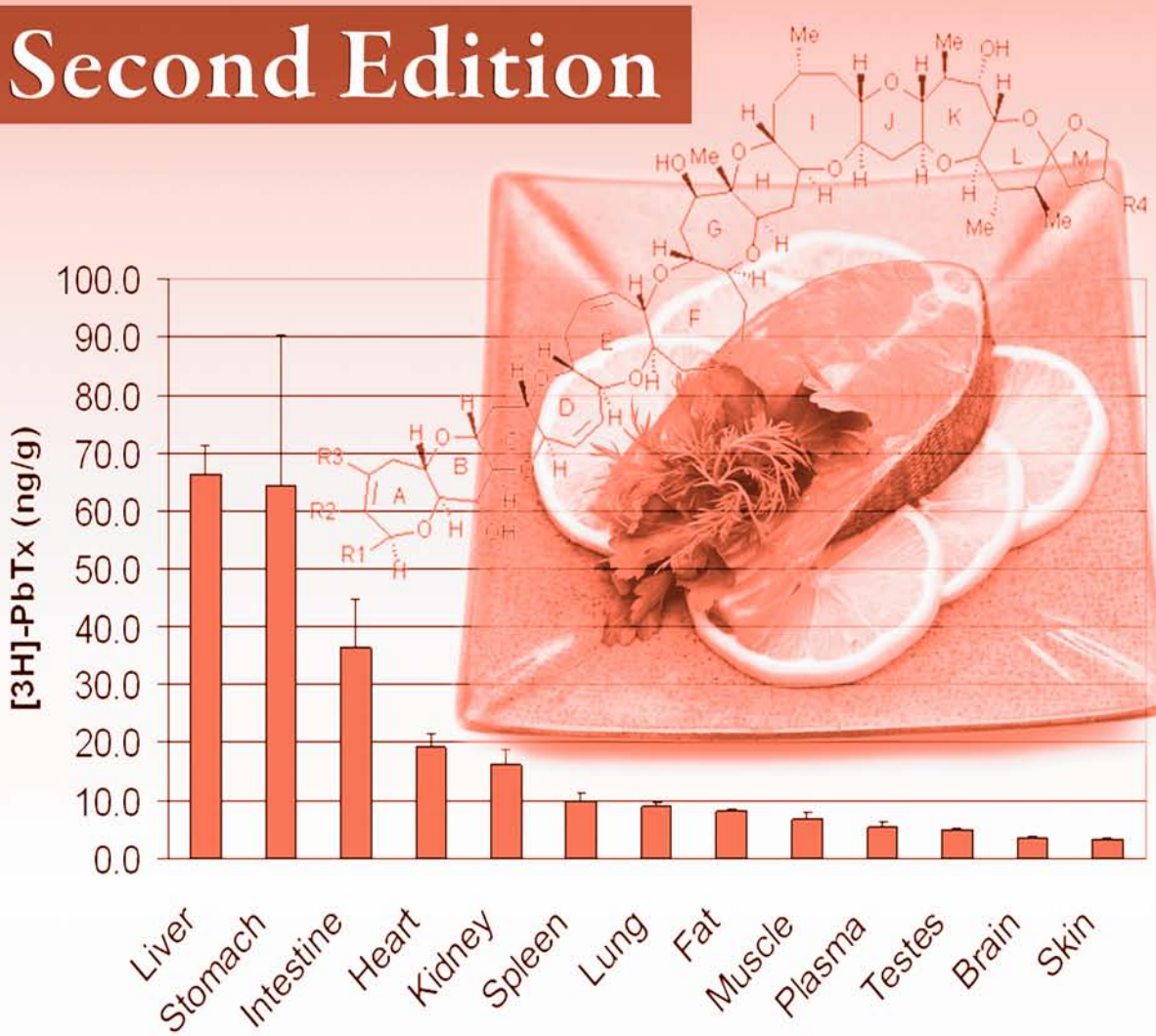
Seafood and Freshwater Toxins

Pharmacology, Physiology, and Detection

Second Edition



Edited by
Luis M. Botana

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FOOD SCIENCE AND TECHNOLOGY

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Preface

The second edition of *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection* is a demonstration that the topic is advancing very fast. In the first edition, *Pfiesteria* did not find mention due to lack of enough information to write a chapter, but this edition shows a part of the mystery already solved. Those toxins that were on the horizon in the former edition are now a well-known problem, and legislation has been developed for some of them. Some other toxins, such as gambierol or polycavernoside, did occupy only a line in the first edition, and now they have their own chapter. All these dramatic changes took place in 6 years, and I am very curious too see what the next years will bring us.

The increasing concern over food safety, combined with a notable advance in analysis technology and toxicological information, is described in this edition. Marine and freshwater toxins are a growing problem, and even the possible relationship with a potentially changing climate is already suggested in some chapters of the book. Therefore, the study of marine and freshwater toxins is extremely complex. International commerce, using large sea cargo vessels, is a source of new toxins in virgin areas, and from burning forest to pollution, everything could be a triggering cause for the appearance of toxic blooms in new places.

This book intends to provide an overall view of the current situation from all points, and as such, it will be of use to food technologists, toxicologists, pharmacologists, and analytical chemists.

The authors who contributed their work to each chapter are world experts in their fields, and I thank them for their dedication and enthusiasm in providing chapters for this book.

I want to thank the collaboration of CRC and T&F for their support to the second edition. I especially want to thank my family, from whom I have taken away sometimes far too much time, and to them I dedicate this work.

Editor

Dr. Luis M. Botana is a full professor of pharmacology at the University of Santiago de Compostela, Lugo, Spain. His group is a world leader in the study of the mechanism of toxicity of marine toxins and the use of toxin targets to develop functional assays. He received his postdoctoral National Institutes of Health Fogarty Fellowship at the Johns Hopkins University, Baltimore, Maryland. He has published more than 200 papers, 15 patents, and has edited several books on pharmacology and marine toxins. Since 2005, he has been the director of the European Community Reference Laboratory for Marine Biotoxins (European Directorate General for Health and Consumer Affairs and Spanish Food Safety and Nutrition Agency). His research is being carried out in collaboration with outstanding research laboratories worldwide.

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Part I

General Considerations

1 Risk Assessment of Marine Toxins

Tore Aune

CONTENTS

1.1 INTRODUCTION

Risk assessment is one of the three elements in the overall method called risk analysis. In addition to risk assessment, risk analysis comprises risk management and risk communication. Risk analysis is the fundamental methodology underlying the establishment of food safety standards. While risk assessments are performed by toxicologists or microbiologists, risk management is performed by persons responsible for regulation and control. It is important that the roles are not mixed and that all the steps in risk analysis are transparent. There should be a functional separation between risk assessment and risk management. This will ensure the scientific integrity of risk assessment and reduce any conflict of interest. Nevertheless, since risk analysis is an integrated process, interaction between risk assessors and risk managers is important for its outcome. Even though the goal is that food be safe, complete absence of risk is impossible, and society accepts some risks associated with food as reasonable in comparison with other risks in everyday life.

1.2 RISK ASSESSMENT IN GENERAL

Risk assessment is usually divided into four steps: hazard identification, hazard characterization, exposure assessment, and, finally, risk characterization [1]. In hazard identification, agents capable of exerting negative health effects are identified. Often, the first indications of toxic potential of a compound or mixture are derived from epidemiological studies. The advantage of epidemiological studies is that extrapolation from experimental animal studies is unnecessary. On the other hand, indications from epidemiological studies show that unwanted exposure has already taken place. For marine biotoxins in bivalve mollusks, all known syndromes have been detected as a result of

intoxication of humans, and not from screening programs. The different surveillance systems in operation mainly protect against repeated poisoning episodes from already known toxins in seafoods.

Hazard characterization consists of qualitative or quantitative evaluation of the adverse health effects associated with different agents, whether they are chemicals or microorganisms. This step comprises several elements, like toxicokinetics (absorption, distribution, metabolism, and excretion of the toxic agent), mechanism of toxic action, dose–response relationships, target organs and different end points, like acute or chronic toxicity, teratogenicity, neoplastic manifestations, and so forth.

In exposure assessment, information is sought on the likely intake of compounds with toxic potential. Knowledge is necessary on both concentrations of the toxic agents and the pattern of consumption of different food items where they appear. This kind of information is very often lacking or at least imprecise. Children are a group of special concern owing to their relatively large consumption on a body weight basis and increased susceptibility for many toxins.

Risk characterization is the final step in the risk assessment process. It comprises quantitative or semiquantitative estimations, including uncertainties, of the probability of adverse health effects in people associated with exposure to the toxic agents. Risk characterization is based on the information gathered through the first three steps in the risk assessment procedure. It is important that the weight of evidence leading to the conclusions be openly discussed. Risk characterization should include a description of the primary causes of uncertainties.

For nongenotoxic chemicals, risk assessment is based on the concept of threshold doses, below which no adverse effect results from exposure. From human or experimental animal data, one tries to establish the no observable adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL). In order to establish "safe" levels of exposure to potentially toxic agents, the NOAEL is divided by a safety factor (often named uncertainty factor). When the risk assessment is based on data from experimental animals, a default safety factor of 100 is usually applied. The safety factor constitutes a factor of 10 for potential differences in susceptibility between animals and man, and another factor of 10 for interindividual differences among humans. The factors are combinations of differences in toxicokinetics and toxicodynamics, both in animals and man. If true factors are known, the size of the safety factor may be changed accordingly. When risk assessment is based on human data, a safety factor of 10 is applied in most cases, for instance, for food additives. However, for natural toxins in food, smaller factors are usually applied. This is a risk management decision, often based on information on the absence of adverse health effects at intake levels close to the estimated LOAELs.

In principle, larger safety factors should be applied when the guidance levels (GLs) are derived from LOAELs instead of NOAELs. In practice, this is often not done, and this is explained by the seemingly safe use of existing GLs for many natural toxins.

For compounds with genotoxic effects, one assumes that there is no safe lower level of exposure, even though the risk may be very low. The term as low as reasonably achievable (ALARA) is often used for toxins without a threshold for effect. In such cases, estimates are made of exposures that constitute a risk of cancer in $1/10^6$ or $1/10^5$ at lifelong exposure. Instead, one may use the principle of margin of exposure (MOE) for such compounds, which means calculation of the ratio between the NOAEL and the estimated exposure.

An adverse effect is defined as changes in morphology, physiology, growth, development, or life span, resulting in impairment of functional capacity, impairment in the capacity to compensate for additional stress, or increased susceptibility to other environmental influences.

In many cases, it is difficult to decide on an exact dose level for the transition from merely an effect to an adverse effect.

1.3 RISK ASSESSMENTS OF MARINE BIOTOXINS IN BIVALVE MOLLUSKS

Since the year 2000, several international expert groups have undertaken risk assessments of marine algal toxins in bivalve mollusks. In 2001, a Working Group (WG) on Toxicology of diarrheic shellfish

poisoning (DSP) and azaspiracid poisoning (AZP) was appointed by the EU Commission [2]. The WG met in Brussels in May and submitted its recommendations to the EU Commission the same year. In 2003, the Codex Alimentarius Committee on Fish and Fishery Products (CCFFP) asked the Food and Agriculture Organization (FAO) and World Health Organization (WHO) for expert assistance associated with establishment of international safe levels of marine biotoxins in bivalves. As a result, a Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Mollusks was established in 2004 [3]. The Expert Consultation was asked to perform risk assessments and to provide guidance on the methods of analysis and monitoring of relevant algal toxins in bivalves. The Expert Consultation classified the marine biotoxins to be dealt with into eight groups, based on their chemical structure: the azaspiracid, brevetoxin, cyclic imines, domoic acid, okadaic acid, pectenotoxin, saxitoxin, and the yessotoxin group.

In 2005, the EU Commission and the Community Reference Laboratory on Marine Biotoxins appointed another Working Group on Toxicology to give further advice on risk assessment of lipophilic marine algal toxins in bivalves [4].

Since most available data on toxicology concerns acute or short-term studies, and since exposure to marine biotoxins in bivalves generally involves only occasional consumption, priority was given to the establishment of acute reference doses (ARfD) for toxin groups by the Expert Consultation [3]. An ARfD is defined as the estimated amount of a substance in food, in mg/kg body weight, that can be ingested in a period of 24 h or less without appreciable health risk to the consumer on the basis of all known facts at the time of evaluation. ARfDs are established from the NOAELs or LOAELs by dividing by the safety factors.

Risk assessment results in the establishment of ARfDs for individual toxin groups. As the final step in the overall risk analysis, risk management GLs for the different toxin groups are established, taking into consideration the estimated consumption of different food items, and by selecting a level of protection after considering health aspects, in addition to economic and other elements.

The portion size of bivalves is a crucial factor. The choice of consumption level will provide the final protection level of different consumers. This is a difficult question, since data on consumption of mussels are scarce, and indicate wide variations. Since this question has such important influence on the outcome of the regulation, both the Expert Consultation [3] and the EU expert group [4] recommended to apply a portion size of 250 g shellfish meat, in order to protect even the high consumers (an estimated 97.5 percentile). Today, regulation of marine biotoxins in bivalves in both the EU and the United States is based on a serving size of 100 g shellfish meat.

The following categories of shellfish syndromes are described today:

- Paralytic shellfish poisoning (PSP), by the saxitoxin (STX) group
- Amnesic shellfish poisoning (ASP), by the domoic acid (DA) group
- DSP, by the okadaic acid (OA) group
- AZP, by the azaspiracid (AZA) group
- Neurotoxic shellfish poisoning (NSP), by the brevetoxin group

In addition, several groups of marine toxins are detected in shellfish by means of different bioassays, but without proven toxicity toward humans [the Yessotoxin (YTX), pecteontoxin (PTX), and cyclic imines group]. The latter groups will be briefly described in this chapter.

The toxicology and chemistry of the different toxin groups are described in detail in other chapters of this book. Consequently, risk assessments described here are mainly summarized outcomes of the most resent assessments undertaken by international bodies (EU WG 2001, FAO/IOC/WHO Expert Consultation 2004 and EU WG 2005), supplemented by assessments on PSP and AZP by national expert groups from the United Kingdom and Ireland, respectively.

1.3.1 Paralytic Shellfish Poisoning Toxins

Hazard identification: The syndrome PSP has been known for several hundred years [3,5]. The intoxications are associated with the intake of toxins from the STX group that consists of about

20 analogues (for chemistry, see Chapter 9). Traditionally, PSP toxins are divided into three main groups, the carbamates (STX, neoSTX, and GTX1-4), the sulfocarbamoyls (B1-2, C1-4), and the decarbamoyl toxins (dcSTX, dc-neoSTX, and dc-GTX1-4). According to the Expert Consultation [3], the PSP toxins constitute the STX group. The carbamates are the most toxic, the decarbamoyl toxins are slightly less toxic, whereas the sulfocarbamoyls are at least one order of magnitude less toxic in mouse bioassays (MBAs) applying intraperitoneal (i.p.) injections [3].

The main producers of STXs are dinoflagellates from the genus *Alexandrium* that has a worldwide distribution.

Hazard characterization: The STX group toxins are quickly absorbed and distributed via the bloodstream to the target tissues since the symptoms appear after a very short time [3].

The information about metabolism of STX group toxins in the human body is scarce. However, since the sulfocarbamoyls have much lower toxicity compared with the corresponding carbamates, and since they are quite effectively converted to carbamates *in vitro* during heating at low pH (step in the extraction method for the PSP MBA), it is of interest to find out whether similar hydrolysis takes place in the human stomach. According to Harada et al., [6], conversion of B1 to STX in artificial gastric juice for 5 h at 37°C and pH 1.1 and 2.2 was 9% and "not detectable," respectively. Similar experiments by Oshima [3] with C1 and C2 toxin incubated for 4 h at pH 1.6 and 2.2 showed that only 5.5% and 1.5% was converted, respectively. On the basis of this, the Expert Consultation [3] concluded that hydrolysis of the sulfocarbamoyls may not be of significance for human health. Data from humans indicate that the STX toxins are excreted via the urine.

The toxic mechanism of action of the STXs is due to binding to voltage-gated sodium channels on excitable membranes and blocking of the passive inward flux of sodium ions [7]. Even though all STX group toxins occupy the same receptor, their affinity differs greatly [3].

Results from studies in mice [8] indicate that oral toxicity is two orders of magnitude lower, compared with i.p. injections (LD_{50} of 260 versus 10 μg/kg body weight).

Observations in humans: The symptoms are described in three categories of increasing severity [3]:

1. *Mild symptoms:* Tingling sensation or numbness around lips, gradually spreading to face and neck, prickly sensation in fingertips and toes, headache, dizziness, nausea, and vomiting.
2. *Moderately severe symptoms:* Incoherent speech, progression of prickly sensation to arms and legs, stiffness and noncoordination of limbs, general weakness and feeling of lightness and floating, slight respiratory difficulty, rapid pulse, backache as a late symptom.
3. *Extremely severe symptoms:* Muscular paralysis, pronounced respiratory difficulty, choking sensation, high probability of death in absence of artificial respiration.

The first symptoms appear within 5–30 min. In more severe cases, further symptoms develop within 4–6 h, while in severe cases death due to respiratory paralysis takes place within 2–12 h [3]. If the patients survive, there are no reports of late effects.

There are many reports on PSP in the literature. However, the toxin levels associated with the different grades of severity vary considerably. This may partly be due to variable quality of the methods of analysis, whether the analyzed material is the same as that causing the intoxications, and whether the results have accounted for the effect of cooking on the toxin level in the food items consumed.

In order to be able to compare results, toxin levels are converted to μg STX.2HCl equivalents. When MBA has been used, a conversion factor of 0.18–0.20 μg STX.2HCl-eq./mouse unit (MU) is normally applied [3].

From all data available to the Expert Consultation [3], they concluded that persons exhibiting mild symptoms had consumed 2–30 μg STX.2HCl-eq./kg body weight, while more severe cases were associated with intakes of >10–300 μg STX.2HCl-eq./kg body weight.

Risk characterization: The Expert Consultation [3] elected 2 μg STX.2HCl-eq./kg body weight as the LOAEL for PSP. Furthermore, they applied a safety factor of 3, and established a provisional

ARfD of 0.7 μg STX.2HCl-eq./kg body weight for the STX group. The small safety factor was based on the availability of toxicity data from a wide variety of consumers with different susceptibilities, in addition to the reversibility of mild symptoms. The Expert Consultation [3] underlined the need for more information, both from patients and on the effects of processing of shellfish on toxin levels. According to Prakash et al. [9], cooking can reduce the STX group toxicity of contaminated shellfish by as much as 70%. However, the toxins are in part leached into the cooking fluids. Consequently, they may contribute to the total exposure in cases where both shellfish and bouillon are consumed (a mild intoxication was reported in a consumer of both blue mussels and bouillon in Norway in 2005, when the toxin level was about 1600 μg STX.2HCl-eq./kg raw mussels [twice the GL], and the meal was prepared from about 100 g raw mussel meat [Aune unpublished]).

According to the author of this chapter, this illustrates the difficulty in applying terms like LOAEL versus NOAEL and the associated choice of safety factors. If mild symptoms are not associated with truly adverse health effects, their use in establishing ARfD may mislead those who are responsible for risk assessment and management. This issue should be further discussed by experts on risk assessment and safety evaluation.

The Expert Consultation [3] made calculations to show how the established provisional ARfD for the STX group could influence the derived GL in shellfish, depending on three different scenarios for portion size. Selection of GLs is the responsibility of risk managers. If the portion size is maintained at today's 100 g, the safety factor will indirectly be eliminated for the most susceptible consumers if their true intake is about 250 g.

The U.K. Committee on Toxicity (COT) [10] has produced a statement on risk assessment and monitoring of PSP toxins in 2006. On the basis of an evaluation of all available data, and given the limitations regarding data on exposure, the committee concluded that the Expert Consultation [3] approach was reasonable. Furthermore, they noted that the ARfD proposed by the Expert Consultation constitute about one-tenth of the lower end of the dose range associated with severe illness and was therefore unlikely to be overly conservative. The COT members noted that a portion size of 250 g was a reasonable estimate for high-level shellfish consumption in the United Kingdom. Given the acute effects of PSP, they considered it essential to refer to high-level potion size as the comparator in the risk assessment. The committee discussed the current regulatory limit for PSP toxins in shellfish, which is 800 μg STX.2HCl-eq./kg shellfish meat, and that this could result in some individuals consuming greater than the proposed ARfD. The committee agreed that it would be imprudent to conclude that mild cases of PSP had not occurred in the United Kingdom, as they may go unreported, and that the ARfD proposed by the Expert Consultation [3] should be supported. The committee concluded that a PSP toxin concentration of 200 μg STX.2HCl-eq./kg shellfish meat would be the maximum concentration considered to be without appreciable health risk, assuming an adult body weight of 60 kg.

1.3.2 Amnesic Shellfish Poisoning Toxins

Hazard identification: A new type of shellfish poisoning was experienced in Prince Edward Island, Canada, in 1987. More than 100 persons were taken ill upon consumption of blue mussels [11]. Within 24 h, the following symptoms appeared: nausea, vomiting, headache, diarrhea, and abdominal cramps. At least one of the neurological symptoms such as confusion, memory loss, disorientation, seizures or coma, and death were observed within the next few days. The syndrome was named ASP. The causative toxins were DA and analogues (for chemistry, see Chapter 20), and the main toxin producer was the diatom *Nitzschia pungens* f. *multiseries* (later named genus *Pseudonitzschia*). According to the Expert Consultation [3], the ASP toxins constitute the DA group.

DA is the dominating toxin in the DA group, and it can be found in a whole series of bivalves, as well as gastropods, crabs, and lobsters [3].

Hazard characterization: Domoic acid is the major component among the ASP toxins. DA is poorly absorbed from the gastrointestinal tract in both rodents and monkeys. Absorption of DA

in cynomolgus monkeys treated orally for 30 days was 4–7%, and plasma half-life about 2 h [12]. Absorbed DA is excreted in the urine, mostly unchanged, indicating minimal metabolism *in vivo* [13]. Impaired renal function results in significant increases in serum concentrations and residence time of DA, and, according to Expert Consultation [3], this presents additional risk to this group of patients.

The toxic mechanism of action of the DA group is exerted by the activation of glutamate receptors in the central nervous system. DA has high affinity to the kainite receptors, a subclass of glutamate receptors. DA leads to the opening of Na^+ channels, leading to Na^+ influx, inducing depolarization. This results in concomitant Ca^+ ion influx, causing toxic effect and cell death [3].

Results from animal studies indicate that oral toxicity is more than ten times lower than via i.p. injections. There is currently no evidence of cumulative toxicity from repeated exposure studies in experimental animals [3].

Observations in humans: During the ASP episode in Canada in 1987, a total of 107 persons were reported with ASP symptoms. The dominating symptoms were nausea (77%), vomiting (76%), abdominal cramps (51%), headache (43%), diarrhea (42%), and memory loss (25%) [11]. Memory loss was only experienced among persons above the age of 50 years. Three among the oldest patients died after 11–24 days.

Data on the mussel consumption were inadequate for most of those with ASP. According to Todd [11], good quantitative exposure data were available for ten elderly persons (60–84 years). Among these, one person was unaffected after consumption of 15–20 mg DA (0.2–0.3 mg/kg body weight). Mild symptoms were recorded among persons consuming 60–110 mg DA (0.9–2.0 mg DA/kg), while the most serious cases exerting neurological symptoms had consumed 135–295 mg DA (1.9–4.2 mg DA/kg). Hospital records for 16 patients indicated that all seriously ill persons less than 65 years of age had preexisting illness [3].

Risk characterization: On the basis of existing data, the Expert Consultation [3] estimated the LOAEL at 1.0 mg DA/kg body weight. Furthermore, they selected a safety factor of 10 to account for interindividual variability and because of the relatively small number of individuals on which to base the LOAEL. A resulting provisional ARfD of 0.1 mg DA/kg body weight was established by the Expert Consultation [3].

The Expert Consultation [3] stated that there is an urgent need for studies on risk during pregnancy, long-term developmental effects, neurological deficits induced by doses below the acute toxic dose, and toxicity in health-compromised individuals.

If the ARfD of 0.1 mg DA/kg is applied, the corresponding derived GLs in shellfish for different portion sizes would be the following: 60 mg DA/kg shellfish meat (100 g), 24 mg/kg (250 g), and 16 mg DA/kg (380 g).

Compared with the risk assessment by the Expert Consultation [3], today's regulation of 20 mg DA/kg shellfish meat is unnecessarily strict if one maintains a portion size of 100 g, while it is in good accordance with the Expert Consultation recommendation if a portion size of 250 g shellfish meat is selected.

1.3.3 Diarrheic Shellfish Poisoning Toxins

Hazard identification: The first well-described episodes of DSP appeared in the late 1970s in Japan [14] and in the Netherlands [15]. The dominating symptoms were diarrhea, nausea, vomiting, and abdominal pain, and hence the name, DSP. The Japanese scientists found the close correlation between dinoflagellates of the genus *Dinophysis*, consumption of shellfish, and DSP in humans. Intoxications associated with the consumption of shellfish have been described before the 1970s, but in those incidences, the association between shellfish and marine algae was not seen. Today, the number of DSP -toxin-producing algae comprises at least seven species of *Dinophysis*, in addition to several species of the genus *Prorocentrum*. The main DSP toxins are OA, and dinophysistoxin1-2 (DTX-1, DTX-2) (for chemistry, see Chapter 10). OA was first isolated from the sponge *Halichondria okadai*, and hence the name of the toxin group. In addition, fatty acid esters of all three

(chain length between C_{14} and C_{22} with varying degrees of unsaturation), named DTX-3 for simplicity, also appear in shellfish, possibly as metabolites from the three toxins. According to the Expert Consultation [3], the DSP toxins constitute the OA group.

Hazard characterization: Information on the toxicokinetics of the OA group is limited. When mice were given a dose of OA at 90 μg/kg body weight, the highest amount was found in intestinal tissues plus its contents (about 50%), and about 12% in urine. OA was found in all tissues examined (brain, lung, spleen, heart, liver, gallbladder, kidney, stomach, skin, blood, and muscle, in addition to intestines) [3]. Elimination of OA from the intestines was slow, and data show that enterohepatic circulation takes place. The results show little metabolism of OA.

Their mechanism of toxic action of the OA group toxins is via inhibition of serine-threonine protein phosphatase 2A (PP2A), and to some extent, protein phosphatase 1 (PP1) [16,17]. Protein phosphatases play an important role in many regulatory processes in cells, like metabolism, membrane transport, secretion, and contractility [18]. A reasonable explanation of the molecular mechanism leading to the DSP symptoms is that the OA group toxins (OA, DTX1-2, and DTX-3 upon hydrolysis) increase the cellular permeability of intestinal epithelial cells [19]. OA has strong cancer-promoting capacity in experimental systems. An important long-term feeding study with OA to rats has been undertaken [20, 21]; 6-week-old rats were initially treated with the cancer inducer *N*-methyl-*N*-nitro-*N*-nitrosoguanin (MNNG) in drinking water at 2 mg/kg body weight for 8 weeks. One week after termination of inducer treatment, they were fed OA at approximately 10 μg/day in the drinking water for 46 weeks, and thereafter 20 μg/day for another 17 weeks. The number of neoplasia (adenomatous hyperplasias plus adenocarcinomas)-bearing rats were MNNG + OA: 75%; MNNG alone: 46%; OA alone: 0%. The number of adenocarcinomas were 18.8%, 14.3% and 0%, respectively. The doses of OA correspond to approximately 15 μg/kg and 30 μg/kg body weight, respectively. This is equivalent to 900 μg and 1800 μg per person in humans (adult, weighing 60 kg). This indicates that the cancer-promoting potential of OA *in vivo* is very low compared with realistic human exposure.

There are a few reports on genotoxic activity of OA. OA was not mutagenic in the Ames test with *Salmonalla typhimurium*, with or without metabolic activation [22], but mutagenicity was found in Chinese hamster lung cells with diphtheria toxin resistance as a selective marker. However, in this study, the cytotoxicity of OA increased in a dose-dependent manner, and in the dose–response area for mutagenicity, cell death was 45–50%. The cytotoxicity has to be considered when evaluating the results [1]. OA induces micronuclei and blocks mitosis in colonocytes from mice 24 h after a single oral dose between 435 and 610 μg/kg body weight [23].

Traditionally, acute toxicity of OA group toxins is measured by i.p. injections of shellfish extracts in mice. OA and DTX-1 have about the same toxicity (LD_{50} 200 and 160 μg/kg body weight, respectively [3]). In a recent report, the corresponding value for DTX-2 is about 350 μg/kg body weight [24]. According to Yanagi et al. [25], homologues of DTX-3 are slightly less active than OA concerning inducing fluid accumulation in mouse intestinal loops, while their i.p. toxicity in the MBA is markedly reduced. The biological activity of the DTX-3 toxins increases with the degree of unsaturation of the acyl side chain. The LD_{50} of DTX-3 varies, but according to the EC [3], the value is 500 μg/kg body weight.

Observations in humans: Since late 1970s, episodes of DSP have been reported from many countries worldwide: Japan, the Netherlands, Norway, Sweden, New Zealand, the United Kingdom, Belgium, Portugal, and Chile. The symptoms mainly comprised diarrhea, nausea, vomiting, and abdominal pain. The onset was from about 30 min, and usually the symptoms vanished within 2–3 days, with no reported late effects. Unfortunately, precise information on toxin intakes is not available. Furthermore, toxin levels have been measured by a variety of methods. The MBA gives only crude estimates of toxins exerting acute effects via the i.p. route. One MU is the minimum amount of toxin injected necessary to kill two of three mice of 20 g [3]. This is estimated to correspond to 4 μg OA or 3.2 μg DTX-1 [26]. If toxins outside the DSP complex are present, like YTXs, pectenotoxins, or spirolides, they may influence the outcome of the MBA. More precise analytical methods are

developed, mainly high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS), and these methods have been used in association with many of the reported episodes of DSP.

Surveys on victims from DSP episodes in Japan in 1976 and 1977 indicate that the minimum amount of toxin to induce DSP symptoms is 12 MU [2], which corresponds to approximately 48 μg OA-equivalent. During the grand opening on a new shellfish farm in Norway in September 1999, 39 of 72 were taken ill with DSP symptoms. Those present were offered several dishes containing local blue mussels. HPLC analysis of leftovers shows a toxin level of 55–65 μg OA-eq./100 g shellfish meat [1]. Even though precise information on how much shellfish the participants consumed is lacking, a crude estimate indicates that those intoxicated were exposed to at least 1–1.5 μg OA-eq./100 g body weight.

In the summer 2002, about 200 persons in southern Norway were taken ill with DSP symptoms associated with the consumption of brown crabs (*Cancer pagurus*). The crabs were caught in the surface. Analysis of crabs by LC-MS showed very low levels of OA and DTX 1 and DTX-2, but high levels in OA-eq./100 g brown meat (digestive gland) after hydrolysis. The crabs had acquired the toxins as a result of feeding on the abundant numbers of blue mussels in shallow waters, and transformed the DSP toxins, mainly OA, to DTX-3 [27]. Analysis of leftovers from crab meals causing DSP indicated DTX-3 levels corresponding with 1050–1500 μg OA-eq./100 g brown meat. If one assumes consumption of two to three crabs of 500 g fresh weight, the DTX-3 level causing DSP symptoms is estimated at 75–150 μg DTX-3 as OA-equivalents [28].

Risk characterization: According to the European WG in 2001 [2], the lowest observed effect level derived from data from Japan and Norway is in the range 48–65 μg OA-eq./100g (minimum 0.8 μg/kg body weight, assuming adults weighing 60 kg). By applying a safety factor of 3, the WG suggested an allowance level of DSP toxins of 0.27 μg/kg body weight as OA-equivalents (equals 16 μg/person). Furthermore, the WG assumed a portion size of 100 g for mussels and other species of shellfish, and stated that if the concentration of these toxins in shellfish is not exceeding 160 μg/kg, there is no appreciable health risk.

The FAO/IOC/WHO Expert Consultation [3] concluded that, based on existing human data, the LOAEL for DSP is 1.0 μg OA-eq./kg body weight, and applied a safety factor of 3, deriving at a provisional ARfD of 0.33 μg OA-eq./kg body weight. The size of the safety factor was chosen because of the relatively large number of persons involved, and because DSP symptoms are readily reversible. According to the Expert Consultation [3], consumption of 250 g shellfish meat (recommended portion size to protect even large consumers) would lead to a derived GL of 80 μg OA-eq./kg shellfish meat. The risk assessment is closely similar to that of the EU WG in 2001 [2], while the choice of portion size differs significantly.

The EU WG in Cesenatico in 2005 [4] agreed with the conclusions from the Expert Consultation [3] concerning both ARfD and the preferred portion size of 250 g shellfish meat.

In the opinion of the author of this chapter, the size of the safety factor of 3 seems reasonable, since the effects are primarily exerted by OA and analogues in the gastrointestinal tract, as a result of inhibition of protein phosphatises (mainly PP2A). Also, a portion size of 250 g shellfish meat is recommended when establishing the GL, since the goal is to protect the majority of consumers.

1.3.4 Azaspiracid Poisoning Toxins

Hazard identification: In November 1995, consumers in the Netherlands were taken ill with symptoms similar to DSP after eating blue mussels from Killary Harbour, Ireland. Since the level of DSP toxins was low, a hitherto unknown group of marine biotoxins, the azaspiracids, was discovered [29], and their structure elucidated [30]. There have been another four outbreaks of AZP, all due to consumption of blue mussels from Ireland [31]. The AZA group comprises several analogues, but AZA1-3 seems to make up the most important ones (for chemistry, see Chapter 35). The AZAs are

detected in seafood in several European countries, but there is still uncertainty about the producer of this toxin group, even though the dinoflagellate *Protoperidinium crassipes* has been suggested.

Hazard characterization: Data on toxicokinetics for the AZA group are lacking, mainly due to lack of availability of pure toxins. Also, information on the mechanism of action is scarce, but they do not inhibit protein phosphatise 2A, which is the main mechanism of DSP toxins [3]. Results from *in vitro* studies have shown multiple effects like cytoskeleton disruption [32], increased levels of cytosolic calcium and cAMP [33,34], and cytotoxicity towards multiple cell types [3].

The acute toxicity of AZAs in mice is as per the following:

Lethal dose i.p.
AZA-1: 200 μg/kg body weight [30]
AZA-2: 110 μg/kg [35]
AZA-3: 140 μg/kg [35]

Acute lethal dose orally for AZA-1 was 250–450 μg/kg, depending of age of the mice [36].

In animal studies, AZAs induce damage to the gastrointestinal tract, liver, and lung [36,37]. From long-term feeding studies in mice, there were indications of tumorigenicity in the lung, but there was no clear dose–response relationship, and the results cannot lead to conclusions concerning carcinogenic potential.

Observations in humans: The symptoms in humans after the AZP episodes in the Netherlands, and in a few other European countries associated with consumption of blue mussels from Ireland, were nausea, vomiting, severe diarrhea, and stomach cramps, similar to those associated with DSP [31,38].

Leftovers of blue mussels from Killary Bay were collected and tested by the rat bioassay for DSP and gave strong positive results. Later, mussels from Killary Harbour were tested for DSP, PSP, and ASP toxins, but the levels of these toxins were at trace amounts or were not detectable. Six months later, mussels from the same location were tested for AZAs and found to contain large amounts of AZA-1 (1.14 mg/kg whole shellfish meat), 230 μg/kg AZA-2, and 60 μg/kg AZA-3 [31]. Unfortunately, there is no information on shellfish consumption.

The next AZP episode took place in Ireland in September/October 1997 upon consumption of blue mussels from Arranmore Island, Ireland. About 20–24 individuals were affected, and eight of them consulted a physician. All patients made a complete recovery after 2–5 days [31]. The best estimate of the concentration of AZA toxins in mussels from Arranmore sampled 1–2 months later was 1.36 mg/kg raw whole shellfish meat. This AZP episode is the best documented, and has been used for estimates of dose–response relationships (they assumed that the concentration of AZAs was reduced during cooking). From this, the Irish expert group made a best estimate of the total concentration of AZAs ingested by one patient with symptoms after consumption of 9.1 μg AZA.

The EU WG in 2001 [2] reevaluated the exposure data, based on new information on the heat stability of the AZAs. The recalculated intake estimates were between 23 μg and 86 μg AZA/person. The same numbers for AZAs causing AZP were used by the Expert Consultation [3].

In a second risk assessment of AZAs by the Food Safety Authority, Ireland [38], new data on AZAs, both concerning distribution in the shellfish, ratios of different AZA analogues, and effect of cooking on AZA levels [39], formed the basis for a reevaluation. According to these estimates, the AZA levels believed to have caused AZP during the Arranmore incidence were between 50.1 μg and 253.3 μg/person.

Risk characterization: The first risk assessment from Ireland [38] concluded that a level of 100 μg AZA/kg raw shellfish meat does not cause AZP.

The European WG 2001 [2] calculated the LOEL of AZAs to be between 23 μg and 86 μg/ person. They suggested a safety factor of 3 to include allowances for individual variation in susceptibility. On the basis of this, they concluded that a level of 80 μg AZA/kg shellfish should result in no

appreciable health effects. They added that the risk assessment was based on several assumptions and therefore cannot be certain that this represents the true LOEL. However, when the WG gave its recommendation for a regulatory limit for AZAs, a regulatory level of 160 μg AZA per kg shellfish meat was proposed in order to allow for detection by an MBA. Furthermore, the WG said that when standards become available, LC-MS should be used to control AZAs, and the limit of 80 μg/kg should be reevaluated when new toxicological data becomes available.

The Expert Consultation in 2004 [3] also used 23 μg AZA per person as the LOAEL. Because of the small number of people involved in the episodes, a safety factor of 10 was used to calculate an ARfD of 0.04 μg/kg body weight (2.4 μg per adult), assuming a body weight of 60 kg. The derived GL of AZAs in shellfish, depending on the portion size, would be 24 μg/kg (100 g), 9.6 μg/kg (250 g), and 6.3 μg/kg (380 g).

The EU WG in 2005 [4] agreed with the choice of the lowest LOAEL of 23 μg per person in accordance with the two previous international risk assessments [2,3]. Owing to the lack of reports on AZP in recent years in spite of marketing of considerable quanta of mussels with AZA at levels close to the current EU regulation of 160 μg/kg, a safety factor of 3 was suggested. The resulting ARfD is 8 μg AZAs per person, in correspondence with the recommendation from the EU WG in 2001 [2]. However, the EU WG in 2005 [4] recommended use of the more appropriate portion size of 250 g instead of 100 g, for deriving a GL of AZAs in shellfish at 32 μg/kg shellfish meat.

In 2006, the Food Safety Authority, Ireland, undertook a reevaluation of the risk assessment of AZAs [38]. Owing to the most recent information about distribution (AZAs accumulate in the digestive gland), effects of cooking (steaming of raw mussels result in a twofold increase of AZAs), and ratios of AZA analogues in mussel [39], the revised LOAEL associated with AZP is estimated at between 50.1 μg and 253.3 μg/person, with a median of 113.4 μg/person. The Food Safety Authority of Ireland (FSAI) applied a safety factor of 3. The size of the safety factor is based on the following: The interindividual safety factor of 10 is subdivided into toxicokinetics (factor 3.2) and toxicodynamics (factor 3.2) [40]. Since the AZAs exert toxicity mainly in the gastrointestinal tract, and it is assumed that metabolism of the toxins is not necessary for toxicity, a safety factor of 3 is appropriate. Furthermore, they applied the median LOAEL (113.4 μg/person) instead of the lowest LOAEL (50.1 μg/person) in calculating the ARfD of 0.63 μg/kg bw (38 μg/person). The derived GLs for AZAs in shellfish with a recommended portion size of 250 g would be 151 μg/kg shellfish meat (and 378 μg/kg for a consumption of 100 g).

If one applies the same principle as in the three international risk assessments, one should use the lowest LOAEL for calculating the ARfD. With a safety factor of 3, this would give an ARfD of 16.7 μg/person or 0.28 μg/kg bw (assuming adults weighing 60 kg). The derived GLs of AZAs in shellfish would be 66.8 μg/kg (250 g) or 167 μg/kg (100 g).

1.3.5 Neurotoxic Shellfish Poisoning Toxins

Hazard identification: Neurotoxic shellfish poisoning (NSP) has primarily been reported from the Gulf of Mexico, the southeastern coast of the United States, and from New Zealand [3]. The syndrome is associated with consumption of bivalves that have accumulated the so-called brevetoxins from primarily dinoflagellates of the genus *Karenia*. In addition, brevetoxins are also reported associated with rhaphidophytes (*Chattonella* spp.). The brevetoxins are cyclic polyethers, grouped according to their backbone structure into types A and B (for chemistry, see Chapter 24 or 25). The most abundant type A-toxins are PbTx-1 and PbTx-7, while the principal B-types are PbTx-2, PbTx-3, and PbTx-9 [41]. PbTx-2 is the most abundant analogue from *Karenia brevis*. In oysters, B-type toxins appear in much higher concentrations than toxins from the A-type. Brevetoxins are metabolized to some extent in shellfish, and the metabolites may contribute to the toxicity.

The NSP symptoms are nausea, diarrhea, and abdominal pain, in addition to neurological symptoms like paresthesia beginning within minutes to hours after consuming shellfish. In addition, inhalation of aerosols containing brevetoxins may induce reversible upper respiratory syndrome [3].

Hazard characterization: Animal studies show that brevetoxins are rapidly absorbed from the gastrointestinal tract and are distributed in the whole body. When rats were exposed orally for sublethal doses of PbTx-3, the highest concentration was found in the liver up to 8 days later [42]. Excretion was about the same in urine and feces.

Their mechanism of toxic action is via binding to the alpha-subunit of voltage-gated sodium channels. The result is depolarization of neuronal membranes due to increased influx of sodium ions [43].

The acute toxicity of two of the most important brevetoxins in mice is as per the following [44]:

1. Oral LD_{50}: PbTx-2: 6600 µg/kg body weight; PbTx-3: 520 µg/kg
2. Intraperitoneal LD_{50}: PbTx-2: 200 µg/kg; PbTx-3: 170 µg/kg

Information on possible subchronic or chronic toxicity including carcinogenicity of the brevetoxins is lacking [3].

Observations in humans: Neurotoxic shellfish poisoning (NSP) associated with consumption of shellfish comprise oysters and clams, in addition to other filter feeders [3]. Those intoxicated, developed symptoms like nausea, diarrhea, and abdominal pain, in addition to one or several neurological symptoms (paresthesia, vertigo, and lack of coordination). The onset of NSP symptoms is between minutes and a few hours. There are no reports of fatalities associated with NSP, but some patients have needed respiratory support. The NSP symptoms are reported to resolve few days after the intake of infested shellfish.

During an episode of NSP in North Carolina in 1987, about 50 persons were taken ill following consumption of cooked and raw oysters. According to Morris et al. [45], 23% reported gastrointestinal symptoms and 39% reported neurological symptoms. The symptoms had rapid onset and lasted for only few days. Unfortunately, there are no quantitative data on the levels of brevetoxins associated with NSP. According to Expert Consultation [3], one NSP episode was associated with the consumption of 100–150 g clams at 120 µg PbTx-3 eq./100 g, indicating an intake of 2–3 µg PbTx-3 eq./kg body weight, but other brevetoxins may have contributed to the symptoms.

Risk characterization: Brevetoxoins were evaluated by the FAO/IOC/WHO Expert Consultation [3]. However, the Expert Consultation decided that there are currently insufficient data to complete a risk assessment and establish an acute reference dose for brevetoxins. There are uncertainties about the true exposure for the different analogues and metabolites of brevetoxins associated with the reported NSP episodes.

The brevetoxins have not been subject to risk assessment by European expert groups so far.

Today, NSP toxins are regulated in the United States and a few other countries, based on i.p. injections in mice (the so-called APHA protocol). The regulatory level in shellfish is 20 MUs/100 g shellfish meat, which equals 80 µg PbTx-2 equivalents [3].

1.3.6 Brief Description of Groups of Shellfish Toxins without Proven Effect toward Humans

1.3.6.1 The YTX Group

Yessotoxins (YTXs) were discovered in shellfish extracts together with toxins from the OA group in 1987 [46]. Since the YTXs contribute to the outcome of the MBA for DSP toxins, they were initially included in the DSP toxin complex. The main producer of YTXs is the dinoflagellate *Protoceratium reticulatum*. YTXs are detected in bivalve mollusks in many parts of the world, like Japan, New Zealand, Australia, Canada, Norway, Italy, and the United Kingdom [3]. Many analogues of YTX are described, and new ones are frequently reported.

Data on acute i.p. toxicity are available for YTX and eight analogues [3] (for chemistry, see Chapter 13). The LD_{50} or lethal dose values for YTX varies between 100 and 750 µg/kg body weight. This is partly explained by possible differences in susceptibilities between mouse strains

and gender, but, in addition, purity and stability of the toxin molecule may play a role. The i.p. toxicity of the other analogues for which information is available, is in the same range. However, relative toxicities of the analogues compared with YTX cannot be established with precision, since the reported LD_{50} for YTX varies so much.

YTX is much less toxic via the oral route. This is explained by low absorption from the gastrointestinal tract and that the gastrointestinal tract is not the target organ of YTX toxicity. In a short-term study, mice were treated by gavage 7 times within 3 weeks with YTX at 1, 2.5, or 5 mg/kg body weight [47]. No pathological changes were recorded in the animals even at the highest dose.

YTX appears to affect isolated cells by multiple mechanisms [3]. However, the YTX group toxins are not inhibitors of protein phosphatases at realistic dose levels, and they do not induce diarrhea.

There are no reports of human intoxications associated with intake of YTXs [3].

Risk assessments: The European WG on Toxicology in 2001 [2] concluded that the YTX group does not belong to the DSP complex. A NOAEL for YTX by single oral administration (gavage) was estimated at 1 mg/kg body weight based on a recent study [48]. Because of the lack of information on repeated administration of toxin and a high risk factor recommended by WHO for substances that injure cardiac muscles, a safety factor of 600 was chosen. By using the traditional portion size of 100 g shellfish meat, the WG [2] recommended an allowance level of 1 mg YTX-eq./kg shellfish meat. This is today's regulation in the European Union.

The FAO/IOC/WHO Expert Consultation in 2004 [3] applied the most recent data from repeated oral gavage of YTX, indicating a NOAEL at 5 mg/kg body weight [47], and used a safety factor of 100, establishing a provisional ARfD of 50 µg YTX-eq./kg body weight. The derived GL in shellfish would be 12 mg YTX-eq./kg for the recommended portion size of 250 g.

The EU WG in 2005 [4], came to the same conclusion as the Expert Consultation [3] concerning the NOAEL from the animal study with repeated oral exposure [47]. They used the same safety factor of 100, and established an ARfD at 50 µg /kg body weight. Since the derived GL is very high (12 mg/kg shellfish meat at the recommended portion size of 250 g) compared with reported levels of YTXs in shellfish, the WG [4] alternatively recommended deregulation of the YTX group.

The YTXs have contributed to closures of harvesting in several countries (Italy, Japan, Norway) on many occasions due to today's regulatory level (1 mg YTX-eq./kg in the EU). According to the recent risk assessments, the GL for YTX group toxins is far too strict.

1.3.6.2 The PTX Group

Like the YTXs, the pectenotoxins were discovered when using the MBA for DSP toxins on shellfish extracts. The PTXs always appear together with toxins from the OA group since they are produced by the same dinoflagellate genus, *Dinophysis*. PTXs are detected in dinoflagellates and/or bivalves in several parts of the world, like Japan, New Zealand, Australia, Portugal, Spain, and Norway [3].

The main pectenotoxin in the dinoflagellates is PTX-2, while several analogues are detected in shellfish, presumably due to metabolism [49,50] (for chemistry, see Chapter 16). According to the Expert Consultation [3], the i.p. toxicity of the most important PTXs, PTX-2, PTX-1, PTX-3, and PTX-11 is in the range 219–411 µg/kg body weight, while similar value for PTX-6 is 500–770 µg/kg. The liver appears to be the main target organ upon i.p. injections of PTX-2 and analogues. PTX-2 is readily converted to PTX-2 seco acid (PTX-2 SA) by enzymes in the hepatopancreas of shellfish [51]. The i.p. toxicity of PTX-2 SA is much lower, compared with PTX-2 (>5000 µg/kg body weight).

The information on the acute toxicity of PTX-2 and analogues in mice by gavage is conflicting, but according to the Expert Consultation [3] it is much lower than their i.p. toxicities (>5000 µg/kg body weight). Studies suggest that PTX-2 and PTX-2 SA are poorly absorbed from the gastrointestinal tract.

Little information is available on the mechanism of toxic action of PTXs but they do affect the cytoskeleton [2]. However, they are not inhibitors of protein phosphatases, and, according to the

Expert Consultation [3], they do not induce diarrhea, in spite of a few studies reporting such effects in experimental animals.

There are no reports proving toxicity in humans associated with exposure to toxins from the PTX group (EC 3).

Risk assessments: When pectenotoxins were evaluated by the WG on Toxicology in 2001 [2], some of the information from experimental studies in mice available to the WG indicated that oral toxicity of PTX-2 was almost at the same level as via the i.p. route [52]. On the basis of this, the LOAEL of PTX-2 orally was estimated at 250 μg/kg body weight. A factor of 10 was applied to convert from LOAEL to NOAEL. From this value (NOAEL 25 μg/kg body weight), a safety factor of 100 was applied to reach a safe exposure level for man, at 0.25 μg/kg body weight. Consequently, a derived allowance level of 15 μg/kg shellfish meat was recommended, assuming adults weighing 60 kg. However, since the MBA was the only method of detection of PTXs at that time, it was concluded that PTXs and OA group toxins would be controlled together using a combined level of 160 μg/kg as OA-equivalents. It was stated that as soon as other methodologies became available, this limit should be reevaluated [2].

According to the Expert Consultation in 2004 [3], the pectenotoxins are considered far less toxic orally compared with the i.p. route (>5000 μg/kg orally against 219–411 μg/kg i.p.), even though the data are conflicting. Owing to the lack or data on repeated oral exposure, the Expert Consultation concluded that there was insufficient data from animal studies to establish an ARfD for the PTX group. However, based on a crude estimate of human exposure to PTX in shellfish (estimated intake level 200 g shellfish meat) from Canada and Norway (0.6 and 1.6 μg PTX-2-eq./kg body weight, respectively), they found that the MOE between humans and toxicity in mice by gavage is on the order of 3100–8000 times.

Pectenotoxins were also evaluated by the European WG in Toxicology in 2005 [4].

The experts were presented with the deviating results from Japan and New Zealand on oral toxicity of PTXs. According to the results from New Zealand, oral administration of PTX-2 and PTX-2 SA at 5000 μg/kg body weight had no toxic effect. In contrast, results from Japan indicated induction of diarrhea in mice receiving 2000 μg/kg PTX-2 by gavage. Furthermore, histological changes were observed in stomach, liver, lung, and kidneys at 1500 μg/kg. The NOAEL from this study was 300 μg/kg body weight.

According to the EU WG in 2005 [4], the reason for these different findings should be further investigated. In the meantime, the WG participants felt they should apply the worst-case scenario, based on an oral NOAEL of PTX-2 at 300 μg/kg body weight. By applying a safety factor of 100, and assuming a portion size of 250 g shellfish meat, the WG recommended a GL for PTXs at 720 μg PTX-2-eq./kg shellfish meat. Because of low toxicity, PTX-8, PTX-9, PTX-10, and the seco- acids should be excluded from regulation.

Owing to the high acute i.p. toxicity of PTXs, and their appearance together with toxins from the OA group, they may result in false positives in the MBA. This MBA was originally established in Japan for control of DSP toxins. From a scientific point of view, PTXs should not be recorded as OA-equivalents since the term toxic equivalents is useful for summarizing the total toxic potential of analogues within a group of toxins with similar mechanisms of action, and OA and PTX group toxins act via different mechanisms.

If PTX group toxins are regulated in the future, a GL in PTX-equivalents should be established, based on an estimated ARfD.

1.3.6.3 The Cyclic Imine Group

The cyclic imine group includes several toxins, like spirolides, gymnodimin, pinnatoxins, pteriatoxins, and others (EC 2004) (for chemistry, see Chapter 26). Toxins from this group were discovered owing to their very high acute toxicity in the MBA for DSP toxins. They are fast acting, leading to death within minutes upon i.p. injections. Few data are available on the toxicokinetics of these

toxins. However, it seems that they are rapidly excreted or metabolized to less toxic compounds since rapid recovery is seen in mice following sublethal doses [53,54].

The i.p. LD_{50} of gymnodimin is about 100 μg/kg body weight, while similar value by gavage is about 755 μg/kg, and by feeding >7500 μg/kg [55].

There are several groups of spirolides. By far the most toxic is desmethyl spirolide C. Its LD_{50} value is 5–8 μg/kg by the i.p. route. Via gavage, its LD_{50} is about 160 μg/kg and for exposure via the feed it is about 500 μg/kg in fasted mice and 1000 μg/kg in nonfasted mice [53]. Less information is available about the other cyclic imines.

