathogenicity island, named Locus Enterocyte Effacement (LEE). EAF is important when speaking of the adherence to epithelial cells and the recruitment of bacteria in the environment through the formation of three-dimensional networks (Giron et al. 1991); intimate adherence is also mediated by a protein, intimin, whose gene is present in LEE.

LEE also encodes a specialised secretion system, which assists in the translocation of relevant proteins to the extracellular environment, and associated proteins. Some strains also produce the EspC enterotoxin which is homologous to members of the autotransporter family of proteins (Mellies et al. 2001), which will be addressed in Sect. 4.2.4.

Among the EPEC, some of the most common serogroups are O20, O25, O26, O44, O55, O86, O91, O111, O114, O119, O125ac, O126, O127, O128, O142 and O158 (Nataro and Kaper 1998).

Usually, infection due to EPEC results in severe watery diarrhoea, together with vomiting and low-grade fever. Diarrhoea likely results from multiple mechanisms, as malabsorption due to loss of microvilli, local inflammation, augmented intestinal permeability and active ion secretion. At present, EPEC strains are no longer as relevant as in the middle of 20th century. Sporadic outbreaks are still observed both in Europe and in the United States (Nataro and Kaper 1998), caused mostly by the so-called atypical EPEC, that contain LEE (but EAF plasmid is absent). However, in contrast to their modest importance in industrialised countries, typical EPEC (i.e. containing the EAF plasmid) continue to be a major cause of diarrhoea in developing countries. In particular, EPEC are responsible for a high mortality in infants younger than 2 years. EPEC strains are often the most frequently isolated bacterial diarrhoeal pathogens in the 0–6-month group (Nataro and Kaper 1998). Adults and

older children are relatively resistant and probably serve as the reservoir being asymptomatic carriers. Various foodborne outbreaks have been reported: prawn mayonnaise, salads and infant formula are described as vehicles, even if in the majority of cases the food sources have been rarely identified (Meng et al. 2001).

4.2.2 *Enterotoxigenic* E. coli *(ETEC)*

The importance of ETEC is correlated with the secretion of at least one group of enterotoxins: heat-stable (ST) and heat-labile (LT) toxins. In fact, ETEC strains may express either one of the two toxins only, or both a ST and a LT. Anyway, both the toxins are under the genetic control of transmissible plasmids, as also colonization factors (CF). CF mediate the colonisation of the small intestine, thus allowing the elaboration of toxins (fimbrial or fibrillar proteins). More than 22 CF have been detected among human ETEC (Qadri et al. 2005), but nonetheless epidemiological studies report that about 75 % of human ETEC express either colonization factor antigens (CFA) I, II or IV. It has also been reported that CF have been detected in less than 10 % of ETEC strains expressing only LT, in comparison to much higher percentages in strains expressing ST or both (over 60 %). These data, together with the more frequent isolation of LT-producing strains from healthy people (in respect to the other two combinations), suggest CF may have a role in pathogenesis. However, the main role is surely due to the production of LT and ST enterotoxins.

LT are closely related to Cholera toxin (CT), an enterotoxin produced by *Vibrio cholerae*, with which they share approximately 80 % amino acid identity (Sixma et al. 1993a). Besides protein sequence, LT and CT have the same enzymatic activity, holotoxin structure, primary receptor and activity in animal and cell culture assays, while they differ in toxin processing and in the immune system response (Nataro and Kaper 1998). LT have an AB_5 multimer structure, where the single A subunit is involved in catalytic activity and the B pentamer is necessary for membrane binding. LT is of ca. 86 kDa; in particular, the A subunit is 28 kDa and each B subunit is 11.5 kDa (Streatfield et al. 1992). In relation to LT, proteins are produced as monomers and the A subunit stimulates the assembly of the five B subunits. The B monomer is small, compact and highly structured, as it consists of a small *N*-terminal helix (α_1), two three-stranded anti-parallel sheets (sheet I: β_2, β_3, β_4, and sheet II: β_1, β_5, β_6) and a long helix (α_2). The two sheets interact with each other through three hydrogen bonds (Sixma et al. 1991). The α_2 helix shows amphipathic behaviour, as on one side it has eight charged residues. On the other side, it is mostly hydrophobic except on the last turn, which has hydrophobic residues on both sides. The loops can be divided in two classes, depending on the end of the sheets: a 'narrow' end of the subunit, where the loops are shorter, involving the connections β_1–β_2, β_3–β_4, α_2–β_5 and the *C*-terminus, and a wider end, with longer loops connecting secondary structure α_1–β_1, β_2–β_3, β_4–α_2, β_5–β_6 and the *N*-terminus nearby (Sixma et al. 1993a).