For those toxins for which data on toxicity in animals is available, the symptoms of intoxication are similar, with death due to respiratory arrest.

Little information is available on chronic toxicity of cyclic imines [3].

The imine function is essential for the toxicity of the cyclic imines. According to Hu et al. [56], the imine ring is easily opened at low pH or upon enzymatic hydrolysis. Since the pH in the human stomach is much lower than in mice (pH 1–3 compared with 3–5), it is speculated that spirolides may undergo hydrolysis to less toxic compounds in humans.

No information of harmful effects is documented on human exposed to gymnodimin, spirolides, or pinnatoxins in shellfish [3].

Risk assessments: The Expert Consultation [3] considered that the database was insufficient to establish an acute reference dose for cyclic imines. However, the Drafting Group for cyclic imines to the Expert Consultation suggested ARfDs based on LD_{50} from feeding experiments in mice; For gymnodimin the NOAEL by feeding was >7500 μg/kg body weight. By applying a safety factor of 100, an ARfD at 75 μg/kg body weight was suggested; For desmethyl spirolider C, the LD_{50} by feeding was 500 or 1000 μg/kg body weight, depending on whether the mice were fasted or not before toxin exposure. The Drafting Group considered nonfasted mice the most relevant to humans, and by applying a safety factor of 100, an ARfD at 10 μg/kg was suggested.

The cyclic imines were also evaluated by the European WG in 2005 [4]. For gymnodimin, a NOAEL from acute toxicity of gymnodimin by feeding at 7500 μg/kg [55] was used, and by applying a safety factor of 100, an ARfD at 75 μg/kg body weight was suggested. Considering a portion size of 250 g shellfish meat, and an adult weighing 60 kg, the derived GL would be 18 mg gymnodimin/kg shellfish meat. However, in view of the high GL and the absence of problems in humans consuming shellfish with gymnodimin, the WG agreed that gymnodimin should not be regulated, or, alternatively, at 18 mg/kg shellfish meat.

For the spirolides, the WG used the level 500 μg/kg body weight of desmethyl spirolide C from feeding experiments [53]. Owing to the high acute toxicity of this group, a safety factor of 300 was applied, reaching an ARfD at 1.67 μg/kg body weight. The derived GL in shellfish, assuming a portion size of 250 g and an adult weighing 60 kg, is 400 μg/kg shellfish meat. The WG agreed to recommend regulation of spirolides at this GL to the European Union.

Data on differences in acute toxicity between gavage and feeding are only available for the very acutely toxic cyclic imines. According to Munday et al. [53,55], there is evidence that the high estimate of the acute toxicity by gavage of rapidly absorbed, fast-acting toxins is an artifact. This important issue should be examined further, including less rapidly acting toxins.

1.4 FINAL REMARKS

Traditionally, MBAs have been used for controlling levels of marine algal toxins except for the DA group. The intension has been to protect the consumers, and this seems to work reasonably well for the STX group since they all act via the same mechanism, blocking of the voltage-gated sodium channel in cellular membranes, even though the potency of the different analogues varies greatly. For the heterogeneous lipophilic toxins, the picture is more complicated, since it comprises several toxin groups with differing mechanisms of toxic action. Some groups exert primarily toxicity in the gastrointestinal tract (OA- and AZA-group toxins), while other groups act in other organs, and,

consequently, depend on toxicokinetic factors like absorption and distribution in order to induce intoxications via normal exposure through food (PTX, YTX, cyclic imine groups). In the latter case, an MBA, based on i.p. injections, will very often overestimate the toxicity in humans.

However, when the methods of analysis are shifted from MBAs to alternative methods, there is need for information about relative toxicities (TEFs) of all toxic analogues within each toxin group. With that information, the total toxicity of each toxin group can be expressed in equivalents (TEQs) by the "signature" toxin for the group, STX-eq., OA-eq., AZA-1-eq., and so on. For analogues where this information is lacking, a relative toxicity of 1 may be applied in the interim.

Today, most information on relative toxicities is based on i.p. injections in mice. It is important to gain information on whether similar relative toxicities exist by the oral route.

Another important issue is whether mixtures of toxins from different groups interact in the consumer if they are present simultaneously or within a short time span. The MBA for lipophilic toxins is not useful for evaluating possible interactions between different toxin groups since the exposure is via the i.p. route. Consequently, experimental studies are urgently needed to address the question of possible interactions between toxin groups via the oral route. During the European WG in Cesenatico 2005 [4], Professor Takeshi Yasumoto referred to preliminary studies with oral exposure of mice to combinations of OA and PTX-2, indicating enhanced toxicity. Similar experiments are ongoing in Italy, concerning OA and YTXs. At our laboratory in Oslo, in collaboration with Marine Institute, Ireland, and AgResearch, New Zealand, studies have started on possible interactions between the AZA group and YTX- and PTX-toxins. One important issue is to study whether toxins damaging the gastrointestinal tract (OA and AZA group toxins) may enhance the absorption of toxins like YTXs and PTXs by changing the toxicokinetics, rendering them toxic via the oral route as well.

If results from studies of combined exposures document additive or synergistic toxicity, this has to be considered when establishing GLs for marine biotoxins in the future.

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2 Analysis of Marine Toxins—Techniques, Method Validation, Calibration Standards, and Screening Methods

Patrick T. Holland

CONTENTS

2.1 INTRODUCTION

Marine toxins pose significant threats to human health through contaminated seafood. Accumulation of toxins in molluscan shellfish from hazardous algae is well researched, and other seafood such as reef fish, crabs, and tunicates can also be affected. Particular phytoplankton blooms can directly affect humans exposed to toxic algae through swimming or aerosols, or cause severe ecological

disruption through widespread killing of sea life including marine mammals. Management systems are in place in most countries to reduce risks to consumers of seafood, and regulations have been promulgated governing maximum acceptable levels of some toxins. Monitoring to enforce the regulations is generally based on regular coastal samplings with testing of water for toxic phytoplankton and testing of seafood (generally shellfish) for marine toxins.

A wide diversity of marine toxins have been discovered, but the ones of major significance to seafood belong to ten classes. Table 2.1 summarizes these toxin groups, their causative algae, and the most widely used classes of test methods. More detailed reviews of the chemistry, analysis, toxicology, and occurrence of these toxins are available in a Food and Agricultural Organization (FAO) monograph on marine biotoxins[1] and the reports from a joint FAO/IOC/WHO expert consultation held in Norway in 2004.[2]

Before 2004, regulatory testing internationally was almost exclusively based on small animal bioassays. The exception was for the domoic acid (DA) group where liquid chromatography-ultraviolet absorption (LC-UV) methods were well accepted. Although a wide variety of alternative methods have been developed for marine biotoxin analysis, in practice, most of these methods remain research tools that cannot meet the often rigid criteria set by regulatory authorities. However, there is widespread recognition that a high degree of reliance on animal bioassays is unsatisfactory. The deficiencies can be summarized under the following headings (the 4-Ss):

- *Sensitivity:* Detection limits are at, or even above, regulatory limits. False negatives are common, although often not recognized. No early warning.
- *Specificity:* Causal toxins cannot be identified and individually quantified. False positives are also common.
- *Speed:* Protocols for lipophilic toxins are lengthy, resulting in low sample throughput and slow reporting.
- *Sustainability:* Killing of animals for routine QC of food is unethical if alternatives exist.

Criticisms of mouse bioassays have been greatest for the diarrhetic shellfish poisoning (DSP) protocols.[3,4] The Association of Official Analytical Chemists (AOAC) paralytic shellfish poisoning (PSP) assay for saxitoxins and American Public Health Association (APHA) neurotoxic shellfish poisoning (NSP) assay for brevetoxins also have limitations with respect to their sensitivity, specificity, sustainability, and validation. There is wide-ranging debate within the European Union (EU) on the status of these assays in relation to the 3-R goals of Reduction, Refinement, and Replacement of animal testing.[4,5] These animal assays are screening tests that cannot have the status of reference methods within the hierarchy established for regulatory methods by Codex Alimentarius.[6] The technical and regulatory constraints in regard to marine biotoxins remain profound, with only slow progress being made toward alternative methods being fully accepted into national legislations. Many marine biotoxin-monitoring programs now make use of instrumental or immunoassay methods, but the formal clearance of food lots generally remains based on the results of animal bioassays.

The EU and the United States have strongly influenced international regulation of marine biotoxins because of their importance as major producers and importers of seafood, and through the comprehensive nature of their food safety legislations. The EU legislation governing testing for marine biotoxins[7] is rather prescriptive in nature, with the details of required test methods and protocols being specified. This is in contrast to other areas of food testing such as veterinary drug residues where the EU requirements for regulatory methods are for performance-based criteria to be met. The report of the joint ECVAM/DG SANCO Workshop held in Italy in 2005[6] provides a summary of the EU regulatory framework and the key pieces of current legislation governing bivalve mollusks. The wide-ranging recommendations from this meeting reflect the urgency of the task to replace animal bioassays and the many technical issues that must be addressed. In the United States, the AOAC International through its official methods program has set many standards for methods used internationally for food testing, including those for marine biotoxins. In 2004, AOAC

TABLE 2.1
Marine Toxin Classes—Sources, Characteristics, and Methods of Analysis

Toxin Class	Main Algal Sources	Main Toxins in Seafood	Characteristics	Methods of Analysis in Seafood
Azaspiracids	Not fully established. Possibly *Protoperidinium crassipes*	Azaspiracid-1 (AZA-1), AZA-2 and AZA-3	Cyclic amine with high acute toxicity; possibly chronic. Cytotoxic and neurotoxic	LC-MS
Brevetoxins	*Karenia brevis* and possibly some other *Karenia* and raphidophyte species	PbTx-1, PbTx-2, PbTx-3 metabolites: BTX-B1, BTX-B2, deoxyBTX-B2, BTX-B4, BTX-B5	Neurotoxic shellfish poisoning (NSP) toxins. Sodium channel activators. Complex metabolism in shellfish	Mouse bioassay receptor binding assay LC-MS ELISA
Ciguatoxins	*Gambierdiscus toxicus*	Caribbean ciguatoxins C-CTX-1, C-CTX-2 Pacific ciguatoxins P-CTX-1, P-CTX-2, P-CTX-3	Ciguatera fish poisoning toxins. Potent sodium channel activators. Complex pattern of metabolites in fish, dependant on region and species	Mouse bioassay Neuroblastoma cytotoxicity assay Receptor binding assay LC-MS ELISA
Domoic acid	Species of *Pseudo-nitzschia* and *Nitzschia*	Domoic acid (DA) Epidomoic acid Range of less common or minor isomeric forms	Amnesiac shellfish poisoning (ASP) toxins	LC-UVD LC-MS ELISA
Gymnodimines	*Karenia selliformis*	Gymnodimine (GYM), GYM-C	Cyclic imine acting at nicotinic AcH receptor. Low oral toxicity	LC-MS
Okadaic acids	*Dinophysis* species *Prorocentrum* species	Okadaic acid (OA) Dinophysis toxin 1 (DTX-1), dinophysis toxin 2 (DTX-2), 7-acyl esters of the above	Diarrhetic shellfish poisoning (DSP) toxins. Inhibitors of protein phosphatases (after hydrolysis of ester forms)	Mouse bioassays Rat bioassay LC-FL (derivatization) LC-MS ELISA Protein phosphatase inhibition (PPIA)
Pectenotoxins	*Dinophysis* species	Pectenotoxin-2 (PTX-2) PTX-11, PTX-12 toxic metabolites in scallop: PTX1, PTX-3, PTX-6	Commonly grouped with DSP toxins but have a different mode of action through actin	Mouse bioassays LC-MS

Continued

TABLE 2.1
(Continued)

Toxin Class	Main Algal Sources	Main Toxins in Seafood	Characteristics	Methods of Analysis in Seafood
Saxitoxins	Several *Alexandrium* species. *Gymnodinium catenatum*, *Pyrodinium bahamense*	Saxitoxin and a wide range of sulfated, *N*-sulfo-carbamoyl, decarbamoyl and other analogues. Proportions very dependent on algal species and shellfish species	Paralytic shellfish poisoning (PSP) toxins. Sodium channel blockers with widely varying potencies between analogues	Mouse bioassay LC-FL (pre- or post-column oxidation) Immunoassays Receptor binding assay
Spirolides	Strains of *Alexandrium ostenfeldii*	Spirolide-C (SPX-C), SPX-D, desmethylSPX-C and other analogues. Proportions of different SPXs depends on strain	Cyclic imines acting at nicotinic AcH receptor. Relative potency and oral toxicity not established	LC-MS
Yessotoxins	*Protoceratium reticulatum* *Gonyaulax polyhedra* *Gonyolaux spinifera*	Yessotoxin (YTX), Homoyessotoxin (homoYX) 45-hydroxyYTX, 45-hydroxyhomoYTX, carboxyYTX A wide range of other analogues and metabolites	YTX induces caspase and phosphodiesterase activities Toxicology uncertain, especially for oral route and for analogues and metabolites	LC-FL (derivatization) LC-MS

established an International Task Force on marine and freshwater toxins with specific goals to advance new methods of analysis toward official acceptance through validation and collaborative studies.[8] This has resulted in methods for saxitoxins by LC and DA by enzyme-linked immunosorbent assay (ELISA) recently receiving AOAC first action status following evaluation of data from collaborative studies,[9,10] the first such new methods for marine biotoxins to be accepted by AOAC for many years.

This review summarizes some key technical aspects influencing the scope, validation, and acceptance of new test methods for marine biotoxins. The coverage of these topics reflects the complex and changing nature of the field and the need to protect the health of consumers through seafood testing that meets suitable performance criteria.

2.2 METHODS OF ANALYSIS WITH EXAMPLES FOR YESSOTOXINS

Techniques that form the basis for methods of analysis for marine biotoxins can be grouped under five broad headings, with some distinctive subsets:

1. *In vivo* animal bioassays—mouse (intraperitoneal injection, i.p.); rat (oral)
2. *In vitro* cell assays—cytotoxicity; ion channel activated; red blood cell haemolysis
3. *In vitro* functional assays—receptor binding; enzyme inhibition/induction
4. Immunoassays—ELISA; lateral flow immuno-chromatography (LFIC); surface plasmon resonance (SPR)
5. Chromatographic assays—thin-layer chromatography (TLC); LC with UV, fluorescence (FL) or mass spectrometric (MS) detection

Examples of these classes can be found for all the toxin groups. Each technique has advantages and disadvantages, which can only be fully evaluated in the context of how they are to be applied. The 4S factors discussed earlier must be taken into account as well as the scope (toxins and matrices), validation status, pathway to full regulatory acceptance, and cost-effectiveness. Within each subclass, for example, LC-MS methods for lipophilic toxins, there are different approaches leading to a range of available protocols. For routine monitoring programs, an integrated suite of methods is needed that provides efficient coverage of all required toxins. If screening methods are being used, then suitable confirmatory methods are also required.

Yessotoxins (YTXs) represent a relatively new toxin class where methods of analysis have been required for integration into monitoring programs. In 2002, the EU set the regulatory limit for YTXs in shellfish whole flesh as 1 mg/kg expressed as the sum of YTX and homoyessotoxin plus their 45-hydroxy metabolites. This limit has been adopted in some other countries. Recent toxicological data may result in the risks from YTXs being downgraded and the regulatory limits raised.[11,12] Nevertheless, this toxin class still provides many interesting examples of the types of technical issues that must be addressed in developing and validating methods for a complex new class of toxins. The following sections review the published methods for YTXs with particular attention to their performance characteristics for use as regulatory methods.

Analysis is conventionally divided into extraction, cleanup, and detection phases. All have proved problematic for YTX and its analogues due to their unusual chemical structures and properties (large lipophilic polyethers with hydrophilic sulfate groups). Exhaustive extraction is required for high recoveries from contaminated shellfish tissues, and the compounds have unfavorable solvent partitioning properties. The deficiencies of animal assays have led to a wide range of research into more specific detection techniques. Concomitantly, the diverse chemistry and shellfish metabolism for YTXs have been revealed and more detailed toxicological studies completed. This has led to reevaluation of regulatory limits and the target analytes.[2] The lack of analytical standards hampered earlier work, and the development of certified reference materials (CRMs) was an important prerequisite for recent work on analytical methods.

2.2.1 Mouse Bioassays for Yessotoxins

Mouse bioassay is defined in EU legislation as the reference method for enforcement of the 1 mg/kg limit for YTXs.[13] Relatively low levels of YTX cause rapid death times and give misleading indications of high levels of DSP toxins—okadaic acid (OA), dinophysis toxins (DTXs), pectenotoxins (PTXs), or azaspiracids (AZAs). These responses led to the initial discovery of YTX.[14] The relatively nonspecific nature of the toxic response by the i.p. route cannot reliably distinguish YTXs from other lipophilic toxins such as the DSP toxins or fast-acting toxins such as gymnodimine and spirolides. Mouse bioassay screening methods for DSP toxins in shellfish are based on the Yasumoto protocol using acetone extraction followed by partitioning with diethyl ether[15–17] or dichloromethane.[18] These methods have not been validated for YTXs. Double extraction with acetone is assumed to be effective at recovering most of the YTXs along with other lipophilic toxins and lipid material. However, no performance data has been reported for this step. Partitioning YTX from aqueous solution requires polar solvents. Butanol was used in the initial preparative studies.[14] Recoveries of YTXs from shellfish in the partitioning steps with diethyl ether or dichloromethane were low[19,20] and likely to be variable due to effects of microemulsions. Mice subjected to i.p injections of YTX were restless and exhibited jumping before death.[21,22] Although these symptoms are different to those from DSP toxins, other neurotoxins and fatty materials can cause similar symptoms. Occurrence of YTX with other toxins, particularly from *Dinophysis* spp., is common in shellfish contamination events involving *Protoceratium reticulatum*.[23–25] The high variability in the measured i.p. toxicity of purified YTX to mice,[22] the low efficiency of extraction, and shellfish matrix effects contribute to the high variability in mouse bioassay results for shellfish extracts containing YTXs.[19]

Revised mouse bioassay protocols for testing whole flesh samples that prepare separate DSP and YTX fractions for mouse assay have been proposed.[17,19,20] Higher recoveries for YTX from mussel tissues have been reported but not specified for a protocol using a chloroform partition.[17,20] The detection limit for YTX was approximately 0.5 mg/kg digestive gland. However, chloroform will not recover the key 45-hydroxy metabolites from aqueous solutions.[26] Yasumoto's revised protocol-1 uses diethyl ether partitioning of crude extracts followed by butanol partition.[19] Quantitative recovery was reported for DSP toxins in fraction-1 but only partial recovery of YTX (approximately 50% in each fraction). Protocol-2 uses methanol/dichloromethane (6 + 4 v/v) partitioning of the crude extract. The lower dichloromethane layer contained the true DSP toxins, and the aqueous methanol layer retained the YTXs. In both protocols, the two fractions are bioassayed separately, requiring a total of six mice per sample. Revised protocol-2 has been proposed as a reference method for DSP toxins by the EU[27] and was implemented in the Italian shellfish monitoring program.

2.2.2 *In Vitro* Cell and Functional Assays for Yessotoxins

Microscope studies on the morphology of treated hepatocytes showed some potential to differentiate toxin groups, including YTX.[28] However, studies were limited to pure toxins and extractives from shellfish have been demonstrated to have a range of cytotoxic effects in the absence of marine biotoxins.[29] The cytotoxic response of YTX involves induction of caspases.[30] This effect has been used to devise a functional assay for YTX in contaminated shellfish.[31] Immunoblotting was used to detect a fragment of E-cahedrin from MCF-7 cells that was specifically related to exposure to YTX. Preliminary validation of the functional assay gave stable calibrations, but quantitative results for YTXs in shellfish extracts were 40% lower than those from LC-FL analysis. Structure–activity relationships for production of the E-cahedrin fragment showed that YTX was 15- and 42-fold more potent than 45-OHYTX and carboxy YTX, respectively.[32]

YTX has also been shown to reduce cAMP levels in exposed cells through enhanced phosphodiesterase activity.[33] This mechanism has been used as the basis for another functional assay for YTXs.[34] Phosphodiesterase and anthranloyl-cAMP (fluorescent) were incubated with YTX or shellfish extracts and the decrease in FL measured. The rate of cAMP hydrolysis was shown to be linearly correlated to YTX concentration (0.5–10 μM). Two shellfish extracts gave the same results

when analyzed by the method and LC-FL within the errors of the methods. The detection limit of the assay was approximately four times lower than that of mouse bioassay. However, fatty coextractives were observed to interfere so a change in protocol from acetone extraction and dichloromethane partition to methanol/water extraction was recommended but not tested with shellfish. A biosensor for YTX has been constructed by immobilizing phosphodiesterase on a resonant mirror.[35] A linear relationship for ligand binding was obtained over the range 1–15 μM YTX. Preliminary data for fortified extracts of a mussel h.p. showed high sensitivity and good repeatability (relative standard deviations, RSDs, 4–15%).

At present, there is limited information about the ability of these *in vitro* assays to detect other analogues of YTX, and there is no evidence that the biological responses detected are directly related to the rapid toxic effects observed when YTXs are injected i.p. into mice. However, functional assays show promise for screening of YTXs in shellfish-provided assay systems, and protocols can be developed with stable and reproducible performance.

2.2.3 ELISA Assays for Yessotoxins

An ELISA for YTX was reported as part of a suite of a immunoassays for comprehensive testing of marine biotoxins.[36] The performance of an ELISA for YTXs has recently been reported in detail.[37] Polyclonal antibodies were raised in sheep to YTX conjugated on the K ring (nonsulfated "right-hand" end). High cross-reactivities were obtained for key analogues: YTX (100%), 45-OHYTX 159%, homoYTX 39%, and 45-OHhomoYTX 51%. Several other analogues with modifications to the side chain also gave high cross-reactivity while the desulfo analogue gave very low cross-reactivity. The calibration range was 70–1300 pg YTX/mL. The recoveries for YTX fortifications of whole flesh were 103–118% using a methanol/water (9 + 1 v/v) extraction. The limit of quantitation (LoQ) in shellfish (diluted methanolic extracts) was 0.12 mg/kg. This assay has been further developed as a kit in a direct competition, 96-well plate format. A preliminary interlaboratory study (ILS)(4 labs; 3 countries) has been conducted using fortified shellfish samples.[38] Mean recovery at 0.5 mg/kg was 111% and reproducibility was 16%.

This ELISA gave consistently much higher estimates of YTX equivalents for extracts of naturally contaminated Norwegian blue mussel samples (*Mytilus edulis*) than those from instrumental analysis (LC-MS for YTX) by a factor of greater than 10.[25,39] LC-MS revealed that these samples also contained 45-OHYTX, carboxyyessotoxin, and hydroxycarboxyyessotoxin. However, the levels of these metabolites could still not account for most of the immunoreactivity, which were presumed to be due to the presence of a range of other analogues or metabolites of YTX whose identity and toxicological significance have yet to be fully evaluated. The high sensitivity of the ELISA has been used to advantage in studies on the production of YTXs by *P. reticulatum*.[40] Analyses were conducted on picked cells (1–20 cells) from Norwegian and New Zealand coastal waters and gave yields of YTXs of 18–79 pg/cell. Analyses of net-haul samples by LC-MS gave a lower estimated content of YTX per cell than by ELISA. *P. reticulatum* is known to produce a range of YTX analogues[41–44] that could account for the higher estimates obtained by ELISA. Metabolism of these analogues by shellfish could also contribute to the higher results by ELISA in mussel extracts.

Although the ELISA has good accuracy and precision characteristics for YTXs, its use in shellfish monitoring programs will be limited to screening until the relationship of the enhanced responses to the regulated levels of YTXs is securely established for different shellfish species.

2.2.4 Liquid Chromatography with Fluorescence Detection for Yessotoxins

The lack of a strong chromophore has hampered use of LC-UV techniques for isolation or analysis of YTXs. The formation of a fluorescent derivative with the dienophile reagent DMEQ-TAD is the basis for a useful LC-FL method for analysis of YTX and homoYTX in shellfish and algal concentrates.[45] The method can also detect some other analogues of YTX including 45-OHYTX,

45,46,47-trinoryessotoxin,[45] and 1-desulfoyessotoxin.[46] However, the method cannot detect analogues lacking the 1,3-dienyl moiety, such as carboxyYTX. Extraction of 1 g DG from naturally contaminated shellfish with 9 mL methanol/water 8 + 2 v/v recovered 90% of the YTX in the first extraction and 10% in the second extraction. The crude extract was cleaned up by SPE and, after derivatization of the YTX fraction with DMEQ-TAD, a further SPE cleanup was carried out before reversed-phase LC with fluorescence detection. Each compound yielded two epimeric peaks, and the calibration with YTX was highly linear. Blank shellfish extracts gave low interferences with detection limits below 0.1 mg/kg DG. Recovery of YTX from fortified samples was 94% (mean, 0.2–20 mg/kg DG). The repeatability of this LC-FL method for the determination of YTX has been tested using contaminated Norwegian blue mussel samples.[20] There was good agreement between duplicates over the range 2–30 mg YTX/kg DG with no significant effect of level on the percent difference.

The LC-FL method for YTX and 45-OHYX has acceptable performance characteristics (sensitivity, accuracy, and precision). However, the cleanup steps and LC determination are time-consuming, and carboxyYTX and toxins from other groups cannot be detected.

2.2.5 LC-MS Methods for Yessotoxins

The power of mass spectrometry for detection and structural elucidation has been widely exploited in the field of novel marine natural products. Initial experiments with YTX used negative ion fast atom bombardment (FAB).[47] Collisional activation of the molecular anion gave fragment ions of structural utility dominated by loss of sulfate $[M\text{-}H\text{-}80]^-$ (where M is considered to be the sulfonic acid form) and a series of cleavages along the polyether ladder. The breakthrough for quantitative analysis of YTXs was the development of an LC-MS method for separation, electrospray ionization (ESI), and tandem mass spectrometric detection (triple quadrupole analyzer) of YTX.[48] C18 reversed-phase chromatography with an acetonitrile/4 mM ammonium acetate (80:20 v/v) mobile phase provided a narrow peak. YTX was insensitive in positive ion ESI, but negative ion ESI provided high sensitivity for YTX with the molecular anion ($[M\text{-}H]^-$) being the main peak in the mass spectrum. The major peaks in the collisional activation spectrum of $[M\text{-}H]^-$ matched those from FAB. Selected ion recording (SIR) on $[M\text{-}H]^-$ or selected reaction monitoring (SRM) of the loss of sulfate both provided excellent signal to noise on a crude extract of contaminated mussel DG. The extension of this method to a multitoxin format for direct detection of three groups of "DSP" toxins in shellfish or phytoplankton was reported,[49] but no precision data was provided.

The reversed-phase chromatography of YTX and analogues can be problematic with tendencies to variations in retention times and peak broadening.[50] Heavily end-capped C8 or C18 column packings and mobile phases based on acetonitrile/water/buffer have been preferred with approximately 80% acetonitrile being required to elute YTX. HomoYTX generally coelutes with YTX. 45-OHYTX and other metabolites elute considerably earlier than YTX. The most stable chromatographic performance has been obtained with neutral ammonium acetate buffers.[26,48,51] Use of acidic ammonium formate/formic acid buffers moves YTX to longer retention times leading to better separation of analogues, and increased intensity of the $[M\text{-}H]^-$ ion at the expense of the $[M\text{-}2H]^{2-}$ ion, and is more suitable for separation and ESI of a wide range of toxins.[52–54] However, the LC retentions of YTX and analogues have been found to be very sensitive to the buffer composition and to subtle changes in column packing selectivity. For example, the retention time for YTX linearly changed from 19.2–21.7 min with decreasing ammonium formate concentrations 4–3.1 mM (formic acid concentrations 46–47 mM). Lower concentrations of ammonium formate result in very long and unstable retention times for YTX.

Japanese workers[26] presented the first validation data for quantitative determination of YTX with other "DSP" toxins in shellfish using LC-MS. Toxins were extracted with methanol/water 9 + 1 v/v (18 mL with 2 g tissue) and partitioned into chloroform. The lipophilic toxins in the chloroform phase were separated into two fractions by silica gel-SPE. YTXs in the second fraction

were analyzed by LC-MS (ESI-, quadrupole) with a neutral buffered mobile phase, and SIR detection of the molecular anions was used. Calibrations were highly linear (R^2 >0.997) for YTX and 45-OHYTX over the range 40–1600 pg injected. Recoveries of YTX from fortified scallop tissues were 80%–90% (45-OHYTX not recovered). YTX and 45-OHYTX were also separately purified from the crude methanolic extract using C18-SPE and gave approximately 70% recoveries. Detection limits for these two toxins were 0.08 mg/kg DG or 0.04 mg/kg adductor muscle. More recently, this multitoxin method was revalidated by two Japanese groups using more sensitive instruments and acidic buffer mobile phases.[55,56] This enabled analysis of crude shellfish extracts without cleanup. YTX, 45-OHYTX, and a range of other lipophilic toxins were detected in a single 30-min run using SIR for the $[M\text{-}H]^-$ species.

Several other groups have published multitoxin methods that use quadrupole LC-MS to detect a range of toxins including YTXs. Norwegian workers[57] reported an LC-MS (SIR) method that determined YTX, 45-OHYTX, carboxyYTX, and several other lipophilic toxins in crude methanol/water extracts (8 + 2 v/v). A water/acetonitrile gradient with acidic buffer gave excellent separation of the YTXs. Detection limits were 0.02–0.08 mg/kg, but no other validation or performance data was provided. A full single-laboratory validation (SLV) has been reported of an LC/MS (SIR) method for all lipophilic toxins regulated under EU legislation.[51] Extraction used 2 g whole flesh with 8 mL methanol/water (8 + 2 v/v). Using a neutral buffer system with gradient elution, the analysis was completed in 13 min and gave high precision for key toxins in four shellfish species. Ion suppression effects necessitated calibration, using standards in shellfish extracts. The LC/MS/MS (SRM) multiresidue method in routine use in testing New Zealand shellfish has been formally published, including the results for an in-depth within-laboratory validation and a small interlaboratory study.[54,58,59] This method enables relatively large numbers of samples to be analyzed routinely for a wide range of toxins with low detection limits and high specificity. YTXs are detected using SRM channels for loss of sulfate from the molecular anions. 45-OHYTX, homoYTX, and carboxyYTX were calibrated using the linear calibration response factor (RF) for YTX. A single extraction of 2 g whole flesh homogenate with 18 mL methanol/water 9:1 v/v was used, followed by a hexane wash to remove lipids. Extractability experiments with contaminated mussel tissues showed a single extraction recovered 75–80% of YTXs with significant proportions in second and third extracts of the pellet. Similar but slightly lower extractabilities were obtained using methanol/water 8 + 2 v/v but this solvent was much less efficient at extracting AZAs and esters of OA, DTX1, and DTX2.

Table 2.2 summarizes the characteristics and performance of the four multitoxin LC-MS methods where validation data was provided for YTX. The methods all were suitable for enforcement purposes with good recoveries and precision for YTX.

An ILS provided further information on the performance of the method of McNabb et al.[54] Eight laboratories obtained data for eight toxins in the methanolic extracts of three contaminated mussel samples.[54,59] Some of the labs had little experience with analysis of YTX and other toxins by LC-MS. The precision estimates for YTX were repeatability 8–12% and reproducibility 15–22% with a Horwitz ratio (HorRat) of 1.3 (two extracts, levels equivalent to 2.9 and 1.7 mg/kg). Reproducibility for most of the other analytes was also acceptable based on the HorRats.

Several groups have used LC-MS with ion-trap mass analyzers for determination of YTX and analogues, although little quantitative data has been reported.[41,50,53,60,61] In addition to the high sensitivity of ion-traps in full scan mode, sequential collisional activation experiments (MS^n) can provide more structural information than conventional MS-MS experiments. For YTXs, the ability to probe the $[M\text{-}H\text{-}SO_3]^-$ ions, which dominate the MS-MS spectra, has been shown to be of particular utility. A range of novel analogues has been identified in algal extracts of *P. reticulatum* using these techniques with ion trap LC-MS. MS^n can, in theory, also provide higher degrees of specificity for quantitative analysis. In practice, no significant increases in signal-to-noise ratio have been demonstrated for analysis of YTXs in shellfish extracts over the large increases obtained in moving

TABLE 2.2
Validated Multitoxin LC-MS Methods for Determination of Yessotoxins and Other Toxins

Method	Extraction[a]	LC Column	LC Gradient	LC Buffer	Rt YTX (min)	MS Detection	Recovery YTX	RSD_r[b]	RSD_R[c]	YTX LoD (μg/kg)	YTX Analogues with Data
Stobo et al. [51]	2 g WF + 8 ml 80% MeOH	50 × 2.1 mm Hypersil C8 3 μm	AcN + water (5:95) to AcN + water (95:5)	5 mM ammonium acetate pH 6.8	9.2	SSQ ESI-SIR $[M-H]^-$	91–105% at 0.20 mg/kg	4.5% $n = 9$	7.0% $n = 9$	100	homoYTX 45OHYTX 45OHhYTX
McNabb et al. [54]	2 g WF + 18 ml 90% MeOH	150 × 2 mm Luna C18 5 μm	AcN + water (13:87) to AcN + water (77:23)	3.3 mM ammonium formate + 46 mM formic acid	19.2	TSQ ESI-SRM $[M-H]^- > [M-H-80]^-$	86–94% at 0.80 mg/kg	12.6% $n = 12$	12.6% $n = 12$	5	hYTX 45OHYTX
Suzuki et al. [55]	5 g HP + 45 ml 90% MeOH	50 × 2.1 mm Hypersil C8 3 μm	AcN + water (95:5) 20%–100% with water	2 mM ammonium formate + 50 mM formic acid	10.9	SSQ ESI-SIR $[M-H]^-$	97% at 0.50 mg/kg HP (spike into extract)	5–6% $n = 10$	NR[d]	<20 (HP)	45OHYTX
Suzuki et al. [56]	1 g HP + 9 ml 90% MeOH	250 × 1.5 mm Capcellpak C18 MGII	AcN + MeOH (80:20) 40–100% with water	2 mM ammonium formate + 50 mM formic acid	12	TSQ ESI-SIR $[M-H]^-$	>90% at 0.02–1.0 mg/kg HP	2%–6%	NR	20 (HP)	45OHYTX

[a] WF: whole flesh; HP: hepatopancreas.
[b] Repeability RSD.
[c] Within lab reproducibility RSD.
[d] NR: not reported.

from SIR (MS) to SRM (MS-MS). YTX and 45-OHYTX were determined in shellfish using ion trap LC-MS.[50] Shellfish tissues were extracted with methanol/water 8 + 2 v/v (1 g + 9 mL) and the supernatant concentrated 20-fold for LC-MS. The preferred LC system was an RP amide-C_{16} column using an acetonitrile/water (60:40) mobile phase and neutral buffer. Linear MS-MS calibrations were obtained for YTX using the loss of sulfate transition from $[M-H]^-$ with a detection limit in a mussel extract of 30 pg injected (0.003 mg/kg). No accuracy and precision data for fortified shellfish tissues were reported. Ciminiello et al. extended the range of YTX metabolites tested in shellfish by ion-trap LC-MS/MS to include 45-OHhomoYTX, carboxyYTX, carboxyhomoYTX, and 42, 43, 44, 45, 46, 47, 55-heptanor-41-oxohomoYTX.[53] The method was used to confirm the types of YTXs present in contaminated shellfish samples from the Italian monitoring program. QTOF LC-MS was used for determination of YTX in picked cells of *P. reticulatum* and structurally useful MS/MS spectra obtained.[62]

Matrix effects are common in LC-MS mainly due to sample coextractives enhancing or suppressing electrospray ionization of analytes. Crude extracts of shellfish tissues (1–2 g/mL) were reported to suppress ionization of YTX by 10–50%.[26,51,54,63] Matrix effects tend to be very instrument dependant as they are influenced by source design, tuning, state of cleanliness, solvent system, and LC separation. SPE cleanup reduced direct and indirect interferences for YTX determination.[26] Calibration of LC-MS response using YTX standards prepared in concentrated extract of a blank scallop did not adequately correct for the approximately 50% suppression of YTX responses for three other scallop samples.[63] It was concluded that it is not always possible to ensure adequate matching of sample and standard matrices. For this reason, the U.S. Food and Drug Administration (FDA) does not approve use of matrix-matched standards for pesticide or veterinary drug residue methods. Standard addition for quantitation of a scallop extract fortified at 0.20 mg YTX/g DG gave 0.213 mg/kg ± 0.020 (Mean ± SD, $n = 6$) compared to 0.134 mg/kg ± 0.014 by external calibration, and similar improvements were reported for quantitation of other "DSP" toxins.[63] Standard addition has also been used for YTX quantitation.[51] The disadvantages of standard addition are that each extract requires two LC-MS runs, and the addition of appropriate levels of standards becomes complex if several toxins are present in the extract. Modern instruments can achieve limits of detection for YTXs that are very low compared to regulatory limits and therefore use of more dilute extracts has become feasible. Immunoaffinity columns are commonly used for sample cleanup in the related field of mycotoxins. Their use for YTX has been suggested.[37] The most satisfactory method for correcting for LC-MS matrix effects is the use of stable isotope labeled internal standards, but these are not currently available for YTX or other marine biotoxins.

LC-MS methods have proved very satisfactory as alternatives to the LC-FL method[45] for precise analysis of YTXs. There are advantages in the simple sample preparation and wider range of analogues detected. However, the greater instrument costs can only be justified if LC-MS is used for quantitative multitoxin analysis where definitive results are obtained for YTXs and a range of other toxin classes in a single run without the need for further confirmatory analyses. Several such methods have been developed and validated.[51,54–56]

2.3 VALIDATION OF METHODS AND REPORTING

Although a wide range of analytical methods have been developed for marine biotoxins in seafood, monitoring programs only use a subset of these methods. The subset of methods recognized by regulatory authorities as fit for the purpose of enforcing toxin limits and passing consignments as safe for human consumption is even smaller. The primary requirement for a method to become accepted is that it has been adequately validated. Although there are anomalies in the status of some currently accepted methods, a variety of rather strict validation criteria are now being applied to new methods. The basis for these criteria is documentation of the performance characteristics of a method to demonstrate that it is fit for the purpose. This is conventionally divided into SLV and ILS phases.

2.3.1 Single Laboratory Validation

The requirements for SLVs for analytical methods have been established by Codex[6] and other authorities such as Eurachem, AOAC, EU, and International Union of Pure and Applied Chemistry (IUPAC).[64–67] Some countries have published specific guidelines for marine biotoxins.[68] An SLV is designed to gather short-term accuracy and precision data and to document a range of other information for the performance of a method in a particular laboratory. The general criteria can be grouped under the following categories:

1. Scope—analytes, matrices, that is, the range of toxins and seafood species
2. Calibrated range—linearity, quantitative concentration range
3. Limit of detection (LoD)—lowest level of toxin that can be reliably distinguished from the blank
4. LoQ—limit of determination; lowest level of toxin where quantitative data can be reported
5. Repeatability precision—RSD for results on same day with same operator
6. Reproducibility precision (within lab)—RSD for results across different days and different operators
7. Accuracy—trueness; closeness of results to the certified or assumed true level (based on recovery of analyte from CRMs or fortified blank samples)
8. Selectivity—degree of specificity for toxin and freedom from interferences confirmation criteria—assurance of the identity of the toxin
9. Robustness—degree of insensitivity of results to minor changes in reagents, procedures, and instrument parameters
10. Uncertainty of measurement—estimate of the confidence intervals for results

Within these criteria, there are significant variations in the detailed requirements depending on the type of method and the end use. For example, the form of the calibration curve and cross-reactivities are very important for ELISA methods[37,69] and functional assays.[70] For a novel extraction system or toxin, the recoveries from seafoods with incurred toxin should be tested. CRMs can be used where available or extractability experiments are performed.[54] Seafoods are highly variable biological matrices and therefore SLVs should gather accuracy and precision data for a range of relevant species. In the field of veterinary drug residues, the EU puts considerable emphasis on establishing the decision limits CCα and CCβ for each analyte. These limits are based on detailed LoD and precision data and are used to determine whether, with defined probabilities, an analyte has been detected or a regulatory limit has been exceeded. Such statistically derived decision limits also have some applicability to marine biotoxins, and it is certainly important that an SLV establish the method accuracy and precision at relevant concentrations of each toxin including the LoQ and the regulatory limit. This precision assessment should extend into the area of overall uncertainty of measurement.

Validation studies must take into account all the above factors while remaining feasible with the available resources. Some key points regarding the design and execution of SLVs are

1. The method protocol should be clearly defined and documented before initiating the SLV. It is a common mistake to attempt to combine method development with validation. Not only are the approaches to these two phases fundamentally different, making significant changes to method protocols during an SLV is likely to invalidate earlier data and result in more work being required.
2. The SLV protocol should also be documented before starting. Careful design can reduce the required work. For example, fortification studies can be used to generate both accuracy and precision data. The degree of replication may be limited by resources, but method development may indicate, for example, that different shellfish matrices behave rather similarly. If an SLV using a minimum of three replicates per matrix proves this to be the

case, then a better estimate of overall method precision can be obtained by combining the data across matrices.

3. Techniques such as ELISA and LC-MS can be exquisitely sensitive. However, a marine biotoxin method does not usually need the lowest achievable LODs. Low levels of toxins are often expected in samples but often have no regulatory significance, unlike pesticide or drug analyses. The method should be established, and the SLV precision data gathered, for levels close to the regulatory limit and at lower levels that might be useful for early warning, for example, 5–10% of the limit.
4. Execution of the SLV protocol should be planned for a set, relatively short, period. Adequate resources should be allocated so the SLV can proceed efficiently and smoothly. Reproducibility data should be gathered on different days, but the aim of the SLV is not to establish the long-term stability of the method.
5. The results of the SLV should be fully reported. Generally, rather basic summaries of data (means, RSDs) will be adequate and testing for outliers should not be necessary. Statistical analyses established for ILS precision data[71] can also be useful to separate within-lab repeatability and reproducibility. Significance tests may be required for some key robustness experiments, but method development should have eliminated most perturbations that could adversely affect reproducibility.

2.3.2 Interlaboratory Studies

Association of Official Analytical Chemists (AOAC) has a long-standing requirement for ILS data in the form of a collaborative study before a new method can be accepted for official first action. A similar requirement is there in EU legislation for methods used to test compliance with food law.[27] The design criteria for collaborative studies have been refined over many years and are broadly summarized in the harmonized IUPAC/AOAC protocol[72] and AOAC guidelines.[73] The minimum requirements are for five materials and eight laboratories reporting acceptable data (10–12 participating labs). Each material is a sample matrix with a certain level of analytes. Two replicates for each material are usually studied, either as blind duplicates or split-levels (Youden pairs). The set of materials must be carefully chosen and prepared for the study so that representative matrices and analyte levels are covered. This is not a trivial task for multitoxin methods to be used with a broad range of seafoods. The main purpose of most collaborative studies is to establish the interlaboratory precision characteristics of a method. The study data can be assessed against the norms established by Horwitz from the precision of a very wide range of analytical methods as a function of analyte concentration.[74] If some materials are CRMs or fortified blank matrices, then the study can also provide accuracy data. A collaborative study is a major undertaking for the lead laboratory that prepares the protocols, provides the test materials, and coordinates data analysis. It also requires a considerable commitment of resources from the participating labs. For these reasons, it is advisable to ensure a satisfactory outcome for the study by

1. Using a method protocol that has been well optimized, characterized, and documented by the lead lab.
2. Keeping the study design as simple as possible.
3. Carefully selecting labs based on their commitment and competence as established from their undertaking elements of an SLV of the method. The main study should not be designed or used as a laboratory proficiency exercise.
4. Including some practice samples with the materials provided for the study.

As with SLVs, it is unwise to incorporate elements of method optimization or comparison of methods into most collaborative studies as this could considerably increase both the workload and the probability that the study will fail to provide precision data that meets ILS statistical criteria.

The reproducibility data from a collaborative study is a more robust basis for the uncertainty of measurement than data from an SLV. However, all significant sources of variation may still not be included. Calibration is an important potential source of method bias and many biotoxin standards are of limited availability or uncertain quality (see Section 2.4). If calibration standards are provided along with the collaborative study materials, then the resulting precision data may significantly underestimate the overall measurement uncertainty for the method as used with different standards.

Two collaborative studies of methods for marine biotoxins accepted by AOAC provide good examples of appropriate designs:

1. *Saxitoxins by LC-FL* (Lawrence et al., 2005).[9] Eighteen participating labs, 9–16 labs reporting acceptable data (depending on material and analyte). Fifteen materials comprising scallop, mussel, oyster, and clam tissues containing incurred saxitoxins and three fortified mussel samples. The materials were supplied as blind duplicates, and each contained five or more saxitoxins with different congeners and levels. Certified reference standards from NRC, Halifax, were supplied. The lead laboratory also analyzed the materials using LFIC immunoassay and mouse bioassay.
2. *Domoic acid by ELISA* (Kleivdal et al., 2006).[10] Sixteen participating labs, 10 reporting acceptable data. Eleven materials comprising scallop, mussel, and oyster tissues fortified at two or three levels plus a blank. The fortified materials were supplied as split-level duplicates. A certified reference standard for DA (NRC, Halifax) was provided as part of the 96-well-plate ELISA kits. Four labs also undertook LC-UV analyses on the materials.

A well-executed collaborative study is the best basis for assessing between laboratory precision of a method. However, this is a costly and time-consuming process that does not directly establish other important method performance parameters. These one-off studies also do not assess ongoing laboratory performance, for example, for ISO 17025 accreditation, or facilitate reoptimization of methods for higher efficiency or to meet changing requirements. These disadvantages have been recognized for multiresidue testing of veterinary drugs and pesticides in food where more emphasis is now put on thorough SLVs, internal laboratory quality control systems, interlaboratory sample exchanges, and proficiency testing. LC-MS methods for marine biotoxins face similar issues. The pool of qualified labs to draw on for a collaborative study is not large, and there is a wide variety of instrument models and configurations. For example, what is achievable using crude extracts on a modern instrument may require cleanup/concentration steps on an older instrument. Although generic method protocols can be drawn up and tested, uncertainties may remain that make running a collaborative study a risky proposition. Replacement of animal bioassays would be hastened if criteria for initial acceptance of new methods more closely matched the performance-based approach used for residue methods.

2.3.3 Reporting

Marine biotoxin testing can be used for a variety of purposes, but results should always be reported on a clear and consistent basis that the end user can follow. The EU and Codex have recently agreed on a set of reporting conventions for data from regulatory testing of foods and feeds.[75] These measures are designed to ensure consistency in interpretation between national and international legislations, and to align with laboratory accreditation requirements under ISO/IEC 17025: 2005. They should be followed in the field of marine biotoxins in seafood:

1. *Units:* SI units should be used for toxin concentrations, both for measurement results and regulatory limits. The most common and clearly understood unit is mg/kg with µg/kg as an alternative (there some potential for confusion for the unindicated with the µ symbol. The use of mixed units such µg/100g should be discouraged. A good case

can be made for use of molar untis e.g. µmoles STX equiv/kg, for group toxins with a common mode of action such as saxitoxins."

2. *Significant figures:* Unless legislation provides clear guidance on the number of significant figures, "the analyst should report to one more significant figure than is given in the specification." The precision of biotoxin methods is unlikely to fully justify application of this rule for limits that are expressed with two significant figures, for example, OA group 0.16 mg/kg.
3. *Measurement uncertainty:* Uncertainty as a term in this context of analytical data has resulted in some confusion. *Uncertainty of measurement* does not imply doubt about the validity of a measurement; on the contrary, knowledge of the uncertainty implies increased confidence in the validity of a measurement result.[76] Estimation of this parameter is a requirement of the ISO 17025 standard and its complexities are well covered elsewhere.[76,77] If one is considering a regulatory limit in legislation, the uncertainty of measurement should be taken into account. "In practice, the analyst will determine the analytical level and estimate the expanded measurement uncertainty U at that level, subtract U from the reported concentration and use that value to assess compliance. Only if that value is greater than the legislation limit can the control analyst be sure beyond reasonable doubt that the sample concentration of the analyte is greater than that prescribed by legislation." If seafood consignments are being cleared for commerce and may be subject to further regulatory testing, then it is prudent not to apply U to test results because, for a result in the vicinity of the limit, there is a significant probability that another test could provide a result that exceeds the limit by more than U.
4. *Recovery:* "When recovery data have been collected using state of the art methods, they should be included in the result." This is in agreement with general ISO requirements and IUPAC recommendations that data should be corrected for known bias.[78] However, international regulations vary in their application of this principle, for example, in general pesticide residue data is not corrected for recovery while veterinary drug residue data is. This issue has been considered in relation to uncertainty of measurement.[77,79] At present, the legislation of the United States and EU regarding marine biotoxins makes no specific reference to correction for recovery, but in terms of consistency it is logical to correct results if a reliable recovery factor has been established. Although, for example, a recovery of 97 ± 9% is practically a recovery factor of 1.0, the reporting procedures of the laboratory and interpretation of results by the end user will be more transparent if the factors are always recorded and applied, independent of their significance.

The following example elaborates the application of these rules:

The common legislated limit for DA in seafood is 20 mg/kg. A small ILS using an LC-UV method gave a mean recovery for DA of 92% and a result of 20.8 mg/kg ± 7.2% (mean ± RSD, $n = 6$) for a scallop tissue.[80] Assume that this ILS precision with a coverage factor of two is a sound estimate for U at the regulatory limit and assume an analyst obtains the following raw data for samples from three lots of scallop: 15.23, 21.13, and 23.78 mg/kg.

Applying the aforementioned rules, this data would be reported, corrected for recovery as

Consignment 1: 16.5 ± 2.4 mg/kg—in compliance (16.5 − 2.4 < 20)
Consignment 2: 23.0 ± 3.3 mg/kg—in compliance (23.0 − 3.3 < 20)
Consignment 3: 25.8 ± 3.7 mg/kg—not in compliance (25.8 − 3.7 > 20)

If the decision is of critical importance, then the laboratory could carry out replicate analyses of the sample and the uncertainty applied to the mean result would be correspondingly reduced by $1/\sqrt{n}$ where n is the number of replicates. In the above example, assume a duplicate analysis of sample 2 gave a mean corrected result of 22.7 mg/kg. U is now $3.3/\sqrt{2}$ mg/kg, and the consignment is not in compliance.

Although the application of these reporting rules to analytical data is logical, the levels of marine biotoxins in seafood tend to be highly variable. Therefore, the adequacy of the sample as tested to represent the lot of seafood in commerce or the marine area being harvested also needs to be taken into account. These issues of sampling plans and number of units that constitute a sample are further aspects of food monitoring that need to be considered.[75]

2.4 CALIBRATION OF ANALYTICAL METHODS

Calibration refers to the procedures used for correlating test method output or response to an amount of analyte (concentration or other quantity). The characteristics of a calibration function and justification for a selected calibration model should be demonstrated during SLV and ILS studies. The performance of a calibration technique and the choice of calibration model (e.g., first-order linear, curvilinear, or nonlinear mathematical function) are critical for minimizing method bias and optimizing precision. The parameters of the model are usually estimated from the responses of known, pure materials. Calibration errors can result from failure to identify the best calibration model; inaccurate estimates of the parameters of the model; errors in the composition of calibration materials; or inadequately studied, systematic effects from matrix components. This section focuses on the critical issue of the traceability and supply of materials used for calibration of marine biotoxin methods.

2.4.1 Traceability

Marine biotoxin testing in seafood should follow the general requirements for traceability established by ISO for chemical measurements. Traceability is defined as "*Property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties.*"[81] These requirements are readily met for the weight and volume components of methods in laboratories where calibrated balances, pipettes, and so forth are being used. Moving beyond uncalibrated bioassays, methods must be regularly calibrated for their response to the toxins of interest. Method protocols specify procedures for preparing a set of calibration standards and their use in determining a regression line or curve of system response, for example, chromatographic peak area versus concentration of toxin. The accuracy and traceability of the results of these methods depend directly on the quality of the reference standards used. Reference materials for chemical measurements can be divided into four levels.[81,82]

2.4.1.1 Primary Standard

A standard with the highest metrological properties and whose value is accepted without reference to other standards. This generally refers to elements such as metals or to simple salts that are available in gram quantities of very high purity ($100 \pm 0.02\%$) and can be assayed by direct means. It is not applicable to complex chemicals available only in small quantities such as biotoxins.

2.4.1.2 Certified Reference Material

Formally defined as "*A reference material, accompanied by a certificate, one or more of whose properties are certified by a procedure which establishes traceability to an accurate realization of the unit in which the property is expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence*."[81] As pure marine biotoxins are rare and expensive, a CRM used for calibration is generally a dilute solution of stated concentration, for example, micromolar, which is traceable in terms of the chemical property (moles) and volume (liter). Methods for preparing biotoxin CRMs including standard solutions and shellfish

tissues have been described.[83–86] Novel techniques such as quantitative NMR and LC with chemiluminescent detection enable determination of toxin concentrations in solution without the need to rely on weights for measuring small amounts of toxin of uncertain absolute purity.[87] Tissue CRMs are very useful for quality assurance of methods, especially with regard to accuracy, but are not recommended for routine system calibration owing to the expense and limited scope. An important part of the preparation of CRMs is validating the homogeneity and long-term stability of the toxin concentrations.[86]

2.4.1.3 Purified Reference Material

For toxins, this generally takes the form of an isolated and purified fraction from biological sources where the identity and purity of the toxin has been established by appropriate techniques such as NMR, MS, and chromatography in various forms. A variety of toxins can be purchased from commercial sources, which have stated weights and purities but are uncertified. Such materials are also frequently exchanged between natural product laboratories and analytical labs. As stated by Eurachem, "*without clear evidence of traceable values of known uncertainty, the adequacy of such material can only be a matter of care and judgment.*"[81] With many biotoxins isolated in small quantities, it is difficult to eliminate or quantify associated water and salts. The stability of such materials during transport and storage are also generally poorly understood. Therefore, purities established by standard techniques may be optimistic and derived concentrations will have high uncertainties. However, solutions from such reference materials can be accurately calibrated using a suitable CRM. If the toxin has a strong chromophore with known extinction coefficient, then a purified reference material, for example, a microcystin, may be directly calibrated by a combination of spectrophotometry and LC-UV (to check purity).

2.4.1.4 Laboratory Materials

There are samples or crude extracts containing the analyte of interest but whose concentration has not been accurately established. For marine biotoxins, such materials may be frozen homogenates of naturally contaminated seafood, crude or partially purified extracts of such seafood, or of harmful algae. Digestive glands of contaminated shellfish are a source of concentrated toxins and their metabolites. Such materials cannot be used to calibrate methods of analysis but can serve very useful functions in method validation and quality control, especially when they contain rare toxins not readily available elsewhere: (i) Retention times and spectral properties can be established on a routine basis for LC-UV, LC-FL, or LC-MS methods; (ii) Partially purified extracts can be used for fortification experiments during method validation and thus establish recovery and precision data;[54] (iii) If concentrations of toxins can be established by reference to CRMs, then the materials can be used as in-house or interlaboratory reference materials for quality control. Issues such as toxin stability and homogeneity then become more important.[86]

2.4.2 Supply of Certified Reference Materials

The supply of CRMs for marine biotoxins has been identified as one of the key factors limiting introduction of alternatives to animal bioassays.[2,5] The advent of instrumental methods with the capability to detect a wide range of toxins and analogues or metabolites has clearly identified the gaps in CRMs. Although the main toxin in each lipophilic group is now covered, there are some key analogues where no suitable materials are currently available internationally for use in routine calibration. Only modest resources have been made available for the effort required to increase the scope and supply of toxin CRMs despite the magnitudes of seafood trade, expenditure on monitoring, and potential human health risks and the pressures to eliminate animal testing. There have been long-standing national efforts at the Japan Food Research Laboratory, important U.S. initiatives for saxitoxins and

brevetoxins, and smaller short-term initiatives within the EC. However, the CRM Program of National Research Council of Canada in Halifax (NRC-CRMP) remains the only significant international supplier of a wide range of fully certified marine biotoxin materials.[83] NRC-CRMP leverages its modest resources through collaborations with other groups for the supply of toxins for certification. This has been a very successful means of speeding up development of CRMs.[84,86]

The lack of CRMs for particular toxins not only affects validation and routine calibration of instrumental methods but is also a severe impediment to the satisfactory validation and implementation of all forms of quantitative assays. For example, ELISAs or functional assays may be able to be calibrated using a CRM for the main parent toxin, for example, YTX. But validation must also securely establish the cross-reactivity to other relevant analogues and metabolites.[37] If CRMs are not available then recourse must be had to less well-characterized materials resulting in significant uncertainties. These issues extend to toxicological studies. If a CRM is not available to calibrate the material used for a dosing study, then there will be significant uncertainties in the derived parameters such as LD_{50} and lowest observable adverse effect level. These uncertainties will affect the validity of the regulatory limits established on the basis of such toxicological data. The uncertainty loop is then closed when analysts cannot accurately calibrate the methods being used to enforce these limits.

2.4.3 Use of Relative Response Factors

When using instrumental method with spectroscopic detection (LC-UV, LC-MS), individual responses from a range of toxin analogues can be observed in samples. The identities of these may be confirmed using available uncertified materials or spectroscopic data but the concentrations cannot be verified. Where a particular toxin analogue that has been detected and a CRM is not available, analysts are faced with difficult decisions. The choices are

1. Do not report the presence of the analogue. This is not a responsible action if the analogue is of toxicological significance and appears to be present at relatively high levels.
2. Report the presence but not concentration of the analogue. While this is a valid response, it is likely to directly lead to questions about the significance of the levels.
3. Estimate concentrations of the analogue based on the calibration established using a purified reference material, if available. This is a common situation, but the analyst must incorporate the uncertainties regarding the purity/concentration of the reference material into the expanded uncertainty of measurement of the results. As discussed (Section 2.4.1.3), these uncertainties are difficult to determine but may be large.
4. Estimate concentrations of the analogue by applying a relative RF to the calibration for a related toxin established using a CRM. This may be the best expedient if a reference material is not available or is of highly uncertain concentration.

The following discussion explores the validity of the relative RF approach and issues arising.

Response factor: The sensitivity or analytical system response per unit of analyte as given by the slope of the linear calibration equation. The calibration curve may be determined using one precisely determined calibration point, but the analytical responses must be demonstrated to be linear. Typically the RF for a toxin in a chromatographic system is expressed as peak area/concentration, for example, as milliAU·sec/μM for LC-UV.

Relative RF (RRF): Ratio of response factors for two compounds. The ratio of the calibration curve slopes.

If the RRF is known for a toxin analogue and a parent toxin, then the concentration of the analogue can be accurately determined from its response using the calibration of the parent toxin, preferably established using a CRM:

$$C_i = A_i \times RF_p \times RRF_{i/p}$$

where C_i and A_i are the concentration and area response, respectively, for the analogue, and RF_p is the response factor for the parent toxin.

Use of RRFs is an accepted concept in analytical chemistry and has found application in diverse areas of trace analysis where a full suite of the CRMs required for regular system calibration is not available, for example, determination of polychlorinated PCBs by GC-MS and fumonisin mycotoxins by LC-FL.[88,89] There are four general scenarios for application of RRFs to marine biotoxin analyses.

2.4.3.1 Use of Small Quantities of Reference Material

A CRM or reliable purified reference material for the analogue toxin is available but in insufficient quantities for use in routine calibration of the method. In this case, one pair of linear calibrations can be carried out to accurately determine the RRF to the parent toxin. This RRF can then be routinely applied to biotoxin testing using the parent toxin calibration. Redetermination at intervals, perhaps using single point calibration, can establish the stability of the RRF over time. By this means, precious standard material can be conserved while the contribution of the RRF to uncertainty of measurement can be estimated. The main point to consider is the reliability of the analogue standard, if it is in such small quantities. Analysis of a tissue reference material can similarly provide data to estimate an RRF for a toxin analogue. For example, analysis of the mussel tissue CRM MUS-1B (NRC, Halifax) by LC-MS for OA and DTX1 provided data to justify the use of an RRF of 1.0 for DTX1 to OA for use in routine testing.[54]

2.4.3.2 Use of Literature RF Data

Literature information may be available that enables calculation of an RRF. This is likely to be applicable only to toxins being analyzed by LC-UV where absolute absorption coefficients are available. For example, tabulated literature data for nine microcystins gave absorption coefficients at 238–239 nm in the range 31,600–50,400 L/mol/cm (mean 38,500, RSD 17%).[90] The main adsorption of microcystins at 238 nm is due to the conjugated diene in ADDA, and there was no apparent trend in the observed coefficients with structural modification. Therefore, the variation can be attributed to experimental errors in the determination of the absorption coefficients and a general RRF of 1.0 can be assigned among microcystins. LC-UV analyses can be calibrated with a CRM of microcystin-LR and the RRF of 1.0 applied to peaks for other known microcystins in sample extracts.[91] The RSD of 17% for the extinction coefficients could be used as an estimate of the contribution of the RRF to the measurement uncertainty. The main point to consider is the positive identification of the microcystin peaks. The minimum requirements would be matching of retention times to those in laboratory reference material, for example, algal extracts and matching of UV spectra (characteristic ADDA band). Unusual microcystins could also be included if LC-MS/MS identification was available.

2.4.3.3 Use of an Independent Method

An alternative method is used to determine the concentration of an analogue in a material. Reanalysis by the method of choice will then enable calculation of an RRF to the parent toxin. For example, certified reference standards are not available for DTX1 and DTX2. These toxins and the "parent" OA were analyzed in selected shellfish materials by LC-FL and by LC-MS using RRFs of 1.0 to OA.[92,93] The concentrations of OA, DTX1, and DTX2 by each technique were very similar and the total toxin amounts for several hundred samples from a Canadian monitoring program were highly correlated (slope 1.0, $R^2 = 0.955$). A good correlation of LC-MS data for DSP toxins to mouse bioassay results was found for the Irish biotoxin program.[94] DTX2 contributed a high proportion of the toxicity in many samples. These correlations are *ipso facto* justifications for use of an LC-MS RRF of 1 to OA for DTX1 and DTX2.

2.4.3.4 Use of Assumed RRFS

The RRF or an analogue is set based on structural similarity to the parent toxin. This is an expedient where there is no reliable means to measure the RRF. For example, an RRF of 1.0 to DA for

LC-UV detection at 242 nm can be assigned for isomers of DA containing the conjugated diene in the side-chain (in analogy to microcystins). This is the current status of the AOAC protocol for DA in shellfish with regard to determination of epidomoic acid. Unfortunately, LC-MS ionization techniques, while of broad applicability to marine biotoxins, do not provide uniform RFs for a wide range of compounds. Ionization efficiency is very dependant on compound structure. Furthermore, the instrument design, operating conditions, and solvents/buffers all affect the overall ionization efficiency and types of ions formed. Only where the structural similarities are very high and the electrospray environment is very uniform can similar sensitivities be expected for the parent toxin and an analogue, that is, a RRF of 1.0 might be assumed.

Some indication of the potential magnitude of errors arising from use of assumed LC-MS RRFs for toxin analogues can be obtained from some comparisons using reference materials. The National Research Council of Canada CRMP programme has relevant CRMs and the Japan Food Research Laboratory has prepared a set of reference materials for calibration of methods used in the Japanese shellfish monitoring programmes.[55,56] Some RRF data obtained in three laboratories using these standards is summarized in Table 2.3. For these instruments using only the parent toxin of each group for calibration and assuming RRFs of 1.00 for analogues, the bias in results would be less than 30% for AZA2, AZA3, iDA-C, DTX1, C16-DTX1, PTX1, and PTX6. The bias for 45-OHYTX would be 10–40% depending on whether multiple reaction monitoring (MRM) or SIR was used (RRFs uncorrected for relative abundance of the monitored peaks in the spectra). The magnitude of these potential biases is not high in the context of other uncertainties surrounding biotoxin testing in seafood. The low RRF for PTX3 has been explained by the formation of a methyl hemiacetal in methanolic solutions.

The application of RRFs has not been explored for the LC-FL methods for saxitoxins using pre- or postcolumn oxidation. The yield of oxidation product and FL RF are very dependent on the toxin structure and the details of the instrument setup. Therefore, use of RRFs may not be feasible. This is unfortunate because it is a huge task to prepare and maintain stocks of CRMs for the wide range of saxitoxin congeners that can be found in seafoods.[83,85]

Without any independent confirmation, the uncertainty may be high for an assumed RRF and thus for any derived concentrations. Whether this is acceptable or not should be decided on a case-by-case basis. Points to consider:

1. The importance of the analogue. If it only occurs in seafood at minor concentrations relative to the parent toxin, then the RRF will not greatly influence the overall uncertainty of measurement for the summed concentration of the toxin group. For example, in New Zealand, *P. reticulatum* produces YTX and not homoYTX, and the predominant shellfish species accumulates the 45-OHYTX metabolite at only 10–15% the level of YTX.[24] Therefore, for the purposes of enforcing the regulatory limit by an LC-MS method, the use of RRFs of 1.0 for homoYTX and 45-OHYTX makes only a minor contribution to the measurement uncertainty for the group.
2. The suitability of alternative techniques to provide the required data. For example, mouse bioassay is designated by the EU as the reference method for lipophilic toxins. However, the technical and ethical limitations are severe, making it increasingly unacceptable. Instrumental methods, with judicious use of RRFs, can provide much more defensible data.
3. Correction of LC-MS RRFs for relative ion abundance. Equal sensitivities for parent and analogue should initially only be assumed for the total ionization. SIR or MRM techniques will be used for trace analysis of toxins. For SIR, the full-scan spectra of parent and analogue should be checked. The assumed RRF of 1.0 should be adjusted if the ions monitored differ in their relative abundances (as % of TIC) due to differences in in-source fragmentation and adduct ion formation. It is probable that the relative intensities in the MS/MS daughter ion spectra will vary between parent and analogue. A cross-calibration

TABLE 2.3
LC-MS Relative Response Factors Established from Toxin Reference Materials

Parent Toxin	OA	PTX2	OA	OA-C16 Ester	PTX2	YTX	YTX	AZA1	DA
MS method	ESI-, SIR	ESI-, SIR	ESI-, SIR	ESI-, SIR	ESI-, SIR	ESI-, SIR	ESI-, SRM	ESI+, SRM	ESI+, SIR
Analogue—RRF to parent	DTX1 1.0	PTX1 1.1	DTX1 0.84 ±16%	DTX1-ester 0.83 ±7%	PTX1 1.00 ±18%	45OH-YTX 1.52 ±17%	45OH-YTX 1.07	AZA2 0.74	isoDA-C 0.73[a]
	DTX1-ester 0.8	PTX6 0.6			PTX3 0.43 ±38%			AZA3 1.05	
					PTX6 1.29 ±22%				
Reference	[55]	[55]	[56, 94]	[56, 94]	[56, 94]	[56, 94]	[95]	[95]	[95]

[a] RRF corrected for relative abundances of the monitored ions in the ESI spectra for parent and analogue.

can be carried out by analyzing a suitable material containing both (concentrations not necessarily known) using SIR and MRM and the peak areas ratios calculated:

$$\text{RRF-MRM}_{\text{analogue}} = (\text{MRM}_{\text{analogue}} \times \text{SIR}_{\text{parent}})/(\text{MRM}_{\text{parent}} \times \text{SIR}_{\text{analogue}})$$

4. Precision and recovery data. Validation data can be gathered for toxin analogues calibrated by RRFs.[54] Blank seafood matrices can be fortified with aliquots of semi-purified extracts of contaminated shellfish or algae containing the toxins of interest. Comparison to an aliquot spiked into solvent enables calculation of recovery and replication enables determination of precision. Thus, several key elements of method validation can be accomplished without a CRM and only minimal revalidation is required when one comes available.
5. Analytical quality control measures. RRFs should always be used in conjunction with general analytical QA/QC measures. Precision and other data can be gathered and maintained over time on a consistent, comparable basis. Laboratory reference materials in the form of an extract containing arbitrary levels of all toxins should be used for daily checks on the retention times and responses. Changes in the response ratio analogue/parent may indicate instrument perturbations affecting the RRF.

2.5 USE OF SCREENING METHODS

Screening methods can be defined as methods that demonstrate the presence of the toxins of interest but which, in the form they are applied, cannot provide data to directly enforce compliance with regulatory limits. They are generally designed to provide high-throughput, relatively lost cost test results. A screening method must be backed by an alternative quantitative method that will be applied when triggered by a positive screen result. The main utility of screening methods is in seafood monitoring programs where a low proportion of samples are expected to be positive. There are also uses where field personnel carry out preliminary screening to establish whether toxins are present at levels of concern for human or environmental health. The important issue of the reliability of the screen test to fulfill its purpose can only be properly established through a thorough SLV. As part of this, the trigger level at which quantitative analysis is initiated must be set—not too low to avoid triggering by inconsequential levels of toxins and not too high to avoid false negatives. The other important issue to consider when setting up monitoring programs based on screening methods is the efficiency of the whole testing system. The combinations of screen and quantitative methods must cost-effectively cover all toxins of interest with varying scenarios of nil, low, and high rates of positives while remaining timely and under quality control. These requirements have proved difficult to meet, and most shellfish monitoring programs internationally are currently based on direct enforcement of regulatory limits using a set of accepted methods. Replacement of mouse bioassays is not leading to widespread use of screening methods because multitoxin instrumental methods can efficiently provide quantitative data. Use of a marker compound to give a quantitative estimate of the total toxic residue has limited application to the direct enforcement of regulatory limits for marine biotoxins in seafoods due to the highly variable and complex nature of the contaminations.[5] However, marker compounds do have utility for screening.

Situations where screening methods are proving useful or have the potential to be more widely used include

1. *LFIC screening for saxitoxins:* The Jellet Rapid PSP kit has been validated and shown to differentiate reliably shellfish containing saxitoxins above or below the regulatory limit of 0.8 mg/kg saxitoxin equivalents.[97] However, there are uncertainties about interpretation of the cutoff point and the effect of different cross-reactivities to saxitoxin analogues. In several countries, it is permitted to be used only to screen that a shellfish area closed to harvest should remain closed due to high saxitoxin levels. Reopening must be by mouse

bioassay. In this case, the main advantage of screening is convenience because the cost is similar to that of the bioassay. The simplicity of LFIC makes it amenable to use in the field by aquaculture facilities or environmental health personnel for screening before regulatory testing in a laboratory is undertaken. LFIC kits are also available for detection of DA.

2. *OA/DTX esters (DTX3):* Seafood contaminated by DSP contain fatty acid ester forms as well as free toxins and (as high as 90%–100% by proportion).[98] Hydrolysis is used to release all the OA, DTX1, and DTX2 to estimate overall compliance with the regulatory limit. Contamination of shellfish by DSP toxins is almost exclusively caused by *Dinophysis* species, which also produce much higher levels of pectenotoxins, mainly PTX2.[99] Rapid hydrolysis of PTX2 in shellfish leads to PTX2 seco acid, which can be sensitively detected by LC-MS.[24,100] Use of PTX2 seco acid as a marker compound for the onset of DSP contamination enables LC-MS multitoxin screening of shellfish using a single run per sample. A second run after hydrolysis of the extract would be required only if PTX2 seco acid was detected.
3. *Brevetoxins: Karenia brevis* produces a range of brevetoxins dominated by PbTx-2. Brevetoxins undergo a complex metabolism in shellfish with oxidations, reductions, and conjugations of the terminal side chain leading to a wide range of derivatives, many toxic.[101–103] The only approved test is the APHA NSP-ether mouse bioassay. Certified standards are available for PbTx-1, PbTx-2, PbTx-3, and PbTx-9 but not for BTX-B5 or any of the conjugates which dominate the toxic residues in contaminated shellfish. Parent PbTx-2 and PbTx-3 (the primary metabolite and minor parent toxin) have been shown to be present in shellfish contaminated by *Karenia* species, although the levels were relatively low.[104] The sensitivity of LC-MS is such that of PbTx-2 and PbTx-3 can be detected in samples contaminated with NSP well below the regulatory limit. LC-MS screening for these marker compounds is used in the New Zealand monitoring program, because toxic *Karenia* blooms are very rare. This has greatly reduced the amount of mouse testing without compromising public safety.
4. *AZAs:* AZAs can be readily detected in shellfish using LC-MS. However, they are a complex class of toxins with three major analogues and a range of minor analogues known.[105] They are also relatively rare internationally. A CRM is available for azaspiracid-1. Therefore, it is convenient for multitoxin screening of shellfish by LC-MS method to use a single channel for this toxin. In the event of detection of this marker compound, samples can be rerun by LC-MS to determine a fuller range of the AZAs and thus enforce the regulatory limit.
5. *Ciguatera toxins:* There is a long-standing and still urgent need for a simple but reliable method to screen tropical reef fish for these dangerous neurotoxins.[106,107] Immunoassay techniques[108] could potentially provide a solution but no assay has been fully validated to meet objective performance requirements. Receptor binding assays and activated cytotoxicity assays have been reported with good performance characteristics.[109,110] However, these *in vitro* research assays are not available in rapid screening formats.

2.6 CONCLUSIONS

Adequate method validation is essential to maintain and improve the quality of testing for marine biotoxins in seafood. Although formal collaborative studies should be conducted where possible, only a few such studies for new marine biotoxin methods have been completed to international standards. More emphasis should be put on introducing new methods to regulatory testing based on thorough SLVs. A range of ongoing laboratory quality control measures, interlaboratory studies, and proficiency testing rounds can be used to ensure no major biases become entrenched in monitoring programs. A wide variety of alternatives to the current unsatisfactory dependence on mouse

bioassays have been explored. A range of instrumental techniques show satisfactory performance capability with the sensitivity, specificity, and multitoxin capability of LC-MS being particularly attractive. Most of the proposed *in vitro* or functional assays have not yet achieved precision/accuracy of analytical quality. ELISAs with excellent precision characteristics are becoming available for some toxin groups. ELISA and functional assays must establish accurate cross-reactivities for analogues and securely relate assay responses to regulatory limits. The issues of the availability and reliability of reference materials are very similar to those for calibration of instrumental analysis. Enhanced international collaboration is required to meet the continuing need for a wider range of biotoxin CRMs. The imperative to reduce reliance on mouse bioassays is a strong argument for use of validated instrumental methods with the judicious use of relative response factors as a transitional measure in a system of continuous improvement of methods. It is not reasonable to interpret current EC legislation for marine biotoxins[7] as making it an absolute requirement for introduction of alternative methods and that CRMs are available for all regulated toxins. This is a suitable long-term goal, but its achievement is a huge and never-ending task due to the wide and increasing range of toxins and analogues.

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Part II

The Epidemiological Impact of Toxic Episodes

3 Epidemiological Impact of Diarrheic Toxins

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CONTENTS

3.1 INTRODUCTION

Since ancient times, there have been references to the awareness of existing toxins related to fish and shellfish consumption.

The first Egyptian plague ("all the water that was in the river turned to blood and the fish that were in the river died; and the river stank and the Egyptians could not drink of the water of the river; and there was blood throughout all the land of Egypt" [1]) could very well have been due to a toxic red tide.

The first Chinese pharmacopoeia (2800 BC) warns against and makes recommendations on balloonfish consumption [2]. Nevertheless, we can assume that humankind knew of the red tide dangers before the written word, given the discovery of the 26-million-year-old fossil *Gonyaulax polyedra*.

Even in pre-Colombian America, the hazards of eating shellfish extracted from the sea when it presented a red color during the day or a glow during the night were already known. To avert this danger, watchmen were placed in affected spots who would alert travelers of such hazards. According to Halstead [3], it is the first known health quarantine in North America.

The first scientific reference to human shellfish poisoning is probably the one in "Ephémérides des curieux de la nature" (1689), quoted in 1851 by Chevalier et al. [4,5]. However, the first written report of an outbreak in British Columbia, which occurred in 1793, was reported by Vancouver in 1801 [6].

In Europe, there are scientific descriptions of paralytic shellfish poisoning (PSP) outbreaks dating back to 1689 [4,5]. It is worth pointing out the Wilhelmshaven case (1885) that sparked off the scientific solving of mussel poisoning, although the precise connection of the poisoning to mussels would not be established until 1927, when an outbreak took place in the central California coast. The ensuing studies led Meyer and Sommer (1937) [7,8] to the discovery that the cause was dinoflagellates and their toxins, and to the beginning of in-depth studies. Halstead [3] compiled all the cases published worldwide up to 1965.

All these episodes refer to poisoning by paralyzing toxins. Awareness of the actual toxins that cause the diarrhea events is more recent. The first known diarrheic poisoning event associated to toxic mussel consumption (described at the time as "mussels that had ingested dinoflagellates") took place in the Easterscheldt area in the Netherlands in 1961 [9]. That same year, there were other cases recorded in Waddensea. The next outbreak in Easterscheldt occurred in 1971 and affected 100 people. There are also references to the ingestion of blue mussels in Scandinavia (Norway) in 1968 [10]. In the following years, several cases were reported in the Oslofjord area. They were classified as "unidentified mussel poisoning."

In October 1976, in the Netherlands, 25 people were taken ill after eating mussels from Waddensea [11]. That year, Yasumoto et al. described diarrheic shellfish poisoning (DSP) for the first time in a food-poisoning outbreak due to the ingestion of mussels and scallops that took place in northeastern Japan [12].

In 1976, we treated the first cases of PSP detected in Galicia (Spain) [13], and in the summer of 1978, there were a series of diarrheic events in the Ría de Ares area. In the town of Lorbé (Oleiros, close to the city of La Coruña), we studied episodes related to mussel consumption. We ruled out microbiological causes and attributed those episodes to an unknown toxin caught by the mussels in their growing zone, as had happened with PSP in previous years.

In the following summers, we studied similar epidemiological outbreaks that, after an incubation period of a few hours, presented as diarrhea, nausea, vomiting, and abdominal pain, without fever. This was associated to the consumption of steamed mussels. Patients would recover in 2 or 3 days. We contacted Dr. Kat and implemented the test she was developing in rats. The test gave inadequate results. This was probably due to methodological problems.

In 1981, the greatest diarrheic poisoning associated with mussel consumption occurred, affecting around 5000 people all over Spain, mostly in Madrid. Once again, the epidemiology analysis associated diarrhea with mussels. By then, Kat's [11] and Yasumoto's [12,14,15] works were

already known. These poisoning cases by the DSP toxin in Galicia as well as in the rest of Spain run parallel to those that occurred in Japan in 1976 and 1977 [12,14,16], and before and after in many other European, Asian, and American countries.

Yasumoto et al. [12,14] reported events in Japan and related them to eating mussels contaminated with the DSP toxin, pointing out that it was a lipophilic toxin referred to as DSP in later works [15].

3.2 EPIDEMIOLOGY OF DSP

The epidemiology of human disease caused by harmful marine phytoplankton is still at an early stage. This lack of progress in the phycotoxin disease epidemiology is attributed to a lack of disease biomarkers and exposure in humans. Epidemiology studies are limited to the mere description of clinically identified cases and little else. More recently, the studies have included laboratory testing of ingested food.

The lack of biomarkers is a hindrance to the discovery of the real incidence, since it is only possible to confirm clinical cases in their acute stage, and as long as there are some food remains available or their origin is known and a sample can be obtained. Asymptomatic cases and those where this possible cause is not considered go undiagnosed.

Biomarkers that measure exposure and effect may be qualitative and quantitative. In order to be useful, they should be detected early in human biological fluids accessible and acceptable. Ideally, biomarkers should also allow for the identification of subclinical cases. Other considerations to be born in mind are the speed in testing, precocity in its application, and price.

In order to have the exposure markers available, it is necessary to develop the toxicological analysis of toxin levels and their metabolites in body fluids. At present, effect markers are based on the clinical picture. It is important to develop markers for subclinical physiological changes.

3.2.1 Relevance

Diarrheic shellfish poisoning is widely spread in the world, affecting particularly Japan and northwestern Europe. It represents a serious economic problem for the shellfish industry and for public health.

3.2.1.1 Socioeconomic Relevance

The problem of toxic bivalve shellfish (mussels, clams, cockles, scallop) affects not only public health but also the tourist industry (exports, markets, advertising, negative publicity), and can therefore cause economic upheaval.

This problem has no easy solution, since the procedures used in the purifying plants are excellent for eliminating potential microbiological contamination, but have no effect on the biotoxin content.

DSP has some relevant economic repercussions that are the object of study in Chapter 47. According to data from Food and Agriculture Organization (FAO), the world's mussel production in 2003 was 1,800,000 tons, 1,620,000 of which came from aquiculture (90%). Europe is the second biggest mussel producer in the world (593,644 tons in 2003) after China (883,237 tons). In Europe, Spain is the main producer, with 248,827 tons (which represents 76,991 M€) (Eurostat data for 2003), followed by Italy with 100,000 tons (65,002 M€), France with 68,000 tons (95,883 M€), and Holland with 56,200 tons (71,544 M€) [16].

Galicia (northwestern region of Spain) produces 95% of mussels in Spain. Galicia has 3337 mussel platforms and generates about 11,500 direct jobs, 8500 of which are fixed-term and 7000 are indirect jobs. Galicia also represents 21% of the fish production (fresh fish) first sale.

The presence of toxic red tides means having to close down the shellfish fisheries in the affected areas and having to endure for long periods a situation of economic hazard for a great number of

families that directly or indirectly depend on these fishing trades. Moreover, if control failures occur and there are cases of human poisoning, the discredit and mistrust created can lead to loss of markets that become hard to recover.

From the beginning of the 1980s, and particularly in the second half of the decade, a progressive increase of poisoning episodes of phytoplankton origin in bivalve shellfish was observed in Galicia. Mussel extraction was prohibited for up to 200 days/year in some areas.

3.2.1.2 Health Relevance

Diarrheic shellfish poisoning is relevant from a health been viewpoint not only because of its acute effects but also because of its potential chronic effects, which are not yet fully understood.

Regarding acute effects, the gastroenteritis caused by okadaic acid (OA) and dinophysistoxins (DTXs) has a favorable evolution toward total recovery in 1–3 days, and no fatalities have been described.

Regarding chronic effects, OA and DTX-1 have been shown to be potent tumor promoters, and given that the stomach, small intestine, and colon have binding sites of OA, this could be implicated in the growth of gastrointestinal tumors [17,18]. Mutagenic [19] and immunotoxic effects due to a marked suppression of interleukin-1 (IL-1) production have also been described [20].

It has been shown in experimental animals that pectenotoxin-1 (PTX-1) is hepatotoxic and induces rapid necrosis of hepatocytes, with a pathological action similar to that of phalloidin. In rats intraperitonially injected with PTXs, the liver finally appears granulated and the hepatocytes contain many vacuoles.

Yessotoxins (YTXs) have been shown to cause heart damage. Almost all cardiac muscle cells of mice inoculated with these toxins were swollen. On the other hand, YTXs do not cause damage in the liver, pancreas, lungs, kidneys, and adrenergic glands.

All these chronic effects need to be studied in depth, and they underline the relevance and dimension of the problem and the need to avoid ingestion of these toxins.

3.2.2 Frequency and Distribution of DSP

The real incidence of human DSP is hard to assess, since its clinical symptoms can be mistaken for diarrhea from other causes, and it can go unrecorded owing to its benign evolution. Isolated cases usually go undetected, and they only become known in countries where outbreaks have to be reported by law.

At present, the appearance of human poisoning cases is something that should not take place, and it only reflects a considerable failure in the watching and preventing process that should be operating and to which we will refer later.

Research on outbreaks in humans has only contributed to prevention, determining their origin, and the potential exposure of other groups of people to shellfish of the same origin. The main interest regarding prevention is the watching and early detection of toxic episodes in the sea that would allow the adoption of measures (forbidding shellfish extraction in the affected areas and informing people against its consumption). In countries where this watch network based on early detection is not possible, the watch on early detection of human intoxication can be useful in spotting the hazard and taking preventive measures that can avoid its spread.

3.2.2.1 Incidence

As pointed out before, the lack of biomarkers does not allow us to know the real incidence of DSP in humans, since our awareness of it is limited to notified outbreaks.

In the last 20 years, the incidence of DSP events in humans has decreased and practically disappeared in developed countries where, as an answer to the problem, watch networks have

been developed to detect the presence of toxic plankton species and poisoning in shellfish. This has not been the case in countries where such a watch network is not available. Nevertheless, poisoning episodes in the sea have increased, and this is partly due to a higher awareness of the disease and to setting watching schemes but also to its spreading to new, and sometimes far apart, geographical zones, aided by international trade of seafood and the chance that cysts of exotic plankton species, producers of toxins, travel with them and settle in these new zones where they were previously unknown. Thus, the phycotoxic hazard becomes a public health problem worldwide.

Although shellfish poisoning is widely spread all over the world, affecting warm and tropical zones, Europe and Japan are the most affected areas. Some reports show that DSP events have also taken place in other parts of the world (Australia, New Zealand, Indonesia, and Argentina) [21,22].

3.2.2.2 Outbreaks in Europe

Various DSP outbreaks were reported in France during the 1980s, and they affected large numbers of people. In the Loire-Atlantique district and in Normandy, 3300 and 150 cases were detected, respectively, in 1983, and 70 and 2000, respectively, in 1984. Other DSP events were reported in 1985 (a few cases) and 1987 (2000 cases) [23]. In 1990, there was an outbreak affecting 415 people due to mussels imported from the north Danish coast. It had a toxic OA load of 170 μg per 100 g of meat [24]. Since the watch network was set in 1984, an increase has been observed in the toxic tides frequency as well as its spreading to other previously uncontaminated areas.

The first outbreak reported in Norway due to a *Dinophysis* spp. toxin [25] took place during a long contamination period (October 1984–April 1985), during which around 400 cases were detected among people living in the southwest coast of Norway. Coinciding with this outbreak, another one took place in October 1984, in the west coast of Sweden, where DSP events had already been taking place since 1983, affecting about 100 people [26]. In 1986–1987, a monitoring program for DSP toxins was established in Norway.

Since 1990, when the watch network was set up in Denmark, mussels contaminated with DSP have been detected during many summers.

In February 2002, an outbreak occurred in Antwerp (Belgium) with 403 cases of DSP, after consumption of boiled blue mussels imported from Denmark [27].

In Spain, the first cases were detected, as we said before, in 1978 in the Ares Estuary [28]. New events also took place in the following years, the main one occurring in 1981 with 5000 cases. Since then, DSP has been regularly detected in seawater.

The first DSP event in Italy was in 1989, where affected cases were found on the north and northwest Adriatic coasts.

In Germany, in September 1978, single cases of DSP intoxication were reported in the Husum area. In November 1986, at least eight people were affected. Since 1986, DSP has regularly been detected on the coast of German Bight, but no large outbreaks of DSP have been described [23], as is the case in Portugal and Ireland.

In Portugal, since 1987, when they were spotted for the first time, DSP toxins have been detected regularly in bivalve shellfish from the northern coast including the Aveiro Estuary and the Mondego Estuary. In 2002, an outbreak with 40 cases occurred in Northern Portugal [29].

In Ireland, since the watch program was setup, DSP has been detected in shellfish samples almost every year. The strictness and the duration of closure of shellfisheries vary according to years. The events are recorded in the summer and autumn months (June–December).

3.2.2.3 Outbreaks in Asia

The first episodes of DSP in Asia, due to the ingestion of toxic blue mussels and scallops, were those mentioned above. They took place in northeastern Japan in 1976 and 1977 and affected 164 people [12,14].

New outbreaks were recorded later. Between 1976 and 1984, Kawabata reports that there were 34 outbreaks affecting 1257 people [30]. The Japanese and European shores are the most affected by toxic blooms.

Toxic red tides have also been described in the Russian East coast (*Dinophysis acuminata*, *D. acuta*, *D. fortii*, and *D. norvergica*) [31] and the presence of DSP toxins in shellfish from India [32] although human cases have not been reported.

3.2.2.4 Outbreaks in America

The first reported DSP event in North America was in 1990 in Nova Scotia off the eastern coast of Canada and was due to DTX-1. It affected 16 people [33]. Other events were reported later in the same region [34]. In 1989, a red tide was detected on Long Island, New York (with a high number of *D. acuminata*). It was of low toxicity in shellfish (0.5 MU), and no human cases were reported [35].

In January 1991, an outbreak affecting 120 people was detected in Chile. *D. acuta* was identified as the responsible toxin [36]. In January 1992, DSP toxins were also detected on the coast of Uruguay [37].

In March 2002, an outbreak with approximately 40 cases occurred in the Chubut Province, Argentina. Those affected had eaten blue mussels and clams with DSP from the North-Patagonian gulfs. This episode coincided with the presence of the *Prorocentrum lima* [22].

3.2.3 Epidemiology Chain

In order to systematize an epidemiological study oriented toward the prevention of DSP, we will follow the epidemiological chain pattern developed in 1931 by Stallybrass, the great scholar of epidemiology.

The reservoirs are the dinoflagellates causing the DSP; the poisoning source and transmission mechanism are the bivalve shellfish with toxins—mainly mussels, although sometimes scallops are also involved. The subjects at risk are the people eating them, who usually live on the coast, have a low education, and are consumers of shellfish; moreover, they pick the shellfish themselves directly from the shore, thus bypassing any health control, which increases the risk.

3.2.3.1 Causal Agent

The DSP group comprised three toxin groups: OA and dinophysotoxins; PTXs (polyether lactone); and YTXs (toxins with sulfate groups).

Toxins from the OA group have been known to cause the disease in humans since the late 1970s. The syndrome was named DSP due to the dominating symptoms.

The first DSP group toxin was isolated from mussel digestive glands and was called DTX-1 [38]. Observation by spectral comparison showed that it was 35-*R*-methyl OA. Later, other OA derivatives were identified (OA had been isolated for the first time from the *Halichondria okadai* sponge in 1981) [39]; later, it was also found in the *P. lima* and *Dinophysis* spp. dinoflagellates, the DTX-3 (7-*O*-acyl-35-(*R*)-methylokadaic acid) in an intoxication by scallops in northeastern Japan [40], and the DTX-2 (31, demethyl-35-methylokadaic acid) in Irish mussels [41].

The presence of PTX in shellfish was discovered because of their high acute toxicity in the mouse bioassay after intraperitoneal (i.p.) injections of lipophilic extracts. The PTX-1 was isolated from the digestive gland of the scallop *Patinopecten yessoensis* in northeastern Japan [40]; later, various homologous ones were described (PTX-2–PTX-4; PTX-6–PTX-9, and PTX-11). Animal studies indicate that they are much less potent via the oral route and that they do not induce diarrhea. In experimental animals, they exert a strong hepatotoxic effect, but their diarrheic effect is mild and even undetectable [42–44]. There are no data indicating adverse effects in humans associated with PTXs in shellfish. Although diarrhea has sometimes been reported in animals dosed with PTX-2

and PTX-2 seco acids, recent studies have shown that PTXs are not diarrheic in humans [45]. PTXs exclusively arise from *Dinophysis* spp. and are always accompanied by toxins from the OA group.

The YTX are a group of bisulfate polyether toxins with a structure similar to brevetoxins (brevetoxin-type polyether), which were isolated from *P. yessoensis* [46]. They are produced by the dinoflagellates *Protoceratium reticulatum* [47] and by the *Lingulodinium polyedrum* [48]. Their presence in shellfish was discovered due to their high acute toxicity in mice after i.p. injection of lipophilic extracts. They are much less potent via the oral route, and they do not induce diarrhea. There are no reports of human intoxications caused by YTXs [45].

Of all these toxins, only OA and its derivatives (DTX) cause acute gastrointestinal toxicity. OA and DTX have been shown to be powerful inhibitors of serine/threonine protein phosphatase PP1 and PP2A activity; they are two of the main cytosol phosphatases in mammal cells, with the subsequent increase in phosphorylated proteins [49]. OA probably causes diarrhea by stimulating phosphorylation of proteins that control sodium secretion in intestinal cells [50–52].

OA was found to be a threshold (indirect) genotoxic compound in various cell types *in vitro*. No genotoxicity data are available for DTX-2 and DTX-3. Animal data indicate that OA and DTX-1 are potential tumor promoters, but the data are insufficient. No data are available for DTX-2 [45].

OA is the predominant toxin in most European countries, although DTX-2 has been reported in Ireland [41,53], Spain [54], and Portugal.

PTXs and YTXs, although they do not present diarrhea symptoms, due to their common lipid soluble properties, are included in the DSP toxins complex (lipophilic toxins), and are subjected to the same regulations as OA and derivatives. A FAO/IOC/WHO work group challenges the toxicity in humans of PTXs and YTXs and suggests that they should be regulated separately. This group considers that only OA and derivatives cause diarrhea [45].

3.2.3.2 Reservoir and Intoxication Source

Dinophysis dinoflagellates in 1989 and later *Prorocentrum* dinoflagellates were identified as the organisms responsible for producing the DSP toxin. In Western Europe, the predominant types are usually *Dinophysis* spp., while *Prorocentrum* spp. are more often found in Japan.

The *D. acuminata* and *D. acuta* species are the most widely spread in European waters [55–57]. *D. acuminata* is the main component in the greatest algal blooms on the northwestern shores in France. *D. acuta* affects the Atlantic coast of Galicia, Portugal, Ireland, Sweden, and Norway. On the European coasts, we also have *D. caudata* and *D. tripos* in the Iberian coast; *D. rotundata* in the whole coast and *D. sacculus* in the Mediterranean Sea, and the Iberian and French coast. In the Adriatic Sea (Italy), *D. fortii* has also been identified [58], as have *D. norvergica* [59] together with *D. acuminata*, *D. acuta*, and *P. micans* in Norway.

Associated with toxic outbreaks, some other species have been detected: *Lingulodinium polyedra* in the Adriatic Sea and *P. reticulatum* in the Norwegian and the Adriatic Sea.

The presence of these dinoflagellates, even in small concentrations (hundreds of cells per liter), can lead to poisoning of shellfish. Their ingestion, or of fish that have previously fed on small herbivorous fish that have themselves fed on toxic algae, causes poisoning in humans.

In the Galician Estuaries of Spain, episodes of DSP appear associated mainly with a proliferation of *D. acuminata* in Ría de Ares and *D. acuminata* and *D. acuta* in Rías Bajas, although, at times, other species such as *D. caudata*, *D. tripos*, and *D. rotundata* can contribute significantly to the level of diarrheic toxins detected.

3.2.3.3 Transmission Mechanism

Bivalve shellfish, mainly mussels and less frequently scallops, are the DSP toxin vectors. They acquire the toxin when there are some species of toxic dinoflagellates in the plankton on which they feed.

Ninety-five percent of the toxin accumulates in the hepatopancreas of the mussel without it suffering any chemical changes, and apparently, the toxin does not alter the mussel's physiological

functioning or organoleptic properties either. The amount of toxin retained depends not only on the number of dinoflagellates present in the medium and its toxic load, but also on the amount of water filtered by the shellfish.

3.2.3.4 Vulnerability Factors

Personal features: People most at risk are those living on the seashore of countries with underdeveloped monitoring systems, with no watch network for sea toxicity events, and a low level of health education. Traditionally, they are consumers of shellfish, which they usually pick themselves from the sea without it undergoing any health control. There are no differences in sex and age.

Time distribution: Toxic episodes generally appear in summer and autumn months, although occasionally they appear earlier—end of winter or early spring. In the Netherlands in the years 1981, 1986, 1987, and 1989, events were recorded during September and October, and once even in December. In the Galician Estuaries (Spain), *D. acuminata* is present practically all year around. It proliferates usually in April, although some years this happens in late February or March, presenting maximum or minimum levels until mid- or late autumn. *D. acuta* usually appears associated with southern winds from September to November due to the advection from towns in the surrounding coastal area, and *D. caudata* is usually found isolated in the plankton.

Space distribution: DSP is widely spread around the world. Europe's western shores and Japan's shores are affected the most.

3.3 EPIDEMIOLOGY OF AZASPIRACIDS

The azaspiracids (AZAs) are a new group of toxins identified in 1995 during an outbreak in the Netherlands, when symptoms of DSP poisoning were observed, but with very low concentration of OA and DTX in shellfish [60]. After purification, it was possible to identify this family of new toxins called azaspiracids [61].

After an incubation period of 3–18 h, azaspiracids cause a clinical illness that is similar to that produced by DSP poisoning. There are no specific laboratory tests that are useful in the diagnosis of AZA poisoning. Diagnosis is based on characteristic symptoms, supported by testing of suspected seafood. There is no specific antidote, and the treatment is symptomatic and supportive only. Complete cure is achieved in 2–5 days.

In animal studies, when the AZA is administered per os, it caused degeneration of epithelial cells and necrosis of the lamina propria in the villi of the small intestine and in lymphoid tissues such as thymus, spleen, and the Peyer's patches; fat accumulation in the liver and degeneration of hepatocytes; reduction of nongranulocytes; and damage to T- and B-cells in the spleen. Overall, AZA1 induced a far greater degree of tissue injury and slower recovery time when compared with OA [62,63].

One study reported that AZA (tumor initiators) and DSP (tumor promoters) toxins in shellfish could cause intoxication concurrently [63].

3.3.1 Outbreaks

Azaspiracid (AZA) poisoning has been reported in five countries, all of them in the European Union and all from consumption of mussels cultivated in Ireland. The first outbreak occurred in Netherlands in November 1995 with eight people affected. The symptoms were similar to those of DSP, but the concentration of the major DSP toxins were very low [60]. No known organisms producing DSP toxins were observed in water samples collected at that time. In addition, a slowly progressing paralysis was observed in the mouse assay using mussel extracts. These neurotoxic symptoms were quite different from typical DSP toxicity. Subsequently, AZA was identified, and the new toxic syndrome was called AZA shellfish poisoning.

The next outbreak occurred in Ireland in September–October 1997, and was caused by consumption of mussels from Arranmore Island. Details of the Arranmore AZA incident were supplied by Dr. Terry McMahon. About 20 individuals were affected in the outbreak, and seven to eight of these were examined by a doctor. Symptoms were vomiting, diarrhea, and nausea. There were no signs of any hepatotoxic effect, and no individuals subsequently presented with illnesses that could be related to the initial intoxication. Some patients reported illness following the consumption of as few as 10–12 mussels. All patients recovered completely after 2–5 days [64,65].

Some episodes occurred later in Italy (10 cases) in September 1998; in France (about 20–30 cases) in September 1998, and in the United Kingdom (12–16 cases) in August 2000. In England, several incidents of AZA poisoning were reported in Sheffield, Warrington, Aylesbury, and the Isle of Wight. AZAs have been found in mussels (*Mytilus edulis*) from Sogneford, Southwest Norway, and Craster on the Eastern coast of England. They have also been found in scallops (*P. maximum*) from Brittany, France; and mussels (*M. galloprovincialis*) from Galicia, Spain [63–66].

So far, outbreaks were limited to Europe. However, given that *Protoperidium* spp. have a widespread distribution, it is possible that other cases occurred in other countries without being detected, as the general mouse bioassay is not specific for the toxins.

However, based on data of the European Commission rapid alert system, Food Safety Authority of Ireland is aware that products contaminated with AZA were available on the market and presumably consumed without any reported AZA syndrome [65].

In accordance with this information, in 2002, AZA-contaminated shellfish arrived on the Belgian market on three occasions: on March 29 (queen scallops from Scotland), June 25 (mussels from Italy), and on October 17 (scallops fished in International Council for the Exploration of the Sea area VIIe by Belgian vessels). In 2005, it has been consumed on four occasions: the first one in the United Kingdom on May 26 (mussels from Canada), and the other three in Norway (crabs from Norway) on November 1, 9, and 14. On these last three occasions, AZA was detected and measured in shellfish in concentrations of 339 µg AZA eq./kg (1045 kg presumed product already consumed), 177–269 µg AZA eq./kg (6200 kg presumed product already consumed), and 217 µg AZA eq./kg (3094 kg, distribution on the market and possibly withdrawn), respectively [65].

3.3.2 Epidemiological Chain

3.3.2.1 Reservoir and Causal Agent

The AZA1 and their derivates (at least 11: AZA1 to AZA11) [63,67] are produced by the dinoflagellate *Protoperidium crassipes*. Owing to the predatory nature of this organism, it cannot be excluded that AZA could accumulate through consumption of another prey species.

3.3.2.2 Transmission Mechanism

In all outbreaks, mussels (*M. edulis*) were the only shellfish responsible. Mussels were the shellfish with the highest toxin concentration: 4.2 µg/g. Only oysters accumulated toxins at levels (2.45 µg/g) comparable to mussels (James et al., 2004). In other shellfish, the concentration detected was much lower: scallops (0.40 µg/g), cockles (0.20 µg/g), and clams (0.61 µg/g).

All reported cases were due to Irish shellfish before 2001, the year in which the EU adopted legal limits for AZA and the biotoxins monitoring program was improved. Since then, no case has been declared.

3.3.2.3 Seasonal Variation and Duration of Toxicity in Shellfish

AZA contamination of shellfish can occur in all seasons; however, it is likely to be prevalent in the summer months (mostly late summer) [63]. In one study, AZA-1–AZA-5 were found in mussels in November 1997.

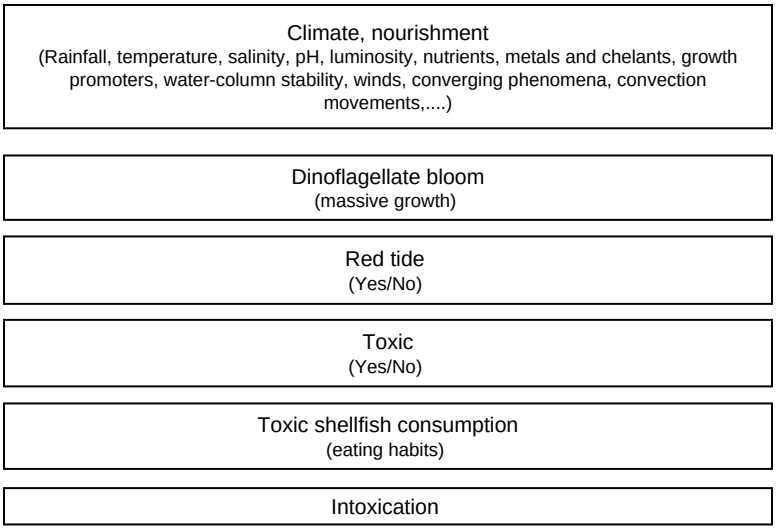

FIGURE 3.1 Factors associated with intoxication.

A long duration of contamination has been reported, with toxins remaining in the mussels for at least 6–8 months after the initial poisoning [63,66–68]. Initially, mussel digestive glands contain most of the AZA; then AZAs migrate to other mussel tissues, leading to persistent contamination. AZA-1 is the predominant toxin in the digestive glands; AZA-3 is predominant in other tissues.

3.4 FACTORS ASSOCIATED WITH TOXIC EPISODES

Studies aimed at establishing epidemiological associations between various factors and an intoxication can be carried out at two levels: The first comprises the study of the conditions that cause the explosive growth of dinoflagellates, resulting in the production of toxins and their concentration in shellfish. The second level studies factors causing the diarrheic event in humans (Figure 3.1).

Knowing the environmental and feeding factors associated with the space distribution and occurrence of poisoning in shellfish allows us to establish risk zones of varying degrees, which together with knowing the seasonal events or cyclical phenomena will contribute to diarrheic toxic episodes prevention in humans.

The retrospective study of previous outbreaks and researching the possibility of future ones allow us to define risk groups and event-associated factors useful for monitoring and preventing toxic events.

3.4.1 Factors Influencing the Explosive Growth of Dinoflagellates

Red tides is a natural occurrence that consists of the massive proliferation of unicellular organisms present in phytoplankton, which presents natural growing-and-decreasing cycles regulated by the chemical and physical conditions of water, such as a mild temperature, a drop in the water salt content, still waters, light, (long days), and concentrations of some organic and inorganic substances (nutrients), as well as biological interactions. Sometimes, and under favorable environment conditions, some of these organisms (dinoflagellates) causing red tides multiply suddenly, causing an explosive growth that gives the well-known coloring of water, and when they are toxic species, they cause the shellfish poisoning.

Dinoflagellates reproduce themselves by simple or multiple partition, and after an intense breeding activity, they encyst and settle for long periods in the bottom of the sea.

The beginning and development of red tides, as well as their later disappearance, depends on the interaction of multiple biological, biochemical, hydrographical, and weather-related factors that are not yet fully known.

In some regions, red tides are frequent and have become yearly events, whereas in others, they appear irregularly or occasionally.

The appearance of red tides is due to two sets of factors: those that favor the growth of the microalga population and those that favor their concentration.

3.4.1.1 Factors Influencing the Increase of Microalga Population

Water that is enriched with nutrients owing to the sea-bottom waters rising to the surface, to land drainage by rainfall, and to urban and industrial waste dumping.

Changes in water temperature influence the encysting (temperature decrease) or exciting (temperature rise) of dinoflagellates.
Sunlight: essential for conditioning photosynthetic vegetative processes.
Salinity: its decrease, caused by fresh water flowing into the sea from rivers or by heavy rainfall, favors the presence of red tides.
Organic substance: comes from land drainage or marine plants, fish, and other decomposing dead matter.
Metals and chelants: metals decrease growth while chelants increase it.
Substances promoting growth (vitamin B_{12}).
Calm seas: favor growth and the accumulation of phytoplankton.

3.4.1.2 Factors Influencing Concentration

Dinoflagellates gather in clusters due to hydrological concentration phenomena. These accumulation processes are influenced by mild winds that blow the surface waters toward the shore. Converging phenomena cause the dinoflagellates concentration along the front line of two masses of water of different density. The convection movements caused by the wind facilitate the dinoflagellate concentration on the converging lines.

When environmental conditions are not favorable, the haploid vegetative cells of very many dinoflagellate species form cysts that can resist very adverse conditions and remain viable in the sediment for long periods of over 15 years.