The B subunits-pentamer is arranged in a 'doughnut' shape, with a pore in the middle. The pore is very polar, being formed by the long α_2 helices, and shows a net positive environment, due to the prevalence of positive residues in comparison to negative ones. The pentamer is stabilised by various interactions, including at least 26 hydrogen bonds between a subunit and the adjacent molecules, and at least four salt bridges between a subunit and each neighbour, with 10 being in the central pore. There is a flat surface on one side of the pentamer, due to the combination of the short loops on the narrow end of the subunits, where A subunit is found. The long loops, instead, form a more convoluted surface, which has been reported to be part of the membrane receptor-binding site (Sixma et al. 1992). Lastly, six-stranded antiparallel β sheets are formed by the combination of the three-stranded sheets with the opposite sheet of the adjacent monomers. The A subunit is responsible for the activity of the toxin and is proteolytically cleaved, generating two peptides: A_1 and A_2. A_1 consists of a wedge-shaped domain with numerous hydrogen bonds, while A_2 has a long helix, that in the complete *A* subunit is packed with one side against the A_1 fragment (Sixma et al. 1993a). A disulphide bridge joins the two fragments; besides, there are also four hydrogen bonds and one salt bridge. Although there are not so many hydrophilic contacts, the packing is not exceedingly hydrophobic with the A subunit surface being 52.9 %-hydrophobic. The *N*-terminal A_1 is the real catalytic moiety, and the *C*-terminal A_2 is inserted non-covalently into the central pore of the B pentamer. Finally, there are also various interactions between *A* and *B* subunits. A_2 is involved mainly in the formation of hydrogen bonds and five salt bridges with one monomer of the B subunit. In addition, there are two regions of hydrophobic interactions between A_2 and the B pentamer: one is located at the narrow end of the central pore and the second is at the wide end of the pore. There are also indirect contacts, mediated by a number of water molecules. Moreover, A_1 forms interactions with the B subunit (Sixma et al. 1993a).

The AB structure of the toxin is important in protecting the A subunit from degradation in a protease-rich environment as the lumen. In fact, it keeps trypsin-sensitive residues in a hidden structural conformation, preventing proteolysis and subsequent degradation of the A subunit. Instead, the arginine 192 residue, whose cleavage is required for LT activation, is located in a surface-exposed loop, allowing the biological function of the toxin.

Purified LT preparations proved stable over pH adjustments and storage at −70 and −20 °C for months; the biological activity of the holotoxin, and in particular of the A subunit, has not been removed completely after heating at 65 °C for 30 min. In addition, the biological activity has been enhanced by incubation with trypsin and has been cancelled by pronase and proteinase K (Kunkel and Robertson 1979).

LT are divided in two serogroups since they do not cross-react immunologically: LT-I and LT-II. LT-I is common as a pathogenic agent both in humans and in animals, while LT-II is found mostly in animals. LT-II has not been previously associated with disease, but recent isolations from diarrheic patients lead to hypothesise its toxicity (Nardi et al. 2005). LT-I and LT-II have highly homologous amino acid sequences for the A subunit, which reflects the evolutionary need to preserve the structure for toxigenic activity. In contrast, the two B subunits display

high divergence (Connell 2007). In fact, the differences in the B subunits reflect the fact that it has to bind gangliosides, which have an interspecific and intraspecific (in different cell types) heterogeneity. From the structural point of view, gangliosides are oligoglycosylceramides, that contain one or more *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid forms of sialic acid, conjugated to the ceramide core by glycosidic linkages (Hajishengallis and Connell 2013). The gangliosides-binding activity of LT-I and LT-II is peculiar. However, different variants exist among both LT-I and LT-II. LTp-I and LTh-I have been discovered in strains isolated from pigs and humans, respectively, and show partial antigenic cross-reactivity. LT-II also shows subclasses; in detail, three variants have been described: LT-IIa, b and c. These members are antigenically distinguishable and have different toxic doses. LT-IIb has a lower isoelectric point (5.2–5.6 if compared with 6.8), a lower toxic dose and its behaviour during purification diverged significantly in comparison to LT-IIa (Guth et al. 1986). The last of these members, LT-IIc, has been only recently detected in an avian host, and it has been shown to be less cytotoxic than the other two variants using mouse bioassay (Nawar et al. 2010). The differences among the variants depend on the multiple amino acid substitutions in the B subunit, which enable the three LT to bind distinct gangliosides, reflecting their capacity to intoxicate several animals.

In contrast to large LT, ST are low-molecular-weight peptides and contain several cysteine residues, which are responsible for their heat-stability. In fact, ST are still active after 30 min at 100 °C. ST include two unrelated classes that differ both in structure and in mechanism of action: heat-stable a (STa) and b (STb). STa are cysteine-rich peptides, highly homologous to proteins found in other enteric bacteria, as *Yersinia enterocolitica*, *Klebsiella pneumoniae* and *Citrobacter freundii* (Guarino et al. 1987; Klipstein et al. 1983; Takao et al. 1984). STa toxic activity is not destroyed after treatment with pronase, trypsin, proteinase K, periodic acid oxidation and several organic solvents (acetone, phenol, chloroform and methanol). In addition, STa is stable at acidic pH (up to pH 1.0), while a pH value greater than 9.0 destroys biological activity (Alderete and Robertson 1978). The isoelectric points of STa range from 3.88 to 4.08 (Dreyfus et al. 1983). Two variants are found among STa. However, they are nearly identical and can be both observed in humans: STp and STh, after their initial discovery in pigs and humans, respectively.