The fact that cysts are so resistant means that they can be carried viable from one zone to another where they can develop their mobile stage, going through an adverse medium in their journey.

The cyst germination is conditioned by internal factors and by external, or triggering, factors (temperature, light, and oxygenation).

Dinoflagellates have maturing and latency periods. When cysts germinate and mobile cells emerge, their survival depends on their capacity to free themselves from the sediment and get into the water column (this is favored by turbulence, which is then harmful to the dinoflagellate population development that needs still waters to avoid dispersion) and the chance of finding a favorable medium in the water column.

The relevance of the cyst population depends on their abundance in the sediment, capacity to germinate, and survival of the emerging mobile cells.

Bivalve shellfish filter water and retain the phytoplankton, which is not toxic to them. Mussels concentrate their DSP toxins in the hepatopancreas, while clams do it in their siphons. The degree of poisoning acquired by shellfish will depend on the toxicity per cell of the toxic phytoplankton organisms.

Toxins are secondary metabolites produced by toxic phytoplankton whose physiological and ecological functions are unknown. The amount and rate relative to toxins produced depends on intrinsic factors of the cells (genotype, age, size, cell cycle moment, and general physiological state),

and on environmental factors (temperature, salinity, pH, light, available nutrients, and relative rate between the various nutrients). Therefore, the toxin content per cell varies and, hence, the difficulty in answering the question: from how many cells per liter can mussels become toxic?

3.5 PREVENTION

In order to systematize the prevention study, we first analyze primary prevention measures based on the sea watch and the market and aimed at effectively foreseeing the toxicity phenomena before it reaches the human intoxication stage. For this, a greater knowledge of the potential dinoflagellate's life cycle and influencing environmental factors is necessary. We also include here the watch for human poisoning cases to be carried out in countries where there is possibility of setting up watching networks on the sea or in the market.

Second, we study the secondary prevention measures or measures to be adopted in a human poisoning outbreak.

Both sets of measures are fundamentally based on epidemiology watching. The weakest links in the epidemiological chain have to be identified in order to focus our attention on them.

It is not possible to act on the reservoir, thus avoiding the proliferation of dinoflagellates by modifying factors favorable to them. We are equally powerless in avoiding the accumulation of toxins in shellfish, or even in accelerating their detoxification. We can only act on the third chain link—the subjects at risk—by informing of the hazards and providing all the means at our disposal to avoid toxic shellfish consumption. As stated earlier, dinoflagellates do not undergo organoleptic changes that would alert the consumer, and ordinary cooking does not destroy the toxins either.

In the epidemiology watch programs, it is essential to have tracers for intoxication prevention. In the case of DSP, the most widely known tracer is visualizing a red tide, which, albeit its limitations, prevents disease outbreak. Nevertheless, it is of very limited efficacy, because events can take place without previous appearance of a red tide. The most efficient watch consists of determining changes in seawater warning of a potential proliferation of toxic plankton species, and watching out for these species as well as the presence of toxins in shellfish.

In short, epidemiology watching is based on the early detection of a problem (presence of the DSP or AZA toxins in shellfish). Each watch program or scheme must be adapted to the area, region, or country where it is going to be applied, bearing in mind the following:

- Incidence of the problem and its effects on the country's population and economy
- Legal infrastructure
- Available resources (human and material)
- Existing means of communication

3.5.1 Marine Biotoxins Monitoring Program

The potential monitoring of blooms requires knowledge of their ecological features. If they show signs that their increase might be due to modifiable factors (such as organic material contribution), the measures to be adopted would not be simple and would have to consider the whole fishery area as a whole.

With the data available nowadays, it seems that most blooms are controlled according to the hydrographical features of the shores, and therefore they cannot be modified, although a better understanding of them and the way they relate to phytoplankton would imply a higher capacity to predict potential events.

Given that, at present, predicting the exact appearance of blooms is not possible, prevention is based on setting a red-tide warning network and a watch scheme in the purifying plant areas and the market in order to determine the absence or presence of DSP, at levels below those established by law, in purified shellfish for consumption.

Self-detection programs are now at an advanced stage through satellite monitoring of temperature changes in seawater.

3.5.1.1 Marine Watching: Red Tide Warning Network

Sea-watching programs must be of several intensity levels, depending on the existence of toxic plankton species or of conditions favoring their proliferation. There must be perfect coordination and a fast and fluent communication network between the authorities responsible for sea watching (Fishing Administration) and for the markets (health, agriculture, and food) and health authorities. There are two subprograms:

1. Studies on plankton and conditions favoring its proliferation with a view to predict when toxic marine blooms are going to take place and to detect as early as possible their existence (red tides).
2. Monitoring shellfish to check the presence of toxins is authorized in fisheries and shellfish farms before gathering and in purifying plants before shellfish is released on the market.

In order to achieve an efficient management, causing the minimum disturbance to producers and allowing a safety warranty to the consumers of seafood, it is important to set zones and subzones in the shellfish farms, as well as fixed primary points that experience has shown to be most rapidly affected in the case of a toxic event; and fixed secondary points supplementing the former and allowing a more detailed knowledge of the affectation degree in the zones.

For sampling programs follow-up and monitoring of toxic phytoplankton to work, better action schemes have to be set according to the species of phytoplankton causing toxicity and to the shellfish and areas affected. Those schemes have to be set before assessing the information gathered in the monitoring program on the plankton and oceanographic conditions and on the shellfish biotoxins. In Galicia (Spain), there are four action schemes set on those bases [69]:

Scheme A (normal situation): Oceanic conditions are not favorable to the development of toxic phytoplankton species, nor are these found in significant concentrations, and there is no toxicity in bivalve shellfish.

Scheme B (alert situation), divided into three subschemes:

B1: When in spite of favorable oceanic conditions, no potentially toxic phytoplankton species are observed in significant concentrations, and there is no toxicity in bivalve shellfish either.

B2: There are favorable oceanic conditions and the presence of potentially toxic phytoplankton species, but no toxicity in bivalve shellfish.

B3: There are favorable oceanic conditions, a significant increase in toxic population, and toxicity is detected in bivalve shellfish but in levels below the limits established by law.

Scheme C (extraction is forbidden). This is applied when toxic levels are above the law-established limits. It is divided into three subschemes:

C1: Oceanic conditions are favorable, and there is a significant increase in toxic population and in bivalve shellfish toxicity levels.

C2: Oceanic conditions are not favorable to the growth of the toxic plankton species whose population is stable or decreasing. Also, toxicity levels in the bivalve shellfish are stable or decreasing.

C3: Oceanic conditions are not favorable, and there is a significant decreasing and disappearing of toxic population, and toxicity levels are close to legal limits.

Scheme D: Oceanic conditions are not favorable to the development of toxic plankton species; potentially toxic phytoplankton species are in insignificant concentrations or absent, and toxicity stays below legal limits as a consequence of a previous event.

Bivalve shellfish samples must be gathered at different depths (1, 5, and 10 m), since toxicity may vary with water depth. Analysis must be carried out separately or integrated, depending on the

uniformity of the degree of mussels and oceanic conditions, and accumulation of toxicity at a given depth. In natural fisheries and fish farms, samples should be as significant as possible, according to the species subjected to monitoring and the area features.

Sampling frequency varies with every action scheme:

Scheme A (all-year round): Weekly sampling of oceanic and phytoplanktonic conditions, as well as of biotoxins in mussels in fixed primary points, and fortnightly sampling in rock mussels

Scheme B (alert situation):

B1: Selective sampling of phytoplankton

B2: Increasing to twice per week the sampling for biotoxins in mussels in fixed primary points

B3: Increasing the sampling to three times per week, and when biotest results advice it, sampling of other species in fixed secondary points, susceptible to being more affected

Scheme C (gathering is forbidden):

C1: The frequency and species to be sampled are dictated by the toxic event intensity and the existing provisions. Subzones (included within the same zone) bordering on a closed down subzone are subjected to daily sampling.

C2: Gathering samples in fixed primary or secondary points to assess the degree of affectation in the zone or subzone.

C3: Sample gathering for biotoxins at least three times per week.

Scheme D: Lifting of extraction prohibition. In fixed points where toxicity remains high, though below legal limits, samples are to be gathered twice a week.

3.5.1.2 Market Watching

Routine controls are carried out throughout the year by veterinary inspectors responsible for monitoring the salubrity and hygienic conditions of food. Surveillance should increase during periods when these problems tend to be present.

3.5.1.3 Surveillance of the Disease

Surveillance of the disease may be particularly relevant as an alternative method to primary prevention in countries that are not able to afford the costs of sustained surveillance programs in shellfish-farming areas. In such circumstances, detected the first cases of the disease have to be used to adopt preventive measures and avoid further cases. At any rate, a minimum public health infrastructure should be available, such as staff with adequate training, and laboratories with staff trained in standard techniques.

The education of medical and public health staff regarding diagnosis, treatment (symptomatic), and notification of suspect cases is very important for the success of a watch program.

Education of populations at risk about preventive measures, such as nonconsumption of shellfish when there are toxic red tides and never to eat mussels picked from cliff rocks or any shellfish picked directly on beaches, is essential and never enough.

People must be well informed and updated through the most suitable means about the presence of toxic red tides, their blooming, and their disappearance.

Finally, education and cooperation with the seafood industry in everything related to poisoning risks by marine toxins as well as in primary and secondary prevention programs is necessary for the effective success of these programs.

3.5.2 European Regulations

In Europe, the Regulation EC 853/2004 of de European Parliament and of the Council, of April 29, 2004, establishes the maximum level admissible of lipophilic toxins [70].

The maximum level of OA, DTXs, and PTXs together in the bivalve mollusks (the whole body or any part edible separately) shall be 160 μg of OA eq./kg; the YTXs shall be 1 mg of YTX eq./kg, and the AZA shall be 160 μg of AZA eq./kg [70].

The Regulation EC 854/2004 of de European Parliament and of the Council, of April 29, 2004, establishes the official controls concerning live bivalve mollusks from classified production areas [71].

Sampling plans to check for the presence of toxin-producing plankton in production and relaying waters and for biotoxins in live bivalve mollusks must take particular account of possible variations in the presence of plankton-containing marine biotoxins. Sampling must comprise of

- Periodic sampling to detect changes in the composition of plankton containing toxins and their geographical distribution. Results suggesting an accumulation of toxins in mollusk flesh must be followed by intensive sampling.
- Periodic toxicity tests using those mollusks from the affected area most susceptible to contamination.

The sampling frequency for toxin analysis in the mollusks is, as a general rule, to be weekly during the periods at which harvesting is allowed. This frequency may be reduced in specific areas, or for specific types of mollusks, if a risk assessment on toxins or phytoplankton occurrence suggests a very low risk of toxic episodes. It is to be increased where such an assessment suggests that weekly sampling would not be sufficient. The risk assessment is to be periodically reviewed in order to assess the risk of toxins occurring in the live bivalve mollusks from these areas.

When knowledge of toxin accumulation rates is available for a group of species growing in the same area, a species with the highest rate may be used as an indicator species. This will allow the exploitation of all species in the group if toxin levels in the indicator species are below the regulatory limits. When toxin levels in the indicator species are above the regulatory limits, harvesting of the other species is only to be allowed if further analysis on the other species shows toxin levels below the limits.

With regard to the monitoring of plankton, the samples are to be representative of the water column and to provide information on the presence of toxic species as well as on population trends. If any changes in toxic populations that may lead to toxin accumulation are detected, the sampling frequency of mollusks is to be increased or precautionary closures of the areas are to be established until results of toxin analysis are obtained [71].

The Commission Regulation EC No 2074/2005, of December 5, 2005, establishes the methods of analysis of the lipophilic toxins [72].

As for the methods of analysis, the regulation considers biologic methods (bioassay in mice and in rats) and alternative methods [high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and phosphatase inhibition].When the results of the analyses performed demonstrates discrepancies between the different methods, the mouse bioassay should be considered as the reference method.

A series of mouse bioassay procedures (biological methods), differing in the test portion (hepatopancreas or whole body) and in the solvents used for the extraction and purification steps, can be used for detection of the toxins DSP. Sensitivity and selectivity depend on the choice of the solvents used for the extraction and purification steps, and this should be taken into account when making a decision on the method to be used, in order to cover the full range of toxins.

A single mouse bioassay involving acetone extraction can be used to detect OA, DTXs, PTXs, and YTXs. This assay may be complemented if necessary with liquid/liquid partition steps with ethyl acetate/water or dichloromethane/water to remove potential interferences. AZA detection at the regulatory levels by means of this procedure requires the use of the whole body as the test portion.

Three mice should be used for each test. The death of two out of three mice within 24 h after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas or 25 g whole body

should be considered as a positive result for the presence of one or more of the toxins DSP at levels above those established.

A mouse bioassay with acetone extraction followed by liquid/liquid partition with diethyl-ether can be used to detect OA, DTXs, PTXs, and AZA, but it cannot be used to detect YTXs, as losses of these toxins may take place during the partition step. Three mice should be used for each test. The death of two out of three mice within 24 h after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas or 25 g whole body should be considered as a positive result for the presence of OA, DTXs, PTXs and AZA at levels above those laid down in Regulation EC No 853/2004 [70].

The rat bioassay can detect OA, DTXs, and AZA. Three rats should be used for each test. A diarrheic response in any of the three rats is considered a positive result for the presence of OA, DTXs, and AZA at levels above those established.

A series of methods, such as HPLC with fluorimetric detection, LC, MS, immunoassays, and functional assays, such as the phosphatase inhibition assay, shall be used as alternatives or supplementary to the biological testing methods, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods, and that their implementation provides an equivalent level of public health protection:

- *OA and DTXs:* A hydrolysis step may be required in order to detect the presence of DTX-3
- *Pectenotoxins:* PTX-1 and PTX-2
- *Yessotoxins:* YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX
- *Azaspiracids:* AZA1, AZA2, and AZA3

If new analogues of public health significance are discovered, they should be included in the analysis. Standards must be available before chemical analysis is possible. Total toxicity shall be calculated using conversion factors based on the toxicity data available for each toxin. The performance characteristics of these methods shall be defined after validation following an internationally agreed protocol. Biological methods shall be replaced by alternative detection methods as soon as reference materials for detecting the toxins are readily available, and the methods have been validated [72].

With a view to eliminating discrepancies between the member states and harmonizing the European market, the European Commission named a reference national laboratory in each member country (LNRS) and a sole community reference laboratory (LCR), in order to set up and coordinate a network for exchanging information, knowledge, and experiences, and create a forum for method and toxicology agreements. The Exterior Health Laboratory in Vigo (Galicia, Spain), under the Ministry of Health and Consumption, was appointed Community Laboratory of Reference 73.

3.5.3 Epidemiological Investigation of an Acute Outbreak of DSP Disease

Human poisoning outbreaks by diarrheic toxins seem to be explosive, localized, and short-lived (holomiantic outbreaks), given the very short incubation period (between 30 min and a few hours—3 h in DSP intoxication to 17 in AZA syndrome), which depends on the amount of toxin swallowed (shellfish toxic load and the amount of shellfish eaten), and the exposure to a common source.

Investigations usually start from the communication of index cases to the health authorities. That reporting, which is mandatory in many countries when there is an outbreak, must be done when clinical suspicion exists, but since this is subjective, the first step will have to be to confirm the case diagnosis. Confirming the diagnosis is easier when there are toxic red tides, and when physicians and populations are alerted. Isolated cases of DSP or AZA will go undetected if DSP or AZA is not considered, given its unspecific symptomatology: diarrhea, nausea, vomiting, abdominal pain, and its mildness. In addition, etiology identification of clusters is even easier (episodes in which two or more cases of the same disease are interrelated).

Urgent notification to the health authorities, from the mere suspicion, so that they can adopt the pertinent administrative preventive measures.

During investigation of outbreak, the three following stages are clearly marked:

1. Setting or verifying diagnosis of recorded cases and confirming the existence of an outbreak
2. Identifying the intoxication source and transmission mode
3. Identifying other people who might have been or are exposed, as well as cases that might have appeared previously; and the description of cases according to the person, place, and time variables

3.5.3.1 Outbreak Confirmation

First of all, in order to establish environmental exposure, existence of a toxic red tide, and then confirm the diagnosis in the laboratory (seafood testing), it has to be decided whether the signs and symptoms (gastroenteritis and no temperature) and their evolution (benign, with complete recovery in 3–5 days) correspond to DSP or AZA; whether the incubation period is short (30 min to a few hours); and whether there are antecedents of shellfish consumption (appropriate seafood ingestion) in its origin.

Other causes, such as toxic infection by *Bacillus cereus* or by *Vibrio parahaemolytic* have to be excluded; the latter is also carried by shellfish. Those affected usually do not present with fever, but their incubation period is longer (12 h), and the germ can be identified in feces or food remains.

Gastroenteritis associated with a clinical picture of no fever, mussel ingestion, and a short incubation period point toward diagnosis of DSP, even more so if there is a DSP toxic red tide at the time.

Based on the study of the Japanese outbreaks, the World Health Organization (WHO, 1984) established the symptons of the following symptoms in DSP disease: diarrhea (92%), nausea (80%), vomiting (79%), abdominal pain (53%), and chill (10%).

In order to obtain laboratory confirmation, food remains should be available (uneaten mussels), or mussel samples should be taken from the same area that the eaten mussels came from (e.g., food market, shellfish purifying plant, fish farm, rocks).

The usual technique used is the bioassay in mice developed by Yasumoto that we described in the European Regulations. One may also use the bioassay in rats developed by Kat [74]. The bioassay in mice is the most widely used because it is more sensitive (OA 4 μg) than exposure by oral ingestion in rats (OA 10 μg), but the bioassay in mice is subject to false positives by interference of nonphycotoxic components, and it is more expensive since mice die or are disposed of, while rats can be used repeatedly, although reading the test in them depends on the subjective examination of feces.

There are also chemical methods such as HPLC, the most widely used method after bioassays; LC-MS; the enzyme-inhibition assays such as protein phosphatase inhibition assays, an inexpensive technique [75,76]; and the immunoassays (monoclonal antibodies to OA and DTX-1, and enzyme-linked immunoabsorbent assay). There are several commercial kits available, and the cytotoxicity assays obviate the morphological changes caused by the DSP toxin activity in various cell lines; for example, human KB cells, salmon and rat hepatocytes, and cultured neurons.

These techniques, and more so the bioassays, HPLC, and phosphatase inhibition, are generally used in health surveillance. Ethical and technical considerations are being focused on the development and use of health-control tests that do not require the use of animals.

3.5.3.2 Identifying Source, Transmission Mechanism, and Subjects at Risk

It is essential to know the kind of shellfish responsible for DSP or AZA intoxication and their origin, whether they were bought in the market or picked directly in fish farms or rocks, and whether there are some not yet consumed. It is also necessary to know whether they were eaten at home, in a restaurant, or in other public places.

It is also important to know whether other people have also eaten the product, searching for other cases or whether there are people who have more shellfish from the same source and have not yet consumed it. Moreover, it has to be investigated whether there have been previous cases in the area or surrounding areas.

3.5.3.3 Describing Cases according to Person, Place, and Time Variables

Regarding the person variable, information must be gathered on age, sex, and occupation; as far as the place variable is concerned, geographical distribution of the cases concerned, noting their addresses; and regarding time, it is interesting to have information on the date, time of exposure (shellfish ingestion), as well as the onset of the first symptoms and sequence of presentation. It is also advisable to collect information leading to an initial idea of the toxic load.

3.5.3.4 Administration Monitoring Measures

In the event of an outbreak, administration measures will have to be adopted forbidding shellfish gathering and setting up an area-monitoring scheme. Continuous vigilance must be exercised for the occurrence of DSP or AZA poisoning, and physicians must be warned of its existence so that they can be alert for the clinical signs of gastroenteritis. At the same time, the population will have to be made aware: industrialists, health professionals, and people in general through the mass media, warning about the hazards and advising that they must not consume the affected shellfish.

3.5.4 Shellfish Detoxification

The ordinary cooking process, either at home or in the industrial setting, does not destroy the DSP toxin. Given that cooking is the first stage in the industrial processing of bivalve shellfish, the application of detoxification is focused on it.

Vieites and Leira [77] have attempted to lower the toxicity of contaminated shellfish by cooking it for 2–5 min at 97ºC in a slightly alkaline medium (pH: 8.22) and adding bicarbonate salts at 2% as a technological coadjuvant. They obtained detoxification percentages (of OA) that range between 24% and 79%, with residual levels of OA in all samples. They tried increasing the cooking time, but with no better results when the increase was moderate—10–15 min. These investigators were successful in some samples with very long cooking times—over 60 min, but this was incompatible with maintaining the optimal market quality of the seafood. Results were not better when the sodium bicarbonate concentration was increased in mid-cooking. Vieites and Leira suggested the possibility of treatment by autoclave sterilization (110–120°C), as used in canning, for better results [77].

We have attempted various manipulations to reduce toxicity—cooking, freezing, canning (sousing) for PSP-contaminated shellfish, but we have been only partly successful. We have reduced toxicity to a half, a third, or a fourth with processes that alter the organoleptic properties and renders selfish unfit for commercialization [13]. These processes are a combination of pressure of 1.5–2 atm and temperature of 113°C or higher [13]. Many other researchers have attempted cooking treatments to destroy these toxins, but without success [78,79].

To date, there is no effective method for eliminating toxins from mussels and other bivalves in an economical and fast manner that can be commercially profitable and free of hazards. The only solution is self-purifying or natural detoxification through metabolizing the toxins. Whereas toxin accumulation in shellfish may only require a few days, its elimination requires several weeks and, on occasion, months. The detoxifying process depends on various factors sometimes related to the shellfish and sometimes to its environment.

Eliminating toxins follows exactly the reverse physiological process. The toxins accumulated, mainly in the hepatopancreas and other parts of the shellfish, have to be catabolized or expelled.

The more active the metabolism and the better the physiological condition of the shellfish, the faster is the elimination to proceed.

The temperature and food available can be considered the two main external factors that determine the detoxification speed. In warm or tepid waters, mussel filtering is more active, and therefore the ingestion of new food and the elimination of old remains will proceed much faster. The availability of abundant nontoxic food is a sine qua noncondition for the shellfish to return to the preintoxication condition.

Very often, toxic episodes take place during the autumn, but winter may arrive without the toxin having been eliminated fully from the shellfish. Water temperature is at its lowest in winter, and then phytoplankton concentrations are very low owing to a combination of excessive turbulence and low light intensity. In such circumstances, mussels may maintain low toxicity levels (possibly below the limit restricting consumption) during these months; it is even possible that the toxicity will never disappear completely before a new toxic episode takes place the following summer or autumn. This circumstance has been observed in Scandinavian mussels, which have a high DSP toxin level during the autumn and have not been able to eliminate these toxins before exposure to the freezing northern waters during the following winter and spring.

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4 Epidemiologic Impact of Toxic Episodes: Neurotoxic Toxins

Bradford D. Gessner and Joseph B. McLaughlin

CONTENTS

4.1 LIMITATIONS OF EPIDEMIOLOGIC DATA

4.1.1 Bias and Reports of Clinical Illness

Bias refers to those factors related to study design and analysis that may affect the results and conclusions of a study. Descriptions of clinical manifestations may have several sources of bias including selection bias, recall bias, diagnostic bias, and information bias. Selection bias occurs at the outset of the study. For example, persons with mild illness usually have a lower likelihood of reporting to a health care facility and thus a lower likelihood of being included in a hospital-based study or evaluation of surveillance data. Consequently, surveillance and hospital-based studies of marine neurotoxins may overstate the importance of severe sequelae such as neurological symptoms and understate the importance of mild sequelae such as gastrointestinal symptoms. One of the important contributions of outbreak investigations is to actively identify persons with mild illness or persons exposed to toxin but who did not develop illness. This, in turn, greatly contributes to the understanding of the spectrum of disease and illness risks associated with a particular toxin.

Recall bias refers to the extent to which patient recall of information affects the accuracy of the data collected. One can imagine that this is particularly a problem for amenestic shellfish poisoning, but it will affect all studies to a greater or lesser degree. Recall bias may also occur if ill persons recall exposures to a greater degree than nonill persons do; one result of this may be to falsely associate exposures with outcomes. The effect of recall bias may be minimized by prospective, systematic studies that use standardized interview forms to collect information from ill persons.

Recording bias is similar to recall bias and refers to the extent to which clinicians accurately and completely record the symptom history of their patients. This is particularly a problem for studies based on retrospective medical chart reviews. For example, a physician who sees a patient with paralytic shellfish poisoning (PSP) may record paresthesias and vomiting as clinical symptoms; the researcher who reviews this chart at some later period will have no way to determine if other symptoms, such as dysarthria or dysphagia, were also present or even assessed. Another way that recording bias may affect the results of a study is through the linguistic and cultural barriers that may exist between the patient and clinician. For example, the floating feeling in PSP and the temperature reversal of ciguatera represent attempts to define a health state in terms that are mutually comprehensible to the patient and clinician. These terms, however, may not have a biologic basis and may not correspond with the way individual patients would describe a particular experiential state if left unprompted.

Diagnostic bias may occur when factors unrelated to the disease influence the diagnosis assigned to a specific constellation of symptoms. For marine toxin ingestions, this problem may be enhanced because of the lack of specific and sensitive diagnostic tests. For example, a patient who has ingested ciguatoxin and presents with gastrointestinal but not neurological symptoms may not receive a diagnosis of ciguatera unless his illness occurred in the context of a more extensive outbreak investigation. Conversely, a patient may have diarrhea during an outbreak investigation and receive a diagnosis of ciguatera even if his illness resulted from a different cause.

4.1.2 Incidence Rates

Incidence is a fundamental component of epidemiologic inference yet notoriously difficult to obtain. The sources of data may be of high, low, or, most often, unknown quality. For example, surveillance systems based on passive reporting of illness from medical providers to government authorities (such as those that exist for individual states in the United States) will identify an unknown but incomplete proportion of all cases. Breakdowns in reporting may occur at several levels: affected individuals may fail to present to a medical provider, because they have mild illness or because they fail to recognize the potentially serious nature of their illness; medical providers may fail to make an appropriate diagnosis because of lack of interest, knowledge, or diagnostic capability; or the provider may neglect to report the illness to the appropriate authorities.

Other factors may influence surveillance data as well. During an outbreak, active case finding will identify additional ill persons, particularly those with mild symptoms. Agencies or institutions that have a researcher or public health official who is particularly interested in a specific disease may pursue case finding more rigorously than other groups. Finally, public awareness and appreciation of the disease as a significant health problem will influence reporting. The most accurate surveillance data derive from systems that are prospective, systematic, active, and which have a high degree of support from the local medical community and the public.

Other methods of estimating incidence exist besides routine surveillance. Retrospective review of hospital or clinic medical charts may provide an estimate of the incidence of relatively severe illness. Unfortunately, these studies are subject to many of the types of bias discussed above. If case definitions with a high degree of specificity are used and clinical acumen in an area is high, incidence data from medical chart reviews may be regarded as lower estimates.

Investigators may design special studies, such as telephone and door-to-door surveys. If a population is systematically sampled, survey studies may provide population-based incidence data and may be the best source of data on less severe cases. The primary limitation of survey data is recall bias, which increases as the retrospective length of the study increases.

Finally, the public health relevance of incidence data may be limited by the investigator's choice of data for the denominator. For example, a study may report the incidence of ciguatera as 100 per 100,000 people per year. Such a figure implies that the entire population is at risk for illness. The true population at risk, however, is that which consumes marine organisms. This population may differ from other populations by proximity to coastal areas, race or culture, income, and other factors. An incidence calculated using as the denominator the number of persons consuming potentially toxic fish would allow more directed and local implementation of public health control programs. It might also allow public health officials to predict the impact on disease incidence of changes in demographic variables and dietary practices. Unfortunately, this information is rarely available.

4.1.3 Risk Factors

Risk factors refer to those factors that increase an individual's risk of a particular outcome. In marine toxin investigations, outcomes have included illness, severity of illness, and particular symptoms. For example, a study of PSP examined risk factors for the development of PSP among persons who ate shellfish [1]. During an outbreak of domoic acid intoxication, investigators examined risk factors for severe illness among ill persons [2]. Finally, during an analysis of ciguatera, investigators identified risk factors for the development of gastrointestinal versus neurological illness [3].

Risk factors for these various outcomes may be placed in the following categories: differences in toxin composition and genetic and nongenetic differences in the host response. For example, toxin composition may differ between outbreaks of ciguatera (ciguatoxin, maitotoxin, palytoxin, gambiertoxin, etc.) or PSP (saxitoxin, neosaxitoxin, gonyautoxin, etc.); although the toxins may be structurally related, their effects are not identical, and thus, the risk of illness may differ depending on the specific toxin or toxins ingested. Genetic differences in the host response may potentially influence the development or progression of illness through slight alterations in binding sites and altered immunological response.

Finally, nongenetic host differences—including diet, dose of toxin ingested, method of food preparation, and ingestion of particular organs—may also influence the development or progression of illness.

4.1.4 Toxic Doses

The estimation of toxic dose is limited in several ways. In particular, samples of the actual ingested animal are rarely available for testing, except for occasional autopsy or vomitus samples. Many investigators estimate the toxin concentration in marine animals implicated in human illness by examining toxin from animals served at the same meal or collected later from a similar location. However, toxin may not be uniformly distributed in an animal and toxin levels may differ between animals of the same species collected at different times or places. Thus, the reported toxin concentration usually represents an estimate (of unknown accuracy) of the toxin concentration in the actual ingested animal. In addition, most studies that attempt to calculate toxic dose do not report dose on a per unit weight basis. Finally, most studies estimate toxic dose based on bioassays that do not differentiate the toxic dose of the different toxin components.

4.1.5 Geographic Distribution

Geographic distribution of reported illness may differ by the distribution of toxin producing and concentrating organisms; it may also differ by factors unrelated to the true distribution of disease. Disease may be underreported in some geographic regions, because the region has other health priorities, a paucity of diagnostic facilities, undertrained medical providers, or a lack of researchers who publish data in scientific journals. For example, Guatemala appears in the medical literature on PSP because of the occurrence of a large outbreak coupled with the involvement of the U.S. Centers for Disease Control and Prevention and Food and Drug Administration [4].

4.1.6 Temporal Distribution

Seasonal and longer-term temporal variations in neurotoxin poisonings have been reported in some instances but not in others. Temporal variations in human illness are the combined product of variations in dinoflagellate blooms, the proportion of dinoflagellate blooms that produce toxin, fish and shellfish depuration patterns, dietary preferences, fish and shellfish availability, public health measures, and surveillance system characteristics. Essentially, in all instances, the relative contribution of these various factors to the presence or absence of temporal variation is unknown.

4.1.7 Literature Sources

The results of any review of existing data naturally depend on the available literature sources. Scientific journals represent the primary and, in general, the most scientifically sound source of widely available epidemiologic information. For many marine toxin-related illnesses, however, a large body of knowledge exists in regional and government publications, the proceedings of scientific meetings, and textbooks. These sources are usually less widely accessible than journals and no attempt has been made to compile a comprehensive list for the current review. Consequently, it is entirely possible that important pieces of information have been excluded. Where possible, an attempt has been made to use only primary data sources. Occasionally, however, information is referenced from a secondary source.

4.2 PARALYTIC SHELLFISH POISONING

4.2.1 Incidence

A substantial increase in the frequency and geographic distribution of toxic plankton blooms has occurred worldwide over the past two decades [5,6]; however, incidence data for PSP

continue to be limited by a lack of surveillance systems, lack of active case finding in areas that have surveillance, and lack of appropriate denominator data. One investigator estimated that 1600 cases of PSP had been identified worldwide between 1689 and 1971 [7], a number, which had increased by another 900 by 1984 [8]. Seven outbreaks were reported in the United States between 1998 and 2002; 43 persons were affected, 13 were hospitalized, and none died. [9]. In two of the largest series of cases from a specific area, Prakesh [5] reported that 80 cases of illness were identified from the Bay of Fundy region in Eastern Canada between 1889 and 1961 and 107 cases were identified from the St. Lawrence region between 1880 and 1970. None of these reports provided data on incidence.

Gessner et al. [1,10] have performed two investigations in Alaska from which incidence could be calculated. The first was based on a retrospective review of surveillance data collected from 1973 to 1992 by the Alaska Division of Public Health. Based on overall population data from Alaska and 117 reported cases during this period, the estimated incidence was 1.2 per 100,000 persons per year.

The second study attempted to identify more clearly the incidence among high-risk populations by using a randomized telephone survey among two coastal populations. This study found an incidence of 150 and 1,500 per 100,000 persons per year in Kodiak and Old Harbor, respectively, and 560 and 1,570 per 100,000 persons per year among persons who reported consuming shellfish collected from unregulated beaches. The incidence calculated from surveillance data from the Department of Health for the same period was 6 and 170 per 100,000 persons per year for Kodiak and Old Harbor, respectively. The large difference in estimated incidence between survey and surveillance data indicates that even in a state with a high awareness of PSP, surveillance data grossly underestimate true incidence.

Recently, investigators have postulated that PSP incidence has increased worldwide due to increased oceanic eutrophication and possibly increased ocean commerce with subsequent dispersal of toxic dinoflagellate cysts [11,12]. Worldwide, compelling evidence exists that observed red tides have increased recently, particularly in Southeast Asia. While Southeast Asia also has seen an increase in reported PSP cases [13], for most of the world, few data points exist to assess whether human illness has increased.

Anderson has summarized data showing that during 1990, more than twice as many areas worldwide reported outbreaks of PSP compared to the number of areas with a reported outbreak during 1970. Summary data from Prakesh in the St. Lawrence region of Canada show that an average of nine cases of illness per decade occurred during 1900–1950 while 47 cases occurred during the 1960s alone. One author suggests that this must represent a true increase since the symptoms of PSP are sufficiently unique that they would immediately be recognized [14].

Despite this faith in surveillance systems and the clinical ability of medical providers, the data of Anderson and Prakesh have several possible explanations other than a true increase in incidence. These include better surveillance systems, an increase in the number of researchers interested in PSP, lower rates of other diseases such that PSP has assumed a greater relative priority, changes in shellfish consumption patterns (which in turn may be related to changes in social conditions and population movements, including tourism), and increased awareness of PSP symptoms through worldwide dissemination of medical knowledge.

Alaska has had a consistent surveillance system in place since the early 1970s. During the 11 years from 1973 to 1983, there were 70 cases of illness and 33 outbreaks; during the 11 years from 1984 to 1994, there were 73 cases of illness and 34 outbreaks; and during the 11 years from 1995 to 2005, there were 42 cases of illness and 22 outbreaks, with no cases being reported since 2002 (Alaska Division of Public Health, unpublished data). Possible explanations for the decreasing incidence of reported cases include fewer toxic human exposures and/or decreased detection/reporting. In the past several years, there has been a decrease in the number of harvest area closure days when compared to previous years (Alaska Department of Environmental Conservation, unpublished data).

4.2.2 Clinical Features

PSP toxins exert a local effect on the oral mucosa, leading to the rapid onset of perioral paresthesias following exposure. For nine studies, where information was available, onset time ranged from a minimum of 5 min to a maximum of 660 min. The median or mean time to illness onset ranged from 8 to 120 min [4,9,15–21].

Unlike ciguatera, the clinical presentation of PSP is reasonably consistent across populations, possibly because individual PSP toxins differ in their quantitative rather than qualitative effects. Three recent published reports include detailed descriptions of clinical symptoms and report on at least 50 ill persons [4,9,22] (Table 4.1). Data from Guatemala and England come from single outbreak investigations while those for Alaska come from 20 years of surveillance. Despite this, and the wide geographic distribution of the three areas, clinical presentation remains consistent.

The most common symptom, occurring in almost all affected individuals, is perioral paresthesias, generally described as either numbness or tingling. Other than in the context of an outbreak, health care providers should be cautious about diagnosing PSP in a person who does not have this symptom. Furthermore, this is likely to be the only symptom that most people with PSP experience, and therefore many patients with mild illness do not seek health care. Among persons who progress to more severe illness, a minority report gastrointestinal symptoms including nausea, vomiting, abdominal pain, and diarrhea.

More severe illness leads to a variety of neurological symptoms culminating, in some instances, in respiratory arrest or death. Neurological symptoms may include weakness, dysarthria, diplopia, ataxia, and vertigo or dizziness. One of the more interesting symptoms is a dissociative feeling,

TABLE 4.1
Symptoms of Paralytic Shellfish Poisoning in Guatemala, England, and Alaska

Symptom	Guatemala [4] *N* = 187 (%)	England [11] *N* = 78 (%)	Alaska [9] *N* = 117 (%)
Gastrointestinal			
Nausea	52		38
Vomiting		36	29
Abdominal pain	38		
Diarrhea			
Neurological	27		9
Paresthesia			97
Oral paresthesia	93	88	
Acral paresthesia	86	83	
Weakness	81	71	28
Ataxia	37	57	27
Dysarthria	66	23	14
Diplopia	39		16
Vertigo	86		24
Transient blindness	53		
Floating sensation		66	21
Headache	80	41	
Dyspnea	74	24	25
Paralysis			
Outcome			3
Death	14	0	1
Hospitalized	70		26

which has been described as floating in various reports. It is unclear why persons from Guatemala did not describe this symptom, although it is possible investigators did not specifically inquire. Limb paralysis is an uncommon event but may occur in severe cases.

Respiratory arrest and collapse represent terminal symptoms in cases of severe poisoning. These symptoms may occur within minutes in a person who otherwise exhibits no evidence of respiratory difficulty [23], emphasizing the need for symptomatic individuals to seek medical attention immediately even though they have seemingly mild symptoms. Few data exist regarding how long after toxin ingestion onset of respiratory arrest may occur: for four patients in Alaska, respiratory arrest occurred from 75 to 240 min after toxin ingestion [20]. It is unclear whether PSP exerts significant effects directly upon the myocardium. In one reported case, where the victim consumed mussels containing a PSP toxin concentration of 19,418 μg/100 g tissue, cardiac arrest and ventricular fibrillation occurred despite prompt initiation of bag and mask ventilation at the onset of respiratory failure [20].

Signs of PSP are nonspecific and may include sluggishly reactive or dilated and fixed pupils, absent or diminished reflexes including deep tendon reflexes, and muscle weakness or paralysis. In severe cases, PSP may clinically resemble brain death. It is possible, however, for patients to retain consciousness despite complete muscular paralysis. The author has interviewed patients who remembered the course of events from the onset of respiratory arrest through intubation. Among other things, this suggests that patients with PSP should receive sedation before intubation. Unlike ciguatoxin, PSP does not cause hypotension or bradycardia. Where blood pressure and heart rate have been measured, patients have instead shown a normal rhythm and hypertension [20,22]. The hypertensive effects reported may result from a direct action of PSP (although by an unknown mechanism), stress, or the presence of other unidentified toxins (e.g., those with calcium channel agonist activity) [24].

Death results from respiratory arrest and occurs in a variable number of cases, ranging from 0% [22] to 14% [4]; the global case-fatality rate has been estimated to be as high as 8.5% [25], but less than 1% in developed countries [10]. Differences in case-fatality rates may reflect different toxin composition, different doses of toxin ingested, or—most critically—differences in access to emergency medical services.

Six studies for which information was available reported a maximum duration of symptoms of from 1 day to 14 days [4,9,15,20,21,26]. Prolonged illness, however, included such nonspecific symptoms as weakness, headaches, memory loss, and fatigue. In general, recovery from neurological symptoms is rapid and complete. Rodrigue et al., for example, reported that neurological symptoms resolved within 24–72 h [4]. Gessner et al. [23] described a person who progressed from an appearance of clinical brain death to almost complete recovery within 28 h. No long-term clinical effects of PSP have been reported, although it is worth mentioning that no reports exist in the literature of rigorous long-term neurological assessment of patients who have had PSP.

4.2.3 Toxic Dose

The minimum toxic dose represents one of the most critical pieces of information for regulatory agencies. Six studies have information available on toxic doses in humans [4,5,9,17,20,22] (Table 4.2). From these, the values of interest are the minimum toxic and lethal doses, the minimum toxic and lethal doses per kilogram body weight, the mean toxic dose, and the maximum asymptomatic and nonlethal doses. The estimated minimum toxic dose varies greatly, from 13 to 2250 μg. Minimum dose estimates from surveillance data, such as the 13 μg presented for Alaska, should be viewed with skepticism since they may represent cases that were not actually PSP. This is particularly true if they occurred as isolated cases and with mild symptoms. If this report is excluded, the minimum toxic dose may be estimated as something greater than 100 μg, the equivalent of eating thirty-one 4 g mussels or four 37 g butter clams at the U.S. Food and Drug Administration's regulatory limit of 80 μg/100 g tissue. It should also be noted that while this is a standard regulatory limit used worldwide, the Philippines has set a lower limit of 400 μg/100 g tissue to protect children better [27].

TABLE 4.2
Toxic Doses (in μg), of Paralytic Shellfish Poison

Category	Alaska [9] *N* = 54	Alaska [20][a] *N* = 10	Guatemala [4] *N* = 5	England [22][b] *N* = 71	British Columbia [17] *N* = 2	Eastern Canada [5] *N* = 37
Minimum toxic dose	13			558		160
Minimum lethal dose	5,863		2,046		2,600	
Minimum toxic dose per kg		21				
Minimum lethal dose per kg		230	89		36	
Mean toxic dose	5,452	9,176				
Maximum nonlethal dose	123,457			5,580		8,272
Maximum asymptomatic dose	36,580			5,580		3,000

[a] Reprinted from Gessner BD, Bell P, Doucette GJ, Moczydlowski E, Poli MA, Van Dolah F, Hall S. *Toxicon* 35:711–722, 1997. With permission from Elsevier Science.
[b] McCollum JPK, Pearson RCM, Ingham HR, Wood PC, Dewar HA. *Lancet* 2:767–770, 1968. © by the Lancet Ltd.

Three studies present the minimum lethal dose and all three are in general agreement that somewhere in excess of 2000 μg represents a potentially lethal dose. There is more variation in the estimated minimum lethal dose per kilogram (Table 4.2). The highest reported shellfish toxin levels are approximately 20,000 μg/100 g tissue [15,20]. At this level, a lethal dose may be ingested from as little as two 4 g mussels. If it is assumed that a lethal dose per kilogram is 36 μg, then a child may consume a lethal dose from eating a fraction of a mussel.

Three studies provide information on the maximum amount of toxin that an individual may ingest without illness. This value ranges from 3,000 to over 36,000 μg, placing these values considerably above the level needed to induce potentially lethal illness. The reason for this is unclear, but what is clear is the consistency of data that suggests that illness and illness severity are not related to estimated ingested toxin dose in any simple way [5,9,22].

One study attempted to determine the toxin concentration in shellfish eaten by healthy people [1]. Following random selection, participants were contacted by telephone and asked if they had shellfish at their home that they had collected from unmonitored beaches. This study found that 29 people had eaten shellfish containing over 80 μg/100 g tissue on multiple occasions, including some who had eaten shellfish that contained over 200 μg/100 g tissue. Of these 29 persons, one may have experienced mild paresthesias.

4.2.4 Geographic Distribution

Toxin-containing shellfish are encountered most commonly along cold-water marine coasts in southern Chile, Japan, the North Sea, Canada, and the northern United States [26,27,29,30], and PSP cases have been reported from as far south as Chile [25,31] and as far north as Alaska [9]. Other cases have occurred in Australia [32], Taiwan [18], South Africa [16], England [22], Guatemala [4], Costa Rico [21], Singapore [19], Canada [5,17], Spain [33], Mexico [34], Japan [35], and other areas of the United States [15,36]. Similar to incidence, Anderson has raised the prospect that the distribution of PSP is increasing. He supports this argument by identifying more than twice as many areas that reported PSP during 1990 as during 1970. As mentioned here, it is problematic trying to

determine if geographic and temporal trends represent changes in the occurrence of PSP or changes in the occurrence of confounding factors.

4.2.5 Temporal Distribution

Most reports of clinical illness come from isolated outbreak investigations making conclusions regarding seasonal and longer-term distribution difficult. A study of 20 years of surveillance data in Alaska found that while the majority of cases occurred during late spring and early summer, cases occurred during all seasons of the year and during every month except November and December [9]. Similarly, a large series in eastern Canada reported outbreaks between March and November with the great majority occurring between June and September [5]. Outbreaks in Costa Rica and Mexico, by contrast, have occurred during October and December [21,34]. Some species, such as mussels, have rapid depuration of toxin, consistent with seasonal variation in outbreaks. Other species, however, such as the butter clam *Saxidomus giganteus*, may retain toxin for up to 2 years following a single exposure to toxic dinoflagellates [5,37].

4.2.6 Risk Factors

No definitive risk factors for illness have been identified. Particularly surprising is the consistency of reports that have failed to identify a correlation between estimated ingested toxin dose and illness. Although no studies have been conducted in humans, animal studies suggest that antibodies or binding proteins may be produced against PSP components [38–42]. Genetic differences [43], differences in toxin components, and poor estimation of toxic dose may also help explain this finding.

Two studies have examined the role of alcohol consumption with one finding alcohol to have no relation to illness [16] and the other study finding a protective role of alcohol [9]. Another study found that Alaska Native race was associated with illness [1]. Finally, while one study suggests that age increases the likelihood of death following exposure [4], another more recent evaluation suggests just the opposite [44], and in one large PSP outbreak, the case-fatality rate was 7% for adults but 50% for children [45]. Although PSP is a heat stable toxin, boiling the shellfish appears to decrease the risk of illness because of elution of toxin into the water.

4.2.7 Public Health Issues

The threat of PSP resulting from the consumption of commercial shellfish has led to the implementation of shellfish monitoring programs in many areas of the world with commercial shellfish harvests. Regulators temporarily close harvest areas when toxin levels exceed the action level [46]. While they are currently the most effective way of protecting the public from PSP intoxication, monitoring programs incur great costs to the farmers. For example, in Alaska the cost to the farmer of sampling for PSP represents approximately 5% of the total crop value. The cost to society is higher because of the added costs associated with the mouse bioassay performed by the State Health Department. Despite these costs, there seems little disagreement among producers that rigorous monitoring must continue to safeguard commercial interests as well as public health. Jensen reports on the economic consequences of PSP outbreaks [47]. Another case, unrelated to PSP intoxication, provides a warning to the shellfish industry: a single death in Belgium associated with canned salmon produced in Alaska led to a decrease in market demand for all types of Alaskan salmon and the loss of an estimated $300,000,000 [48].

More problematic is monitoring of subsistence and recreational shellfish harvesting. In Washington State, a marine Biotoxin Bulletin lists beaches that are closed to recreational harvest due to significant levels of biotoxins. This bulletin is updated as changes occur [49]. The implicit message from this program is that shellfish are safe to eat when a particular beach is not closed. In Alaska, however, outbreaks of illness have occurred during all seasons [9]. Moreover, shellfish toxin levels may vary widely from one section of a particular beach to another (Gessner BD, unpublished data) and as demonstrated in the preceding section, one mussel may have sufficient toxin to cause death. Thus in

Alaska, the Health Department has taken the position that shellfish harvested from noncommercial beaches are unsafe to eat regardless of the season. Unfortunately, this position may conflict with the cultural and nutritional benefits that derive from subsistence food harvesting [50] and, by its very inflexibility, lead people to ignore it [1]. It may also lead to conflict between different government agencies that are charged with different tasks, such as protecting the public health versus promoting tourism. If recreational shellfish monitoring programs are implemented, they should consider the following issues [37,48]:

- The geographic and temporal sampling frame, taking into account issues of safety, cost, and fairness to a multitude of local communities.
- The representativeness of toxin levels in tested shellfish for an entire beach.
- The impact of a monitoring program on the public's belief in shellfish safety; an unrealistic expectation of shellfish safety may lead to an increase in PSP cases.
- Given the expense and ethical issues associated with the current mouse bioassay, the sources of funding and support for the program.
- The criteria that will be used to reopen a beach that has been closed.

Education programs also have the potential to impact the occurrence of PSP cases. A study in Alaska found that residents held erroneous beliefs regarding PSP including that it could only occur during the winter or following a red tide and that cooking or eviscerating the shellfish rendered it safe [9].

4.3 CIGUATERA

4.3.1 Incidence

Ciguatera is estimated to affect more than 50,000 persons annually [51,52], and is the most common foodborne illness related to finfish consumption in the world [53]. In 2000, Lewis produced a table demonstrating ciguatera incidence rates by region (Table 4.3), and in 2000, Lehane and Lewis updated ciguatera incidence data [54]. In the United States, approximately 5–70 cases of ciguatera per 10,000 people are estimated to occur each year in ciguatera-endemic states and territories, including Hawaii, Florida, Puerto Rico, Guam, the U.S. Virgin Islands, American Samoa, and the Commonwealth of Northern Mariana Islands [55]. A study from Hawaii reported that the incidence varied among different islands from 3 to 34 per 100,000 persons per year during 1988 [51]. Within islands, the incidence stayed relatively constant during 1984–1988 but on Kauai varied from a low of 17 to a high of 105 per 100,000 per year. The South Pacific showed variation between nations and territories, ranging from a low of 1 to a high of 653 per 100,000 per year [56]. As with Hawaii, the incidence remained relatively constant within areas during 1973–1983. In 2000, Lehane and Lewis produced an excellent visual image of the global distribution of ciguatera [54]. In the Eastern Caribbean, disease incidence may vary from 3.3 to 730 per 100,000 persons per year on Cuba and St. Thomas, Virgin Islands, respectively [57,58]. Incidence data are also available from Australia [59] with a reported of 30 per 100,000 persons per year.

Certain islands or island groups in the South Pacific have extraordinarily high ciguatera incidence rates. For example, on the island of Atiu in the Cook Islands, 19% of persons reported having experienced ciguatera at some point in the past and 12% of the population had developed ciguatera during the 2-year period before the study (estimated incidence, 6,243 per 100,000 persons per year) [60]. Even more dramatically, the population of the Gambier archipelago in French Polynesia had an incidence of 22,700 per 100,000 per year during 1960–1984 [61]. The author of this study emphasizes that the incidence in the South Pacific varies dramatically among different islands and has increased among certain islands during 1960–1984 [61].

These latter two observations may be related to "decreasing diversity of marine and terrestrial fauna" that in turn may be related to nuclear test explosions [61] and other environmental disruptions

TABLE 4.3
Ciguatera Incidence by Area

Location	Incidence (per 100,000 per year)
Hawaii, 1988 [51]	
Oahu	3
Kauai	13
Maui	11
Hawaii island	34
Hawaii state	8
South Pacific, 1973–1983 [56][a]	
American Samoa	87
Cook Islands	1
Fiji	16
French Polynesia	545
Guam	8
Kiribati	324
Nauru	7
New Caledonia	200
Niue	130
Papua New Guinea	>1
Solomon Islands	2
Tokelau	653
Tonga	21
TIPI	173
Tuvalu	439
Vanuatu	25
Wallis and Futuna	9
Western Samoa	54
Virgin Islands [57]	365–730
Australia, 1965–1985 [59]	30

[a] Reprinted from Lewis ND. *Soc Sci Med* 23:983–993, 1986. With permission from Elsevier Science.

of marine ecosystems [62,63]. As has been pointed out, however, significant disruptions to marine ecosystems have occurred without an increase in ciguatera [64]. In addition, increases in ciguatera poisoning may reflect changes in diagnostic capability and surveillance systems. Other possible causes of regional increases in the incidence of ciguatera poisoning include: increased number of persons fishing for and consuming contaminated fish, such as those employed on oceanic oil rigs, which provide new habitats for dinoflagellates and the reef fish that feed upon them [65]; increasing importation of contaminated fish in areas that have no naturally occurring ciguatoxin [66]; increasing numbers of international travelers who may not possess local knowledge of which fish are safest to eat [67]; and increasing ocean water temperatures, which might enlarge the global distribution of ciguatera-producing dinoflagellates and provide a temporary increase in available nutrients for dinoflagellates by killing portions of coral reefs [68,69].

During outbreaks of ciguatera, some authors have attempted to determine attack rates. Where these data are presented, the attack rates following consumption of the same fish have been high. Among five reviewed studies, the attack rates varied from 63% to 100% emphasizing the consistent, although not necessarily uniform, distribution of ciguatoxins among the edible flesh of toxic fish [70–74].

4.3.2 Clinical Features

A plethora of studies exists documenting the clinical presentation and course of ciguatera. In addition, at least 175 symptoms have been reported to occur during ciguatera [75], likely reflecting the variety of toxins that may cause illness [76,77]. The present analysis does not attempt a comprehensive review of all studies and all symptoms. Instead, a representative sample of large case series from different areas has been gathered. Ciguatera presents with neurological, gastrointestinal, and cardiac symptoms. The median or mean time from ingestion to onset of illness is longer than for PSP and in different studies has varied from 5 to 7 h [71–73,78]. The minimum time reported (except for cases where palytoxin is implicated) is 30 min while the maximum time is 48 h.

Five large series [59,78–81]—two from the Atlantic and three from the Pacific Ocean areas—were reviewed (Table 4.4). Gastrointestinal effects include nausea, vomiting, diarrhea, and

TABLE 4.4
Ciguatera Symptoms by Area

Symptom	South Pacific [79] *N* = 3009 (%)	Australia [59] *N* = 527 (%)	Hawaii [80] *N* = 203 (%)	Miami, FL [78] *N* = 129 (%)	Virgin Islands [81][a] *N* = 33 (%)
Gastrointestinal					
Diarrhea	71	64	65	76	91
Nausea	43	55	38		
Vomiting	38	35	37	68	70
Abdominal pain	47	52	28		39
Neurological					
Acral paresthesias	89	71	63	71	33
Circumoral paresthesia	89	66	61	54	36
Temperature reversal	88	76	48		36
Vertigo or dizziness	42	45	21		21
Ataxia	38	54			
Diaphoresis	37	43	15	24	18
Tremor	27	31			9
Salivation	19	10			
Dyspnea	16	28	2		
Paresis	11	27			
Cardiovascular					
Bradycardia	13				
Hypotension	12		2		
Other					
Myalgia	81	83	64	86	
Arthralgia	86	79	4		52
Weakness	60		69	30	58
Chills	59	49	24		24
Headache	59	62	12	47	
Pruritus	45	76	21	48	58
Dental pain	25	37			
Neck stiffness	24	27			24
Watery eyes	22	41	4		21
Rash	21	26	3		9
Dysuria	19	22			
Death	0.1	0.2			

[a] Morris JG, Lewin P, Hargrett NT, Smith W, Blake PA, Schneider R. *Arch Inter Med*, 142:1090–1092, 1982. Copyright 1992, American Medical Association.

abdominal pain. The predominant neurological effects include acral and circumoral paresthesias, vertigo or dizziness, ataxia, and diaphoresis. Temperature reversal (also known as paradoxical dysaesthesia) is a well known feature of ciguatera, but one author points out that while cold objects may feel hot, no reports exist of hot objects feeling cold [82]; these authors suggest that the symptom may more closely resemble hyperesthesia than dysesthesia. Other common symptoms include myalgia, arthralgia, weakness, chills, headache, and pruritus. Cardiovascular symptoms include hypotension [83] and bradycardia, as well as less commonly arrhythmias. Death is rare with only three occurrences out of 3009 cases documented by Bagnis [79] and one occurrence out of 527 cases documented by Gillespie [59].

Unlike PSP, there is a firm body of evidence that documents the occurrence of hypotension and bradycardia with severe ciguatera. Katz, for example, reported bradycardia for 66% and hypotension for 27% of cases in Hawaii [65] while Bagnis reported bradycardia for 14% and hypotension for 12% of cases in the South Pacific [79]. One report suggests that hypotension in ciguatera results from parasympathetic excess and sympathetic failure [83]. Occasionally tachycardia has occurred as well.

Others have compared the symptoms reported in different regions and have noted that some areas of the South Pacific (e.g., French Polynesia) have more severe symptoms than Hawaii, including more temperature reversal and cardiovascular manifestations [80]. To take the studies compared in Table 4.4 at face value, it appears that the South Pacific and Australia have a higher proportion of persons with neurological symptoms (other than paresthesias) than persons in Hawaii or the Atlantic region have and a lower proportion of persons with gastrointestinal symptoms. A geographic difference in the distribution of symptoms is consistent with the variety of toxins that may cause ciguatera. One should exercise caution, however, in making a direct comparison. The studies of Bagnis and Lawrence were designed prospectively while the others were retrospective. Moreover, it is not clear from the reports whether symptoms that were not reported did not occur or were not assessed.

Some reports have suggested that symptoms may vary by the type of fish consumed. Bagnis associated consumption of herbivores such as surgeonfish with gastrointestinal illness and consumption of carnivores such as grouper with neurological illness [3]. Kodama and Hokama [84] and Glaziou and Martin [85] found that, compared with consumption of herbivores, consumption of carnivores resulted in more severe disease including increased cardiovascular symptoms; in addition, symptoms may differ following consumption of different carnivorous species. Presumably, these findings represent the accumulation of different toxins or the same toxin at different concentrations within different species.

In contrast to PSP, prolonged duration of symptoms in ciguatera is well documented. For two studies where this information was available, the mean or median length of illness was 24 days and 72 days [65,86], but both of these studies included a small number of cases. Gastrointestinal symptoms resolve relatively quickly over several hours to a week [71–73,81]. Neurological symptoms—including weakness, paresthesias, and temperature reversal—have been reported to last many months [72,78,81,87], with one woman reporting paresthesias in her hands 18 months after eating barracuda contaminated with ciguatoxin [88]. In one extreme example, polymyositis was documented 11 years after a case of ciguatera [89], although the causal link in this case is questionable.

4.3.3 Geographic Distribution

Dinoflagellates that produce one or more of the toxins associated with ciguatera are found worldwide within coastal waters between 35° north and south of the equator [90]. As has been pointed out, however, ciguatera occurs more commonly on islands rather than continental coasts, the most notable exceptions being Florida and the Great Barrier Reef of Australia [64]. It is hypothesized that the implicated dinoflagellate thrives in areas most exposed to oceanic flows and does not thrive near continents or other major landmasses with land runoff.

Several reviews have documented an expanding range for ciguatera poisoning [90–92]. Cases of ciguatera associated with consumption of tropical fishes are being reported more commonly in

temperate regions, in association with an expansion of travel and trade [67,93–95], including recent new documentation of disease along coastal West Africa and its regional island archipelagos [96]. In the United States, cases have now been reported from fish caught as far north as North Carolina [73] and a recent report documents the first outbreak in California, which was traced to fish caught off the coast of Baja California, Mexico [97].

With increases in interstate fish transport, more outbreaks have occurred in areas without risk of indigenous ciguatera such as Canada [86], Rhode Island [98], California [99], and Vermont [100]. In addition, clinicians in any part of the world may see patients who present after acquiring illness during travel [101,102]. Finally, one report identified a case of ciguatera that resulted from the consumption of farm-raised salmon, raising the possibility of ciguatera occurring in novel locations [103].

4.3.4 Temporal Distribution

Temporal differences may occur in the rate of ciguatera fish poisoning but, as with PSP, the occurrence of seasonal variations and the seasons of high risk vary by location. In Hawaii, the greatest number of cases occurred during July, but no overall seasonal distribution was identified [80]. Similarly, no seasonal variation was found in the Virgin Islands [57]. In Miami, Florida, the majority of cases occurred during May with a clear increase in cases during the spring and summer [78] while in Puerto Rico, the majority of cases occurred during January, March, and April [87]. In Puerto Rico, investigators have found consistent increases in the number of ciguatoxic barracuda during January through April, but also have found less consistent increases during the summer and fall [104]. As one review points out, fish may remain toxic for years following exposure to ciguatoxin, an observation that likely explains the reported increased risk of illness following consumption of older and larger fish [76]. This finding may also help to explain the lack of seasonal variation in human illness despite variations in dinoflagellate blooms. In Hong Kong, investigators have documented no seasonal patterns to ciguatera food poisoning; interestingly, however, they have observed that the annual peak activity of cholera follows the annual peak activity of ciguatera food poisoning by 2–3 months, suggesting that related environmental factors might affect both dinoflagellate and *Vibrio cholerae* proliferation [105].

4.3.5 Risk Factors

While consumption of contaminated fish is the primary risk factor for ciguatera, transmission has also been shown to occur via consumption of breast milk from an affected mother to her infant and across the placenta to the embryo/fetus [106–108]. Numerous studies have attempted to identify factors that put exposed persons at increased risk for symptomatic ciguatera, although the outcomes and risk factors measured have differed widely between these studies. One study reports an association between illness and age [65], but three other studies report no association [66,78,80]; in addition, cases have been reported among persons from less than 1 to 83 years of age [57]. Similarly, Bagnis [79] and Glaziou [85] found 50–60% more males than females with ciguatera, but numerous other studies have found no association with gender [57,65,71,78,81]; it is possible the described association represents gender-specific differences in fish consumption practices in the South Pacific.

Perhaps the strongest documented association is between previous exposure to ciguatera and either severity of illness relative to presumed dose [79,85] or illness [57,79]. Glaziou has explained his findings by suggesting that humans may accumulate toxin. It should be kept in mind that two smaller studies report no association with previous exposure [65,71]. Fish evisceration does not appear to be protective [65, 81], despite concentration of toxin in specific organs, and, because the toxin is heat stable, cooking also provides no protection.

Two studies suggest that alcohol increases the severity or chronicity of symptoms [73,102] while a third found no association with severity of illness [71]. Two studies report no association with race [78,81]. Other reported risk factors include exertion and eating large fish. All of the studies that

examined risk factors, suffer from one or more methodological problems including lack of a control group, retrospective design, lack of clear case definitions, failure to use appropriate denominators (i.e., the population of persons who eat fish rather than the overall population), and lack of a systematic approach to measurement of risk factors and outcomes. The discrepancies between studies may be attributable to these methodological flaws, to differences in population characteristics, or to differences in toxin components among different areas.

4.3.6 Public Health Issues

The gold standard test for ciguatoxin detection is the mouse bioassay, which is expensive and time consuming; therefore, research has been ongoing to develop a practical screening method for fish. Because ciguatoxin bioaccumulates in humans, somebody who consumes fish containing ciguatoxin levels that are too low to trigger a positive test result, might still cause clinical effects in some previously exposed persons. Therefore, regulatory hazard analysis and critical control point plans have been difficult to approve. In 1997, a latex bead immunoassay test for the detection of ciguatoxin in fish was developed (Cigua-Check®). The test is being used by private parties and nonregulatory agencies in several countries, and is currently being reviewed by the Association of Official Analytical Chemists–International and the U.S. Food and Drug Administration for chemical validation; the present price is impractical for mass testing, however (approximately $23–28 for three tests) [109].

Regulatory alternatives to direct testing of fish include banning the sale of fish suspected to cause ciguatera such as grouper, snapper, barracuda, and surgeon fish in French Polynesia [110] and barracuda in Miami [78]; provision of education regarding ciguatera case identification to medical providers [78]; and using, despite its limitations, an assay for fish sampling with subsequent withdrawal of toxic fish from the market [100,111–113]. In addition, where governments have not acted, the combination of legal decisions and insurance industry pressure has prompted interventions such as the placement of warnings on restaurant menus in endemic areas [114].

Ciguatera undoubtedly limits subsistence seafood harvesting in endemic regions, but the extent of its impact is unclear and likely varies by location. Factors that affect whether the presence of ciguatera limits seafood harvesting may include the economic scale of the fisheries industry relative to other industries, the availability of other protein sources, and the perceived alteration of a state of well-being from ciguatera poisoning relative to other health events. In the Pacific region, Lewis [56] and Bourdy et al. [115] report a number of strategies that islanders have adopted to avoid ciguatera including avoidance of high risk species, discarding the internal organs of fish, and feeding fish to a pet and observing the reaction [56]. Additional strategies have been employed that are less effective or ineffective, such as cooking the fish with plant materials or feeling the texture of the fish. Another report documents similar practices among the residents of the Dominican Republic [116]. It is unclear, however, how many people avoid fish because of the presence of ciguatoxin. In Puerto Rico, an area with relatively high levels of health and a diversified economy, the threat of ciguatera has been shown to lead people to avoid eating fish entirely [87].

Similar to the case with PSP, Bagnis and Lewis have suggested that ciguatera results in the loss of hundreds of thousands of dollars in commercial fish harvesting in French Polynesia [56,105,117]. The threat of ciguatera may also adversely affect the tourist industry, particularly hotels and restaurants. A single well-publicized outbreak may adversely affect income not only at one or several businesses but also for an entire circumscribed location that becomes associated with the outbreak in the public's viewpoint [117].

If this occurs, more specific regulatory intervention will not be far behind. Public health and regulatory measures to control ciguatera should consider the relative value of a fish diet compared to the risk of ciguatera, the economic and social importance of fish harvesting to a community, the anticipated intervention when toxic fish are identified (particularly if toxic fish represent a considerable portion of the total harvest), and guidelines for relaxation of specific restrictions (e.g., import or export restrictions) once implemented.

4.4 AMNESIC SHELLFISH POISONING

4.4.1 Incidence

The incidence of amnesic shellfish poisoning (ASP) is unknown, but appears to be low as only one confirmed outbreak of human illness has been reported, which occurred in 1987 and involved over 150 people, 19 hospitalizations, and 4 deaths. Following the detection of domoic acid in razor clams in the State of Washington, an epidemiologic investigation was conducted to identify possible cases of human illness [118]. Among 127 persons who had recently eaten razor clams, no illness was identified.

4.4.2 Clinical Features

Almost all of the information regarding clinical features derives from the originally described outbreak in Canada involving 107 people [2,119]. Illness onset during this outbreak varied from 15 min to 38 h (mean, 5.5 h). The most common symptom was nausea (77%) followed by vomiting (76%), abdominal cramps (51%), diarrhea (42%), headache (43%), and memory loss (usually but not exclusively anterograde) (25%). Among hospitalized patients, symptoms included confusion, disorientation, coma, mutism, grimacing, seizures, hiccups, and emotional lability. Physical findings included no response to painful stimuli, piloerection with miosis or mydriasis, paresis, ophthalmoplegia, unstable blood pressure, and arrhythmias [2,119]. Three patients died. During examination, several months after exposure, prolonged symptoms included memory deficits, atrophy and mild weakness of the extremities, and hyporeflexia [119].

Quick used a case-control study to identify symptoms resulting from exposure to lower doses of domoic acid [118]. Initial analysis suggested that persons with mild gastrointestinal and neurological symptoms had eaten razor clams with a higher concentration of domoic acid than persons without symptoms had. Unfortunately, this study was not completed and further studies have not been conducted. Consequently, the effect of low-dose exposure to domoic acid remains unknown. The hypothesis exists that exposure to environmental chemicals, such as domoic acid, underlies some human neurodegenerative disorders including Parkinson's disease and dementia of the Alzheimer type. A recent article, however, suggests that it is unlikely that progressive neurodegenerative disorders are linked to environmental toxins [120].

4.4.3 Toxic Dose

During the Canadian outbreak, the implicated mussels contained from 31 to 128 mg of domoic acid per 100 g of tissue and total ingested dose ranged from 60 to 290 mg [2]. Although not reported in the original article by Perl, a subsequent article reported that a dose per kilogram could be calculated for seven persons with mild symptoms and ranged from 0.9 to 2.0 mg/kg [121]. Primate studies also suggest that 1 mg/kg represents a toxic dose [122]. Quick found that cases and controls had eaten razor clams with 3.7 and 2.6 mg of domoic acid per 100 g of tissue, respectively; the doses for these two groups were 12 mg (range, 4.2–29) and 6.5 mg (range, 0–24.4) [118]. The interpretation of this latter data is uncertain. It seems clear that ingesting 60 mg will lead to illness in some patients, but the lowest toxic dose remains to be determined. In the United States, shellfish beds are closed to harvesting when the domoic acid concentration reaches 20 μg/g of shellfish meat [123].

4.4.4 Geographic Distribution

While the geographic distribution of confirmed illness remains limited to eastern Canada, domoic acid-producing diatoms have been isolated on the east and west coast of the United States and Canada, Europe, Australia, New Zealand, Korea, Japan, and Vietnam [124], and therefore, monitoring programs are becoming increasingly more common in these areas [30,125–127].

4.4.5 Temporal Distribution

No temporal trend data for human illness exists. Similar to the case with other marine biotoxins, some authors have argued that toxic blooms are increasing [123].

4.4.6 Risk Factors

Among ill persons, males and the elderly had an increased risk of memory loss and hospitalization [2]. Perl has suggested that the association with age was due to increased renal disease in the elderly and thus that domoic acid is excreted through the kidneys. By contrast, Auer has suggested that increased susceptibility with age is related to the dendritic location of excitatory receptors and the increased branching of neuronal dendritic trees among the elderly [128]. Because specific parts of different species may concentrate toxin—for example, the viscera of dungeness crabs or the foot of razor clams [129]—selective consumption of these parts may increase the risk of illness. In the Canadian outbreak, cooking was not protective [2]; another study, however, suggests that boiling dungeness crabs significantly reduces the visceral toxin level [130]. Some ethnic groups may have an increased risk of toxin exposure because of different patterns of seafood consumption, for example, the practice of eating the viscera of crabs among persons of Chinese descent in Washington State [121].

4.4.7 Public Health Issues

Based on current knowledge, domoic acid is primarily of public health concern because of its potential for widespread illness via commercial shellfish. During the original outbreak, 68% of persons became ill in 1 of 45 different restaurants [2]. Based on this fear, regulatory limits for commercial shellfish have been established. The current level of 20 μg/g of tissue (20 ppm) [131] was established based on animal studies. A recent study suggests that, based on consumption patterns and toxic dose estimates, a tolerable regulatory level would equal 20 ppm for razor clams and 32 ppm for dungeness crabs [121]. It remains unclear whether the current regulatory limits are excessive or too low for preventing human illness. It is clear, however, that these regulatory limits will result in periodic fisheries closures as toxin levels in excess of 20 ppm have been found on numerous occasions [132]. An updated 10-year frequency map for ASP in the United States is available at the Harmful Algae Page, which is supported by a National Oceanic and Atmospheric Administration Center for Sponsored Coastal Ocean Research Coastal Ocean Program grant to the National Office for Harmful Algal Blooms [133].

4.5 TETRODOTOXIN

4.5.1 Incidence

No reliable incidence data exist for tetrodotoxin poisoning. The most extensive data on tetrodotoxin poisoning comes from Japan where 6386 cases of puffer fish poisoning were reported during the 78-year-period 1886–1963 (59.4% were fatal) [134, 135]. If the average population during this time is assumed to have been approximately 60,000,000, this implies a minimum incidence of 0.14 cases per 100,000 persons per year. Another report from Japan identified 2688 *deaths* due to puffer fish ingestion during 1927 through 1949 [136]. Using the same denominator, this implies that 0.2 deaths per 100,000 population per year occurred due to tetrodotoxin poisoning during this period. Interestingly, with the exception of the period during World War II, the number of reported puffer fish poisoning episodes in Japan during 1886–1963 remained relatively constant at 100–300 per year. In addition, during the same period no systematic decrease in the case fatality rate occurred. More recently, 495 persons became ill from puffer fish ingestion during 1977–1986 [36].

Other Southeast Asian countries have also reported cases of tetrodotoxin poisoning. A report from the Poison Control Center in Taiwan, with a 1989 population of approximately

20,000,000, identified 20 outbreaks involving 52 patients during 1988 through 1995 [137]. This suggests a minimum incidence of 0.03 cases per 100,000 population per year. Similarly, in Thailand, with a 1989 population estimate of approximately 55,000,000, 71 persons developed tetrodotoxin poisoning from horseshoe crab ingestion during January 1994 through May 1995 [138]. This suggests a minimum incidence related to ingestion of this animal of 0.09 cases per 100,000 persons per year. Forty-two outbreaks of tetrodotoxin-associated paralytic snail poisoning, involving 309 cases of illness, occurred from 1977 to 2001 in Asia, especially in China [139].

4.5.2 Clinical Symptoms

Symptom onset occurs within minutes and only rarely more than 6 h after eating a toxic animal [137,140,141]. Perioral paresthesia is the most immediate and, with acral paresthesias, the most common symptom (Table 4.5). Nausea and vomiting may or may not occur. Disease may progress to dizziness or vertigo, weakness, ataxia, dyspnea, diaphoresis, and death from respiratory failure. Similar to PSP, affected persons may report a floating sensation [141]. Clinical findings may include mydriasis, motor paralysis, respiratory paralysis, tachycardia, and bradycardia [142]. In addition, although hypotension has been a classic finding, at least three reports from Taiwan have documented the occurrence of hypertension [137,143,144]; in one case the blood pressure rose to 300/140 with death occurring 2 h after consumption of the implicated fish [144].

The mortality rate is dependent on, among other things, timely access to intensive care facilities. In some series, it has approached 60% [133,145]. When death results, it usually occurs within 6 h, and sometimes as rapidly as 17 min, following toxin ingestion. Persons who have not died within 24 h generally recover completely. Similar to PSP, symptoms of tetrodotoxin poisoning usually resolve within 1–2 days and residual impairment has not been reported. The mortality rate from the retrospective analysis of 42 outbreaks of tetrodotoxin-associated paralytic snail poisoning in Asia was 5.2% and 16% had respiratory arrest [140].

Fukuda and Hani, as reported by Halstead [134] have divided tetrodotoxin intoxication into four stages of progression. Stage 1 includes oral paresthesias with or without gastrointestinal symptoms. Stage 2 includes paresthesias of other areas and motor paralysis. Stage 3 includes muscular

TABLE 4.5
Tetrodotoxin Symptoms in Two Different Areas

Symptoms	Taiwan (Mainly Puffer Fish) [137] *N* = 52 (%)	Thailand (Horseshoe Crab) [138] *N* = 71 (%)
Acral paresthesias	54	87
Perioral paresthesias	48	94
Vomiting	40	30
Dizziness	37	
Weakness	29	44
Headache	25	
Dyspnea	17	
Vertigo	12	42
Diaphoresis	9.6	
Respiratory paralysis	33	27
Ataxia	27	
Hypertension	25	
Mydriasis	15	13
Hypotension	13	
Cyanosis or tachycardia	9.6	
Death	13	2.8

incoordination, aphonia, dysphagia, respiratory distress, precordial chest pain, cyanosis, and hypotension. Stage 4 includes depressed mental status, respiratory paralysis, and severe hypotension. As measurement of tetrodotoxin levels in implicated seafood has not usually been performed, it remains unknown whether this disease classification corresponds to either the toxin dose or other biological parameters.

4.5.3 Toxic Dose

The toxic and lethal doses are not known. In Taiwan, 30 persons became ill following consumption of the ovaries of an unknown species of fish. Subsequent testing of uneaten ovaries revealed toxin levels of 54 mouse units (MU)/g and 287 MU/g of tissue with an estimated intake of no more than 74,000 MU [144]. A second study in Taiwan measured toxin levels in the implicated marine organisms for six outbreaks with toxin varying between 13 (for gastropod mollusks) and 1200 (for puffer fish roe) MU/g of tissue; unfortunately the amount of fish or mollusk eaten was not reported [137]. A study in Madagascar found that four persons were ill and one died following ingestion of tissue with a toxin level of 16 MU/ g of tissue [146]. The lethal dose for humans has been estimated as 200,000 MU [144,147].

4.5.4 Geographic Distribution

Human intoxication from tetraodontiform has occurred in a variety of species that live in diverse ecosystems [148] including puffer and other tetraodontiforme fish, the blue-ringed octopus [149,150], mollusks [137], horseshoe crabs [138], and the Oregon newt [151]. Moreover, tetrodotoxin-containing fish exist in tropical waters throughout the world [140]. For most populations, however, species that contain tetrodotoxin do not constitute a significant part of the diet. Consequently, llness generally occurs in areas where potentially toxic animals such as puffer fish, gastropod mollusks, and horseshoe and other crabs are commonly eaten: Southeast Asia and, more specifically, Japan. In addition to Japan, Taiwan, and Thailand, intoxication has also been reported from the South Pacific [152], Malaysia [153,154], Hong Kong [155,156], Singapore [157], Australia [149,158], Madagascar [146], China [140], and Bangladesh [159]. It is possible that fatalities from eating some species of crab on Negros Island, Philippines, also resulted from tetrodotoxin poisoning [160,161].

Locally acquired tetrodotoxin poisoning has been reported in Mexico [162], and the United States, where cases have occurred in Florida due to the consumption of locally caught puffer fish, which were assumed to be contaminated with tetrodotoxin [163,164].

4.5.5 Temporal Distribution

Cases occur during all months of the year. It is not known whether the proportion of tetrodotoxic fish has increased. Simple incidence data would not necessarily answer this question since public health measures such as education and regulation of fugu chefs in Japan may effect incidence estimates regardless of changes in the proportion of animals containing toxin. In addition, it is possible that improved medical care has lowered the number of lethal cases. The retrospective analysis of 42 outbreaks of tetrodotoxin-associated paralytic snail poisoning in Asia found no temporal variation [140].

4.5.6 Risk Factors

No risk factors for tetrodotoxin poisoning are known. It is likely that intoxication and its severity are dose dependent [137]. Age has not been shown to increase the risk of illness; in Taiwan, illness occurred in persons from 9 months to 71 years of age [137]. The toxin is heat stable so that cooking is not protective. Tetrodotoxin concentrates in the viscera and roe of some animals, such as puffer fish [148]. Presumably,

removal of viscera will provide some measure of protection although cases have been reported where only the flesh of the fish was eaten [137]. Previous exposure does not provide protection [140].

4.5.7 Public Health Issues

No regulatory limits for tetrodotoxin have been established in the United States as personal importation of puffer fish is prohibited. An agreement between the U.S. Food and Drug Administration and the Japanese Ministry of Health and Welfare has been adopted, which allows importation of fugu for special occasions provided the fish is certified safe by the Japanese government before export [139]. Japan and Taiwan have attempted to control tetrodotoxin poisoning through licensing of restaurants and chefs or by establishing regulatory limits for the sale of puffer fish [36]. As people may eat tetrodotoxin from fish not served at restaurants, this approach will prevent only a portion of cases. Some countries, including Japan, have enacted laws restricting the sale of certain species known to cause tetrodotoxin poisoning [140].

4.6 NEUROTOXIC SHELLFISH POISONING

4.6.1 Incidence

No data on neurotoxic shellfish poisoning (NSP) incidence exist in the literature. Less than 100 cases have been reported in the United States, approximately half of which came from a single outbreak in North Carolina [165].

4.6.2 Clinical Symptoms

The most rigorous analysis of data on NSP comes from an outbreak in North Carolina involving 48 persons [162]. The median latent period between ingestion and onset of illness was 3 h (range, 15 min–18 h) with a similar onset for both gastrointestinal and neurological symptoms. The most common symptoms were paresthesias (81%), vertigo (60%), malaise (50%), abdominal pain (48%), nausea (44%), diarrhea (33%), weakness (31%), ataxia (27%), chills (21%), headache (15%), myalgia (13%), and vomiting (10%). Illness lasted from 30 min to 3 days (median, 17 h) and no long-term symptoms have been reported. The symptoms reported from the North Carolina outbreak in general agree with other investigations of illness from Florida [166, 167], although cases in Florida tended to have a shorter incubation period and less associated nausea and vertigo. Among other causes, reported differences in symptoms may result from qualitative or quantitative differences in toxin consumption or more rigorous identification of milder cases. Death, if it occurs, is exceedingly uncommon.

The above documents the consequences of toxin ingestion. A few reports suggest that inhalation of aerosolized toxin may cause conjunctival irritation, rhinorrhea, respiratory irritation, and possibly exacerbate or cause symptoms similar to reactive airways disease [168, 169]. Several recent studies have provided additional evidence of adverse respiratory effects, including upper airway irritation and discomfort, decreases in pulmonary function parameters, and worsening asthma symptoms, due to occupational and environmental exposures to aerosolized brevetoxins.

4.6.3 Toxic Dose

During the outbreak in North Carolina, implicated oysters had a toxin level of 35–60 MU/100 g of tissue. Two persons became ill after consuming less than 12 oysters, but in this group, the attack rate was only 13%. At 12 oysters and above, the attack rate equaled 65%. If 12 oysters are used in the calculation, and we assume a weight of 10 g per oyster, a low (but not minimum) toxic dose estimate equals 42–72 MU. No lethal toxic dose estimates based on human intoxication episodes exist.

4.6.4 Geographic and Temporal Distribution

As summarized by Fleming, the causative agents of NSP have been found in Florida, North Carolina, the Gulf of Mexico, Brazil, Spain, Japan, New Zealand, and the Solomon Islands [173]. Most reports of illness and dinoflagellate blooms come from Florida, although this finding may represent differences in surveillance rather than true differences in occurrence. No information on temporal distribution exists as published reports have relied on outbreaks rather than systematically collected surveillance data.

4.6.5 Risk Factors

No risk factors for illness have been identified other than estimated ingested dose [162]. The investigation in North Carolina examined age, gender, the presence of chronic illness, medication use, and alcohol consumption during the implicated meal and found no association with illness. As with other marine neurotoxins, brevetoxin is heat stable and thus, cooking contaminated seafood will not alter the risk of intoxication. Furthermore, the toxin is lipid rather than water-soluble [174] and thus, boiling or steaming contaminated food is similarly unlikely to alter the risk of intoxication.

4.6.6 Public Health Issues

As with ASP, NSP is primarily of public health concern because of its potential for large outbreaks via distribution in commercial seafood products. Because of this concern, public health agencies in Florida have routinely monitored coastal waters for the presence of *Ptychodiscus brevis* since the mid-1970s.

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Part III

Diversity of Marine Toxins as Pharmacological Tools

5 Calcium Channels for Exocytosis: Functional Modulation with Toxins

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CONTENTS

5.1 INTRODUCTION

The combination of patch-clamp techniques, ω-toxins, and molecular strategies has revealed a great heterogeneity of voltage-dependent Ca^{2+} channels in neurons. Peptide toxins derived from the venoms of marine snails *Conus geographus* (ω-conotoxin GVIA) and *Conus magus* (ω-conotoxins MVIIA, MVIIC, and MVIID), as well as from *Agelenopsis aperta* spider venom (FTX; ω-agatoxin IVA) are powerful diagnostic pharmacological tools to discriminate between different subtypes of neuronal Ca^{2+} channels. Thus, the so-called high voltage-activated (HVA) Ca^{2+} channels are selectively recognized by ω-conotoxin GVIA and MVIIA (N-type), by low concentrations (nanomolar) of ω-agatoxin IVA (P-type), or by high concentrations of ω-agatoxin IVA (micromolar) or the ω-conotoxins MVIIC and MVIID (Q-type). L-type HVA Ca^{2+} channels present in neurons, cardiovascular tissues, skeletal and smooth muscle, and in endocrine cells are targeted by so-called organic Ca^{2+} antagonists such as the 1,4-dihydropyridines (DHPs) nifedipine or Bay K 8644, the benzylalkylamine verapamil, or the benzothiazepine (BTZ) diltiazem; they are also specifically blocked by snake toxins calciseptine and calcicludine. Wide-spectrum ω-toxins (ω-conotoxin MVIIC, ω-agatoxin IA, IIA, and IIIA) and organic compounds (flunarizine, dotarizine, cinnarizine, fluspirilene, R56865, and lubeluzole) can block several classes of HVA Ca^{2+} channels, including the L-type. A neuronal R-type HVA channel seems to be sensitive to SNX-482, a peptide from the venom of the African tarantula *Hysterocrates gigas*. Low-voltage-activated (LVA) channels (T-type) are blocked by 1-octanol, amiloride, and mibefradil, and are more sensitive to Ni^{2+} than to Cd^{2+}; no toxins that recognize these channels are known.

It is interesting that a single cell can express different subtypes of HVA Ca^{2+} channels and that the quantitative expression of each channel subtype differs with the animal species. The example of adrenal medulla chromaffin cells is illustrative. In the bovine, P/Q-type (45%) and N-type (35%) are predominant; the L-type Ca^{2+} channel carries a minor component of the whole-cell current (20%). In the rat and the mouse, the L-type predominates (50%), together with the N-type (35%), whereas the P/Q family accounts for a minor component (15%). In cat chromaffin cells, L-type Ca^{2+} channels carry 50% of the current and N-type channels carry 45%; P/Q account for only 5%. In human chromaffin cells, P/Q-type Ca^{2+} channels dominate (60%) while in pig chromaffin cells N-type channels are predominant (80%). The functional significance of this variety of Ca^{2+} channels begins to be understood.

Ca^{2+} channels consist of a multiple subunit protein complex with a central pore-forming α_1 subunit and several regulatory and/or auxiliary subunits, which include β subunits, γ subunits, and the disulfide-linked α_2/δ subunit. The α_1 subunit contains the Ca^{2+} conductance pore, the essential gating machinery, the receptor sites for the most prominent pharmacological agents, and modulatory

sites for G-protein subunits, protein kinase-induced phosphorylation, or exocytotic machinery protein binding sites. The mammalian family of Ca^{2+} channel α_1 subunits is encoded by at least ten genes. These subunits are grouped in three families, Cav1, Cav2, and Cav3 that give rise to inward Ca^{2+} currents termed HVA or L, N, P/Q, and R channels, and LVA or T-type channels.

Marine toxins have been invaluable tools to recognize the role of each channel subtype in controlling the Ca^{2+}-dependent exocytotic release of a given neurotransmitter. Thus, N-type Ca^{2+} channels are highly involved in the control of norepinephrine release from sympathetic neurons, as well as acetylcholine release from the electric fish muscle end plate, the myenteric plexus, and detrusor muscle. Also N-channels partially control the nonadrenergic noncholinergic (NANC) neurotransmission in smooth muscle, γ-aminobutyric acid (GABA) release in cerebellar neurons, glycine release in dorsal horn neurons of the spinal cord, epinephrine release from the dog adrenal, dynorphin release in dentate gyrus, and the synaptic neurotransmission in retinal ganglion neurons and the hippocampus. P channels dominate the release of GABA from deep cerebellar neurons, glycine from dorsal horn neurons of the spinal cord, and acetylcholine from the mammalian neuromuscular junction. They also seem to participate partially in the control of the release of other neurotransmitters. Up to now, Q channels have been implicated in the control of neurotransmission in the hippocampus and in the release of catecholamines from bovine chromaffin cells. L-type Ca^{2+} channels dominate the release of catecholamines in rat and cat chromaffin cells, and partially control the secretory process in bovine chromaffin cells.

A critical question is why a neurosecretory cell expresses several Ca^{2+} channel subtypes. In bovine adrenal chromaffin cells L, N, P, Q, and R channels have been found; depending on the stimulus and the experimental conditions, all of them seem to be involved in the control of catecholamine release induced by depolarizing stimuli. It is uncertain whether a given Ca^{2+} channel subtype colocalizes more than others do with the secretory machinery of chromaffin cells. Many other questions remain unanswered, for instance, to find a selective blocker for the R type channel. A third question relates to the number of Ca^{2+} channels yet unrecognized. The functions of the Ca^{2+} channels not related to exocytosis (i.e., the neuronal L-type channels) are beginning to be discovered; thus, Ca^{2+} entry through these channels may cause gene induction, apoptosis, or preferentially activate endo- over exocytosis, in bovine chromaffin cells [1]. Finally, it is important to stress the need of finding nonpeptide molecules to target specifically different channel subtypes; these compounds should cross the blood–brain barrier and thus serve as therapeutic drugs to treat different brain diseases. We will review all these aspects in this chapter, emphasizing on the use of ω-toxins as tools to identify Ca^{2+} channels, Ca^{2+} signals, and cell function, particularly exocytosis.

5.2 ω-TOXINS AS DIAGNOSTIC PHARMACOLOGICAL TOOLS

Some static or slow animals, both terrestrial (snakes, spiders) and marine (snails), have developed venoms containing potent neurotoxins to capture their prey with high efficiency and speed. The efficiency of the method used to capture the prey will influence the venom content evolved by a predator.

One of the most representative examples of venomous animals is constituted by the *Conus* marine snails [2]. Of the approximately 500 *Conus* species, about 40–100 prey primarily on fish (fish-hunting species), and these species use two parallel physiological mechanisms requiring multiple neurotoxins to immobilize fish rapidly [3]: neuromuscular block and excitotoxic shock. Fish-hunting *Conus* snails use a harpoon-like device to inject their venom in their preys. The venom contains a cocktail of neurotoxins that will cause a double-phase paralytic process (Table 5.1), with an initial phase characterized by a fast paralysis with tetanus and a second phase characterized by a flaccid paralysis. Finally, the fish will be engulfed by the snail.

The fast paralysis of the phase I is mediated by two groups of neurotoxins, the δ-conotoxins, that suppress the inactivation of the voltage-dependent Na^+ channels, thus causing an increase in Na^+ influx; and the κ-conotoxins that block K^+ channels not allowing the cells to repolarize.

TABLE 5.1
Paralytic Process Induced by the Venom of *Conus* Marine Snails and Neurotoxins Implied with Their Mechanisms of Action

Phase I: Fast paralysis with tetanus (rapid immobilization)	
δ-Conotoxins	Suppression of Na^+ channel inactivation (increases Na^+ influx)
κ-Conotoxins	Blockade of K^+ channels
Phase II: Flaccid paralysis	
α-Conotoxins	Blockade of nicotinic acetylcholine receptors
μ-Conotoxins	Blockade of voltage-dependent Na^+ channels
ψ-Conotoxins	Blockade of nicotinic acetylcholine receptors
κ-Conotoxins	Blockade of K^+ channels
δ-Conotoxins	Suppression of Na^+ channel inactivation
ω-Conotoxins	Blockade of voltage-dependent Ca^{2+} channels

This combination of toxins lead to hyperactivity of the fish, followed by a continuous contraction and extension of major fins, without death. The second phase consists in a flaccid state and is caused by a different cocktail of neurotoxins (see Table 5.1): the α-conotoxins that block nicotinic acetylcholine receptors; the μ-conotoxins that block voltage-dependent Na^+ channels; the ψ-conotoxins that also block nicotinic acetylcholine receptors; the κ-conotoxins that cause the blockade of K^+ channels; the δ-conotoxins that suppress the inactivation of the voltage-dependent Na^+ channels, and the ω-conotoxins that block voltage-dependent Ca^{2+} channels, and are the subject of this chapter.

Another example of venomous animals is the funnel-web spider *A. aperta* that has a potent venom with paralytic properties. As in the *Conus*, the venom of this spider possesses a mixture of toxins with different targets, with the polyamines and the polypeptides being the main components of such venom. The polyamines group is composed of the FTX, which targets voltage-dependent Ca^{2+} channels [4] and the acylpolyamines (α-agatoxins), most of which are blockers of glutamate receptors. The other group, the polypeptide toxins, is composed of the ω-agatoxins that selectively block different subtypes of voltage-dependent Ca^{2+} channels [5], and the μ-agatoxins that are potent activators of voltage-dependent Na^+ channels. This combination of toxins secures a fast and reversible paralytic effect (induced by the α- and μ-agatoxins) with a slower but irreversible paralysis of the prey, induced by the ω-agatoxins.

Finally, venoms from different snakes from the *Elapidae* and *Hydrophidae* families also contain a cocktail of different paralytic toxins, some of which are selective for voltage-dependent Ca^{2+} channels. For instance, the venom of the black mamba *Dendroaspis polylepis polylepis* contains a toxin termed calciseptine, which selectively blocks L-type Ca^{2+} channels [6] and the venom from the green mamba *D. agusticeps* contains calcicludine, a toxin that acts as a potent blocker of most of the HVA Ca^{2+} channels [7].

5.3 ω-TOXINS FOR THE CHARACTERIZATION OF VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

As indicated, ω-conotoxins are found in almost all fish-hunting *Conus* species examined up to now. The most thoroughly studied are the venoms from *C. geographus*, *C. magus*, and *C. striatus*. Several of these peptides have been purified, sequenced, and synthesized (with similar potency as the natural toxins) and they have become important tools for the identification and characterization of the different subtypes of voltage-dependent Ca^{2+} channels found in neuronal tissues.

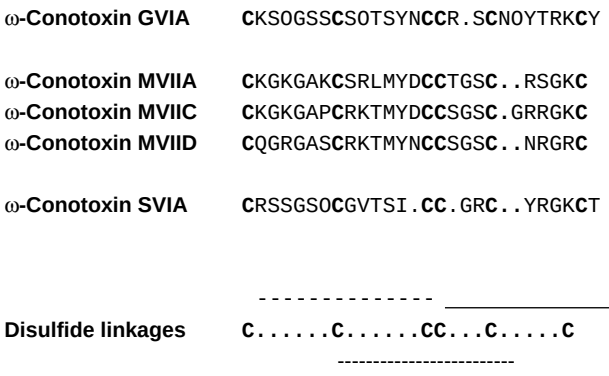


FIGURE 5.1 Upper panel shows the sequence of ω-conotoxins isolated from *Conus geographus* (GVIA), *Conus magus* (MVIIA, MVIIC, and MVIID) and *Conus Striatus* (SVIA). Lower panel shows the arrangement of the *cys* residues that constitutes the "four-loop" structure.

5.3.1 ω-Conotoxins

ω-Conotoxins are small peptides containing 24–29 amino acid residues (Figure 5.1); they share several features, which are common to all ω-conotoxins. More characteristic is the presence of six *cys* residues, with three intramolecular disulfide bridges, forming a structure known as "four-loop framework" [8,9]. This arrangement of Cys residues is similar to that observed in δ-conotoxins, which target voltage-gated Na^+ channels [10].

Although the sequence of different ω-conotoxins has great interspecies variations, they can compete for the same Ca^{2+} binding site and show similar physiological effects. For instance, ω-conotoxin GVIA [11] and ω-conotoxin MVIIA [12] have a homology lower than 30% in the non-Cys residues, but both target N-type Ca^{2+} channels (as described below) and elicit similar biological effects; the major differences are that ω-conotoxin GVIA blocks N-type Ca^{2+} channels in an irreversible manner [2,13], whereas ω-conotoxin MVIIA does it in a reversible manner [14,15].

Other ω-conotoxins have broader Ca^{2+} channel blocking properties than ω-conotoxin GVIA and ω-conotoxin MVIIA. Complementary DNA (cDNA) clones encoding a previously unknown ω-conotoxin were identified from a cDNA library made from the venom duct of *C. magus* [16]. The predicted peptides ω-conotoxin MVIIC and ω-conotoxin MVIID were chemically synthesized and characterized. Both peptides inhibit N-type Ca^{2+} channels and P-type Ca^{2+} channels, but also other Ca^{2+} channels resistant to DHP, ω-conotoxin GVIA, and ω-agatoxin IVA [16], and thus, they constitute actually an important tool for the characterization of P/Q-types of Ca^{2+} channels, as described below. Some differences between the ω-conotoxins relate to the reversibility of its blocking effects, and thus, N-type Ca^{2+} channels can be blocked in an irreversible manner by ω-conotoxin MVIIC but in a reversible manner by ω-conotoxin MVIID [17].

5.3.2 ω-Agatoxins

ω-Agatoxins derived from the venom of *A. aperta* are also a heterogeneous group of polypeptides (5–100 kDa) that specifically target voltage-dependent Ca^{2+} channels. Four subtypes of ω-agatoxins have been identified up to now [5,18,19]. Type I ω-agatoxins (ω-Aga-IA, ω-Aga-IB, and ω-Aga-IC) are potent blockers of neuromuscular transmission in insects. Of these, the most studied is ω-agatoxin IA, which seems to block both L- and N-type Ca^{2+} channels [20]. Type II ω-agatoxins have a spectrum of action on neuronal Ca^{2+} channels in vertebrates similar to that of ω-agatoxin IA, although they may block Ca^{2+} channels by a different mechanism [21]. ω-Agatoxin IIA has been shown as a potent blocker of both L- and N-type Ca^{2+} channels [22].

Type III ω-agatoxins (ω-Aga IIIA, ω-Aga IIIB, ω-Aga IIIC, and ω-Aga IIID) have a broader spectrum of blockade than other agatoxins and block several subtypes of voltage-dependent Ca^{2+} channels. Of these, ω-agatoxin IIIA has been shown to be a potent inhibitor or L-, N-, and P/Q-type Ca^{2+} channels in neurons of rats and frogs [23–25]; it shows a very high potency (IC_{50} <1 nM) for both inhibiting L- and N- channels, being more potent than ω-conotoxin GVIA for blocking N-type channels [26]. Efficacy of blockade induced by ω-agatoxin IIIA is higher for L-type channels and decreases for N- and P/Q-type Ca^{2+} channels [24]. In these latter channel subtypes, ω-agatoxin IIIA seems to act as a high-affinity partial antagonist, blocking less than 50% of Ca^{2+} conductance [24].

Type IV ω-agatoxins (ω-Aga IVA and ω-Aga-IVB) show a different pharmacological effect to that described for other ω-agatoxins, and, in addition to N-type Ca^{2+} channels, they also block P-type Ca^{2+} channels with a K_d of 2–3 nM [22,27,28].

5.3.3 FTX

The toxin fraction (FTX) of *A. aperta* spider venom can be also used as a P-type Ca^{2+} channel blocker. In fact, this toxin was initially used to describe and characterize P-type Ca^{2+} channels in Purkinje cells [4,29,30]. Although FTX was initially considered to be selective for P-type Ca^{2+} channels, later it was shown to block other ionic channels [31].

5.4 DIVERSITY OF VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

Two approaches are mainly responsible for the discovery of the rich diversity of voltage-dependent Ca^{2+} channels. On the one hand, the characterization of the biophysical properties of Ca^{2+} channels (kinetics of activation, inactivation and deactivation, voltage-range for activation, and conductance), both at the single-channel and at the whole-cell level has been possible, thanks to the improvement of the patch-clamp techniques [32]. On the other hand, the isolation, purification, and synthesis of different neurotoxins have provided ligands with remarkable discrimination for different subtypes of high-threshold DHP-resistant Ca^{2+} channels [2].

With the combination of the patch-clamp techniques and these pharmacological probes, at least five subtypes of voltage-dependent Ca^{2+} channels have been described up to now: T, L, N, P/Q, and R (Table 5.2). These channels can be classified according to their range of activation in two main groups: one with a low threshold for activation (low-voltage-activated, LVA) and the other with a high threshold for activation (high voltage-activated: HVA).

5.4.1 LVA Channels: T-Type Ca^{2+} Channels

The first attempt to identify different subtypes of voltage-dependent Ca^{2+} channels was carried out by Carbone and Lux [33], who identified two types of channels, those that open with small depolarizations from a hyperpolarized holding potential, so-called LVA channels; and those that require higher depolarizations to open, so-called HVA channels.

In addition to its low threshold for activation, LVA Ca^{2+} channels [33] are characterized by a similar permeability for Ca^{2+} and Ba^{2+} [34,35]. This channel was termed T (for "Transient" or "Tiny"), with its fast inactivation, which generates a transient current, and their inactivation when the holding potential is fixed between −60 and −50 mV as the main characteristics of this channel. The single-channel conductance has been estimated to be around 8 pS.

Pharmacologically, T-type channels can be distinguished from other subtypes, because they are more sensitive to blockade by the inorganic Ca^{2+} channel blocker Ni^{2+} than to Cd^{2+} [34,35]. It has also been described that T-type channels can be blocked by l-octanol, amiloride, the antihypertensive drug mibefradil [36], and ethosuximide [37]. Interestingly, a new scorpion toxin (kurtoxin) that binds to the α_{1G}T-type Ca^{2+} channel with high affinity was identified; it inhibits the channel by modifying voltage-dependent gating [38].

TABLE 5.2
Calcium Channel Subtypes according to Their α_1-Containing Subunit

Calcium Channel Type					
(Novel Nomenclature)	**(Traditional Nomenclature)**	**Type of Current**	**Blockers**	**Activators**	**Tissue Location**
Cav 1.1	α_{1S}	L	Nifedipine Nisoldipine Nitrendipine	Bay K 8644 FPL64176	Skeletal muscle
Cav 1.2	α_{1C}	L	Nifedipine Nisoldipine Nitrendipine	Bay K 8644 FPL64176 PCA50941	Heart Smooth muscle Brain Pituitary Adrenal medulla
Cav 1.3	α_{1D}	L	Nifedipine Calcicludine	Bay K 8644 FPL64176 PCA50941	Brain Pancreas Adrenal medulla Cochlea Kidney Ovary
Cav 1.4	α_{1F}	L	Nifedipine	Bay K 8644 FPL64176	Retina
Cav 2.1	α_{1A}	P/Q	ω-aga- IVA ω-ctx-MVIIC ω-ctx-MVIID		Cerebellum Pituitary Cochlea Adrenal medulla
Cav 2.2	α_{1B}	N	ω-ctx-GVIA ω-ctx-MVIIA		Brain Peripheral nervous system Adrenal medulla
Cav 2.3	α_{1E}	R	SNX-482		Brain Cochlea Retina Heart Pituitary Adrenal medulla
Cav 3.1	α_{1G}	T	Mibefradil		Brain Peripheral nervous system Adrenal medulla
Cav 3.2	α_{1H}	T			Heart Brain Kidney Liver Adrenal glomerulosa Adrenal medulla
Cav 3.3	α_{1I}	T			Brain

ω-aga-IVA, ω-agatoxin IVA; ω-ctx-GVIA, ω-conotoxin GVIA; ω-ctx-MVIIA, ω-conotoxin MVIIA; ω-ctx-MVIIC, ω-conotoxin MVIIC; ω-ctx-MVIID, ω-conotoxin MVIID.

T-type currents are difficult to record in chromaffin cells. Although we have detected T-type channel messenger (mRNA) in bovine chromaffin cells [39], we have been unable to record T-type currents. However, there are three studies reporting T-type Ca^{2+} currents in bovine [40] and rat chromaffin cells [41,42]. It has been suggested that T-type Ca^{2+} channels are mainly expressed in immature developing chromaffin cells [41]. Recently, T-type channels of the α_{1H} class have been found to be expressed in rat chromaffin cells exposed to cyclic adenosine monophosphate (cAMP) [43]; those channels were found to trigger a secretory response [44]. This α_{1H} T-type Ca^{2+} channel has also been identified in rat adrenal glomerulosa zone [45].

5.4.2 HVA Channels

High-voltage activated (HVA) channels are characterized by their activation by strong depolarizing steps [34,35], a higher permeability to Ba^{2+} than to Ca^{2+}, and a higher sensitivity to Cd^{2+} than to Ni^{2+}, in contrast to LVA channels. Up to now, five major subtypes (L, N, P, Q, and R) of HVA channels have been identified. The major differences between them are related to their inactivation kinetics and their pharmacological properties.

5.4.2.1 L-Type Ca^{2+} Channels

L-type (for "long lasting") Ca^{2+} channels are kinetically characterized by showing little inactivation during depolarizing steps (τ_{inact} >500 ms) and their lower sensitivity to depolarized holding potentials. Single-channel conductance was estimated to be around 18–25 pS. This subtype of Ca^{2+} channel seems to be present in all excitable cells and in many nonexcitable cells, and they constitute the main pathway for Ca^{2+} entry in heart and smooth muscle, serving also to control hormone and transmitter release from endocrine cells and some neuronal preparations. Four different α_1 subunits (α_{1C}, α_{1D}, α_{1F}, and α_{1S}) are responsible for L-type Ca^{2+} currents in different tissues (see Table 5.2).

Pharmacologically, L-type Ca^{2+} channels are highly sensitive to DHPs (Table 5.2), both agonists (i.e., Bay K 8644) and antagonists (i.e., nifedipine, nimodipine, and furnidipine). DHP agonist effects are characterized by the prolongation of the mean time for channel opening [46,47], typically observed in whole-cell electrophysiological recordings as a prolongation of tail currents [48].

Other organic compounds have been described to effectively block L-type Ca^{2+} channels [49,50]: the arylalkylamines (i.e., verapamil) and BTZs (i.e., diltiazem) are particularly useful in cardiac and smooth muscle cells, where they exert negative inotropic effects. Some piperazine derivatives (cinnarizine, flunarizine, dotarizine, and R56865) also block L-type Ca^{2+} channels, but they block other subtypes of Ca^{2+} channels and thus, have been proposed as "wide-spectrum" Ca^{2+} channel blockers [51–53]. The same is true for imidazole antimycotics [54]. Some toxins have also been shown to block L-type Ca^{2+} channels, either selectively (calciseptine and calcicludine) or in a nonselective manner (ω-agatoxin IA, ω-agatoxin IIA, and ω-agatoxin IIIA).

L-type currents have been characterized in bovine [55–60], rat [47,61], mouse [62], pig [63], cat [64], and human chromaffin cells [65]. Recent studies have presented molecular evidence that L-type currents in chromaffin cells are carried out by two different Ca^{2+} channels: α_{1C} and α_{1D} [66].

5.4.2.2 N-Type Ca^{2+} Channels

N-type Ca^{2+} channels display faster inactivation kinetics (τ_{inact} 50–80 ms) than that of L-type channels. This relative fast inactivation usually leads to their inactivation when maintaining a depolarizing holding potential, although in some preparations, N-type Ca^{2+} channels can contain a noninactivating component, even at the end of long depolarizations, for instance, in bovine chromaffin cells in which N-type channels have been described as "nonclassical N-type" [57]. Single-channel conductance of N-type channels has been estimated to be around 13 pS.

Pharmacologically, N-type Ca^{2+} channels are characterized by the irreversible blockade induced by the *C. geographus* toxin ω-conotoxin GVIA [2,46,67] and the reversible blockade induced by the *C. magus* toxin ω-conotoxin MVIIA (Table 5.2) [14,15]. Other wide-spectrum toxins as ω-conotoxin MVIIC and ω-conotoxin MVIID [16,68] can also block N-type Ca^{2+} channels in a nonselective manner. This is also the case for ω-agatoxin IIA, ω-agatoxin IIIA, and ω-grammotoxin SIA (isolated from the venom of the tarantula *Grammostola spatulata*).