STa is initially synthesised as a 72-amino acid precursor peptide, while the mature protein contains 18 (STp) or 19 (STh) amino acids, with a molecular weight of 2 kDa. The 18–19 amino acid peptide contains the toxin, which reaches the final sequence after cleavage during translocation to the periplasm. In fact, the highly conserved region (residue 29–38) in the precursor protein increases the translocation of STa across the membrane (Yamanaka et al. 1993). The toxic domain (14 residues) is located at the *C*-terminus, while the last residues at *N*-terminus are not required for biological activity. The mature toxin contains six cysteines that form three disulphide bridges. Disulphide linkages are fundamental to enterotoxicity, as they play a crucial role in determining the strength of the bond with the receptor; consequently, biological activity is completely abolished by treatment with reducing and oxidising agents. Moreover, they contribute to stability of the

molecule, together with hydrogen bonds. In the crystal structure, the Cysteine 5–Cysteine 10 disulphide bridge has quite a rare geometry, adopting a right-handed conformation, whereas the other two bridges have a more common left-handed spiral conformation (Sato et al. 1994). The crystal structure of STp has shown good agreement with the structure of STh, determined by NMR (Matecko et al. 2008). A ring-shaped self-associated hexamer is formed by STa (Sato and Shimonishi 2004).

STb is associated prevalently with diseases in cattle while rare cases have been reported for humans (Lortie et al. 1991; Okamoto et al. 1993), where STb had probably been acquired from animals (e.g. pigs). Its sequence shows no homology when compared with STa. Moreover, STb can induce the loss of villus epithelial cells and partial villus atrophy (Nataro and Kaper 1998). STb is a highly basic molecule, with a determined isoelectric point of 9.6: in fact, it contains nine basic amino acids. In particular, lysine residues seem to contribute in an important way to the toxicity of STb (Fujii et al. 1994).

ETEC produce enterotoxins in the early stages of growth. Although they are apparently secreted in all conditions that enable cell growth, the release of LT in enriched culture media seems to be favoured at neutral to alkaline pH values if compared to acid pH values (Gonzales et al. 2013), while a pH-dependent release of ST has not been observed (Johnson et al. 1978).

Among the ETEC, some of the most common serogroups are O6, O8, O11, O15, O20, O25, O27, O78, O128, O148, O159 and O173 (Stenutz et al. 2006).

Known infections result in watery diarrhoea, usually without blood, mucus or pus, whereas vomiting and fever are present rarely. Clinical records may vary from mild, brief and self-limiting diarrhoea, to severe and life-threatening cases, especially in weanling infants.

The majority of cases are thus associated with childhood diarrhoea in the developing world, but also people who travel in these areas also frequently experience the infection. In fact, available data suggest 20–40 % of traveller's diarrhoea cases may be caused by ETEC. The infection requires quite high infectious doses: up to 10^8–10^{10} colony forming units (CFU), mainly because of faecal contamination of food and water sources (Neill et al. 2001). Indeed, an inadequate sanitation of drinking water is common in the developing countries; sampling in the endemic areas has shown impressive rates of contamination, above all during warm months and in the rainy season, when growth and multiplication in food and water are more efficient (Qadri et al. 2005). Transmission of ETEC diarrhoea in developed countries is also possible, due to asymptomatic travellers importing these microorganisms. Food-borne outbreaks have been reported in the United States and Sweden, with ETEC strains present in processed meat and cheese products (Danielsson et al. 1979; Sack et al. 1977). In addition, other types of foods have been involved with ETEC outbreaks, including curried turkey mayonnaise, crabmeat, salads and tuna paste, maybe because of infected food handlers or contaminated water used during preparation (Meng et al. 2001).

4.2.3 *Enterohaemorrhagic* E. coli *(EHEC)*

EHEC are a frequent source of foodborne outbreaks and may cause life-threatening complications. However, EHEC denote a subset within verotoxigenic *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC). The term STEC is correlated with the production of a Shiga toxin (Stx) which is quite identical to the Stx produced by *Shigella dysenteriae* I, while VTEC indicates the toxicity of Stx for Vero cells (African green monkey kidney cells). Whereas not all STEC are considered pathogens, EHEC strains are all pathogens by definition, as they have other virulence factors besides Stx.

In fact, EHEC denote strains possessing also the so-called 'eae' gene, responsible for A/E lesions in the intestinal epithelial cells, and a 60-MDa plasmid (i.e. typical EHEC). A/E-positive STEC strains contain in their chromosomes the LEE pathogenicity island, as seen in EPEC (Sect. 4.2.1).

The key virulence determinant is Stx. Two types of Stx are produced by STEC: Stx1 and Stx2, immunologically non cross-reacting. Stx1 is identical to Shiga toxin from *Shigella dysenteriae;* there may be a difference in one residue (a serine instead of a threonine), and is highly conserved. Three Stx1 variants—Stx1a, Stx1c and Stx1d—have also been reported (Burk et al. 2003; Zhang et al. 2002). In particular, Stx1c has been related to mild or no disease in humans, and is more frequently isolated in ovine. Conversely, Stx2 includes numerous antigenic variants—Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, Stx2 g and Stx2d-activable—which vary in their biological activity, immunological reactivity and association with disease. Stx2e and Stx2f are the two most divergent variants from Stx2 at the amino acid level and are usually associated with animals (Stx2e and Stx2f are specifically linked with oedema disease of swine and feral pigeons respectively). Instead, Stx2a, Stx2b, Stx2c and Stx2d variants are more frequently associated with human illness. Stx2 g has been isolated from a bovine strain of *E. coli* and displays the highest homology with Stx2a and Stx2c.