N-channel currents have been characterized in chromaffin cells of various species including bovine [57,69], pig [63], cat [64], rat [47], mouse [62], and human [65]. This current suffers voltage-dependent inactivation [34,70], but see reference [57], and is irreversibly blocked by ω-conotoxin GVIA [11] and ω-conotoxin MVIIC [16,17] or reversibly blocked by ω-conotoxin MVIID [17,68].

5.4.2.3 P-Type Ca^{2+} Channels

P-type Ca^{2+} channels were first described by Llinás et al. [29] in cerebellar Purkinje cells, in which Ca^{2+} currents were resistant to blockade by DHPs and ω-conotoxin GVIA. The toxin fraction from the venom of the funnel web spider *A. aperta* (FTX) was found effectively to block this resistant current, and these results led these authors to suggest the existence of a new subtype of HVA Ca^{2+} channel, which was termed P (for "Purkinje").

P-type Ca^{2+} channels are characterized by their relative insensitivity to changes in the holding potential, and do not inactivate during depolarizing steps [22,71,72]; multiple single channel conductances have been described for P-type Ca^{2+} channels [73,74].

Pharmacologically, P-type Ca^{2+} channels can be blocked by FTX and its synthetic analog synthetic funnel-web toxin (sFTX) and by ω-agatoxin IVA at concentrations in the nanomolar range (<30–100 nM). This toxin is actually accepted to be the selective probe to identify the presence of P-type Ca^{2+} channels (see Table 5.2). P-type Ca^{2+} channels can be also blocked in a nonselective manner by ω-conotoxin MVIIC [16,68], ω-conotoxin MVIID, and by ω-grammotoxin SVIA [75–78].

Nanomolar concentrations of ω-agatoxin IVA known to fully and selectively block P-type channels [22,72], cause only a 5–10% blockade of Ca^{2+} channel current in bovine chromaffin cells [79]. Previous studies reported larger contributions of P-type channels to the whole chromaffin cell Ca^{2+} currents; however, this blockade is now attributable to inhibition of Q-type channels [80] by sFTX [81] or large concentrations of ω-agatoxin IVA [55,82]. In cat chromaffin cells, combined ω-conotoxin GVIA plus nisoldipine blocked 90% of the current, leaving little room for P-type channels [64]. In rat [47] and mouse chromaffin cells [62], the ω-agatoxin IVA-sensitive current fraction was only 10–15%. Thus, in all species studied, P-type channels are barely expressed in chromaffin cells. This, together with the difficulty of separating the α_{1A} subunit into P- and Q-type channels [83] suggests the convenience of speaking of P/Q-type channels rather than of two separate Ca^{2+} channel subtypes.

5.4.2.4 Q-Type Ca^{2+} Channels

In many neuronal preparations, a significant component of the whole-cell current through Ca^{2+} channels is resistant to blockade with DHPs, ω-conotoxin GVIA, and ω-agatoxin IVA (<100 nM), suggesting the presence of a subtype of Ca^{2+} channel different from L-, N-, and P-types. The isolation, purification, and synthesis of the toxin from the marine snail *C. magus* ω-conotoxin MVIIC [16,68] led to the identification and characterization of a new subtype of HVA channel termed Q [80,84].

Characterization of Q-type Ca^{2+} channels is mostly based on pharmacological criteria. As described, Q-type channels are resistant to blockade by DHPs, ω-conotoxin GVIA, and low doses (<100 nM) of ω-agatoxin IVA, but they are sensitive to ω-conotoxin MVIIC (1–3 μM). Increasing concentrations of ω-agatoxin IVA (up to 2 μM) can also block Q-type Ca^{2+} channels [80]. It should

be noted that these toxins, used to identify Q-type channels, are not selective for this subtype of channel, and they also block N- and P-types in a nonselective manner. Other toxins that can also block this subtype of Ca^{2+} channel include the *C. magus* snail toxin ω-conotoxin MVIID [68] and the *G. spatulata* tarantula toxin ω-grammotoxin SIA [75–78].

In chromaffin cells, the P/Q component of the whole-cell Ca^{2+} channel current has been widely studied in chromaffin cells. This component is voltage inactivated [70] and it is pharmacologically isolated by 2 ⊠M ω-conotoxin MVIIC, ω-conotoxin MVIID, or ω-agatoxin IVA. In bovine chromaffin cells, ω-conotoxin MVIID reversibly blocks the N current but blockade by ω-conotoxin MVIIC is irreversible [17]. Thus, the use of ω-conotoxin MVIID followed by its washout can be a convenient tool to isolate the P/Q channel. The blocking effects of ω-conotoxin MVIIC are extraordinarily slowed down and decreased in the presence of high concentrations (i.e., more than 2 mM) of Ba^{2+} [79,85] or Ca^{2+} [15].

5.4.2.5 R-Type Ca^{2+} Channels

In neuronal tissues, a residual Ca^{2+} current, characterized by its insensitivity to blockade by DHPs, ω-conotoxin GVIA, ω-agatoxin IVA, and ω-conotoxin MVIIC has also been described and termed "R-type" (for "resistant") [84]. This new subtype of Ca^{2+} channel belongs to the HVA group, is rapidly inactivating ($\tau = 22$ ms), and more sensitive to blockade by Ni^{2+} ($IC_{50} = 66$ μM) than to Cd^{2+}.

Newcomb et al. [86] described the first selective R channel blocker, SNX-482, a peptide from the African tarantula *H. gigas*. We found, however, that this toxin also blocks P/Q channels in the bovine chromaffin cell [87]. Thus, caution should be exerted when using this toxin to target R-type currents.

Differences have been reported in various laboratories concerning the expression of R-type Ca^{2+} channels in chromaffin cells, and they may be due to the configuration of the patch-clamp technique used (whole-cell vs. perforated-patch recordings). In some initial studies, an R-type component of I_{Ca} could not be detected in bovine [55,56,79,81,82,88–90], cat [64], human [65], pig [63], or mouse chromaffin cells [62,91]. In contrast, using the perforated-patch configuration instead of whole-cell patch configuration of the patch-clamp technique, an R-type component was found in slices of mouse adrenal medulla and mouse chromaffin cells [91,92]. The most obvious explanation for this finding is that some soluble cytosolic factor, which is necessary for chromaffin cell R channel activity, is dialyzed with the whole-cell, but not with the perforated-patch configuration.

5.5 SOME CURIOUS DIFFERENCES AMONG SPECIES

Drastic species differences in the subtypes of Ca^{2+} channels expressed by different cell types have been found. For instance, the K^+-evoked Ca^{2+} entry in brain cortex synaptosomes is controlled by N channels in the chick and by P channels in the rat [93]. On the other hand, neurotransmitter release at the muscle end plate is controlled by N channels in fish [94–96] and amphibians [97] and by P channels in mammals [98].

Detailed comparative electrophysiological studies among six mammalian species have been performed only in adrenal medullary chromaffin cells (Figure 5.2). L-type Ca^{2+} channels account for near half of the whole-cell Ca^{2+} channel current in the cat [64], rat [47], and mouse chromaffin cells [62]. In pig [63], bovine [55,81], and human species [65] L channels carry only 15–20% of the whole-cell Ca^{2+} current.

The N channel also shows a high interspecies variability. In the pig it carries as much as 80% of the whole-cell Ca^{2+} channel current [63] and in the cat 45% [64], in bovine [69], rat [47], mouse [62], and human chromaffin cells [65], the N type fraction accounts for 30% of the whole-cell Ca^{2+} channel current.

P channels have proven difficult to characterize in chromaffin cells. Through the use of the sFTX [81], 1 μM ω-agatoxin IVA [55], or 100 nM ω-agatoxin IVA [82] as much as 40–55% of the

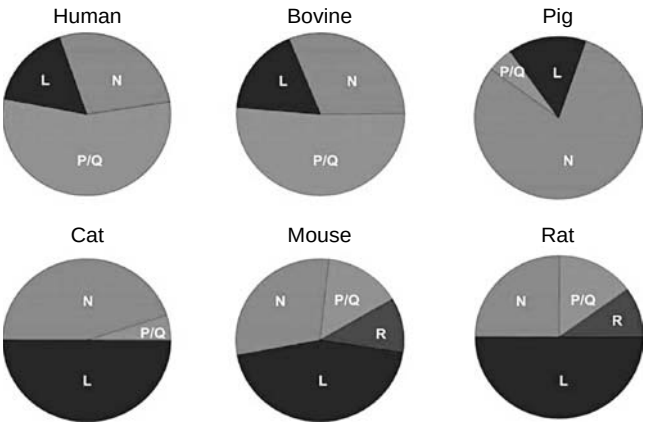


FIGURE 5.2 Species differences between the relative densities of HVA Ca^{2+} channels expressed by adrenal chromaffin cells.

whole-cell Ca^{2+} channel current was attributed to P channels. Later on we learned that concentrations of ω-agatoxin IVA higher than 10–20 nM in excess of P channels [72] also block Q-channels [80]. Thus, nanomolar concentrations of ω-agatoxin IVA known to block p channels fully and selectively [22] cause only 5–10% blockade of Ca^{2+} channel current in bovine chromaffin cells [79]. In cat chromaffin cells, combined ω-conotoxin GVIA plus nisoldipine blocked 90% of the current, leaving little room for P channels [64]. In rat [47] and mouse [62], the ω-agatoxin IVA-sensitive current fraction was only 10–15%. Thus, in all species studied, it seems that P channels are barely expressed if at all, in their chromaffin cells. This, together with the difficulty of separating the α_{1A} subunit into P and Q channels [83], suggests the convenience of speaking of P/Q channels rather than of two separate Ca^{2+} channel subtypes.

The P/Q channel component is pharmacologically isolated by 2 μM ω-conotoxin MVIIC or ω-conotoxin MVIID, or by 2 μM ω-agatoxin IVA. In bovine chromaffin cells, ω-conotoxin MVIID blocks the N current reversibly while ω-conotoxin MVIIC does so irreversibly [17]. Thus, the use of ω-conotoxin MVIID followed by its washout can be a convenient tool to isolate the P/Q channel. The blocking effects of ω-conotoxin MVIIC are extraordinarily slowed down and decreased in the presence of excessive concentrations (i.e., more than 2 mM) of Ba^{2+} [79,85] or Ca^{2+} [15]. Taking into consideration these methodological problems, we believe that the fraction of current carried out by P/Q channels in bovine chromaffin cells amounts to 50% [79]. This fraction is even higher (60%) in human chromaffin cells [65]. The opposite occurs in pig [63] and cat chromaffin cells [64] where P/Q channels carry only 5% of the current. Finally, in rat chromaffin cells, P/Q channels contribute 20% to the current [47] and in the mouse 30% [62]. More recent studies show that this component is about 15% in mouse chromaffin cells [92].

We do not know yet what the physiological relevance of these drastic species differences is. But it is sure that it has clear consequences for the fine control of the differential exocytotic release of epinephrine and norepinephrine in response to different stressors. Different autocrine/paracrine regulation by catecholamines and other coexocytosed vesicular components of L- and non-L-types of Ca^{2+} channels might be a reason. Other regulatory mechanisms, that is, voltage-dependent [70] or Ca^{2+}-dependent inactivation of Ca^{2+} channels [99] could also explain the preferential expression of one or another channel type in a given specie. Also, the selective segregation of a given channel type to exocytotic microdomains and the uneven geographic distribution of other channel types might also enable a given neurosecretory cell to express preferentially one or another channel type. The drastic difference of channel type expression provides different models of chromaffin cells to study the dominant role of a Ca^{2+} channel subtype in controlling exocytosis [100].

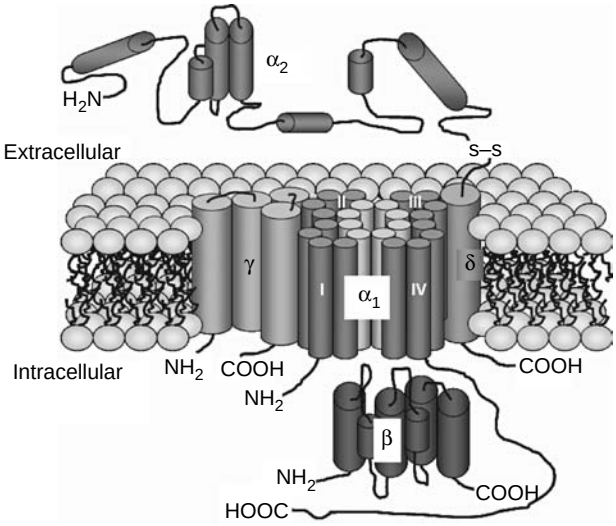


FIGURE 5.3 Subunit arrangement for a typical HVA Ca^{2+} channel.

5.6 MOLECULAR STRUCTURE OF Ca^{2+} CHANNELS

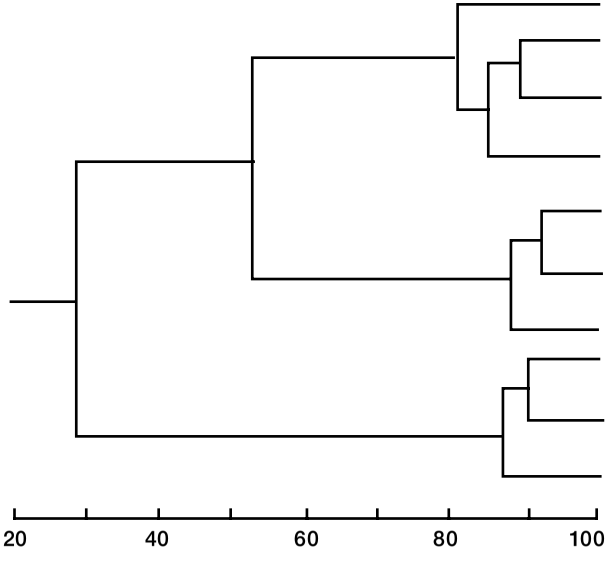
Voltage-gated Ca^{2+} channels are oligomeric complexes composed of up to five distinct proteins (α_1, β, $\alpha_2\delta$, and γ) encoded by four genes [101–103]. The α_1 protein incorporates the conduction pore, the voltage sensor and gating apparatus, as well as most of the known binding sites of channel regulation by second messengers, drugs, and toxins. Associated with the pore-forming α_1 subunit are the cytoplasmic β, the membrane anchored extracellular $\alpha_2\delta$, and the transmembrane γ-subunits, which drastically influence the properties and surface expression of these channels. Parallel to the channel protein complex, additional proteins, such as kinases, Ca^{2+}-binding proteins, and GTPases, can eventually interact with the main α_1 protein to modulate the complex activity and, in turn controlling Ca^{2+} influx. Figure 5.3 represents the hypothetical subunit arrangement for a typical HVA Ca^{2+} channel.

5.6.1 Diversity of Ca^{2+} Channel α_1 Subunits

Several individual voltage-gated Ca^{2+} channels have been identified and classified by their biophysical and pharmacological profiles as L-, N-, P-, Q-, R-, or T-types. This diversity arises predominantly from the nature of the principal pore-forming α_1 subunits, which are encoded by at least ten distinct genes (Table 5.3). Historically, various names have been given to the main pore-forming α_1 subunit of Ca^{2+} channels, giving rise to distinct and sometimes confusing nomenclatures. A unified, but arbitrary, nomenclature was adopted in 1994 [104]. Thus, α_1 subunits were referred to α_{1S} for the original skeletal muscle subunit and α_{1A} through α_{1I} for those discovered subsequently.

In 2000, a more rational nomenclature was adopted [105] in which Ca^{2+} channels were named using the chemical symbol of the principal permeation ion (Ca) with the principal physiological regulator (voltage) indicated as a subscript (Ca_V). The numerical identifier corresponds to the Ca_V channel α_1 subunit gene subfamily (1–3 at present) and the order of discovery of the α_1 subunit within that subfamily (1 through n). According to this nomenclature, there are three different families of Ca_V channel α_1 subunits (Table 5.3). The first Ca_V1 subfamily ($Ca_V1.1$–$Ca_V1.4$) includes HVA channels containing α_1 subunits that mediate L-type Ca^{2+} currents (α_{1S}, α_{1C}, α_{1D}, and α_{1F}). The second Ca_V2 subfamily ($Ca_V2.1$–$Ca_V2.3$) comprises HVA channels containing α_1 subunits that mediate P/Q-type (α_{1A}), N-type (α_{1B}), and R-type (α_{1E}) Ca^{2+} currents. Finally, the third Ca_V3 subfamily ($Ca_V3.1$–$Ca_V3.3$) includes LVA channels containing α_1 subunits (α_{1G}, α_{1H}, and α_{1I}); members of this subfamily mediate T-type Ca^{2+} currents. In contrast to the Ca_V3 channels, which express

TABLE 5.3
Diversity of Voltage-Activated Ca^{2+} Channel α_1 Subunit Genes



Subunit (Channel Name)	Channel Gene	Current Type	Localization
α_{1S} ($Ca_v1.1$)	CACNA1S	L	Skeletal muscle
α_{1C} ($Ca_v1.2$)	CACNA1C	L	Cardiac and smooth muscle myocytes; endocrine cells; neuronal cell bodies
α_{1D} ($Ca_v1.3$)	CACNA1D	L	Endocrine cells; neuronal cell bodies and dendrites; cochlear hair cells
α_{1F} ($Ca_v1.4$)	CACNA1F	L	Retinal rod and bipolar cells; spinal cord; adrenal gland; mast cells
α_{1A} ($Ca_v2.1$)	CACNA1A	P/Q	Nerve terminals and dendrites; neuroendocrine cells
α_{1B} ($Ca_v2.2$)	CACNA1B	N	Nerve terminals and dendrites; neuroendocrine cells
α_{1E} ($Ca_v2.3$)	CACNA1E	R	Neuronal cell bodies and dendrites
α_{1G} ($Ca_v3.1$)	CACNA1G	T	Neuronal cell bodies and dendrites; cardiac and smooth muscle myocytes
α_{1H} ($Ca_v3.2$)	CACNA1H	T	Neuronal cell bodies and dendrites; cardiac and smooth muscle myocytes
α_{1I} ($Ca_v3.3$)	CACNA1I	T	Neuronal cell bodies and dendrites

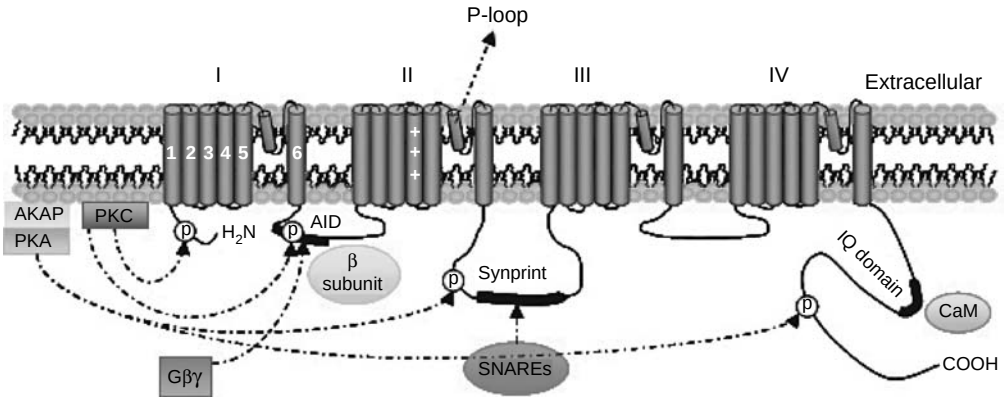


FIGURE 5.4 Membrane topology of α1 subunit of the Ca^{2+} channel illustrating major protein interaction sites.

by themselves as typical T-type Ca^{2+} channels in heterologous systems, HVA Ca_V channels function as oligomeric complexes containing auxiliary subunits (Figure 5.3).

Table 5.3 summarizes the sequence similarity among the diverse Ca_V α_1 subunits known to date as well as the name of the gene encoding for each subunit. The amino acid alignment was constructed using the CLUSTAL program. Only the membrane-spanning regions of α_1 sequences were included into the analysis. The table also shows the major sites of expression for each gene product. The diversity of α_1 genes found so far, together with the alternative splicing from each single gene, adds a large structural diversity to the multitude of Ca^{2+} channel α_1 gene subproducts.

The α_1 subunits are large proteins with molecular weight between 212 and 273 kDa. Each α_1 subunit of Ca_V channel is organized in four homologous repeats (I–IV) of the six transmembrane structures. Each repeat contains an S4 region that acts as the voltage sensor, a P-loop that forms the selective filter, and S6 segments that form the channel pore (Figure 5.4). The four domains are connected through cytoplasmic linkers, and both C- and N-termini are cytoplasmic. These regions contain sites of interaction with auxiliary subunits, binding sites for various activators and blockers, including G-proteins, as well as several putative phosphorylation sites.

5.6.2 Molecular Pharmacology of Ca_V Channels

The pharmacology of the three subfamilies of Ca_V channels is quite distinct. Channels of the Ca_V1 family (L-type) are the molecular targets of the organic agents including DHPs, phenylalkylamines (PAAs), and BTZs. Different techniques, including photoaffinity labelling and mutation analysis have been used to localize potential binding sites of these drugs on the Ca^{2+} channel complex. Results reveal that they act at three separate, but allosterically coupled, receptor sites [103,106,107]. Thus, PAAs (i.e., verapamil) are intracellular pore blockers, which are thought to enter the pore from the cytoplasmic side of the channel and block it. Their receptor site is formed by amino acid residues in the S6 segments in domain III and IV [108–110].

On the other hand, DHPs can be activators (i.e., Bay K 8644) or inhibitors (i.e., nifedipine or nitrendipine) and, therefore, are thought to act allosterically to shift the channel toward the open or closed state, rather than by occluding the ion-conducting pore. Their receptor sites consist of amino acids located in the S6 segments of domains III and IV and the S5 segment of domain III [108,110,111]. Interestingly, the DHP receptor site shares some common amino acids with the PAA receptor site. Finally, BTZs (diltiazem and related compounds) bind to a third receptor site, but the amino acids that are required for their interaction overlap also those required for PAA binding [112,113].

Members of the Ca_V2 family of channels are relatively insensitive to DHPs but are specifically blocked by peptide toxins from spiders and marine snails [106,107]. P- and Q-type ($Ca_V2.1$) channels are blocked with high affinity by ω-agatoxin IVA from the funnel web spider venom and by ω-conotoxin MVIIC from the marine snail *C. geographus*, respectively [16,22]. $Ca_V2.2$. channels are blocked selectively by ω-conotoxin GVIA [114,115]; the receptor site for the toxin comprises amino acid residues in the extracellular loop between segments S5 and S6 of domain III, consistent with a direct pore-blocking mechanism. $Ca_V2.3$ channels are blocked by the synthetic peptide toxin SNX-482 derived from tarantula venom; the presence of domains III and IV are necessary for toxin-mediated inhibition [86,116].

Finally, the Ca_V3 family of channels is insensitive to the above-mentioned blockers. Although there are no pharmacological agents that specifically target T-type Ca^{2+} channels, some clinically useful drugs are able to block this channel subtype [117]. These Ca_V3 channel antagonists include antihypertensives such as mibefradil and amiloride, antiepileptics (i.e., ethosuximide), and antipsychotics (i.e., pimozide). In addition, Ni^{2+} is somewhat specific for T-type versus other classes of Ca^{2+} currents. Interestingly, a new scorpion toxin (kurtoxin) that binds to the α_{1G} T-type Ca^{2+} channel with high affinity was identified; it inhibits the channel by modifying voltage-dependent gating [38].

5.6.3 Interaction of Ancillary Subunits with α_1 Subunits

The α_1 subunits of the Ca_V1 and Ca_V2 channels associate with ancillary β subunit (encoded by four different genes), α_2/δ subunits (four genes known), and possibly γ subunits (ten genes known). Although α_1 subunit has been considered as the central actor in these Ca_V channel complexes, auxiliary subunits aid membrane expression and alter the biophysical properties of the α_1 subunit [102,109,118,119]. In contrast, the subunit composition of the Ca_V3 channels remains an open controversial issue [120].

The β subunit of all HVA Ca_V channels is an intracellular auxiliary subunit that binds to a conserved alpha-interaction domain (AID) of the α_1 subunit to modulate channel gating properties and promote cell surface trafficking (Figure 5.4). This interaction site of both subunits was identified on the connector between I and II domains of the α_1 subunit [121,122]. Interestingly, it has been recently demonstrated that Gem, a small guanosine triphosphatase (GTPases) of the Rem-Gem-Kir (RGK) family, binds directly to the β subunit; this interaction inhibits the association of the β with the α_1 subunit, decreasing channel abundance by inhibiting transport to the plasma membrane [123].

Although the above data suggest that the only function of β subunit is to modulate the expression, targeting, gating, and activity of the main α_1 subunit, recent experimental evidence indicates that this function could represent a "part-time" job for some isoforms of the β subunit. In fact, the identification of a Src homology type (SH3) and guanylate kinase (GK) domains in the structure of the β subunit indicates that this subunit belongs to the membrane-associated guanylate kinase (MAGUK) family, thereby suggesting a role for the β subunit in scaffolding multiple signaling pathways around the channel [122]. Moreover, a recent study of Berggren et al. [124] reveals that β_3 subunits directly reduce glucose-induced Ca^{2+} oscillations in pancreatic β cells. Although far from being clearly demonstrated, two signaling pathways are proposed in this β_3 subunit-mediated effect: direct regulation of the inositol trisphosphate (IP_3) receptor, and indirect reduction of phospholipase Cβ. Thus, the β subunit now claims the status of independent regulatory protein.

The α_2/δ subunit is translated as a single protein but cleaved into δ (a single transmembrane-spanning helix) and α_2 (the extracellular domain) subunits, which are linked by a disulfide bond; however, its interaction site on the α_1 subunit is unknown. The γ subunit, characterized by four predicted transmembrane domains, was formerly found in skeletal muscle and later in heart and brain Ca^{2+} channels [119]. One member of the γ subunit (γ_6) has been shown reducing the activity of a subtype of T-type Ca^{2+} channel in cardiomyocytes [125]. Further, functional studies have

suggested a dual role for another member of this family of proteins (γ_2, also known as stargazing), both as a modulatory γ subunit for Ca^{2+} channels and as a regulator of postsynaptic membrane targeting for alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type glutamate receptors [126,127].

5.6.4 Modulation of Ca_V Channels by Protein Kinase-Dependent Phosphorylation

Families of Ca_V1 and Ca_V2 channels are substrates for phosphorylation by cAMP-dependent protein kinase A (PKA, Figure 5.4). Single channel recordings have suggested that phosphorylation by PKA is necessary for the channels to become active, and once these channels are active, phosphorylation can increase their open probability. Ser1928 located in the C-terminal region of the cardiac α_1 subunit of the $Ca_V1.2$ channel is the only detectable phosphorylation site for this kinase, while another phosphorylation site in the intracellular loop connecting domains II and III has also been found for the $Ca_V1.1$ α_1 subunit. Interestingly, it has been proposed that PKA may be in close proximity to the Ca_V channel thanks to the A-kinase anchor protein (AKAP), an adapter protein that directs PKA to a variety of substrates and intracellular locations (see review by Felix [107]).

Protein kinase C (PKC) can also modulate Ca_V channels; moreover, this regulation is believed to be of substantial physiological importance since it mediates the effects of several hormones and intracellular messengers. It has been shown that this activated-PKC pathway mediates the regulation of L-type Ca^{2+} currents by α-adrenergic agonists, adenosine triphosphate (ATP), and glucocorticoids among others [107]. Residues in the N-terminal and in the intracellular loop connecting domains I and II seem to be necessary for the modulation by PKC of some members of the Ca_V1 and Ca_V2 channel families. Moreover, PKC can reverse G protein inhibition of these channels by phosphorylating the intracellular loop connecting I and II domains (Figure 5.4). This characteristic cross-talk between G protein and PKC is thought to allow the α_1 subunit to integrate multiple modulatory inputs [128].

5.6.5 Molecular Determinants of the G-Protein-Dependent Inhibition of Ca_V Channels

Neurotransmitters and hormones can regulate directly or indirectly (via second messengers and/or protein kinases) Ca_V channels. The former possibility is exerted by a physical interaction between G-protein subunits and the α_1 subunit of the channel complex. Numerous functional studies have firmly established that G-protein activation by α-adrenergic and μ-opioid receptor agonists reversibly inhibits neuronal non-L-type (Ca_V2) channels. This effect is mediated by the $G\beta\gamma$ dimer, whereas the role of the $G\alpha$ subunit on the channel regulation remains poorly understood. Moreover, experimental evidence indicates that the $G\beta\gamma$ complex directly interacts with a site in the linker region connecting domains I–II of the Ca_V channel subunit (Figure 5.4). Interestingly, this binding site partially overlaps the AID site where the Ca_V channel β subunit binds. Because the β subunit increases Ca_V channel activity drastically [129, 130], this overlap suggests a mechanism for the antagonism between the Ca_V channel β subunit and the $G\beta\gamma$ complex [131]. Moreover, it has been shown recently that G protein might also inhibit LVA channels, in particular, $Ca_V3.2$ channels, by a different mechanism from the established for Ca_V2 channels through the interaction of the $G\beta\gamma$ dimer with the intracellular loop connecting domains II and III [132].

5.6.6 Regulation of Ca_V Channel by Ca^{2+}/Calmodulin

High $[Ca^{2+}]_c$ in the vicinity of a Ca_V channel affects the gating, limiting its ability to reopen after a period of activity. This inactivation process of the channel is controlled by the association of Ca^{2+}

with calmodulin (CaM), a ubiquitously expressed Ca^{2+}-binding protein containing four E–F hands (Ca^{2+}-binding sites), which constitutes the Ca^{2+} receptor tethered to the channel (Figure 5.4). In fact, there is experimental evidence indicating that Ca^{2+}/CaM complex regulates $Ca_V1.2$ and $Ca_V2.1$ channels due to CaM interaction with an amino acid sequence, called the IQ motif, located in the C-terminal of the α_1 subunit [133, 134].

In contrast, little is known about the regulation of the Ca_v3 family of channels. However, in the case of Ca^{2+}/CaM regulation it seems to depend on the activity of the Ca^{2+}/CaM-dependent protein kinase II. Thus, activation of this kinase in cells expressing recombinant $Ca_v3.2$ channels increases current amplitude at negative test potentials as the result of Ser1198 phosphorylation within the linker connecting domains II and III in the α_1 subunit [135,136].

5.6.7 Functional Interactions of Ca_V Channels and SNARE Proteins

The brief rise in $[Ca^{2+}]_c$ to the level required for exocytosis likely occurs only in the vicinity of Ca_v channels, because $[Ca^{2+}]_c$ falls off steeply as a function of distance. Therefore, there must be a physical link between the channel and the release mechanism. Moreover, several specialized proteins that mediate exocytosis have been identified [107, 137, 138]. Thus, three membrane proteins, syntaxin, synaptosome-associated protein of 25 kDa (SNAP-25), and synaptobrevin (VAMP) have shown to assemble into a stable ternary complex and participate actively in the exocytosis process. In addition, synaptotagmin, a vesicle protein that is thought to serve as a Ca^{2+} sensor for exocytosis, binds Ca^{2+} and interacts with syntaxin in a Ca^{2+}-dependent manner. A detailed analysis of exocytosis using recombinant proteins has shown a specific binding site for syntaxin 1A and SNAP-25 in a region (called synprint) of the II–III linker of $Ca_v2.1$ and $Ca_v2.2$ channels (Figure 5.4).

5.7 CALCIUM CHANNELS AND NEUROSECRETION

It has been long demonstrated that Ca^{2+} is essential for neurotransmitter release. The existence of multiple types of Ca^{2+} channels and the fact that several of them can coexist in the same cell type has raised questions about which channel (or channels) contribute to the control of the delivery of the Ca^{2+} necessary to trigger a secretory signal in a particular synapse. We will therefore review throughout this section how the different Ca^{2+} channel subtypes (defined by ω-conotoxin blockade of neurosecretion) control the release of neurotransmitters depending on the synapse, the neurotransmitter, and the animal species. Tables 5.4 through 5.7 summarize how Ca^{2+} entry through different Ca^{2+} channel subtypes control neurotransmitter release at different sites of the central and peripheral nervous system, motor nerve terminals, and chromaffin cells.

5.7.1 Brain Synaptosomes

Transmitter release from brain synaptosomes is controlled by different Ca^{2+} channels and is greatly dependent on the animal species studied. In chick brain synaptosomes, inositol phosphate production together with norepinephrine release is highly sensitive to ω-conotoxin GVIA [139]. Ca^{2+} transients measured in chick brain synaptosomes loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 demonstrated that increases in the $[Ca^{2+}]_c$ induced by high K^+ was almost completely suppressed by ω-conotoxin GVIA [93]. On the other hand, in rat brain synaptosomes the production of inositol phosphate and secretion of norepinephrine are insensitive to ω-conotoxin GVIA, but sensitive to ω-agatoxin IVA [140]. Glutamate release from rat brain synaptosomes is blocked 56% by ω-agatoxin IVA and 23% by ω-agatoxin IIIA, an L-N-P-type Ca^{2+} channel blocker [28]. These results indicate that in chick brain synaptosomes Ca^{2+} entry and therefore, neurotransmitter release is predominately controlled via an N-type Ca^{2+} channel. In rat brain synaptosomes L- and

TABLE 5.4
Control of Neurotransmitter Release by N-type Ca^{2+} Channels

Neurotransmitter	Preparation	ω-Conotoxin GVIA [μM]	Inhibition %	Reference
Acetylcholine	Electric fish (*Torpedo marmorata*)	5	30	[95]
	Electric fish (*Gymnotus carapo*)	2.5	>95	[96]
	Myenteric plexus (Guinea pig)	0.01	92	[154]
	Myenteric plexus (rat)	0.1	70	[98]
	Detrusor (Guinea pig)	0.1	88	[157]
	Urinary bladder (Guinea pig)	1	71	[155]
	Urinary bladder (rat)	1	25	[155]
	Urinary bladder (rat)	0.3	54	[156]
	Atria (Guinea pig)	IC_{50} = 0.42 μM		[161]
	Phrenic nerve (rat)	0.1	47	[98]
	Brain slices (rat)	1	60	[232]
ATP	Vas deferens (rat)	IC_{50} = 0.20 nM		[160]
Epinephrine	Urethra (rabbit)	0.1	77	[157]
	Chromaffin cells (dog)	0.4 μg/min	33	[233]
Catecholamines	Chromaffin cells (bovine)	5	17	[82]
	Chromaffin cells (bovine)	1	10	[69]
	Chromaffin cells (bovine)	3	30	[175]
	Chromaffin cells (cat)	1	20	[164]
	Chromaffin cells (rat)	1	42	[177]
Dopamine	Striatum (rabbit)	0.005	39	[143]
	Striatum (rat)	1	38	[142]
	Brain slices (rat)	1	30	[232]
Dynorphin	Dentate gyrus dendrite (Guinea pig)	1	79	[146]
	Dentate gyrus axon (Guinea pig)	1	50	[146]
EPSCs	Retinal ganglion neurons (rat)	5	67	[151]
EPSP	Hippocampal synaptic transmission (rat)	1	46	[80]
GABA	Deep cerebellar neurons (rat)	0.1	50	[145]
5-HT	Brain slices (rat)	1	30	[232]
Glutamate	Hippocampal CA1 pyramidal cells (rat)	3	80	[145]
	Hippocampal synaptosomes (rat)	1	16	[144]
Glycine	Dorsal horn neurons of the spinal cord (rat)	3	50	[145]
Norepinephrine	Brain synaptosomes (rat)	1	>90	[139]
	Neocortex (rabbit)	0.005	46	[93]
	Sympathetic neurons (rat)	0.1	92	[149]
	Vas deferens (rat)	1	100	[155]
	Anococcygeus (Guinea pig)	0.01	98	[155]
	Vas deferens (Guinea pig)	0.01	97	[154]
	Atria (Guinea pig)	IC_{50} = 0.200 μM		[15]
	Atria (Guinea pig)	0.1	80	[234]
	Atria (Guinea pig)	10	49	[235]
	Chromaffin cells (dog)	0.4 μg/min	32	[233]
	Mesenteric artery (rat)	0.01	92	[158]
	Right atria (mouse)	0.1	100	[158]
	Right atria (rat)	0.1	100	[158]
NANC	Detrusor (rabbit)	0.1	85	[157]
	Anococcygeus (Guinea pig)	0.05	64	[155]
	Urinary bladder (Guinea pig)	0.1	58	[155]
	Urethra (rabbit)	0.1	47	[157]
	Jejunum (Guinea pig)	0.1	33	[154]
	Taenia caecum (Guinea pig)	0.05	20	[154]

TABLE 5.4
(Continued)

Neurotransmitter	Preparation	ω-Conotoxin GVIA [μM]	Inhibition %	Reference
Oxytocin	Neurohyhophysial terminals (bovine)	0.8	32	[147]
Vasopressin	Neurohyhophysial terminals (bovine)	0.8	32	[147]

EPSCs, excitatory postsynaptic currents; EPSP, excitatory postsynaptic potentials; NANC, nonadrenergic-noncholinergic neurotransmission.

TABLE 5.5
Control of Neurotransmitter Release by P-type Ca^{2+} Channels (Blockade with Low Concentrations of ω-Agatoxin IVA)

Neurotransmitter	Preparation	ω-Agatoxin IVA [μM]	Inhibition %	Reference
Acetylcholine	Phrenic nerve (Guinea pig)	0.02	>95	[235]
	Phrenic nerve hemidiaphragm (mouse)	0.1	92	[158]
	Phrenic nerve hemidiaphragm (rat)	0.1	0	[158]
Catecholamines	Chromaffin cells (bovine)	0.1	35	[82]
GABA	Deep cerebellar neurons (rat)	0.2	98	[145]
Glutamate	Brain synaptosomes (rat)	0.2	56	[28]
	Cortex synaptosomes (rat)	IC_{50} = 12.2 nM		[141]
	Hippocampal synaptosomes (rat)	0.2	40	[144]
	Hippocampal CA1 pyramidal cells (rat)	0.2	25	[145]
Glycine	Dorsal horn neurons of the spinal cord (rat)	0.2	98	[145]

GABA, gamma-aminobutyric acid.

TABLE 5.6
Control of Neurotransmitter Release by Q-type Ca^{2+} Channels

Neurotransmitter	Preparation	ω-Conotoxin MVIIC [μM]	Inhibition %	Reference
Acetylcholine	Urinary bladder (rat)	3	54	[156]
	Atria (Guinea pig)	IC_{50} = 0.28 μM		[161]
	Phrenic nerve hemidiaphragm (mouse)	1	80	[158]
	Phrenic nerve hemidiaphragm (rat)	1	57	[158]
	Chromaffin cells (bovine)	IC_{50} = 218 nM		[174]
ATP	Vas deferens (rat)	IC_{50} = 200 nM		[160]
Catecholamines	Chromaffin cells (bovine)	3	50	[69]
Glutamate	Cortex synaptosomes (rat)	IC_{50} = 35 nM		[141]
EPSPs	Hippocampal synaptic transmission (rat)	5	100	[80]
Norepinephrine	Atria(Guinea pig)	0.5	100	[235]
	Atria(Guinea pig)	IC_{50} = 0.19 μM		[15]
	Atria (mouse)	1	100	[158]
	Atria (rat)	1	100	[158]
	Chromaffin cells (bovine)	IC_{50} = 182 nM		[174]
Vasopressin	Neurohyhophysial terminals (bovine)	0.3	25	[147]

ATP, adenosine triphosphate; EPSPs, excitatory postsynaptic potentials.

TABLE 5.7
Control of Neurotransmitter Release by P/Q-type Ca^{2+} Channels (Blockade with High Concentrations of ω-Agatoxin IVA)

Neurotransmitter	Preparation	ω-Agatoxin IVA [μM]	Inhibition %	Reference
Acetylcholine	Urinary bladder (rat)	3	46	[156]
	Atria (Guinea pig)	3	37	[161]
	Brain slices (rat)	1	50	[232]
GABA	Brain slices (rat)	1	100	[232]
Glutamate	Brain slices (rat)	1	100	[232]
Dopamine	Brain slices (rat)	1	70	[232]
5-HT	Brain slices (rat)	1	50	[232]
Norepinephrine	Atria (Guinea pig)	3	21	[161]

N-type Ca^{2+} channel blockers do not modify $[Ca^{2+}]_c$ levels or transmitter release; therefore, another Ca^{2+} entry pathway seems to be involved in the control of neurotransmitter release.

Turner and Dunlap [141] measured [^{3}H]-glutamate release from rat cortical synaptosomes as an assay for presynaptic Ca^{2+} channel activity. In this system, they observed that the efficacies of ω-agatoxin IVA and ω-conotoxin GVIA and MVIIC were increased when Ca^{2+} influx was decreased by decreasing the KCl concentration to diminish the extent of depolarization, by decreasing the external concentration of Ca^{2+} or by partially blocking Ca^{2+} influx with one of the other toxins. Using these ω-toxins, they found at least three types of pharmacologically distinct Ca^{2+} channels that participate in exocytosis. The largest fraction of glutamate release was blocked by ω-agatoxin IVA with an IC_{50} of 12.2 nM and ω-conotoxin MVIIC with an IC_{50} of 35 nM, consistent with the pharmacology of a P-type Ca^{2+} channel. The N-type Ca^{2+} channel blocker, ω-conotoxin GVIA, inhibited a significant portion of the release (IC_{50} < 1 nM) but only under conditions of reduced Ca^{2+} concentrations. These results suggest that the N-type channel in nerve terminals is different from that found in hippocampal somata, since it appears to be resistant to ω-conotoxin MVIIC. The combination of ω-conotoxin GVIA (100 nM) and either ω-agatoxin IVA or ω-conotoxin MVIIC (1 μM) blocked approximately 90% of release when the Ca^{2+} concentration was reduced (0.46 mM or less), but 30%–40% of release remained when the concentration of Ca^{2+} in the stimulus buffer was 1 mM or greater, indicating that a resistant channel also participates in exocytosis.

5.7.2 Striatum

In the striatum, neurotransmitter release is controlled by different Ca^{2+} channels. Dopamine release induced by K^+ is blocked around 30% by ω-conotoxin GVIA [140,142] although dopamine release evoked by electrical stimulation is almost completely inhibited by ω-conotoxin GVIA [142]. Turner and coworkers, using subsecond measurements of glutamate and dopamine release from rat striatal synaptosomes, showed that P-type Ca^{2+} channels, which are sensitive to ω-agatoxin IVA, trigger the release of both neurotransmitters although dopamine (but not glutamate) was also partially blocked by ω-conotoxin GVIA-sensitive Ca^{2+} channels. Another interesting observation by these authors is that the blockade of neurotransmitter release is voltage-dependent. With strong depolarizations (60 mM K^+), neither ω-agatoxin IVA nor ω-conotoxin GVIA were effective alone, although a combination of both produced a synergistic inhibition of 60–80% of Ca^{2+} dependent dopamine release. With milder depolarizations (30 mM K^+), ω-agatoxin IVA (200 nM) blocked over 80% dopamine and glutamate release, while ω-conotoxin GVIA (1 μM) blocked dopamine release by 25% and left

glutamate release unaffected. The results suggest that multiple Ca^{2+} channel subtypes coexist to regulate neurosecretion under normal physiological conditions in the majority of nerve terminals, while P-type and ω-conotoxin GVIA- and ω-agatoxin IVA-resistant channels coexist in glutamatergic terminals. Such an arrangement could lend a high degree of flexibility in the regulation of transmitter release under diverse conditions of stimulation and modulation.

5.7.3 Hippocampus

In the hippocampi of rabbits, Dooley at al. [143] demonstrated that electrically-induced release of dopamine, 5-hydroxytryptamine, and acetylcholine was similarly blocked (around 40%) by nanomolar concentrations of ω-conotoxin GVIA. Under the same experimental conditions, dopamine release from the corpus striatum and norepinephrine release from the neocortex was also blocked by 40% by ω-conotoxin GVIA (5 nM). Using a superfusion system with subsecond temporal resolution, Luebke et al. [144] studied the effects of ω-conotoxin GVIA and ω-agatoxin IVA on glutamate release from rat hippocampal synaptosomes. K^+-induced release of glutamate was inhibited by 16% by ω-conotoxin GVIA and by 40% by ω-agatoxin IVA; such blockade was increased when lower concentrations of K^+ were employed to induce secretion. The amplitude of excitatory postsynaptic potentials (EPSPs) in CA1 pyramidal neurons was reduced by ω-conotoxin GVIA and ω-agatoxin IVA, although ω-agatoxin IVA was more rapid and more efficacious [144]. Thus, at least two Ca^{2+} channels seem to control glutamate release from hippocampal neurons, but P-type channels seem to play a major role.

Synaptic transmission between hippocampal CA3 and CA1 neurons is mediated by N-type Ca^{2+} channels together with Ca^{2+} channels whose pharmacology differs from L- and P-type channels but resembles that of Q-type Ca^{2+} channels encoded by the α_{1A} subunit gene. Using rat hippocampal slices, Wheeler et al. [80] showed that ω-conotoxin GVIA blocked EPSP by 46%, while P- and L-type Ca^{2+} channel antagonists had no effect. In contrast, ω-conotoxin MVIIC (N–P–Q Ca^{2+} channel blocker) inhibited 100% of the EPSP. This suggests that hippocampal synaptic transmitter release is regulated by N- and Q-subtype of Ca^{2+} channels. Measuring excitatory postsynaptic currents (EPSCs) from hippocampal CA1 pyramidal neurons, Takahashi and Momiyama [145] demonstrated that synaptic transmission at this level is predominantly controlled by N-type Ca^{2+} channels (80% block of EPSPs by ω-conotoxin GVIA) and to a lesser extent by P-type Ca^{2+} channels (25% inhibition by ω-agatoxin IVA).

The release of the neuropeptide dynorphin is controlled by different Ca^{2+} channels, depending on the release site (dendrite or axon). L-type Ca^{2+} channels mediate dynorphin release from dendrites and N-type Ca^{2+} channels mediate dynorphin release from the axons of hippocampal granule cells [146].

5.7.4 Cerebellum

Inhibitory postsynaptic currents (IPSCs) evoked in neurons of the deep cerebellar nuclei by stimulating presumptive Purkinje cell axons were reversibly abolished by bicuculline, indicating that the responses were mediated by GABA. The application of ω-agatoxin IVA (200 nM) blocked IPSCs amplitude by 50%, while the L-type Ca^{2+} channel blocker nicardipine had no effect [145], indicating that GABA release from Purkinje cell axons is mediated via Ca^{2+} entry through P-type Ca^{2+} channels.

5.7.5 Neurohypophysis

Neurohypophysial terminals exhibit, besides L- and N-type currents, another component of the Ca^{2+} current that is blocked by low concentrations of ω-conotoxin MVIIC or by high concentrations of ω-agatoxin IVA indicating the presence of a Q channel. In the study performed by Wang and coworkers

[147], they demonstrate that secretion of vasopressin is controlled by N, L, and Q channels while that of oxytocin is regulated mainly by N and L channels with no participation of Q channels.

5.7.6 Sympathetic Neurons

In rat sympathetic neurons, whole cell recordings have provided evidence for two subtypes of Ca^{2+} channels, the N and the L-type [46], although norepinephrine release is predominantly blocked by ω-conotoxin GVIA [148,149]. In contrast to sympathetic neurons, release of substance P from peripheral sensory neurons is highly dependent on Ca^{2+} entry through L-type Ca^{2+} channels. In the sympathetic nerve endings of the iris, ω-conotoxin GVIA (1 μM) blocked over 80% of norepinephrine synthesis induced by high K^+ while nicardipine had no effect, indicating that Ca^{2+} entry through N-type Ca^{2+} channels play a major role in norepinephrine synthesis [150].

5.7.7 Retinal Ganglion Neurons

Glutamatergic synaptic responses in rat retinal ganglion neurons are partially sensitive to ω-conotoxin GVIA (30% block) and insensitive to ω-agatoxin IVA [151]. These results indicate that the major part of synaptic glutamate release in retinal ganglion neurons is governed by a novel toxin-resistant Ca^{2+} channel that could possibly be of the Q or R type.

5.7.8 Spinal Cord and Dorsal Root Ganglion Neurons

In cocultures of fetal neurons from ventral half of the spinal cord (VH neurons) and from the dorsal root ganglion (DRG neurons) the synaptic transmission between pairs of spinal cord neurons from ventral half of the spinal cord (VH–VH connections) or between DRG neurons and VH neurons (DGR and VH connections) were studied with two cell recording and stimulation techniques. In 70% of the VH–VH connections and in 50% of the DGR-VH connections, Bay K 8644 failed to affect transmitter release. ω-Conotoxin GVIA produced no consistent effect on EPSPs or IPSPs elicited by VH neurons by stimulation of the nearby neurons. VH EPSPs elicited by stimulation of the nearby DGR neurons were reduced by 50% by ω-conotoxin GVIA. Therefore, neither sustained nor inactivating HVA Ca^{2+} channels sensitive to Bay K 8644 or ω-conotoxin GVIA such as those measured in the neuronal cell body are responsible for action-potential-evoked transmitter release from the majority of the VH neurons; these channels may be involved in transmitter release in approximately 30% of these neurons [152].

In rat dorsal horn neurons of the spinal cord, release of glycine induces IPSCs. The IPSCs were almost completely blocked by ω-conotoxin GVIA (more than 95%) and partially inhibited by ω-agatoxin IVA (50%), while nicardipine had no effect.

5.7.9 Intestinal Tract

Electrically evoked release of acetylcholine is predominantly controlled through N-type Ca^{2+} channels at the myenteric plexus [98,153,154]. ω-Conotoxin GVIA markedly reduced (70%) the evoked release of [^{3}H]-acetylcholine from the myenteric plexus of the small intestine, with an IC_{50} of 0.7 nM; the potency was similar at 3 and 10 Hz stimulation. An increase in the extracellular Ca^{2+} concentration attenuated the inhibitory effect of ω-conotoxin GVIA [98]. No species difference was observed as to the channel controlling Ca^{2+} entry for transmitter release.

In the guinea-pig jejunum, ω-conotoxin GVIA blocked only partially (33%) the inhibitory NANC transmission upon electrical stimulation. This was also the case at the *taenia caecum* (20% inhibition) [154]. In the proximal duodenum the NANC transmission was insensitive to ω-conotoxin GVIA [155].

Therefore, cholinergic transmission at this level seems to be regulated by Ca^{2+} entering through N-type Ca^{2+} channels while NANC transmission is regulated by another Ca^{2+} entry pathway besides N channels.

5.7.10 Lower Urinary Tract

In the rat- or guinea pig-isolated bladder, ω-conotoxin GVIA produced a concentration- and time-dependent inhibition of twitch responses to field stimulation without affecting the response to exogenous acetylcholine. In the rat bladder, the maximal effect did not exceed 25% inhibition while a much larger fraction of the response (70%) was inhibited in the guinea pig bladder. In the rat bladder, the effects of ω-conotoxin GVIA were frequency dependent; maximal effects of ω-conotoxin GVIA were observed at 2–5 Hz. Frew and Lundy [156] have demonstrated that neurotransmission in the rat urinary bladder is supported by both N- and Q-type Ca^{2+} channels. In their experiments, the resistant portion (non-N non-P) was sensitive to ω-conotoxin MVIIC, which, in addition to N and P also blocks Q channels. Further experiments carried out by Waterman [181] in mouse bladder using Ca^{2+} channel toxins demonstrates that acetylcholine release in these parasympathetic neurons depends primarily on N-type channels, and to a lesser extent on P- and Q-type channels, whereas ATP release involves predominantly P- and Q-type channels.

In the rabbit urethra and detrusor, Zygmunt et al. [157] have studied the effects of ω-conotoxin GVIA on adrenergic, cholinergic and NANC responses induced by electrical stimulation. The adrenergic contraction (25 Hz) and NANC relaxation (10 Hz) in the urethra and the cholinergic and NANC contractions (10 Hz) in the detrusor were inhibited in a concentration-dependent manner by ω-conotoxin GVIA. The adrenergic contraction of the urethra was ten times and the cholinergic contraction in the detrusor was three times more sensitive to ω-conotoxin GVIA than the NANC responses. These results suggest that NANC transmission is less sensitive to ω-conotoxin GVIA than transmission mediated by adrenergic and cholinergic nerves in the rabbit lower urinary tract.

5.7.11 Vas Deferens

In rat- or guinea pig-isolated vas deferens ω-conotoxin GVIA (1 nM–1 μM) produced concentration- and time-dependent inhibition of the response to electrical field stimulation, while the response to K^+, norepinephrine, or ATP was unaffected. A concentration as low as 1 nM produced almost complete inhibition of twitches, but this effect took about 1 h to be completed. With higher concentrations the time course of the inhibition was much faster [155]. In a study performed by Wright and Angus [158] in rat and mouse vas deferens, they observe that ω-conotoxin GVIA (10 nM) and ω-conotoxin MVIIC (1 μM) block completely the twitch responses when they are induced at low frequencies (0.05 Hz); but when higher frequencies are used (20 Hz) there is a ω-conotoxin GVIA resistant component that can be blocked by 1 μM ω-agatoxin IVA or ω-conotoxin MVIIC. These results indicate that sympathetic transmission in the vas deferens is mainly controlled by Ca^{2+} entering N channels, although when high frequency stimulation is employed (20 Hz) P–Q-type channels are also implicated [158].