Stx1 and Stx2 may be present in a single EHEC strain both together or singularly. One strain may produce more than one Stx as it may harbour more than one Stx-encoding bacteriophage. These phages are important for the spreading of Stx genes and other species expressing Stx have been described, as some strains of *Citrobacter freundii* and *Enterobacter cloacae*. Both Stx1 and 2 have an AB_5 structure, in which the A subunit (32 kDa) is bound to 5 B subunits (each 7.7 kDa). However, Stx1 and 2 share only 55 and 57 % amino acid homology, respectively, in the A and B subunits (Jackson et al. 1987).

Stx has a complex structure (as LT in ETEC) and the two toxins share some similarities, although they have almost no sequence identity (three identical residues in 52 superimposed residues, Sixma et al. 1993b). Stx, in fact, shows a pentameric ring formation for the B subunit (Stein et al. 1992), which is associated to the A subunit, that can be cleaved in two smaller fragments: A_1 (27.5 kDa) and A_2 (4.5 kDa). The two portions are covalently associated through a disulphide bond, with A_1 retaining the catalytic activity. The ring formed by the B subunits encircles

a helix at the C-terminus of the A subunit, while the rest of the A subunit lies on one side (Fraser et al. 1994). The A subunit interacts with the B pentamer via the A_2 fragment, that is inserted in the central pore. The B monomer is formed by two three-stranded antiparallel β-sheets and the α-helix. Therefore, unlike LT B-monomer, Stx lack an additional a-helix at its N-terminus. The β-sheet interaction between adjacent monomers is similar to LT but it is exposed due to the shorter N-terminus, forming six-stranded antiparallel β-sheets around the outer surface of the pentamer. Moreover, the interface between monomers is not as large as in LT, making Stx less stable (Sixma et al. 1993b). In Stx, the central pore is delineated by the five helices and shows no charges (on the other hand, LT pore is highly charged).

As previously indicated, Stx1 is structurally different from Stx2, which is 400 times more lethal to mice (Tesh et al. 1993) and 1,000 times more toxic for human renal microvascular endothelial cells than Stx1 (Louise and Obrig 1995). Stx2 is associated with high virulence in humans, and it is a predictor of HUS if associated with the presence of the eae gene. Structural differences account for the greater pathogenicity of Stx2.

STEC strains have the potential to produce Stx in food. The Stx2 molecule is quite heat stable, as it resists pasteurisation processes (Rasooly and Do 2010). Stx2 retains almost full activity after heating at 60 °C for one hour, while a significant reduction occurs after heating at 80 °C for one hour (He et al. 2012). Consequently, it may account for those disease cases in which no live bacteria have been found after mild heat treatments.

In addition to Stx, EHEC contain numerous virulence factors, for instance a variety of fimbrial and non-fimbrial adhesins, all involved in various stages of adhesion, and other toxins that may contribute to EHEC pathogenesis (Dautin 2010; Kaper et al. 2004; Schmidt et al. 1995; Taneike et al. 2002).

Infection with STEC may result in no clinical symptoms, but in most cases it causes diarrhoea which can evolve in bloody diarrhoea (haemorrhagic colitis, HC), abdominal cramps, accompanied by vomiting and fever in some patients . The incubation period is on average 3–4 days, even if shorter (1 day) to longer (up to 8 days) period has been described (Nataro and Kaper 1998). In some cases, patients may develop haemolytic uremic syndrome (HUS) which is a potentially lethal complication, characterised by haemolytic anaemia, thrombocytopenia (decreased number of platelets) and acute kidney failure. The onset of HUS is more likely to occur (Gyles 2007) with O157 serotype—up to 10 % of individuals—and in patients affected by haemorrhagic colitis. In fact, production of enough Stx results in damage to blood vessels in the colon. Should a sufficient level of toxin be concentrated in the blood, Stx could reach various districts of the body causing HUS and/or impairment of other organs, as central nervous system and heart. In particular, children under five years of age and the elderly usually are more involved in these progressions.

The most famous EHEC serotype is O157:H7, because of its prevalence and importance in human disease, even if there are several other common serogroups: O4, O5, O16, O26, O46, O48, O55, O91, O98, O111ab, O113, O117, O118, O119,

O125, O126, O128, O145, O157 and O172. Recently, new EHEC serogroups—O176, O177, O178, O179, O180 and O181—have been isolated (Stenutz et al. 2006). On the basis of serogroups, STEC have been divided in classes—from A to E—depending on the virulence and the frequency of the outbreaks, where O157 belongs to seropathotype A while STEC serotypes that have not been implicated with outbreaks belong to seropathotype E (Karmali et al. 2003).