As to the purinergic transmission in the vas deferens, Hata et al. [159] showed that the ATP-mediated component of the biphasic contraction was found to be more susceptible to ω-conotoxin GVIA than the adrenergic component. In a study performed 5 years later by Hirata and coworkers [160], electrically induced twitch responses of the prostatic segment of the rat vas deferens, which depends mainly on ATP release, was fully blocked by nanomolar concentrations of ω-conotoxin GVIA, MVIIA, and MVIIC, most likely by inhibiting Ca^{2+} entry through presynaptic N-type Ca^{2+} channels that control ATP release. The main conclusion we can draw from these studies is that sympathetic and purinergic transmission in the vas deferens is predominantly controlled by N-type Ca^{2+} channels.

5.7.12 Heart

The innervation in mammalian atria is both sympathetic and parasympathetic, which regulates the heart rate and the contractile strength. The subtypes of Ca^{2+} channels involved in neurotransmitter release have been studied by various investigators. Vega et al. [15] have shown that electrically

stimulated guinea pig left atria are sensitive to N-type Ca^{2+} channel blockers. Thus, ω-conotoxin GVIA and ω-conotoxin MVIIA blocked the inotropic response in a concentration-dependent manner with IC_{50} values of 0.20 μM and 0.044 μM respectively. The N–P–Q-channel blocker, ω-conotoxin MVIIC showed an IC_{50} of 0.19 μM; ω-agatoxin IVA had no effect. These results have been confirmed later on by Wright and Angus [158] in the right atria of mouse and rat, where they see full inhibition of contraction with 100 nM ω-conotoxin GVIA.

Hong and Chang [161] have studied the Ca^{2+} channel subtypes mediating the cholinergic and adrenergic neurotransmission in the guinea pig atria. In left atria paced at 2–4 Hz, the negative inotropic effect induced by electrical field stimulation on parasympathetic nerves (in the presence of propranolol) was abolished by ω-conotoxin MVIIC. On the other hand, the inotropic response resulting from electrical field stimulation of the sympathetic nerves (in the presence of atropine) was abolished by ω-conotoxin GVIA and ω-conotoxin MVIIC. None of the peptide toxins affected the chronotropic and the inotropic responses evoked by carbachol, isoprenaline, or norepinephrine [15,161].

These results suggest that under physiological conditions, the release of acetylcholine from parasympathetic nerves to the heart is dominated by a P/Q subfamily of Ca^{2+} channels, while that of norepinephrine from sympathetic nerves is controlled by an N-type Ca^{2+} channel.

5.7.13 Motor Nerve Terminals

Neurotransmitter release at this level is controlled by different Ca^{2+} channels depending on the species. The electroplax of marine electric fish is highly rich in motor nerve endings; this is the reason why it has been so widely used as a model to study transmitter release from motor nerve endings. ω-Conotoxin GVIA blocks the release of acetylcholine and Ca^{2+} uptake induced by depolarization in electric organ nerve terminals of the ray; the IC_{50} values were 3 μM for blocking transmitter release and 2 μM for blocking Ca^{2+} entry [94]. Sierra et al. [96] have also shown that N-type Ca^{2+} channels mediate transmitter release at the electromotoneuron-electrocyte synapses of the weakly electric fish *Gymnotus carapo;* ω-conotoxin GVIA (2.5 μM) blocked over 95% of the end plate potential (EPP) while ω-agatoxin IVA and nifedipine had no effect. In contrast to these data, in *torpedo* synaptosomes, Fariñas et al. [95] showed that ω-conotoxin GVIA (10^{-8} to 5×10^{-5} M) showed a differential effect on acetylcholine and ATP release: nucleotide release was inhibited by 90% at the highest concentration tested while acetylcholine release was only moderately decreased (30%). In the frog neuromuscular junction, Jahromi et al. [97] have demonstrated that synaptic transmission is also governed by Ca^{2+} entry through an N-type Ca^{2+} channel.

In contrast, in mammalian motor nerve terminals, Ca^{2+} entry serving to discharge acetylcholine release seems to be ruled by a P-type Ca^{2+} channel rather than an N-type Ca^{2+} channel, as in fish and amphibians. So in the rat phrenic nerve, [^{3}H]-acetylcholine release was only partially inhibited by ω-conotoxin GVIA [98]. In the mouse, EPP were almost completely abolished (>95%) with 200 nM ω-agatoxin IVA. The twitch responses of the phrenic nerve hemidiaphragm were blocked in a different manner depending on the animal species. In the mouse, ω-agatoxin IVA at 100 nM blocked 92% of the twitches while in the rat, ω-agatoxin IVA (≤100 nM) and ω-conotoxin GVIA (≤1 μM) had little effect although ω-conotoxin MVIIC caused 57% blockade [158]. In normal human muscles, Protti et al. [9] have shown that transmitter release at the motor nerve terminals is mediated by a P-type Ca^{2+} channel.

5.7.14 Chromaffin Cells

As described above, different Ca^{2+} channel subtypes are found on the plasmalemmal membrane of chromaffin cells. This coexistence raises the question as to whether or not all of the channel types participate in the control of exocytosis and how their density and properties would condition their participation, if any. Furthermore, the presence and proportion of the various Ca^{2+} channels subtypes varies widely between animal species (Figure 5.2). Therefore, catecholamine secretion

from these cells will presumably be controlled differently, in accordance with the Ca^{2+} channels expressed by the cells. In this section, we will review how catecholamine secretion is controlled in different animal species and how some subtypes of Ca^{2+} channels are more directly implicated in the control of exocytosis. It is important to emphasize that, depending on the type of stimulus used (i.e., K^+ depolarization, acetylcholine, step depolarizations, and action potentials), one type of channel may be more favored over another in secretion. For this reason, the type of stimulus used is indicated in each of the following subsections.

5.7.14.1 Cat Chromaffin Cells

The K^+-evoked secretion of catecholamines is effectively blocked in a concentration-dependent manner by DHPs and other drugs acting on L-type Ca^{2+} channels like verapamil and diltiazem [162]. Measuring differential secretion of epinephrine and norepinephrine, Cárdenas et al. [163] demonstrated that secretion of both amines are completely blocked when it is induced by either high K^+ or the nicotinic agonist dimethylphenylpiperazinium (DMPP). Initially, these data indicated that an L-type channel controlled secretion in these cells. But, Albillos et al. [64] showed that cat chromaffin cells also contained ω-conotoxin GVIA-sensitive channels in addition to the L-type channels. It was then demonstrated that HVA L and N Ca^{2+} channels in cat chromaffin cells were present in an approximate proportion of 50–50%, and that the increase in $[Ca^{2+}]_c$ induced by short (10 s) depolarizing pulses (70 mM K^+) could also be reduced 44% by furnidipine and 43% by ω-conotoxin GVIA. In a perfused adrenal gland or isolated cat chromaffin cells, catecholamine release induced by 10 s pulses of 70 mM K^+ was blocked by more than 95% with furnidipine and only 25% with ω-conotoxin GVIA. These results show that though Ca^{2+} entry through both channels (N- and L-type) leads to similar increments of the average $[Ca^{2+}]_c$, the control of the K^+-evoked catecholamine release response in cat chromaffin cells is dominated by the Ca^{2+} entering through L-type Ca^{2+} channels [164]. However, more recent data suggest that when exocytosis is measured using capacitance techniques, and the membrane potential is held at −80 mV, and the N-type channels also contribute to exocytosis (G. Arroyo, M. Aldea, A. Albillos, and A.G. García; unpublished). It may be that previous experiments using cell populations or intact cat adrenal glands [162,163] and long-duration (seconds) depolarizing stimuli inactivated the N-type Ca^{2+} channels.

5.7.14.2 Bovine Chromaffin Cells

K^+-evoked catecholamine secretion from bovine chromaffin cells is greatly potentiated in the presence of the DHP L-type channel agonist Bay K 8644; the rise in secretion parallels the increase in ^{45}Ca uptake [165]. Ceña et al. [166] showed that nitrendipine completely blocked catecholamine release ([^{3}H]-norepinephrine) in bovine chromaffin cells stimulated with high K^+. These results do not agree with those obtained by other authors who found that in bovine chromaffin cells, DHP did not block more than 40–50% of the secretion [167–169]. The differences may be based on different stimulation patterns and the use of cultured chromaffin cells, fast superfused cell populations, or the intact perfused adrenal gland.

When toxins were available to selectively block specific subtypes of Ca^{2+} channels, it was demonstrated that these cells contain other Ca^{2+} channel subtypes besides L, that is, N and the P/Q-type [55,58,59,81,170]. ω-Conotoxin GVIA was ineffective or just barely effective in blocking K^+-evoked catecholamine secretion [69,82,88,168,169,171,172] in bovine chromaffin cells.

As the contribution of P-type Ca^{2+} channels to catecholamine secretion, we find different results in the literature. Thus, Granja et al. [173] showed that catecholamine secretion induced by high-K^+ is not affected by ω-agatoxin IVA (100 nM); nevertheless, when secretion was activated by nicotine, the ω-agatoxin significantly decreased catecholamine release by 50%. Thus, Granja et al. [173] concluded that ω-agatoxin IVA could also affect the nicotinic receptor. Duarte et al. [171] showed that FTX decreases K^+-evoked norepinephrine release to 25% and epinephrine release to 39% of the control levels; the combination of FTX plus nitrendipine further decreases norepinephrine and

epinephrine release to 12% and 24% of the control levels. Baltazar et al. [174] showed that bovine chromaffin cells contain two types of ω-agatoxin IVA-sensitive Ca^{2+} channels and that the contribution of the P-type channels to secretion is higher at low levels of depolarization.

The L-N-P-insensitive portion of catecholamine release in bovine chromaffin cells seems to be ω-conotoxin MVIIC sensitive. López et al. [69] observed that catecholamine release from superfused bovine chromaffin cells (stimuli: 70 mM K^+ for 10 s) was inhibited by 50% by DHP furnidipine (3 μM). ω-Conotoxin MVIIC (3 μM) also reduced the secretory response by 50%. The combination of furnidipine with ω-conotoxin MVIIC completely abolished secretion. On the other hand, these authors also demonstrated that ω-conotoxin GVIA and ω-agatoxin IVA have no effect on secretion. These results strongly suggest that secretion in these cells is predominantly controlled by Ca^{2+} entering through the L- and Q-type Ca^{2+} channels.

Further studies performed by Lara et al. [175] suggest that Q-type channels are coupled more tightly to active exocytotic sites that are the L-type channels. This hypothesis was suggested by the observation that the external Ca^{2+} that enters the cell through a Ca^{2+} channel, located near chromaffin vesicles, will saturate the K^+ secretory response at both $[Ca^{2+}]_e$, that is 0.5 and 5 mM. In contrast, Ca^{2+} ions entering through more distant channels will be sequestered by intracellular buffers and will therefore not saturate the secretory machinery at a lower $[Ca^{2+}]_e$.

5.7.14.3 Rat Chromaffin Cells

1,4-Dihydropyridines (DHPs) block secretion in perfused rat adrenal glands in a concentration-dependent manner. The magnitude of this blockade is related to the type of stimuli employed to induce secretion. The DHP isradipine can fully block secretion when the stimuli used are K^+ or nicotine. In contrast, when electrical field stimulation is used, the DHPs can only obtain a partial blockade and the inhibition is frequency dependent [176]. Measuring Ca^{2+} currents and capacitance, Kim et al. [177] have shown that ω-conotoxin GVIA (1 μM) blocks 40%, and nicardipine around 60% of the total capacitance increase in rat chromaffin cells. Therefore, in these cells secretion would be controlled by L as well as by N-type Ca^{2+} channels.

The role of each Ca^{2+} channel subtype in secretion has also been studied in intact whole adrenal glands from rats. Secretion evoked by depolarizing stimuli like high K^+ was strongly inhibited (80%) by L-type Ca^{2+} channel blockers, whereas acetylcholine-evoked responses were inhibited equally by either furnidipine or ω-conotoxin MVIIC [178]. Electrical field stimulation of intact glands releases acetylcholine and other cotransmitters from the splanchnic nerves [179]. Under these conditions, N-type Ca^{2+} channels seem to contribute to the maintenance of the secretory responses, probably by acting on presynaptic channels at the splanchnic nerve terminals [178]. In rat chromaffin cells treated with cAMP, a "low-threshold" exocytotic response was triggered at very low depolarizations; this unusual secretory response is associated with the α_{1H} subtype of Ca^{2+} channels [44,180].

5.7.14.4 Dog Chromaffin Cells

Kitamura et al. [63] have studied the effects of ω-conotoxin GVIA and L-type Ca^{2+} channel blockers (nifedipine and verapamil) on catecholamine release in anesthetized dogs. Catecholamine release into the blood stream was induced either by electrical stimulation of the splanchnic nerve or by intra-arterial injection of acetylcholine. Administration of 0.4 μg/mL of ω-conotoxin GVIA reduced catecholamine secretion by 30% in response to the electrical stimulation; nifedipine or verapamil had no effect under these experimental conditions. However, when catecholamine release was induced by acetylcholine, ω-conotoxin GVIA blocked secretion by around 50% and nifedipine also reduced it by 50%. These results suggest that N- and L-type Ca^{2+} channels contribute to the release of catecholamines in the dog adrenal gland. To our knowledge, a patch-clamp study that determines the subtypes of Ca^{2+} channels expressed by dog chromaffin cells is not available.

5.7.14.5 Mouse Chromaffin Cells

Simultaneous recordings of I_{Ca} and ΔCm in isolated mouse chromaffin cells indicate that exocytosis is proportional to the relative density of each Ca^{2+} channel subtype: 40% L, 34% N, 14% P/Q, and 11% R [92]. This indicates that under the perforated-patch configuration the secretory response elicited by 200 ms depolarizing pulses is a strict function of the amount of Ca^{2+} entering the cell, by whatever Ca^{2+} channel subtype, L, N, P/Q, or R. In addition, it seems that any Ca^{2+} channel type colocalizes with the secretory machinery in a similarly random manner, and shows the same relative efficacy in activating exocytosis, depending on its density [92]. This conclusion differs from that obtained in another study in acutely isolated adrenal mouse slices. In this latter study [91], the proportion of channel subtypes differs from that obtained in cultured mouse chromaffin cells [62], that is, 27% L, 35% N, 22% P, 23% Q, and 22% R. It is curious, however, that the R channels (22% of total current) control as much as 55% of the rapid secretion. Thus, Albillos et al. [91] conclude that "R-type Ca^{2+} channels in mouse adrenal slice chromaffin cells are in close proximity to the exocytotic machinery and can rapidly regulate the secretory process."

5.7.14.6 The Blocking Effects of ω-Toxins Varies with the Stimulation Pattern and the Ca^{2+} Gradient

The efficacy of the different channels in controlling exocytosis varies with the degree of depolarization and the concentration of external Ca^{2+} used in the experiments. There are different examples in the literature that demonstrate this fact. For instance, Turner and co-workers [78,141] observed that the efficacies of ω-agatoxin IVA and ω-conotoxin GVIA to block glutamate release from rat cortical synaptosomes increased when Ca^{2+} influx was reduced by decreasing the external concentration of KCl, decreasing the extent of depolarization, decreasing of the external concentration of Ca^{2+}, or by partially blocking the Ca^{2+} influx with an antagonist or another. For example, glutamate release was inhibited by ω-conotoxin MVIIC with an IC_{50} of 200 nM when stimulation of secretion was induced with 30 mM KCl; however, the same toxin had no effect when synaptosomes were stimulated with 60 mM KCl. The same investigators also found that dopamine release from rat striatal synaptosomes [140] could be blocked by ω-agatoxin IVA and ω-conotoxin GVIA when they used mild depolarizations with KCl. In contrast, with strong depolarizations, neither toxin alone was effective, although a combination of both toxins together produced a synergistic inhibition of 60%–80% of the Ca^{2+}-dependent dopamine release.

Transmitter release in parasympathetic neurons in the mouse bladder shows a similar pattern; bladder strip contraction was stimulated by single pulses or trains of 20 pulses at 1–50 Hz. Waterman [181] observed that ω-conotoxin GVIA and MVIIC inhibited contractions in a concentration-dependent manner with IC_{50} values of approximately 30 and 200 nM, at low stimulation frequencies; the same toxins had little effect at high stimulation frequencies.

Dunlap et al. [182] try to explain these puzzling findings: (a) with strong depolarizations neurotransmitter exocytosis is not affected when a single Ca^{2+} entry pathway is blocked; (b) a synergic inhibitory effect is observed when a combination of toxins is used to block two Ca^{2+} entry pathways; (c) in synapses with several Ca^{2+} channel subtypes, when one tries to sum up the individual inhibitory effects of the toxins, the values obtained are greater than 100%. They suggest that these findings could be explained by the presence of "spare" channels. Under conditions in which the $[Ca^{2+}]_c$ is saturating for the acceptor, participation of multiple Ca^{2+} channels might increase the reliability of excitation–secretion coupling, since activation of a single channel will be sufficient to maximize the release probability. This "spare channel" model might describe excitation–secretion coupling under conditions of relatively strong stimulation, such as high frequency trains of action potentials, or with prolonged depolarizations using increasing concentrations of K^+. Biochemical modifications (such as phosphorylation), which increase the sensitivity of the Ca^{2+} acceptor, would also predict an increased probability of release elicited by entry of Ca^{2+} through a single channel. Under these

conditions, the binding affinity of the Ca^{2+} channel antagonists would be underestimated by their effect on synaptic transmission, since blockade of one of several channels at the active zone would have little or no effect on release. This would produce a rightward shift in the concentration–response relationship relative to the binding curve.

A Ca^{2+} dependency of the blockade of different Ca^{2+} channel subtypes is also shown in the study performed by Lara et al. [175] in bovine chromaffin cells, where it was demonstrated that L and Q channels predominantly control catecholamine release. These investigators observed that blockade of secretion mediated by L-type channels is not dependent on the extracellular concentration of Ca^{2+} while blockade of Q-type channels is. The explanation that these authors give to these findings is that Q-type channels could be more tightly coupled to exocytotic active sites in comparison to L-type. The physiological meaning given to this channel distribution might be found in considering the need for regular secretory rate during normal activity of the body (L-type secretion) and explosive catecholamine secretion that occurs under stressful conditions (P/Q-type secretion). An additional effort should be made to understand further how and why the combined blockade of two channels and/or the Ca^{2+} gradient have synergic effects on secretion in chromaffin cells.

A number of studies performed in voltage-clamped bovine chromaffin cells have also produced contradictory results. For instance, Artalejo et al. [82] measured ΔCm elicited by a train of 10 depolarizing pulses of 50 ms to +10 mV separated by 500 ms (5 s of stimulation) in bovine chromaffin cells; 10 mM Ba^{2+} (or Ca^{2+}) was used as charge carrier. They found that N or P channels contributed about 20% to exocytosis; so-called "facilitation" Ca^{2+} channels (DHP-sensitive L-type channels), that were recruited by previous pretreatment with D1 receptor agonists or cAMP, contributed 80% of the exocytosis. The authors suggest that "facilitation Ca^{2+} channels may be closer to the docking and release sites than either of the other two channels."

Lukyanetz and Neher [89] also measured ΔCm in response to single 200-ms depolarizing pulses applied to bovine chromaffin cells under the whole-cell configuration of the patch-clamp technique, using 60 mM Ca^{2+} as charge carrier. They could not obtain the facilitation of ΔCm observed by Artalejo et al. [82]. In addition, they could not observe a preferential role of any Ca^{2+} channel subtype (in eliciting exocytosis) either; the action of the Ca^{2+} currents was proportional to the Ca^{2+} charge, irrespective of channel type. Contrary to Artalejo et al. [82], Lukyanetz and Neher [89]) reported that "participation of N-type channels (in exocytosis) is higher than that of L-type." The P/Q channel contributed little to I_{Ca} and ΔCm; this may be due to the fact that under conditions of excess divalent cations (60 mM Ca^{2+} was used as charge carrier by Lukyanezt and Neher [89], ω-conotoxin MVIIC binds and blocks P/Q channels poorly [79]. Ulate et al. [90] studied voltage-clamped bovine chromaffin cells, measuring I_{Ca} and ΔCm elicited by single 100-ms depolarizing pulses in 10 mM Ca^{2+}. They found that "all Ca^{2+} channel types (20% L, 48% N, and 43% P/Q) contributed to the secretory response in a manner roughly proportional to the current they allow to pass, thus implying a similar efficacy in triggering catecholamine release." Finally, Engisch and Nowycky [183] found that ΔCm evoked by single-step depolarizations "was strictly related to the integral of the voltage-clamped Ca^{2+} currents, regardless of the Ca^{2+} channel subtype."

Different views are also obtained from experiments performed using other preparations and stimulation procedures. For instance, with brief depolarizing K^+ pulses (seconds), L channels contribute more than N or P/Q channels to trigger secretion in populations of cat and bovine chromaffin cells [69, 164]. Also, by changing the Ca^{2+} concentration of the superfusion medium it was suggested that P/Q channels colocalize closer to the secretory machinery than L channels [175]. Furthermore, O'Farrell et al. [184] obtained evidence in perfused bovine adrenal glands suggesting that "N-type Ca^{2+} channels are largely responsible for catecholamine release induced by nerve stimulation."

Neither, depolarizing pulses in the range of milliseconds applied to voltage-clamped cells, nor pulses in the range of seconds applied to cells with their membrane potential free, are representative of the physiological conditions in which chromaffin cells are being stimulated *in situ*. It is true that the first approach has a time resolution closer to the duration of action potentials triggered by endogenously released acetylcholine [185]. However, by holding the membrane potential at

hyperpolarizing voltages, the voltage-inactivation of N and P/Q, but not L channels [70], might be prevented. The second approach has a more limited time resolution, but cells keep their "physiological" membrane potential free at the moment of application of the depolarizing pulse. At their resting membrane potential [186,187], chromaffin cells might well have partially inactivated the N and P/Q channels; thus, their role in exocytosis could be underestimated when using K^+ depolarization, giving more protagonism to L channels [69,164].

The real sequence of events leading from stimulus to release of catecholamines at the adrenal medulla is unknown. However, several studies make the following sequence feasible. Acetylcholine depolarizes the chromaffin cell [188], and this causes the firing of action potentials [189]. This recruits Ca^{2+} channels, triggering Ca^{2+} entry and exocytosis. However, the various Ca^{2+} channel subtypes suffer different degrees of inactivation, depending on the cytosolic Ca^{2+} concentration [99] and on the membrane potential [70]. Furthermore, chromaffin cells express Ca^{2+}-dependent K^+ channels of small conductance [190] that will also contribute to the regulation of action potential firing and exocytosis [191,192]. In summary, all these factors suggest that each type of Ca^{2+} channel could exhibit different efficacies to trigger and control the secretory process. Selection of the appropriate experimental conditions might reveal these differences.

5.8 BASIC AND CLINICAL PERSPECTIVES

In over two decades of ω-toxins use, at least six subtypes of HVA Ca^{2+} channels have been identified and characterized. New toxins are needed to target selectively the Q-type Ca^{2+} channel without affecting the N or P. The R- or T-type channels also need new toxins to characterize their functions. Whether the P and Q channels are the same or separate entities in various cell types remains to be clarified. The question of how many Ca^{2+} channel subtypes remain to be discovered is also relevant. In addition, differences among tissues and cell types for a given Ca^{2+} channel are emerging; L-type Ca^{2+} channels differ from skeletal, to cardiac, to smooth muscles and the brain. Are the Q channels from hippocampal and chromaffin cells identical? What about the N, P, or R channels? Why different Ca^{2+} channels are required to control exocytosis of the same transmitter (i.e., acetylcholine and catecholamines) in the same cell type, and in different animal species? Another important question relates to the expression of various channel subtypes in the same cell. Why does exocytosis require Ca^{2+} from different pathways? Is it a safety valve to secure the efficiency of the process? If the N channel is a part of the secretory machinery, what about the L, P, or Q channels? How close are they from exocytotic active sites? And most interesting, are the channels of a paraneuronal cell such as the chromaffin cell equally organized than those of brain synapses? Why is the release of norepinephrine controlled by N channels in sympathetic neurons and by L or Q channels in chromaffin cells? Do action potentials recruit different Ca^{2+} channel subtypes in those two catecholaminergic cell types? Furthermore, do Ca^{2+} channel subtypes that dominate secretion in cultured chromaffin cells differ in intact adrenal glands or in adrenal slices? Will a K^+ depolarizing stimulus recruit Ca^{2+} channels different from those recruited by action potentials in neurons, or by acetylcholine receptors in chromaffin cells? Is the electrical pattern of different excitable cells causing different secretion patterns by simply recruiting specific Ca^{2+} channels with particular gating and kinetic properties?

Another critical question relates to the development of a pharmacology for neuronal Ca^{2+} channels. While L-type Ca^{2+} channels have a rich pharmacology that has provided novel therapeutic approaches to treat cardiovascular diseases, nonpeptide molecules, which block or inactivate the N, P, Q, T, or R channels, are lacking. Thus, a major goal for research in this field is the search for selective blockers or modulators of specific Ca^{2+} channel subtypes that could eventually be used as therapeutic tools in disease. The recent introduction of mibefradil as a T-type Ca^{2+} channel blocker opened new possibilities to study the functions of these channels. The knowledge of the three-dimensional structure in solution of the different toxins is very important for studying the specificity of their interactions with Ca^{2+} channel subtypes, and to define active sites that can

serve as models to design and synthesize nonpeptide blockers. The ω-conotoxins are small peptides containing 24–29 amino acid residues. It is interesting that the amino acid sequence of ω-conotoxin MVIIA is much more similar to that of ω-conotoxin MVIIC than to ω-conotoxin GVIA; yet the pharmacology of ω-conotoxin MVIIA is much closer to that of ω-conotoxin GVIA (blockade of N-type channels). Thus, it will be very important to define structural differences determining the toxin selectivity for N- or Q-type Ca^{2+} channels. The three-dimensional structures of ω-conotoxin GVIA [193–196], ω-conotoxin MVIIA [197], and ω-conotoxin MVIIC [198, 199] have been elucidated. Other new toxins will facilitate their comparisons and the definition of structural determinants for specific binding to Ca^{2+} channel subtypes to identify a pharmacophore and to facilitate the synthesis of nonpeptide HVA Ca^{2+} channel modulators of therapeutic interest [200].

Nonpeptide blockers for neuronal Ca^{2+} channels are emerging, but they lack selectivity. For instance, the piperazine derivatives flunarizine, R56865, lubeluzole, and dotarizine are "wide-spectrum" Ca^{2+} channel blockers [51,52,201]. Fluspirilene, a member of the diphenylbutylpiperidine class of neuroleptic drugs (which also includes pimozide, clopimozide, and penfluridol) has antischizophrenic actions and block N-type Ca^{2+} channels in PC12 cells [202]. It may be that its neuroleptic properties are due, at least in part, to an inhibition of neuronal N-type Ca^{2+} channels. Thus, inhibition (or facilitation) of specific neurotransmitter release by selective blockers (or activators) of Ca^{2+} channels may have functional and therapeutic consequences. For instance, synthetic ω-conotoxin MVIIA protected hippocampal CA1 pyramidal neurons from damage caused by transient, global forebrain ischemia in the rat [14].

Channel opathies are increasingly being associated to specific diseases [203]. For instance, mutations of the Cav2.1 gene that encodes the α_{1A} subunit of the P/Q Ca^{2+} channel are responsible for the human familial hemiplegic migraine, episodic ataxia type 2, and spinocerebellar ataxia type 6 [204–206]. Also, natural P/Q mutations have been reported for the tottering and leaner mice, of which the homozygous rodents exhibit symptoms of ataxia and epilepsy [207,208]. In addition, P/Q knockout mice display progressive ataxia and dystonia until they are finally unable to walk, and eventually die [209].

In patients suffering paraneoplastic Lambert–Eaton syndrome and in passive transfer animal models of the disease, owing to an autoimmune reaction against the P/Q-type Ca^{2+} channel located at the presynaptic motor nerve terminal, a reduction of neurotransmitter release is observed [210]. It is interesting that L-type Ca^{2+} channels that are normally absent at the muscle endplate become coupled to neurotransmitter release after neuromuscular junctions were treated with immunoglobulins from either Lambert–Eaton patients [211] or amyotrophic lateral sclerosis patients [212] during reinnervation [213] and during functional recovery from botulinum toxin type-A poisoning [214].

In hair cells, the L-type Ca^{2+} channel current is associated to a gene expressing a Cav1.3 α_1 subunit. The central role of these currents in auditory transduction was shown through deletion of the gene expressing Cav1.3 that caused complete deafness [215].

ω-Toxin Ca^{2+} channel blockers have recently entered the clinic as therapeutic tools. Such is the case of ω-conotoxin MVIIA, also known as SNX-111 or ziconotide [216]. The recent approval of Prialt®, a synthetic version of ω-conotoxin MVIIA, by several drug regulatory agencies, for the treatment of severe chronic pain associated with cancer, acquired immune deficiency syndrome (AIDS), and neuropathies represents a significant advancement in analgesia. Ziconotide has shown potent efficacy in a postsurgical setting [217] as well as in patients suffering from a variety of chronic, and otherwise intractable, severe pain syndromes [218]. The drug has to be given intrathecally to prevent important side effects such as orthostatic hypotension [219]. Its potent analgesic effects that are manifested even in patients resistant to opioids is due to inhibition of proprioceptive neurotransmitter and neuromodulators from the central nerve terminals of primary afferent neurons in the dorsal horn of the spinal cord [220,221].

Another target that is intensely being studied for pain treatment is the T-type Ca^{2+} channel [216,222]. So Cav1.3 knockout mice are hyperalgesic in a model of visceral pain, which is likely related to a T-type Ca^{2+} channel-dependent antinociceptive mechanism operating in the thalamus

[223]. On the other hand, ethosuximide is a selective T-type Ca^{2+} channel blocker [37]. The drug reverses tactile allodynia and thermal hyperalgesia in nerve ligated rats [224].

An interesting Ca^{2+} channel therapeutic target is the auxiliary subunit $\alpha_2\delta$. Gabapentin is an approved analgesic and antiepileptic drug that binds with high affinity to $\alpha_2\delta_1$- and $\alpha_2\delta_2$-subunits of HVA Ca^{2+} channels [225,226]. This causes a blockade of Ca^{2+} currents, particularly after an injury such as constriction of the sciatic nerve in rats [227]. Its antinociceptive effects in rats in the absence of tolerance [228,229] led to clinical trials showing moderate efficacy in postherpetic neuralgia, diabetic neuropathy, trigeminal neuralgia, low back pain, and cancer pain. Recently, another $\alpha_2\delta$ ligand, pregabalin, has shown higher efficacy in animal models and in diseases developing neuropathic pain. Its association with the $\alpha_2\delta_1$-subunit has been clearly demonstrated because pregabalin loses its antinociceptive effects in transgenic mice expressing $\alpha_2\delta_1$-subunit with a point mutation that prevents pregabalin binding [230]. Pregabalin is presently being used in the clinic to treat neuropathic pain of various origins (see review in Reference 231).

Many questions have been answered over two decades of intense research in the field of neuronal voltage-dependent Ca^{2+} channels. Most of the studies performed were possible only because the group of Professor Baldomero Olivera particularly made available to many groups of researchers potent ω-toxins to type and characterize those channels. The same is true for the patch-clamp techniques, which exploded at the same time as ω-toxins, thanks to the efforts of the group of professor Erwin Neher. We are sure that some references on this topic escaped our review; we apologize to those authors not cited here involuntarily.

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6 The Mouse Bioassay as a Universal Detector

Luis M. Botana

CONTENTS

6.1 ISSUES ABOUT BIOASSAY

6.1.1 Ethical

About 11 million mice are killed every year in Europe for research and diagnostic purposes. This number is further increased with the sacrifice of larger and far more expensive animals (cats, rabbits, dogs, etc.) that are also needed for many research areas, in drug development, toxicology, and so forth. Therefore, animal sacrifice is really needed for scientific advance. It is clear that ethics should be first applied to animals that live in the wild, along with those animals that are being grown in animal houses only for research. The human species has been historically rather aggressive to others, and if we find it unbelievable that some beautiful species became extinct in recent decades, it is also true that the white dolphin became officially extinct a few months ago, that big gorillas are in real danger, and that rather advanced societies in Europe find it acceptable to kill whales, foxes, bulls, bears, or deer in some cases just for fun. But in parallel with this, humans also spend large amounts of money to save the lynx in Spain, tigers in Asia, or Tasmanian devil in Australia. The odd conclusion to this, is that big businesses are sustained around animal sacrifice (hunting, fishing, etc.), and also there is increasing business on the other hand to sustain and support nearly extinct animals.

Ethical pressure to avoid research with animals comes from a combination of a city culture that is not in touch with animals (where no one questions that a hamburger comes from a cow that was actually killed for us to eat) and the request to avoid unneeded suffering in animals. It is true that a farmer sees animal sacrifice as a tool for a business, and therefore to question the ethics of killing

a cow or a pig is totally out of place. On the other hand, it is also true that many animal experiments provide similar information to *in vitro* assays, and therefore it is only logical to replace one thing for the other. The demand to not use animals in experiments has been a boost to research of *in vitro* models, and has proven to be a leading force in scientific advance. Therefore, the ethical issue of animal experimentation is a plus for research, since what may be lagging because of not using animals, is progressing in other aspects of science.

6.1.2 Legal

But ethics aside, from a legal point of view, in Europe animals in research are to be replaced because current European legislation requires just that. Therefore, since European Directive 86/609/EEC was published,[1] animals are to be used in the minimum, optimized, and less painful way. The European Commission did create, within the Joint Research Center (JRC), the European Center for the Validation of Alterative Methods (ECVAM) that seeks animal replacement in research, with a rather successful track in cosmetics, and now taking a lead in marine toxins owing to their support of the activities of the European Community Reference Laboratory for Marine Biotoxins (CRLMB). The ruling guide to ECVAM is the three R approach (Reduction, Replacement, Refinement).[2] *Council Directive 86/609/EEC* makes provision for laws, regulations, and administrative measures for the protection of animals used for experimental and other scientific purposes. This includes the use of live animals as part of testing strategies and programs to detect, identify, and quantify marine biotoxins. Indeed, the scope of Article 3 of the Directive includes the use of animals for the safety testing of food and the protection of consumers.

Directive 86/609/EEC sets out the responsibilities that the European member states must discharge. As a result of the use of prescriptive language, there is little room for flexibility, and most of the provisions of the Directive must be applied in all cases.

Live animals are not to be used in procedures that may cause pain, suffering, distress, or lasting harm, "if another scientifically satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonably and practicably available." Therefore the number of animals used for experimental and other scientific purposes is to be reduced to the justifiable minimum, such animals must be adequately cared for, and no unnecessary or avoidable pain, suffering, distress, or lasting harm is to be caused in the course of such animal use. When animal use can be justified, *Directive 86/609/EEC* specifies a range of safeguards that European member states must put in place to avoid or minimize unnecessary pain, suffering, distress, and lasting harm. It must be ensured that user establishments undertake experiments as effectively as possible, with the objective of obtaining consistent results, while minimizing the number of animals and any suffering caused.

This latter requirement demands minimum severity protocols, including appropriate observation schedules, and the use of the earliest humane end points that prevent further suffering, once it is clear that the scientific objective has been achieved, that the scientific objective cannot be achieved, or that the suffering is more than can be justified as part of the test procedure. It is also required to encourage research into, and the development and validation of, alternative methods that do not require animals, use fewer animals, or further reduce the suffering that may be caused, while providing the same level of scientific information. Chapter 43 provides a detailed explanation of actual legislation.

6.1.3 Technical

Mouse bioassay is the current detection method for most of the marine toxins, with the exception of domoic acid. The reason for this is that it has been protecting consumers for the past decades, ever since monitoring systems were implemented in many countries. Therefore, food safety authorities are reluctant to allow replacement of the mouse bioassay, unless a solid evidence is provided to sustain current levels of consumer protection. This evidence is legally sustained in Europe by the requirement of an internationally recognized validation study for any method that could be an alternative to the bioassay.[3,4] A recent outbreak of mice toxicity in bivalves took place in Arcachone, France, and the source of this toxicity was unknown. This was a very good example of universal

protection to consumers from toxic marine products by the use of animals. Since the source of toxicity was unknown, it remains to be seen how alternative methods would behave, but clearly the system did work. A similar example could be applied for the first outbreak of domoic acid in Canada, where the symptoms of dead mice did provide information on something other than the paralytic shellfish poisoning (PSP) toxins the technicians were expecting and seeking.[5] These symptoms ended up in a major restructure of monitoring laboratories worldwide, accompanied by legislation and technology to detect the presence of this new group of compounds. The domoic acid outbreak could not be detected by liquid chromatography-mass spectrometry (LC-MS) or any other functional method, which is designed to cope with a full group of toxins, but cannot identify the presence of a new group. Therefore, the mouse bioassay is useful for some purposes, even in the case that all toxin groups are detected by alternative validated methods. In terms of consumer protection, it should not be eliminated from a monitoring protocol for this practical and very useful reason.

However, the main source of uncertainty from using bioassays, is the fact that the method is probably impossible to be validated, due to the large number of variables that must be controlled, and cannot be reproduced in different laboratories [diet for the animals, environmental conditions (such as temperature, quality of the air, acoustic levels, light), strain of rodent, sex, weight, other animals nearby, number of animals per cage, etc.]. From a technical point of view, the current reference method is one that cannot be validated, but that clearly works for consumer protection, due to the fact that it is the only one that can detect any potential toxic compound. For this reason, any option for replacement must prove, through a full validation, that it is reliable enough to replace the use of rodents for a given group of toxins.

A long and sustained collective effort is taking place to replace the mouse bioassay, but several problems remain very elusive to solve. Table 6.1 summarizes the current options for method alternatives.

Although the bioassay has been a reliable option to control the presence of toxins and protect consumers, the fact today is that a replacement is needed, and encouraged, by current European legislation. “A lack of reference material and sole use of non-bioassay tests currently means that the level of public health protection provided in respect to all toxins specified is not equivalent to that afforded by biological tests. Provision should be made for the replacement of biological tests as soon as possible.”[3] A different issue, regarding how to replace the bioassay, is the toxicological information. One of the consequences of the use of mice to monitor the presence of marine toxins is that current regulatory limits are actually set in some cases to fit the sensitivity of the bioassay (yessotoxins, azaspiracids). Therefore, once a method is offered as an alternative to replace the use of the bioassay, it will create instantly the need to know how regulatory limits are implemented for that specific method, and this could be rather complex. In the case of chromatographic methods, a good example is the already accepted high performance liquid chromatography (HPLC) method for PSP toxins;[6] it did create the problem of how to perform the interpretation of chromatographic peaks into toxic values, and integrate the total toxicity suggested by the chromatographic peaks according to the current regulatory level. This turns out to be a rather complex topic, with no easy solution,[7] since toxicological information is not available for all toxins in the PSP group, and the method has been validated to few individuals in the group. This sets a limitation for the wide use of the method that needs further validation extensions, plus complete toxicological information of the PSP group. To make things more complicated, toxicology studies require a rather large supply of pure toxins, and this has been the major drawback of the field, the lack of enough (or even any) supply for most of the toxins. Therefore, to achieve advances in the marine toxins field is proving to be a formidable task.

6.1.3.1 Methods

6.1.3.1.1 Paralytic shellfish poisoning bioassay

The bioassay for saxitoxin and analogues is well described[8] and provides a very reliable semiquantitative method,[9] although care must be taken owing to interferences caused by salts. The major

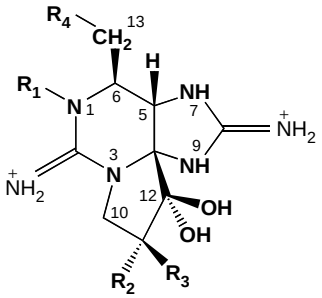
TABLE 6.1
Summary of Current Alternative Method Strategies

Method Strategy	Advantages	Disadvantages	Technology	Status	Pros	Cons
Antibody-based	Easy to use Sensitive	Unable to detect all individuals in each group	ELISA Optical biosensor[61]	Several kits nearly available	Inexpensive Sensitive Quantitative	Limited to few compounds. Only useful for toxin groups with few individuals (i.e., domoic acid). Needs a legal toxic level for each individual
		Enough toxin to obtain antibody is difficult (if possible) to get	Lateral immunoflow strip	Under development Under development	Fast Sensitive Quantitative Easy to use	Expensive Qualitative
Receptor-based	Detects all toxins in a group Sensitive	Difficult to develop Difficult to obtain the receptor Difficult to identify the receptor	Fluorescence[62] (includes polarization fluorescence[63]) Absorbance[64] Luminescence Optical biosensor[65] Others (i.e., Patch clamp[66])	Fluorescent kits available and in the process of validation	Inexpensive Sensitive Quantitative	Difficult to validate Requires to define a legal toxin level for each group
Cell-based	Sensitive Detects all toxins in a group	Probably not possible to validate Slow	Fluorescence[67] Absorbance[68] Luminescence			
Separation methods (HPLC, thin layer chromatography,[69] capillary electophoresis[70])	Allows quantification	Slow Needs standards Needs toxicology information for each toxin	Precolumn reaction[71] Postcolumn reaction[72] UV detection[73] Fluorescent detection[74,75]	PSP-HPLC fluorescence validated[6,7,76]	Available now	Tedious Validated for few matrices and toxins[7] Limited available toxicological information

LC-MS[77]	Allows quantification Sensitive	Slow Complex Expensive Needs standards	Ion trap Single to triple quadrupole	Some protocols under validation	Allows multitoxin detection and quantification	Needs validation Very complex validation
Mouse bioassay	Universal detection Easy to perform The method itself is cheap	Detection limit is low. Error % high. Slow for lipophilic toxins Interference of extracts and salts Several nonregulated marine compounds provide false positives Animal house is expensive Validation is probably not possible	Not possible to automatize. Many variables to the animal: strain, age, sex, weight, diet, environment (light, air , temperature), size of cage, number of animals, and so forth Many variables to the method: Weight ratio body/ hepatopancreas due to seasonal changes on the bivalve, pH, salt concentration, solvents used, extraction volume, temperature, and so forth	Lipophilic bioassay has been harmonized (CRL/NRL working group on bioassay harmonization report) PSP bioassay is an official AOAC method[8]	PSP bioassay allows quantification if dead takes less than 5 min	Lipophilic bioassay does not allow quantification, and it is very slow (24 h observation time), and several nontoxic/ nonregulated marine toxins may give false positives (spirolides, gymnodimine, etc.) Coextracted compounds could modify the results (metals such as Zn, Mg, Ca, chlorophyll derivatives, etc.)

source of discrepancy in this mouse bioassay comes from the modifications in the protocol at the heat/acidic extraction. At one step, all PSP toxins are heated in acidic pH to convert some of the toxins to more toxic compounds (Table 6.2). Therefore, depending of the efficiency of this conversion, toxicity can be increased by a factor of 10; the more acidic the pH, the more complete the conversion. If a laboratory performs this conversion at pH 1, and another one uses pH 4, the difference in the response could make a difference, to the point that a commercial product sold as safe (pH 4) could be identified as very toxic (pH 1). Therefore, the National Reference Laboratory (NRL) network in Europe agreed to carry out the extraction at pH 3, in order to harmonize the results. Another minor modification also agreed on is to increase the weight range of animals by 10%, so that weekends are not a limitation to the use of the animals and to avoid discarding them for excess or less than desired weight before or after the weekend (Figure 6.1).

TABLE 6.2
Acidic pH Used in Extraction can Increase Toxicity Several Fold



Toxin	R_1	R_2	R_3	R_4	Compound	Toxicity Reported	With Heat (90°C/15 min) and Acid pH, Converts to
	Carbamate				GTX I	752–2,468	
STX	H	H	H	$CONH_2$	GTX II	793–1,150	
Neo STX	OH	H	H	$CONH_2$	GTX III	1,465–2,234	
GTX I	OH	H	OSO_3^-	$CONH_2$	GTX IV	602–1,803	
GTX II	H	H	OSO_3^-	$CONH_2$	**GTX V ---->**	150–354	**STX**
GTX III	H	OSO_3^-	H	$CONH_2$	**GTX VI --->**	175–180	**Neo STX**
GTX IV	OH	OSO_3^-	H	$CONH_2$			
	Decarbamoyl				dc-GTX I	950	
dc-GTX I	OH	H	OSO_3^-	H	dc-GTX II	380–1617	
dc-GTX II	H	H	OSO_3^-	H	dc-GTX III	380–1872	
dc-GTX III	H	OSO_3^-	H	H	dc-GTX IV	950	
dc-GTX IV	OH	OSO_3^-	H	H			
dc-STX	H	H	H	H	**C1 ----->**	17–25	**GTX II**
dc-Neo STX	OH	H	H	H	**C2 ----->**	180–430	**GTX III**
	***N*-Sulfocarbamoyl**				**C3 ----->**	8–33	**GTX I**
GTX V	H	H		$CONHSO_3^-$	**C4 ----->**	57–143	**GTX IV**
GTX VI	OH	H		$CONHSO_3^-$			
C_1	H	OSO_3^-		$CONHSO_3^-$	STX	1,656–2,483	
C_2	H	H		$CONHSO_3^-$	Neo STX	1,038–2,295	
C_3	OH	H		$CONHSO_3^-$	dc-STX	1,175–1,274	
C_4	OH	OSO_3^-		$CONHSO_3^-$	dc-Neo STX	900	

Note: There is a wide margin of reported toxicities.[78–84] See also Chapter 7.

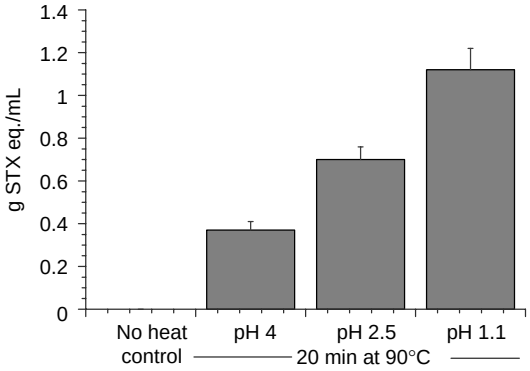


FIGURE 6.1 Effect of pH on toxicity increase in an extract with C toxins.

6.1.3.1.2 Lipophilic toxins bioassay

The lipophilic bioassay is a precious development by Yasumoto et al.,[10,11] and the only method internationally used to control the presence of toxic compounds in molluscs. There has been an intense activity of the CRLMB/NRL network in Europe to harmonize the different protocols carried out in the routine monitoring laboratories, and this harmonized protocol is shown in Scheme 6.1. Although the oral rat assay performed mainly in Holland[12] has some advantages, such as fewer false positives caused by fatty acids from molluscs, and avoidance of animal sacrifice, it is less used owing to the subjectivity of the symptoms [normal feces (rated-), soft (+ to ++), soft to diarrheic (+++), and diarrheic (++++)], and the cost and difficulty in handling larger animals.

A thorough study on extraction methods has been recently reported to selectively eliminate the effects of false positives caused by yessotoxins.[13]

6.2 UNIVERSAL DETECTOR: SYSTEMS AND TARGETS AFFECTED

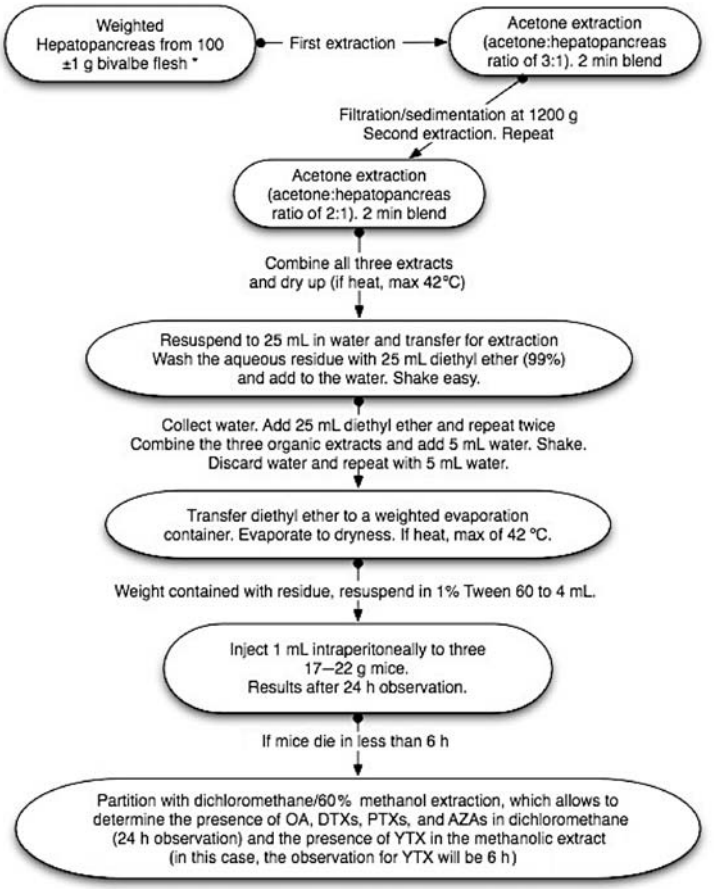
Although for a detailed mechanistic explanation of each toxin mentioned in this part it is recommended to see specific chapters in this book, the description here refers to the targets to marine toxins that could be used as the base for the development of functional assays.

6.2.1 Sodium Channel

Membrane potential is very sensitive to the effect of several marine toxins.[14] The voltage-gated sodium channel is the target to several marine toxins. Brevetoxins and ciguatoxins are channel activators that bind to site 5 of the alpha subunit of the channel.[15–17] Ciguatoxins and brevetoxins have a dual effect: (1) activation voltage for channel opening shifts to a more negative value, and (2) the inactivation of opened channels is inhibited.[18] Saxitoxin and analogs (gonyautoxins, neosaxitoxin) also bind and block this channel, although the binding site for this toxin group is 1.[19,20] Gambierol seems to also interact with this channel, possibly at the ciguatoxin site.[21]

6.2.2 Protein Phosphatase

The type of compounds that target protein phosphatase is rather large. Protein phosphatases are the receptors to the polyether fatty acid structures okadaic acid and dinophysistoxins,[22] cyclic heptapeptide microcystins,[23] as well as calyculins,[24,25] cyclic pentapeptide nodularins,[26] and motuporin.[27] The main phosphatases affected are PP1 and PP2A, and the interaction can be covalent (microcystin) or noncovalent (nodularin).[27] The binding site for okadaic acid, microcystin, and calyculin is the same, but the kind of bonds with which they interact are unique to each compound group.[28,29]



SCHEME 6.1 European harmonized protocol for lipophilic bioassays. Hepatopancreas is to be used in all molluscs. Whole flesh should be used for: (a) large molluscs (scallops), where there are at least 12 individuals, and weight of hepatopancreas is to be proportional to 100 g whole flesh, (b) small molluscs, where removal of the digestive apparatus is difficult.

Recent observations suggest that at least okadaic acid may have some unknown activities outside the inhibition of phosphatase. This is based on the observation that methyl okadate does show a similar toxic effect in cell cultures than okadaic acid, but methyl okadate is 500 times less potent than okadaic acid to inhibit phosphatases (unpublished results) (Figure 6.2).

6.2.3 Phosphodiesterase

Several lines of experiments point clearly to yessotoxins as a group that binds this family of enzymes. The interaction is reversible and yessotoxins target the cyclic adenosine monophosphate (cAMP) phosphodiesterases in a more selective manner.[30–32]

6.2.4 Calcium Channels

Calcium channels that are activated by potential were thought to be the receptor of maitotoxin, but nonexcitable cells are also the target to this toxin. Although the mechanism of action of maitotoxin is not fully understood, a nonselective cation channel seems to be the effecter to maitotoxin.[33,34] Calcium influx has been also proposed as the main effect of polycavernoside.[35]

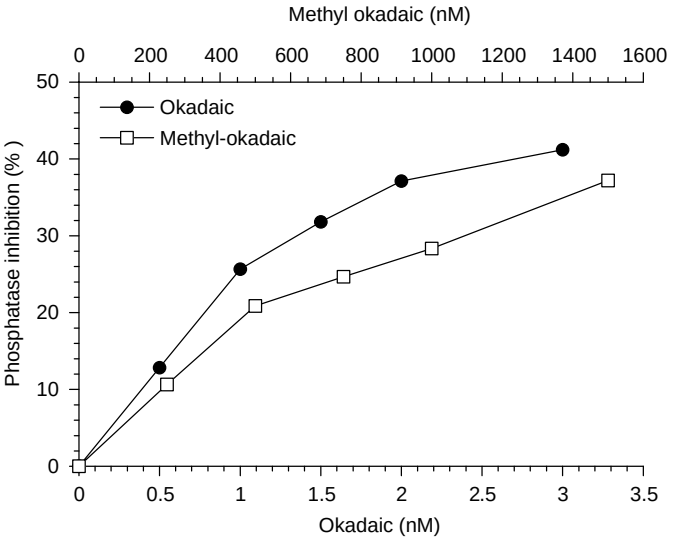


FIGURE 6.2 Inhibition of phosphatase caused by okadaic acid and methyl okadate.