Escherichia coli O157:H7 is the leading cause of STEC infections worldwide: the United States Center for Disease Control and Prevention has estimated that it is responsible for more than 50 % of all EHEC infections in North America. Additionally, the O157:H7 serotype is also very common in Europe, Japan, Australia, Argentina, Chile and South Africa, even if non-O157 EHEC—O26, O103 and O111—are often causing outbreaks in these countries (Brooks et al. 2005). However, O157:H7 is statistically the serotype most often associated with haemorrhagic colitis and HUS. Interestingly, EHEC are less frequently isolated in developing countries if compared with other pathotypes as EPEC and ETEC (Nataro and Kaper 1998).

Therefore, EHEC epidemiology is very complex, involving animal reservoirs, a long duration of shedding and a very low infectious dose. Specifically, STEC are shed in stools of infected patients for weeks. In fact, duration ranges from two to 62 days, with an average of 13; the range is far longer for HUS patients (5–124 days). The infective dose for *E. coli* O157:H7 is reported by the Food and Drug Administration (FDA) to be 10–100 cells, while the number is slightly higher for other EHEC. Transmission is by the faecal-oral route and occurs by direct contact with the infected animal, from person to person and by ingestion of contaminated food or water. In particular, edible materials are the most relevant way, as it has been estimated that up to 85 % of STEC infections are transmitted by food (Meng and Schroeder 2007). In fact, most EHEC outbreaks can be traced from food that had previously entered into contact with animal faeces. STEC strains have been isolated from a variety of animals, such as cattle, sheep, pigs, birds, goats, as well as horses, cats and dogs, though ruminants are thought to be the prevalent reservoir (Hussein 2007). They can host several strains simultaneously and the rate of colonisation is high, with reported prevalence of up to 70 % in bovine herds (Pradel et al. 2000), and concentrations of 10^7 CFU/g (Fegan et al. 2004). Furthermore, shedding seems to be related to age and period of the year with the highest frequency in young animals and in warmer months (Gyles 2007). Additionally, STEC are also commonly detected in cattle feedlots.

Thus, meat derived from these animals may be contaminated with STEC strains if there is any deficiency in hygiene practices. Food associated with STEC hence includes firstly undercooked beef and other types of meat, as porcine and sheep meat, but also fresh produce, salami, raw milk and cheeses. STEC outbreaks have been increasingly associated with consumption of fruits and vegetables, as lettuce, cantaloupes, alfalfa sprouts and radish sprouts, bagged salads and even potatoes in one case in Great Britain (Meng et al. 2001). In these situations, it can be speculated that contamination has occurred in the field due to irrigation or the use of soil that had been treated with farm effluent (Fremaux et al. 2008). Subsequently, the contamination has

reached the whole batch. Sources of infection include as well chicken, turkey and seafood (i.e. fish and shellfish), maybe due to cross-contamination.

STEC can also survive for long periods in water. As a result, almost every water source, as drinking water, streams, ponds, well water and even municipal water system, has been linked to outbreaks (WHO 2011). Notably, also food that has been regarded as safe and ready-to-eat for its acidity, as mayonnaise and unpasteurised apple cider and juice, has been found implicated. *E. coli* has a wide range of acid tolerance, being capable of growing at pH 4.4–10, but many STEC strains—in particular, O157:H7—have shown to survive in a really acidic environment, with a pH of 2.5–3 for more than 4 h (Molina et al. 2003). Leyer and co-workers have found that *E. coli* O157:H7 is able to survive for several days at pH 3.4, even if acid tolerance is dependent on the type of acid and is increased in microorganisms which had been previously exposed to weaker acids (Leyer et al. 1995). Furthermore, Stx production is also influenced by environmental factors. In fact, Stx amounts are more abundant in strains grown at 37 °C than at lower temperatures (Abdul-Raouf et al. 1995); higher concentrations have been obtained in minced meat in respect to milk.

4.2.4 *Enteroaggregative* E. coli *(EAEC)*

EAEC are the most recently described category of diarrheagenic *E. coli*. In contrast to the EPEC localised adherence pattern, EAEC show an aggregative adherence in Hep-2 cells (Nataro and Kaper 1998). The aggregative adherence pattern is mainly due to fimbrial structures, known as aggregative adherence fimbriae (AAF), related to the Dr family of adhesins (Nataro et al. 1992). Genes coding for AAF are located on a 60-MDa plasmid; four variants of AAF exist at least, each expressed in only a minority of EAEC strains. However, some EAEC strains with the characteristic adherence pattern have been found to lack AAF, suggesting the existence of other adhesion mechanisms. For instance, a novel adhesion pilin has recently been reported in EAEC strains lacking known AAF (Boisen et al. 2008). Another protein instead, dispersin, acts to disperse the bacteria, hence partly counteracting the aggregating effect mediated by AAF (Sheikh et al. 2002).

EAEC pathogenesis is also due to the elaboration of several toxins. The enteroaggregative heat-stable toxin 1 (EAST-1) is present in ca. 40 % of EAEC strains and seems to play a role in EAEC pathogenicity, even if its role is still questioned, given that it can be detected also in commensal strains. In addition, this toxin is associated with diarrhoea also in cattle and pigs. EAST-1 is a 38-amino-acid protease-sensitive enterotoxin, with a molecular weight of 4.1 kDa and a calculated isoelectric point of 9.25 (Savarino et al. 1993). It contains four cysteines (at positions 17, 20, 24 and 27), forming two disulphide bridges with a C1–C2 and C3–C4 conformation (Menard et al. 2004). EAST-1 is homologue to STa, as they share the small molecular weight, the disulphide bridges and 50 % identity in their enterotoxic domain. However, they differ immunologically as no cross-neutralisation occurs. Moreover, unlike STa, EAST-1 is only partially heat-stable, as after heating at 65 °C

for 15 min it retains 63 % of activity. Two main variants of EAST-1 have been described, one present in 17–2 strain and the other in strain O 42 (Yamamoto et al. 1997). Other variants have been described but they are considered rare since they have been isolated only once in epidemiological studies (Menard and Dubreuil 2002). Because of the similarities between EAST-1 and STa, they are considered to act through the same mechanism, with diarrhoea effects.

The *Shigella* enterotoxin 1 (ShET-1) has been firstly described in *Shigella flexneri* 2a but is also found as well in many EAEC strains. This toxin is encoded by chromosomal genes and has an A–B_5 configuration consisting of a single 22 kDa- A subunit associated with a pentamer of five 7 kDa- B subunits.

Several other virulence factors are associated with EAEC, as haemolysin E (Mueller et al. 2009). Another relevant class of virulence factors is the serine protease autotransporter of *Enterobacteriaceae* (SPATE) class of cytotoxins, which is present in the overwhelming majority of EAEC strains (Boisen et al. 2009). SPATE proteins are produced by *E. coli* and *Shigella* species.

SPATE are subdivided in two classes: I SPATE all have a cytotoxic effect in epithelial cells (Henderson and Nataro 2001). Class II SPATE consists of more phenotypes that are diverse and include different proteins and compounds (Boisen et al. 2009).

SPATE production is generally regulated by temperature, as more protein is observed in cultures grown at 37 °C than both at lower and higher temperatures. In some situation, pH is also relevant in SPATE expression, with the optimum at quite alkaline values (Dautin 2010).

Within the EAEC, identified serogroups are O3, O7, O15, O44, O77, O86, O111, O126 and O127 (Stenutz et al. 2006).

EAEC infection usually elicits watery diarrhoea, often with mucus, with or without blood, vomiting and low-grade fever. The pathogenesis of EAEC is complex due to the high heterogeneity of the strains and it involves an increased mucus secretion with the formation of thick biofilms. These biofilms could be responsible for the persistency of the colonisation (Nataro and Kaper 1998). Moreover, EAEC infection causes also cytotoxic effects on the intestinal mucosa, and a mild inflammatory response. EAEC strains are increasingly recognised as an emerging diarrheagenic pathogen, both in industrialised and in developing countries. They are a highly common cause of acute diarrhoea, but they may also provoke a persistent (>14 days) diarrheal illness above all in newborns, children and in Human Immunodeficiency Virus- infected patients. In addition, EAEC is actually the second most common cause of traveller's diarrhoea. Besides asymptomatic carriers, food is also a vehicle of transmission of EAEC. Some restaurant- and canteen-associated outbreaks have occurred, for instance, in Great Britain, Mexico and Japan, even if the type of food has not been identified. In Italy, two consecutive EAEC outbreaks are linked to contaminated unpasteurised cheese (Feng 2013; Kaur et al. 2010). Interestingly, in 2011 an unusual EAEC strain has been involved in one of the largest HUS outbreaks ever recorded.

4.2.5 *Enteroinvasive* E. coli *(EIEC)*

EIEC strains are characterised by the invasiveness of the colonic epithelium. They are closely related by the biochemical, genetic and pathogenic point of view with *Shigella spp*. In fact, they share a 140-MDa plasmid that carries the genes necessary for invasiveness and virulence.

The pathogenic scheme has yet to be fully understood, though it seems that the first stages of infection coincide with those of *Shigella* and are represented by epithelial cell penetration, lysis of the endocytic vacuole, multiplication in the intracellular environment, directional movement through the cytoplasm and extension into adjacent epithelial cells (Sansonetti 2002). These stages determine a strong inflammatory reaction. EIEC and *Shigella* infections cause in most cases a watery diarrhoea, even if occasionally they induce the dysentery syndrome, with blood and mucus in the stool.

Among the EIEC, some of the most common serogroups are O28ac, O29, O112ac, O124, O136, O143, O144, O152, O159, O164 and O167 (Stenutz et al. 2006).

Various outbreaks have occurred due to EIEC, even if the incidence in developed countries is lower. The transmission is primarily food-borne or water-borne; inter-human transmission has been reported. It is currently unknown as to what type of food may carry EIEC, but any food contaminated with human faeces directly or via contaminated water could elicit the disease. Outbreaks have been associated with hamburger meat, cheeses and vegetables in the United States, Europe and Japan (Feng 2013; Meng et al. 2001).

4.2.6 *Diffusely Adherent* E. coli *(DAEC)*

DAEC is a category of diarrheagenic *E. coli* (Kaper et al. 2004). However, little is known about pathogenesis of DAEC.

Epidemiological studies do not always confirm the association between the presence of DAEC strains and watery diarrhoea. In fact, in some studies, certain DAEC strains have been isolated from diarrheal patients and asymptomatic controls in similar frequencies; however, a certain age-dependent susceptibility has been observed. In particular, the association is stronger in children older than 1 year. However, the hypothesis of the presence of additional factors triggering the disease is to be considered. Besides being isolated from developing countries, DAEC strains have been isolated in France in several diarrhoeal cases among hospitalised patients (Jallat et al. 1993), showing that they may account for a number of cases also in the developed world. Currently, no food vehicles have still been reported.

4.3 Detection Methods

Culture methods have represented the 'gold standard' for a long time but they are time-consuming. In addition, they do not allow to discern among pathogenic forms and commensal and/or environmental *E. coli*. Moreover, phenotypic traits are often equivocal because of their high variability; consequently, they may lead to evaluation errors. For instance, many *E. coli* strains—about 90 %—are lactose-positive; as a result, the presumptive identification on agar plates can be misleading in the residual 10 %, given that many laboratories assume that all *E. coli* strains are lactose-positive. An exception could be O157:H7, for which particular biochemical characteristics have been observed. In fact, *E. coli* O157 strains do not produce β-glucuronidase, do not grow well at the temperature commonly used for environmental strains (44 °C) and do not ferment sorbitol rapidly. The last feature is used to isolate O157 in sorbitol-MacConkey (SMAC) agar, where it grows without production of coloured pigments. SMAC selectivity is improved by adding cefixime and tellurite that are supposed to inhibit the growth of most other strains.

More specific methods are based on immunology. Immunological methods use specific antibodies to identify homologous antigens. Latex agglutination is one of the fastest and most common techniques and it involves the in vitro aggregation of microscopic carrier particles (i.e. latex), mediated by the reaction with the suspected antigen. In addition, antibodies can be labelled with a fluorescent compound to emit fluorescence if they are bound by the complementary antigen, or with a radioactive label. However, these techniques are not widely used for the advent of molecular techniques and require costly equipment as the fluorescence microscope. Enzyme-linked immunosorbent assay (ELISA) is another immunoassay: the antibody is bound to an enzyme, typically peroxidase or alkaline phosphatase, and it gives a quantitative colorimetric reaction depending on the amount of enzyme. ELISA technique is extensively used to identify microorganisms, their serotype and their metabolites such as toxins in food: for instance, LT, ST and Stx (Cryan 1990; Downes et al. 1989; Yolken et al. 1977).

In some situations, strains are present in the sample with a low concentration—especially for O157:H7 serotype—making it more difficult to detect bacteria. A very sensitive method that allows to isolate microorganisms, particularly used for *E. coli* O157, is immunomagnetic separation (IMS), requiring numbers as low as 10^2 CFU/g (Karch et al. 1996). IMS employs paramagnetic beads conjugated with antibodies against *E. coli* O157 and exploits the formation of a complex between the antigen and antibodies when bound to beads. The complex is separated from the rest of the sample by using a magnet. The complex is then suspended again and cultivated in appropriate media. Beads coated with antibodies against serogroups O103, O111 and O145 are commercially available.

Traditional methods are based on the phenotypic identification of the microorganism of interest by means of procedures such as culture and biochemical methods. However, these methods suffer principally from a major drawback: they often do not consider the interspecific variability. In fact, some strains exhibit

different biochemical characteristics that do not fit into the predetermined pattern. Indeed, the current trend in research is oriented towards an increasing use of molecular methods, which involve examining the DNA of the bacterium. In addition, molecular detection has two main advantages over traditional techniques. The identification can be completed using a very small amount of material and is more accurate than with previous methods, allowing reliable differentiation of species and pathotypes, when speaking of *E. coli*.

Two major approaches are used in identification: probes (sequences that bind to the DNA of targeted organisms only) and polymerase chain reaction (PCR). Probes are used for a quick detection and identification of bacteria and rely on the hybridisation of a designed species-specific oligonucleotide probe with a precise region of the targeted gene. Each probe is tagged with a radioisotope, enzymes (e.g. alkaline phosphatase) or fluorescent dyes that give a detectable reaction upon hybridisation. For instance, the detection of ETEC may be performed using probes for LT and ST enterotoxins (Moseley et al. 1982). In relation to EHEC, the detection of Stx1 and Stx2 genes has been made easier thanks to the development of probes (Willshaw et al. 1987).

PCR has become one of the major advances in molecular diagnostics thanks to its high specificity and sensitivity, including tests for several pathogens as *E. coli*, *Salmonella*, *Staphylococcus* and *Listeria*. PCR is based on gene amplification that multiplies, creating millions of copies, specific regions of the particular segment of a DNA strand of interest. There are several variants among PCR and the most useful adaptations include multiplex PCR. In the last method, the amplification reaction is directed towards two or more target genes. There is also real-time PCR that allows determining the presence and quantity of DNA. PCR may be performed both on stool, food or environmental samples and on pure subcultures; target genes can be located in the chromosome or in plasmids, even if plasmids can be lost during subcultures and storage (O'Sullivan et al. 2007).

Several PCRs have been developed to identify the different pathotypes among *E. coli* (Tornieporth et al. 1995), based on the principal genes responsible for pathogenic activity (Cerna et al. 2003; Fratamico et al. 2000; Stacy-Phipps et al. 1995).

Other detection methods are sometimes used in laboratories, including animal assays (e.g. the suckling mouse assay for detection of ST in ETEC) and assays on specific cellular lines, but they fall outside the scope of this book.

4.4 Regulatory Norms and *E. coli*

Coliform bacteria, including *E. coli*, are the most extensively used microbiological indicators of faecal contamination both in water for human consumption and in foodstuffs. Nevertheless, the term 'coliform' includes many bacterial species, ranging from enteric commensals to environmental members as *Serratia* and *Aeromonas*. Therefore, recovery of coliforms in food is not always easy to interpret.

E. coli, which is regarded as a permanent member of intestinal flora, can grow in a variety of extra-intestinal niches, including processing plants (Cox et al. 1988). However, the presence of *E. coli* in raw materials is often due to a direct or indirect contact with human or animal faeces. Specifically, bacteria enter roots or leaf surfaces and can colonise the internal plant compartment (Solomon et al. 2002), making it difficult to remove them by washing and disinfection. Microorganisms can further spread from raw materials to uncontaminated products during processing and/or packaging steps. In fact, it is objectively difficult to isolate completely environments where raw materials arrive from processing areas: anything may serve as vehicle, even operators clothing. *E. coli* may also be present in processed foods, preserved through lethal treatments (pasteurisation), due to an inadequate thermal process or to a re-entry of bacteria in the product via cross-contamination. In addition, a number of *E. coli* can undergo a rapid increase in processing environments starting initially from a minimum contamination, depending on the presence of favourable environmental factors. *E. coli's* principal limiting factor is the availability of water (Ottaviani 1996). The minimum water activity required for growth is 0.95, even if the optimum is 0.995; tolerance to lower values diminishes as other factors become sub-optimal, as temperature and pH. As said in Sect. 4.2.3, *E. coli* has a wide range of pH tolerance, growing at pH 4.4–10.0 (but with optimum around neutral pH values, between 6.0 and 7.0). Temperature may be important: *E. coli* is able to grow between 7–8 and 46 °C. The optimum temperature is 35–40 °C, but its heat resistance increases when it is in the stationary phase of growth (Desmarchelier and Fegan 2003). Finally, *E. coli* does not require oxygen for growth being a facultative anaerobe, even if aerobic conditions are preferred.

However, the presence of *E. coli* in processing rooms and in the proximity of equipments is rather controllable—even if not completely eliminable—through the application of Good Manufacturing Practices and Good Sanitation Practices and does not necessarily imply the simultaneous presence of pathogens.

In fact, the dilemma whether hygiene deficiency is associated or not to a real risk of the presence of pathogens is not determined by the presence of *E. coli* itself, but by the severity of the productive defect and by epidemiological considerations, depending on the type of raw materials.

Currently, the tendency in European legislation regards prevention and control according to hazard analysis and critical control points (HACCP), which has been reassessed with Regulation (EC) No. 852/2004, and to microbiological criteria expressed in the Regulation EC No. 2073/2005 and subsequent amendments. These regulatory protocols have defined different limits for *E. coli* in food; in addition, *E. coli* can be considered as a faecal indicator when speaking of live fishery products, as bivalves and gastropods, and meat products (e.g. minced meat and preparations). On the other hand, this bacterium is also a hygiene indicator, specifically in cheeses that have undergone heat treatment, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation. Other products are cooked fishery products, ready-to-eat (RTE) pre-cut fruits and vegetables, unpasteurised RTE fruit and vegetable juices.

Therefore, *E. coli* can play a pivotal role in pointing out the need to perform corrective actions. On the other side, RTE-sprouted-seed sprouts are a high-risk food, as the sprouting process provides the ideal condition for bacterial multiplication, like humidity and temperature. The recent outbreak of diarrheagenic *E. coli*, occurred in 2011, is one of the biggest events in Europe. A total number of 4,321 cases, including 852 HUS cases and 50 deaths, have been reported (RKI 2011). It has been concluded that many fenugreek seeds, imported from Egypt, were the most likely source of the outbreak (Soon et al. 2013). The factor responsible was a particular O104:H4 *E. coli* strain, which can combine virulence properties of enteroaggregative and enterohaemorrhagic pathotypes. As a result, a heightened virulence of the strain has been observed: in fact, the number and combination of SPATE and the enhanced adherence due to the EAEC phenotype may have had a role in the high prevalence of the HUS syndrome, by facilitating the adsorption of Shiga-toxin (Bielaszewska et al. 2011). After the outbreak, the European Food Safety Authority has published a scientific opinion on the risk posed by the potential presence of Shiga-toxin-producing *E. coli* and other pathogens in seeds and sprouted seeds, suggesting that contamination should be detected and mitigated earlier in the production chain, with the aim of avoiding bacterial multiplication. In fact, short shelf-life values of these products would not consent an in-time withdrawal from the market. The new Regulation (EC) No. 209/2013 was then issued amending the Regulation (EC) No. 2073/2005 as regards microbiological criteria for sprouts and the sampling rules for poultry carcases and fresh poultry meat. The new protocol states that the following STEC: O157, O26, O111, O103, O145 pathotypes and O104:H4 must be absent in 25 g in sprouts for the whole shelf life, adding a new step for the consumer protection.

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