6.2.5 Potassium Channels

These channels are targeted by at least some ciguatoxins and conotoxins,[36–38] but due to the fact that membrane potential is sensitive to several other marine toxins, in future more developments on this topic could be expected.[14,39] A potent interaction between gambierol and potassium channels is currently being investigated (J. Molgó, personal communication).

6.2.6 Nicotic Receptor

Gymnodimine (J. Molgó, personal communication) and conotoxins are known to antagonize the nicotinic receptors.[40–42]

6.2.7 Mitogen-Activated Protein Kinases

Although at first the target was thought to be calcium channels, azaspiracids are a rather elusive toxin group in terms of receptor identification,[43–49] but all preliminary results are aiming at mitogen-activated protein (MAP) kinases as the target to this group of compounds, and some recent observation, more specifically, to c-Jun-*N*-terminal kinase (unpublished results).

6.2.8 Kainate Receptors

Kainate receptors, a part of the glutamate receptor complex, are the receptors to domoic acid (see Chapter 20).[50–52]

6.2.9 Actin Proteins

Actin is considered to be the receptor to pectenotoxins.[53–55] The interaction decreases the formation of filamentous actin, and increases the amount of globular actin, the most important factor to the potency of pectenotoxins being an intact lactone ring.[56] The interaction between actin and pectecnotoxin follows a stoichiometry of 1:1.[57] From indirect observations, actin is a sensor to the effect of several other toxins.[43,58]

6.2.10 Na-K ATPase

The Na-K ATPase is the known receptor to palytoxin and ostreocins. The interaction shows lower potency for ostreocins, the nature and site of the binding of palytoxin is unknown, it acts as a functional antagonist of ouabain,[59] and their binding sites on the receptor are probably different.[60]

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Part IV

Paralytic Shellfish Poisoning (PSP)

7 Metabolism of Paralytic Shellfish Toxins Incorporated into Bivalves

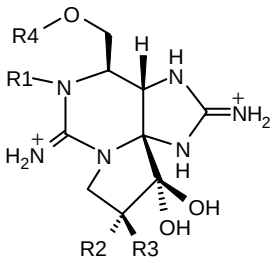
Masaaki Kodama and Shigeru Sato

CONTENTS

During a bloom of toxic dinoflagellates such as *Alexandrium tamarense*, plankton feeders such as bivalve shellfish accumulate toxin by ingesting them. Human consumption of toxic shellfish causes severe food poisoning and is hence called paralytic shellfish poisoning (PSP). The accumulation of PSP toxins in shellfish poses a severe problem to public health and the fisheries industry. In the areas where causative dinoflagellates occur, the toxicity of bivalve is monitored regularly. When shellfish toxicity exceeds the safety consumption level, the shellfish market is banned to avoid food poisoning by consumption of toxic shellfish. Because of the costly countermeasures, no serious poisoning has occurred in these areas. In 1980s, regular monitoring of shellfish toxicity was carried out in association with the survey on the occurrence of causative dinofagellates [1]. It has often been observed in these surveys on shellfish toxicity in association with the abundance of causative dinoflagellates that both parameters are not parallel to each other. When the causative dinoflagellates bloom in high density and reach their maximal abundance, the level of shellfish toxicity is still low, but it increases to a higher level and reaches a maximum after most of the dinoflagellates disappear from the environment. There is always about a 1-week time lag between the peaks of these parameters [1,2]. This phenomenon is difficult to explain if the toxins in the dinoflagellates transfer to the shellfish via food chain. However, it is hard to evaluate the balance of the toxin amount between dinoflagellates and shellfish in a field survey in which samples of shellfish and plankton are collected periodically at a station set in the field, even though the frequency of the sampling is increased. Thus, the nature of the balance of the toxins between shellfish and dinoflagellates has not been clarified by data from field surveys. In this chapter, the kinetics of PSP toxins in shellfish on the results of feeding experiments and the fate of PSP toxins are discussed.

7.1 STABILITY AND CHEMICAL TRANSFORMATION OF PARALYTIC SHELLFISH POISONING TOXINS

Paralytic shellfish poisoning toxins consist of a group of toxin components, the structures of which are similar to each other. Since Schantz et al. [3] succeeded in determining the chemical structure of saxitoxin (STX), various derivatives of STX have been isolated from toxic dinoflagellates and toxin-contaminated shellfish [4–16]. Currently, more than 20 components are known [17].



	R1	R2	R3	R4
Saxitoxin (STX)	H	H	H	$CONH_2$
Neosaxitoxin (neoSTX)	OH	H	H	$CONH_2$
Decarbamoyl saxitoxin (dcSTX)	H	H	H	H
Decarbamoyl neosaxitoxin (dcneoSTX)	OH	H	H	H
Gonyautoxin1 (GTX1)	OH	OSO_3^-	H	$CONH_2$
Gonyautoxin2 (GTX2)	H	OSO_3^-	H	$CONH_2$
Gonyautoxin3 (GTX3)	H	H	OSO_3^-	$CONH_2$
Gonyautoxin4 (GTX4)	OH	H	OSO_3^-	$CONH_2$
Gonyautoxin5 (GTX5) B1	H	H	H	$CONHSO_3^-$
Gonyautoxin6 (GTX6) B2	OH	H	H	$CONHSO_3^-$
Decarbamoyl gonyautoxin1 (dcGTX1)	OH	OSO_3^-	H	H
Decarbamoyl gonyautoxin2 (dcGTX2)	H	OSO_3^-	H	H
Decarbamoyl gonyautoxin3 (dcGTX3)	H	H	OSO_3^-	H
Decarbamoyl gonyautoxin4 (dcGTX4)	OH	H	OSO_3^-	H
C1	H	OSO_3^-	H	$CONHSO_3^-$
C2	H	H	OSO_3^-	$CONHSO_3^-$
C3	OH	OSO_3^-	H	$CONHSO_3^-$
C4	OH	H	OSO_3^-	$CONHSO_3^-$

FIGURE 7.1 Structures of saxitoxin and its derivatives.

Figure 7.1 shows the structure of the components that are often observed in toxic dinoflagellates and toxin-contaminated shellfish. All of these components are derivatives of STX, but different names were given to some of them by different research groups. In Figure 7.1, the structures are shown with the names, including synonyms.

Generally, the toxins are considered to be water soluble and heat stable. However, the stability varies greatly depending on the pH and the structures of toxins [18]. At an alkaline pH, all the toxin components degradated quickly, even at room temperature. Generally, the toxins are stable under acidic conditions. However, the stability depends on the structures. STX is extremely stable, and the hydrochloride solution can be stored without loss of potency for decades. On the other hand, C11-*O*-sulfate toxins such as gonyautoxin (GTX) 2,3 are more heat labile, even at a lower pH. Toxins belonging to the N1-H group, such as GTX2,3 and STX, are more stable than those belonging to the N1-OH group, such as GTX1,4 and neoSTX. Carbamoyl-*N*-sulfate toxins, such as B and C toxins, are easily hydrolyzed by dilute mineral acid treatment to give desulfated toxins [4, 6, 7].

It is noteworthy that the potency of a toxin is different among toxin components. Table 7.1 shows the specific toxicity of each toxin component expressed as mouse units (MU) per μmole of toxin, in which 1 MU is a dose of toxin to kill a male mouse (ddY strain) in 15 min [19]. STX shows the highest toxicity. The toxicity is different between the toxins of N1-H and N1-OH groups. It is also different between 11-epimers of toxins such as GTX2 and GTX3. It is noteworthy that the potency of carbamoyl-*N*-sulfate toxins, such as B and C toxins, is remarkably low, whereas that of the corresponding desulfated toxins, such as STX, neoSTX, and GTX 1-4, is high. This means

TABLE 7.1
Specific Toxicity of Each Toxin Component (MU/μmol)[a]

Component	Specific Toxicity	Component	Specific Toxicity
STX	2483	dcGTX2	1617
neoSTX	2295	dcGTX3	1872
dcSTX	1274	GTX5	160
GTX1	2468	C1	15
GTX2	892	C2	239
GTX3	1584	C3	33
GTX4	1803	C4	143

[a] According to Oshima (1995).

that mild acid treatment of carbamoyl-*N*-sulfate toxins leads to remarkable enhancement of the toxicity.

As described above, the carbamoyl-*N*-sulfate of B and C toxins is easily hydrolyzed by the treatment of mild mineral acids, which result in the formation of the corresponding desulfated toxins, such as STX, neoSTX, and GTX1-4. Interestingly, these carbamoyl-*N*-sulfate toxins are easily converted to decarbamoyl toxins when heated under neutral conditions [17], although conversion of STX to dcSTX requires prolonged heating in 7.5 N hydrochloric acid [20]. When a water solution of an isolated stereoisomer is left at an ambient temperature, it is gradually converted to another stereoisomer to reach an equilibrium mixture of α- and β-isomer with a mole ratio of approximately 3:1 [21]. The higher pH and temperature accelerate the epimerization [17]. When the solution is boiled at neutral pH, the epimerization is complete within 5 min.

7.2 CHANGES OF TOXIN COMPONENTS IN BIVALVES

The profile of the toxins accumulated in shellfish during a bloom of toxic dinoflagellates reflects that of the causative dinoflagellate. However, the toxin components accumulated in the shellfish change after the causative dinoflagellates disappear from the environment [17]. This finding suggests that the bioconversion of toxin components occurred in the shellfish. In an earlier study, Shimizu and Yoshioka [21] first showed that transformation of PSP toxins occurs in the shellfish. They incubated a homogenate of the scallop *Patinopecten magellanicus* contaminated with neoSTX, GTX2, and 3, and found that the proportion of STX increased. These facts show that neoSTX and GTXs are converted to STX in the shellfish. They suggested the presence of enzymes in the shellfish, which are involved in the transformation of toxin components, although the rate of conversion is very low. Oshima [17] screened the enzyme activities that transform C toxins in the shellfish, and detected the activity to hydrolyze *N*-sulfocarbamoyl of C toxins to form dcGTXs in two species of clams, *Mactra chinensis* and *Peronidia venulosa*. Enzyme activity to hydrolyze carbamate toxins was also detected in some species of shellfish [22]. However, the enzyme involved in the transformation of GTXs to STX has not been found in shellfish, although this transformation is observed generally in shellfish.

In a trial to solve this problem by feeding dinoflagellates to shellfish, interspecific difference in the ability to accumulate PSP toxins were examined in four species of bivalves and an asdian [23]. The specimens of each species were reared in separate tanks and fed a known number of *A. tamarense* cells. As shown in Figure 7.2, the toxicity of each species increased during the feeding period. The toxicity decreased after the cessation of feeding. However, marked individual variations were observed in accumulated toxin levels of these species sampled at the same stage of the experiment, although the average toxin levels showed a trend toward increasing during feeding. These differences are considered to be due to physiological changes owing to individual sensitivities to handling

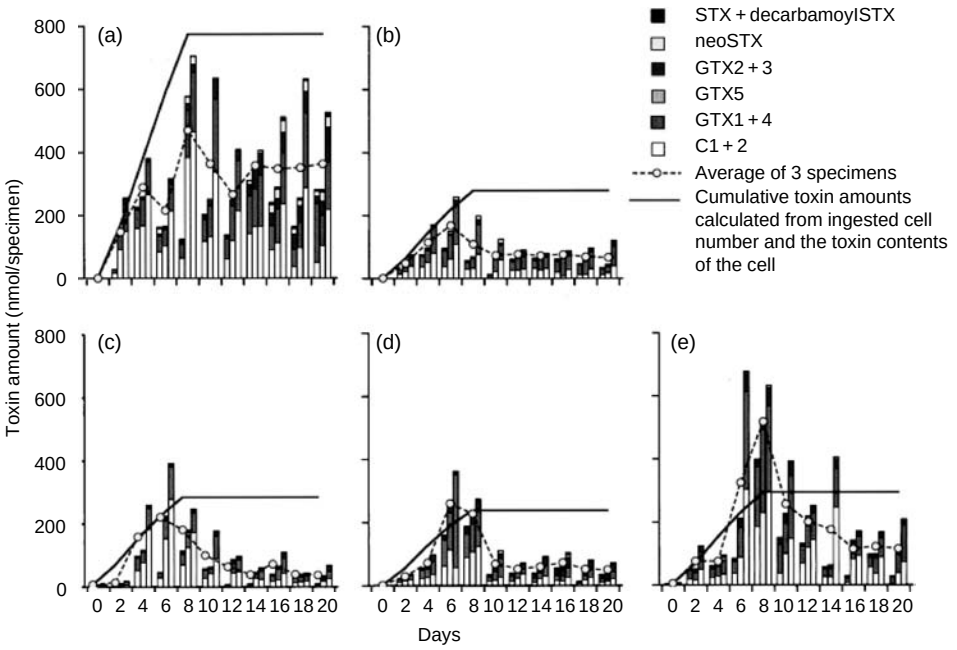


FIGURE 7.2 Toxin accumulation in four species of bivalves and ascidian fed on cultured cells of *Alexandrium tamarense*. (a) Scallop *Patinopecten yessoensis*, (b) mussel *Mytilus galloprovincialis*, (c) oyster *Crassostrea gigas*, (d) short-necked clam *Ruditapes philippinarum*, (e) ascidian *Halocyntia roretzi*. (From Sekiguchi, K., Sato, S., Kaga, S., Ogata, T., and Kodama, M., *Fish. Sci*., 67, 301, 2001.)

or a sudden change in the environment during the experiments. As shown in Table 7.2, similar individual difference in toxicity is observed even in the scallop *P. yessoensis* cultured in the same area, indicating that the feeding habitat of the shellfish in natural conditions is also different among the specimens (unpublished data). These findings show that feeding experiments on accumulation and depuration kinetics of the toxins should be designed to take feeding behavior of shellfish into consideration. Therefore, a single specimen of scallop was reared in a single tank, and the known amount of cultured cells of dinoflagellate was fed to each scallop specimen [24]. These experiments showed that scallop accumulates toxins by ingesting the toxic dinoflagellate, and they release a part of the toxins into the environmental water even while ingesting the dinoflagellate. When the feeding stopped, the scallop excreted the toxins continuously. The profile of excreted toxins was similar to that accumulated in the scallop, that is, the scallop releases the toxin components nonselectively.

Interestingly, the amount of toxins accumulated in the scallop was not parallel to that of dinoflagellate cells fed to the scallop (Figure 7.3). At the earlier period of the experiment when scallop were ingesting the cells, the amount of toxins accumulated in the scallop was often more than that in the dinoflagellates fed to the scallop. In the later period, when the feeding was stopped, the sum of the toxins in the scallop and rearing water had decreased to a level that was less than that introduced from the dinoflagellates cells, showing that some amount of toxins disappeared from the experimental system. However, this level recovered to almost the same level as that derived from the fed cells of the dinoflagellate, when they were further reared. These facts indicate that a part of the toxins was transformed to an unknown form that could not be detected by chemical analysis, such as high performance liquid chromatography (HPLC). The unknown form of the toxins is gradually transformed to toxins again in the scallop, which can be detected by the chemical analysis, indicating that toxins incorporated to shellfish undergo metabolism in which biological components of the shellfish are

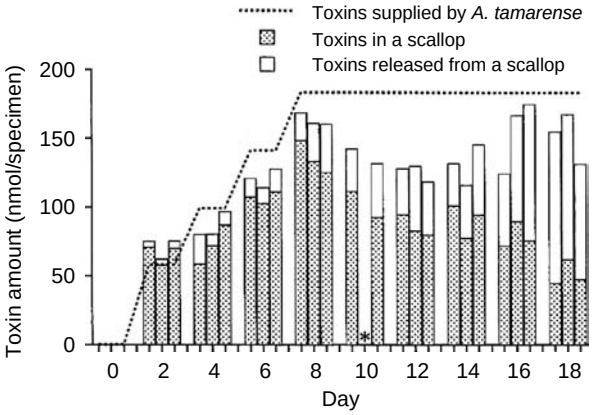


FIGURE 7.3 Change in amounts of PSP toxins accumulated in scallop specimens reared separately in individual tanks. The integrated amounts of toxins released from a single specimen and those supplied to each specimen by feeding *Alexandrium tamarense* cells are also shown. *The sample was accidentally lost during analysis. (From Sekiguchi, K., Sato, Ogata, T., S., Kaga, S., and Kodama, M., *Mar. Ecol. Prog. Ser.*, 220, 213, 2001.)

involved. The unexpected high level of toxins accumulated in the scallop, more than the fed amount of toxins, may show that an unknown form of toxins occurs also in the dinoflagellate fed to the shellfish.

7.3 INTERMEDIATE CONJUGATES IN THE REDUCTIVE TRANSFORMATION OF GONYAUTOXINS TO SAXITOXINS BY THIOL COMPOUNDS

As described above, GTXs accumulated in the shellfish gradually transform to STXs. The rate of transformation is reported to be very slow [17], suggesting that the reaction is not enzymatic. On the other hand, Kotaki et al. [25] reported that a part of GTXs incubated with bacterial cells are transformed to STXs, showing that these bacteria possess an ability to transform GTXs to STXs. These also indicate that the factors involved in toxin transformation occur widely in the ecosystem. Thus, we incubated GTXs in the water-soluble extract of a bacterium (PTB-1) [26] and found that the extract prepared with phosphate buffer often convert GTXs to STXs. However, this transformation activity was not always observed, indicating that the activity of the extract is not stable. Asakawa et al. [27] reported that glutathione (GSH), a biological reductant occurring widely in organisms, transforms GTX1,2,3 to STX. Bacteria are known to possess a membrane-bound enzyme that degradates GSH [28]. The bacterial extract prepared with buffer containing the inhibitor of the enzyme was found to transform GTXs to STXs, showing that the active substance is GSH [29]. In addition, the same reaction was found to occur with other thiols such as 2-mercaptethanol (ME). Interestingly, it was found that conjugates of the toxins and thiols are formed during the reaction. Figure 7.4 shows the changes of the toxin components in the reaction mixtures consisting of GTX1,4 (equilibrium mixture of GTX1 and GTX4) and thiols that were incubated at 70°C under neutral pH. Most of GTX 1,4 disappeared within 2 h, whereas neoSTX increased gradually to 15–35% of the initial amount of GTX1,4. More than half the toxins disappeared from the reaction mixtures. When the reaction mixtures were boiled for 5 min in 1 M ME, almost 100% of the initial amount of GTX1,4 was recovered as neoSTX, indicating the formation of the conjugate of toxins and ME in the process of the reaction. This suggestion is supported by thin-layer chromatography (TLC) analysis of the reaction mixture in which a spot other than GTX1,4, neoSTX, and ME appeared [29]. In a trial to purify the

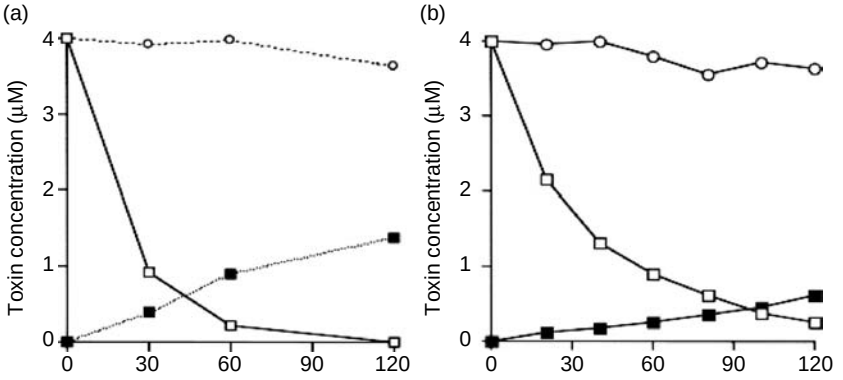


FIGURE 7.4 Change of toxin components in the mixture of GTX1,4 and thiols. GTX1,4 (4 μM) was incubated with 8 mM glutathione (a) or 2-mercaptoethanol (b) in 0.1 M phosphate buffer, pH 7.4 at 70°C. GTX1,4 in the phosphate buffer was used as a control. (From Sakamoto, S., Sato, Ogata, T., Kodama, M., *Fish. Sci*., 66, 136, 2000.)

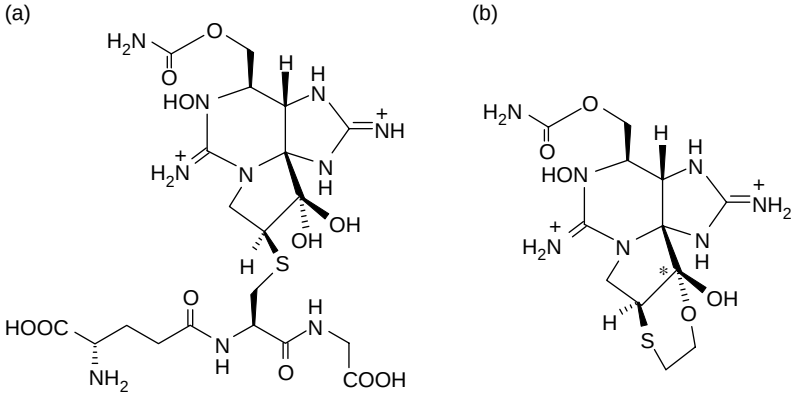


FIGURE 7.5 Structure of the intermediates in the transformation of GTX1,4 to neoSTX. (a) glutathione-neoSTX conjugate, (b) 2-mercaptoethanol-neoSTX conjugate. ∗, Stereochemistry is tentative.

conjugate by chromatography on Bio-Gel P-2, it was isolated in a pure form. The high-resolution fast atom bombardment mass spectrometry (FABMS) and nuclear magnetic resonance (NMR) revealed that the conjugates consist of neoSTX and thiols in which the C11 atom of neoSTX is bound with a sulfur atom of thiols covalently [30]. When GTX2,3 was incubated with thiols, the corresponding conjugates of STX and thiols were obtained (Figure 7.5).

There has been no report on the reductive elimination of sulfate ester by thiols. Thus, the transformation of GTXs to STXs via the intermediate conjugates is considered to be quite a unique reaction. Formation of the conjugates does not occur under acidic conditions. Furthermore, it was confirmed that GTXs-12-ol, analogues in which the gem diol at C12 is reduced, did not react with thiols at all. These facts indicate that the characteristic keto-gem diol structure at C12 of the toxins is essential for the reaction [29]. When the equilibrium mixture of α- and β-*O*-sulfate toxins is incubated with thiols, the α-*O*-sulfate toxin is consumed faster than the β-*O*-sulfate toxin in the formation of the conjugates, showing that α-*O*-sulfate toxin is a "reactive isomer" [30].

Treatment of the conjugates with an excess amount of thiols results in the formation of disulfides and STX or neoSTX, indicating that the transformation of GTXs to STXs is a two-step reaction (Figure 7.6). In the first step, the sulfur atom of the thiols attacks the electrophilic C12 of GTXs

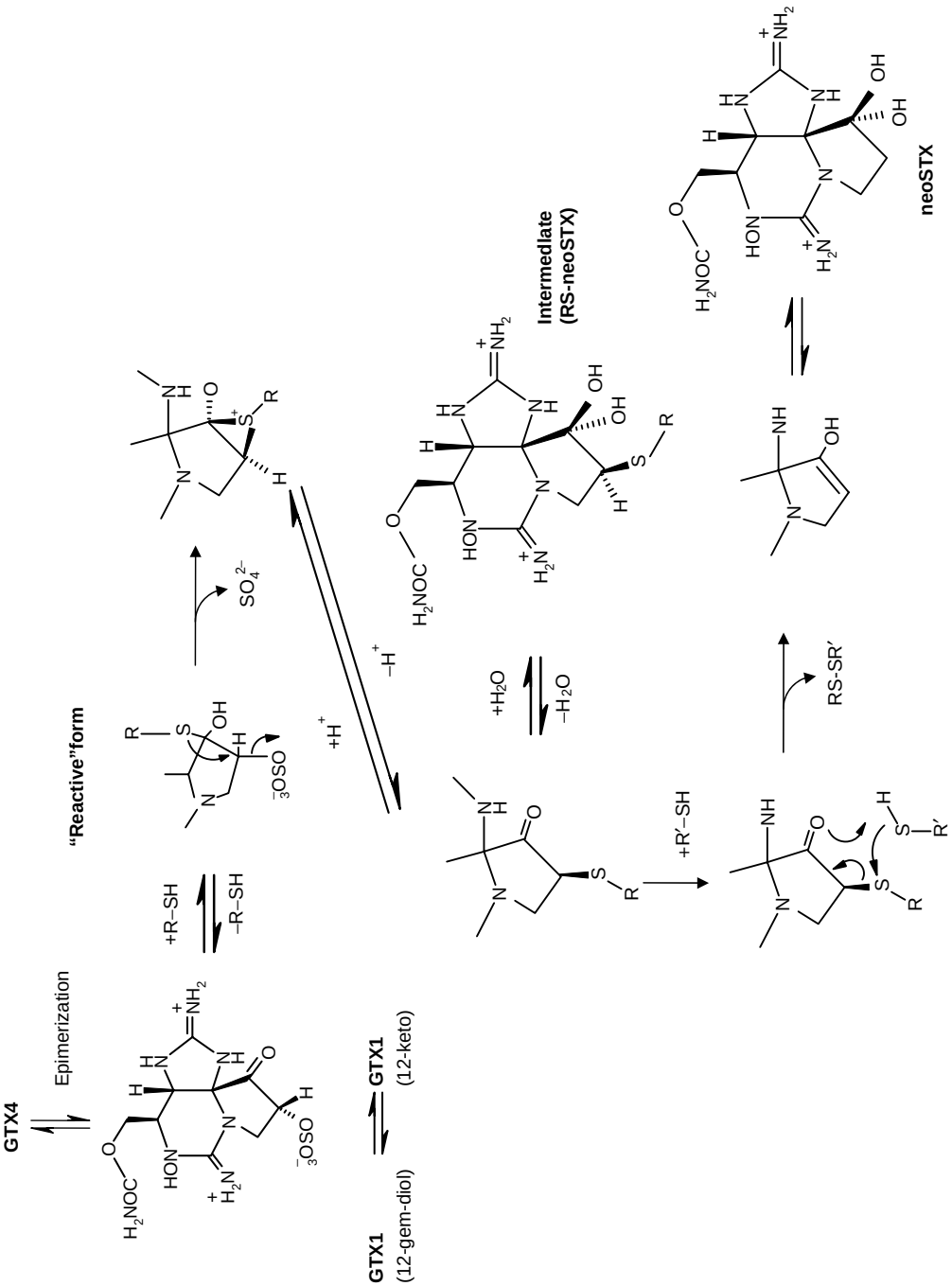


FIGURE 7.6 Mechanism of reductive transformation of GTX1,4 to neoSTX by thiols.

to form a thiohemiketal, followed by the formation of stable thioether conjugates by a 1,2 shift. This is an intramolecular reaction in which GTXs easily react with a low concentration of thiols at a low temperature. The second step is an intermolecular reaction between the conjugates and thiols. Therefore, it requires more energy than the first-step reaction. In order to complete the second-step reaction within a short period, heating with an excess amount of thiols is necessary. Actually, the conjugates are formed even in a solution of GTXs in a low concentration of thiols, such as 1 mM or lower at a room temperature. However, only a trace amount of STXs is obtained under the condition. In contrast, almost 100% of STXs in the conjugates can be recovered when conjugates dissolved in 1 M or higher concentration of thiols are heated for several minutes.

It is noteworthy that thiols do not reduce N1-OH-type toxins. This fact does not coincide with the results of Asakawa et al. [27] in which GSH transforms GTX1 to STX. In their experiments, they used partially purified toxins containing GTX2,3 with a trace amount of GTX1. The reaction mixtures were analyzed by electrophoresis. Probably, most of GTXs in the reaction mixture formed conjugates with GSH and disappeared from the mixture. The amount of neoSTX derived from the conjugate was too low to be detected by electrophoresis.

The conjugate of PSP toxins with thiol compounds, especially with GSH, a biological reductant distributed widely in various organisms, is important in the metabolism of PSP toxins accumulated in various organisms as well as that of toxin-producing ones. The reaction with toxins and GSH seems to be involved in the bioconversion of GTXs to STX in the shellfish, as observed by several researchers [17,21].

7.4 FATE OF PSP TOXINS INCORPORATED TO SHELLFISH FROM DINOFLAGELLATES

As described above, the mechanism for bivalve to become toxic during a bloom of toxic dinoflagellates is hardly explained by a simple food chain mechanism. [1,2]. Similar phenomena are also observed in the feeding experiments of scallop *P. yessoensis* on accumulation and depuration kinetics of the toxins [24]. These observations suggest that the toxins incorporated into the shellfish from toxic dinoflagellate are considered to undergo metabolism in the scallop through which the toxins are changed to derivatives undetectable by chemical analysis such as HPLC. Actually, GTXs react with GSH, a biological thiol, to form conjugates [29,30]. This reaction occurs with various thiols under physiological conditions. Possibly, GTXs bind with proteins via cysteine residues. In order to detect PSP toxins bound with biological substances, specific antibody against STX was developed by immunization of STX-protein conjugate prepared by utilizing STX-thiol conjugate [31]. The obtained antibody showed almost equal affinity to all the toxin components of PSP toxins.

Figure 7.7 shows the light micrographs of the sections of digestive gland of scallop from Ofunato bay prepared for electron microscope and stained with antibody against STX [32]. In the section stained with antibody (Figure 7.7b), clear staining was observed, showing that toxins are binding with some parts of the tissues. The adjacent section stained with toluidine blue (Figure 7.7a) shows that the areas that were stained with antibody are membrane and connective tissues that encircle the nutrient cells. No staining was observed in the membrane and contents of nutrient cells themselves as well as enzyme cells of the diverticula. These results suggest that a part of toxins that accumulated in the digestive gland bind to these tissues.

In an attempt to confirm that toxins are bound with membrane proteins, the membrane suspension prepared from scallop digestive gland was treated with protease to digest the protein moiety and examined whether toxins are released. Figure 7.8a shows the change of toxins that appeared in the membrane suspension of scallop digestive gland that was digested by protease [32]. The level of toxins increased with increase of digestion period, showing that toxins are released from

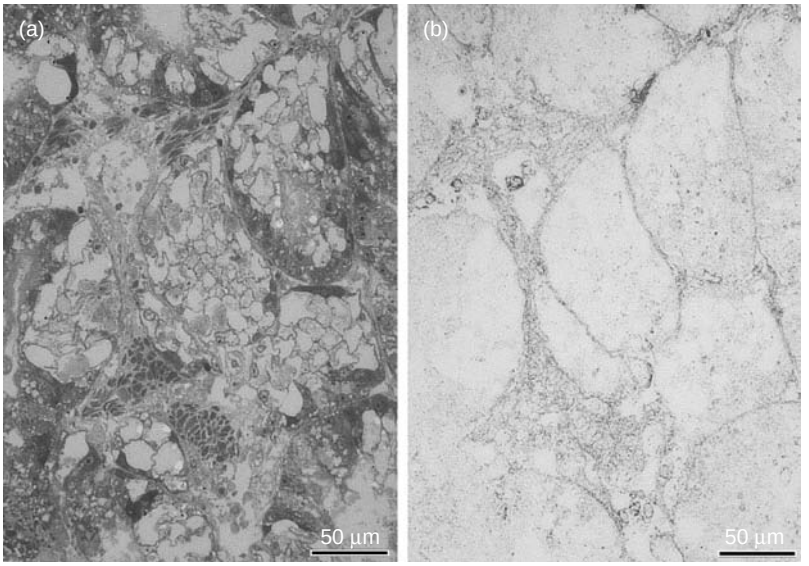


FIGURE 7.7 Micrographs of the digestive gland of toxic scallop from Ofunato Bay with toluidine blue stain, (b) immunostain with silver enhancement. (From Montojo, U.M., Study on paralytic shellfish poisoning in the Philippines, PhD thesis of Kitasato University, p. 139, 2006. With permission.)

membrane when membrane proteins were digested. Toxin profile of released toxins was similar to that of free toxins in the digestive gland in which GTX1,4 are dominant. These results show that a part of toxins accumulated in the digestive gland binds with membrane proteins. Similar results were obtained when the suspension without protease was treated in a same way (Figure 7.8b). Toxin components with almost the same profile were released with incubation time, although the level of toxins is lower. The release of toxins stopped after 3 h incubation. These results suggest the occurrence of protease activity in the membrane-bound proteins. Thus, the same experiments were repeated with the addition of a commercially available protease inhibitor mixture to the suspension (1 tablet/25 mL of the suspension) (complete, Boeriger Manheim). Figure 7.8c shows one of the results. Toxins were also observed in the suspension during incubation, although the amount was very low. These suggest the occurrence of membrane-bound protease that is not inhibited by the inhibitors used. The toxin profile of this case was different from that in the digestion with protease (Sigma). These facts suggest that various types of proteases are present in the membrane proteins, and that toxin components released from proteins are due to the type of protease.

Generally, it has been considered that bivalves accumulate PSP toxins by ingestion of toxic dinoflagellates such as *A. tamarense* when these dinoflagellates bloom in the environment. Toxins transferred from dinoflagellates to bivalves are gradually released from the bivalves. On the other hand, it is often observed in the field survey that the toxin level accumulated in bivalves is not always correlated with abundance of causative dinoflagellates [1,2,33]. It is also pointed out in the regular monitoring on bivalve toxicity, in association with the abundance of causative dinoflagellates, that bivalve toxicity often increases under absence of the causative dinoflagellates. A similar phenomenon was observed in the feeding experiments in the laboratory [23]. These results show that at least a part of toxins incorporated to bivalve via dinoflagellates binds with biological components such as proteins and that the bound toxins are released from the proteins as free forms when the proteins are catabolized. These results suggest that the fate of PSP toxins accumulated in bivalves is more complex than has been considered.

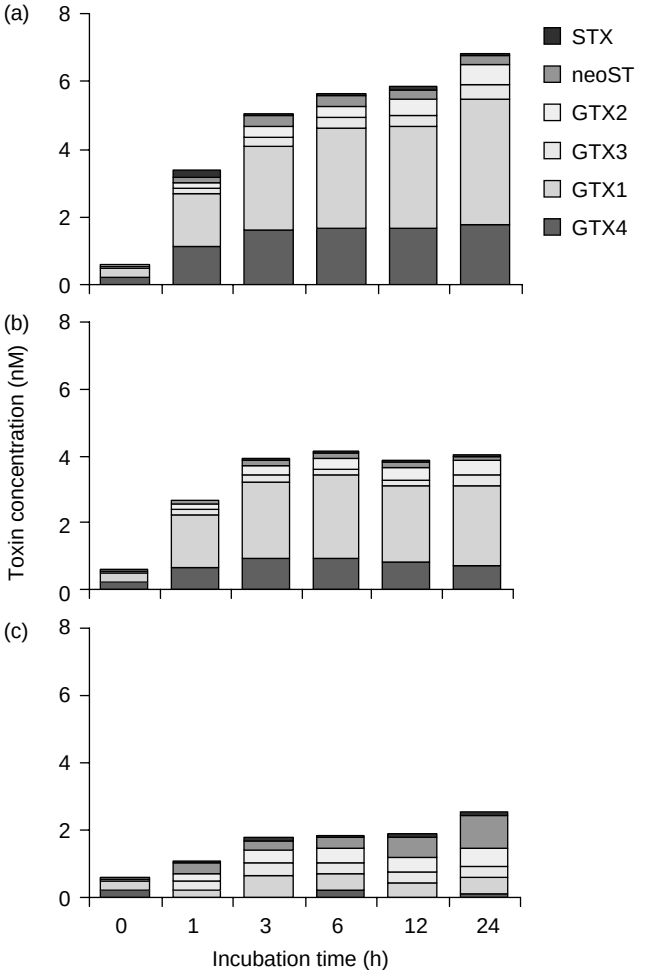


FIGURE 7.8 Release of toxins from membrane protein fraction of scallop digestive gland. (a) Incubated with nonspecific protease, (b) incubated in phosphate buffer, (c) incubated with protease inhibiter mixture. (From Montojo, U.M., Study on paralytic shellfish poisoning in the Philippines, PhD thesis of Kitasato University, p. 139, 2006. With permission.)

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8 Chemical Analysis

Begoña Ben-Gigirey and Adriano Villar-González

CONTENTS

8.1 INTRODUCTION

Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins produced by several species of dinoflagellates. The concentration of PSP phycotoxins in the sea is highest during algae blooms. The phycotoxins are taken up by predators feeding on plankton, either directly as with mollusks or through several tropic levels as in fish [1]. Ingestion of contaminated shellfish or fish can lead to PSP, a serious life-threatening illness. The mechanism of action of PSP toxins is the blockage of sodium channels in excitable membranes, which results in stoppage of the propagation of neuronal impulses [2]. The highly potent and unpredictable nature of PSP toxins necessitates constant monitoring of the toxin content of shellfish in affected areas. PSP monitoring programs rely on relatively intensive sampling and analysis protocols that require the availability of rapid, sensitive, accurate, and precise analytical techniques for the PSP toxins.

Sommer and Meyer [3] were the first to develop a method to determine PSP toxins. Their mouse bioassay method established the basis for the standardized assay [4]. This reference method is internationally recognized for quantifying PSP toxicity and it is used worldwide in PSP monitoring programmes, albeit with some variation in the acceptable regulatory limit for toxicity [5]. The mouse bioassay detects lethal toxicity in a sample, regardless of the chemical structure of the toxins and this may be advantageous from a regulatory standpoint. However, important drawbacks are the need to maintain a large supply of mice, interferences from high salt concentrations, low sensitivity, and variability of ±20%. Moreover, ethical considerations against sacrificing a large number of animals place an increasing pressure on regulatory bodies and researchers to provide alternative methods.

Chemical methods used for the determination of PSP toxins are a fluorimetric assay, high performance liquid chromatography (HPLC) methods with fluorescence detection (using precolumn or postcolumn derivatization), liquid chromatography/mass spectrometry (LC/MS) methods, and capillary electrophoresis (CE) [6,7]. Chemical methods have the advantage of detecting individual PSP toxins. However, their major disadvantage is the lack of standards (i.e., GTX6, C3, C4, dc-GTX1,

dc-GTX4) for the correct identification and quantitation of all the PSP toxins. Immunochemical techniques and enzyme-linked immunosorbent assays (ELISA) based on the interaction of PSP toxins and antibodies have been also developed and used for PSP toxins determination.

Functional assays that measure parameters reflecting the activity of the toxin can also show some potential for PSP toxin determination. Among these are *in vitro* tissue culture bioassays, membrane potential–sensitive methods, and receptor-based assays [6]. Their major advantage is the possibility of relating the parameter being measured with the sample toxicity.

8.2 SPECTROSCOPIC DETECTION

In 1975, Bates and Rapoport [8] described the initial fluorimetric method for the measurement of saxitoxins in shellfish samples. The method was based on the oxidation of the toxin in alkaline solution to fluorescent pyrimidino purins. After acidification, the fluorescence intensity of oxidation products was measured in the solution. The results obtained by this method were compared with the results from the bioassay and the authors indicated that the proposed fluorimetric method was 100 times more sensitive than the existing bioassay. Indrasena and Gill [9] tested three different oxidants for the fluorimetric detection of PSP toxins with the aim of developing a method for saxitoxin (STX) analysis but with similar sensitivity to the *N*-1-hydroxide derivates. After evaluating periodic acid, *tert*-butyl hydroperoxide, and hydrogen peroxide, they reported that hydrogen peroxide was found to be the most convenient and efficient oxidant since the fluorescence can be detected after incubation of toxins at 100°C for 3–5 min. On the basis of fluorimetric method proposed by Bates and Rapoport [8], Gerdts et al. [10] reported a fast fluorimetric assay (FFA) for the detection of STX in natural phytoplankton samples. Despite the fact that the initial fluorimetric method had been replaced by more sensitive methods, they decided to develop a simple and fast assay for the determination of STX with the aim of having a simple tool for monitoring STX levels. In this work, the results obtained by FFA (as total fluorescence) were compared with HPLC results for a number of carbamoyl, decarbamoyl, and *N*-sulfocarbamoyl STXs using commercial PSP toxin standards. The correlation between the results was significant for most of the carbamoyl STXs.

Recently, Kele et al. [11] reported a novel spectroscopic method based on the surface modification of quartz with a coumaryl–aza-crown-6 derivative to detect STX using fluorescence enhancement through photoinduced electron transfer (PET). The authors presented this method as an alternative to mouse bioassay for STX analysis. In the investigations performed by this group, they found that fluorescent sensor molecules that possess a crown ether receptor unit were effective for signaling the presence of STX. These sensing molecules belong to the PET sensor family. Their work with coumarin-based PET sensors has shown that the STX can be detected in buffered aqueous solution in the presence of Na^+, K^+, and Ca^{2+} ions. Moreover, these coumarin sensors showed larger fluorescence enhancement in the presence of the toxin than the anthracyl derivatives [12–15]. Therefore, they reported results with a sensor monolayer that is covalently bound on a quartz substrate and used for STX detection. A bromoalkyl-modified coumaryl-crown derivative was bound on thiol-modified quartz substrate by thioether formation. For the detection of STX, the covalently modified quartz slide was placed under a bifurcated optical fiber and the fluorescence changes in the presence of different STX concentrations were tested (λ_{Exc} = 332 nm, λ_{Em} = 415 nm; Figure 8.1). STX was administered from 10^{-6} to 10^{-4} M concentrations in 0.01 M phosphate buffer. Fluorescence enhancement was observed with a detection limit of 10^{-5} M STX concentration. The authors indicated that the sensitivity of the assay is within one order of magnitude of the mouse bioassay with their experimental design using only one side of the modified slide. However, the developed method has not been tested for the analysis of naturally contaminated samples with the aim of presenting this method as an alternative to the existing methods for the analysis of PSP toxins.

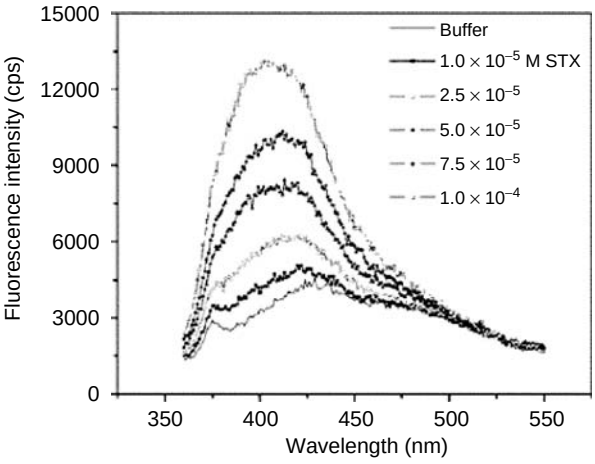

FIGURE 8.1 Fluorescence changes of the self-assembled monolayer in the presence of saxitoxin. (From Kele, P. et al., *Chem. Commun*., 14, 1494, 2006. With permission.)

8.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography methods are widely used to quantify the PSP toxins present in a shellfish sample, but they can also serve to identify the toxin profile of a PSP bloom. PSP toxins have only a weak natural chromophore and must be modified before detection [16]. When oxidized in alkaline solution, they form a purine, which becomes fluorescent in acidic solution [6]. This derivatization reaction can be carried out precolumn or postcolumn, and the purines are monitorized in a fluorescence detector.

8.3.1 Postcolumn Derivatization Methods

The fluorimetric method of Bates and Rapoport [8], based on the oxidation of PSP toxins in alkaline conditions to form fluorescent derivatives, was incorporated into a detection method with the PSP toxins separated in a chromatographic column by Buckley et al. [17]. This method set the basis for the development of a high pressure liquid chromatography with postcolumn reaction system that was subsequently improved to achieve a better toxin separation and adequate sensitivity [18]. Sullivan et al. [19] evaluated its applicability to shellfish toxicity monitoring, by comparing the results obtained by the HPLC method and the standard Association of Official Analytical Chemists (AOAC) mouse bioassay. They found, in general, a good correlation between the two methods. However, C1 and C2 toxins could not be separated and individually quantified. Further improvements and modifications [20] were not able to solve the problems in C1, C2, C3, and C4 toxins coelution, these toxins being difficult to distinguish from coeluting artifacts at the solvent front. Apart from that, the decarbamoyl toxins were not resolved from their respective carbamate analogs [7].

Luckas et al. [21] proposed ion-pair chromatography on a RP-C18 column with octanesulphonic acid in the phosphate buffer eluent and isocratic elution to overcome the problem of dc-STX and STX separation, but this led to interferences in the chromatograms at the retention times of gonyautoxins. Posterior modifications involved the use of the RP-C18 column and two phosphate buffers containing octanesulphonic acid and acetonitrile-tetrahydrofuran (Thielert's method). With a two step-elution system, separation of carbamate and decarbamoyl toxins was possible, and there was a good resolution for NEO, dc-STX and STX, but GTX1 and GTX4 coeluted [1,7]. Yu et al. [22] improved the method and combined two eluents in a step gradient to enable the determination of GTX, NEO, dc-STX, and STX in one chromatographic run. Good separation of GTX1, GTX2,

GTX3, GTX4, NEO, dc-STX, and STX was achieved. The determination of GTX5, GTX6, and dc-GTXs toxins was also possible.

In 1984, Oshima et al. [23] proposed an HPLC method based on the alkaline oxidation of PSP toxins with *tert*-butyl hydroperoxide to produce highly fluorescent derivatives. Since NEO and STX showed stronger basicity than GTX1-6, the two groups of toxins were analyzed under different conditions, each group with a particular mobile phase. Separation between GTX2 and GTX3 was possible, but was unsuccessful for the epimers GTX1 and GTX4. GTX3 and GTX5 were also inseparable. Later Oshima et al. [24,25] described a method that was able to separate almost all the PSP toxins by applying three chromatographic systems, with three isocratic runs, according to the basicity of the three toxin groups (group I: C1–C4; group II: GTX1–4, GTX5 (B1) and GTX6 (B2), dc-GTX1-4; group III: NEO, dc-STX, and STX). Figures 8.2 and 8.3 show chromatograms of several PSP standards obtained with the method developed by Oshima et al. [25]. Franco and Fernández-Vila [26] reported the improvement of this method to adequately resolve PSP toxin profiles in dinoflagellate cultures, mollusks contaminated by such algae, and net samples of phytoplankton from the field. They described a RP-HPLC method for the separation of PSP toxins employing three

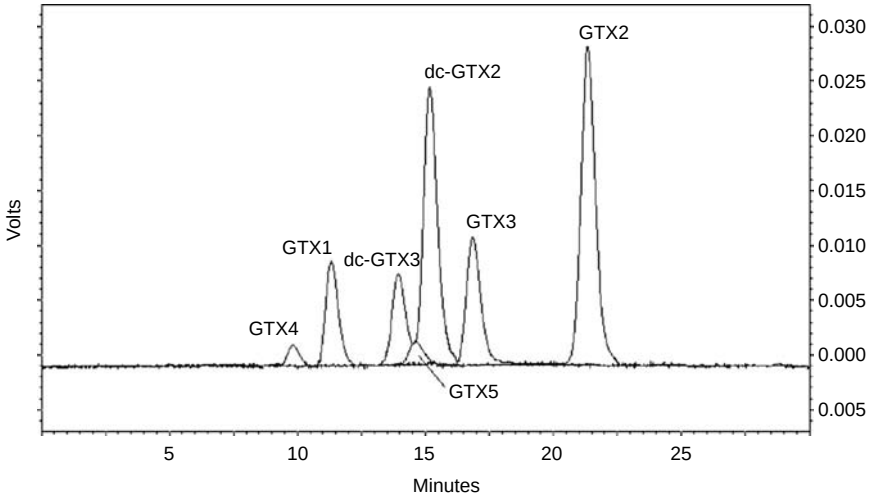

FIGURE 8.2 Chromatographic profile of GTXs and dc-GTXs obtained with Oshima et al. [25] method. (Departamento de Farmacologia, Universidad de Santiago de Compostela, Campus de Lugo, Spain.)

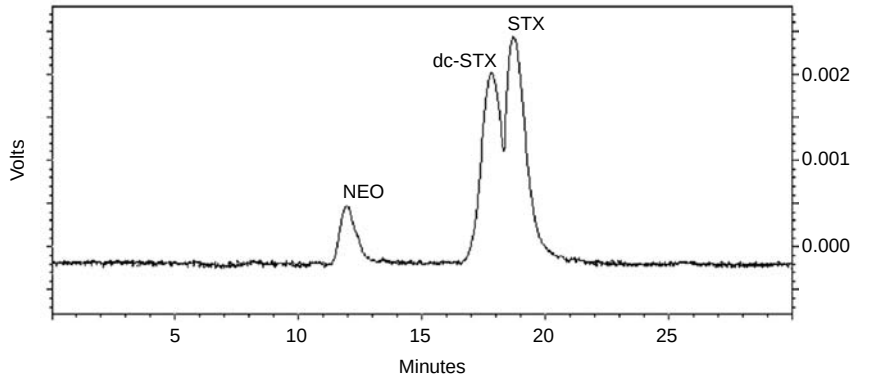

FIGURE 8.3 Chromatographic profile of NEO, dc-STXs and STXs obtained with Oshima et al. [25] method. (Departamento de Farmacologia, Universidad de Santiago de Compostela, Campus de Lugo, Spain.)

isocratic systems, which separates PSP toxins before postcolumn reaction and fluorimetric detection. In contrast to the Oshima's method, Franco's method used acetic acid for sample extraction. They also included a SPE-C18 sample extract cleanup step. Later, Oshima [27] reported further improvements to his system and the inclusion of an extract cleanup procedure. Using this method it is possible to separate most of the PSP toxins; however, GTX5 and dc-GTX3 have been shown to coelute [28]. Nevertheless, the main drawback of the method is that it is time consuming because three chromatographic runs are required for the quantitation of all the PSP toxins in a sample.

Hummert et al. [29] employed a sensitive HPLC method based on ion-pair chromatographic separation for the analysis of the characteristic PSP profiles of *Pyrodinium bahamense* and several strains of *Alexandrium.* The method is comprised of a gradient elution using two eluents in a C18 DB column and postcolumn oxidation with an ammonia and periodic acid solution. The method was able to separate dc-STX, STX, GTX2, GTX3, GTX5, and NEO with good detection limits. In 1998, the same authors developed an effective alternative HPLC method [7]. The method employed three mobile phases and gradient elution to achieve the separation of GTX1, GTX2, GTX3, GTX4, GTX5, GTX6, NEO, dc-STX, and STX.

A single-run HPLC method utilizing ion exchange as the separation mode with a novel mobile phase has been developed by Papageorgiou et al. [30]. It was successfully used to determine PSP toxins, present at low ppb levels, in raw fresh waters and cyanobacterial extracts. The chromatographic system was able to separate C1, C2, GTX1, GTX4, GTX2, GTX3, NEO, and STX. However, the resolution of dc-STX and STX seems to be poorer and an unidentified peak (suspected to be another PSP toxin) was seen to coelute with NEO in a *Cylindrospermopsis raciborskii* extract.

Recently, Diener et al. [31] reported the development of an ion-pair chromatography method that allows the determination of very low amounts of PSP toxins in different matrices of novel food. It also allows comparing the PSP profiles of various algae-based dietary supplements. The PSP toxins are eluted in the following sequence GTX4, GTX1, dc-GTX2, dc-GTX3, GTX2, GTX5, GTX3, NEO, dc-STX, and STX, and its main improvement over previous methods is the lowering of detection limits [31].

A method that uses HPLC coupled to a postcolumn electrochemical oxidation system (ECOS) was described by Boyer et al. [16] and Boyer and Goddard [32]. A coulometric electrochemical cell, placed inline between the column outlet and the fluorescence detector, oxidizes PSP toxins into fluorescent derivatives. The advantages of this system over HPLC with conventional postcolumn reaction system are that it has a simpler instrumental set up, provides information about naturally fluorescent compounds present in the sample, and avoids the problems of instability of the reagents. However, it has drawbacks related to the care and maintenance of the oxidizing electrode. This method is less sensitive to the *N*-1 hydroxy carbamoyl toxins (NEO, GTX1, and GTX4) than to their corresponding STX analogs [16], and was successfully used to analyze cyanobacteria, dinoflagellate, and shellfish samples [32]. By employing electrochemical postcolumn oxidation, Jaime et al. [33] developed a method that can separate the PSP toxins in a single chromatographic run, suitable for the analysis of biological materials. They used anion- and cation-exchange chromatography with an aqueous ammonium acetate mobile phase (two eluents, gradient conditions). The elution order of PSP toxins was C1, C2, GTX1, GTX4, dc-GTX3, dc-GTX2, GTX2, GTX3, NEO, dc-STX, and STX. The toxins could be detected with a fluorescence detector, and also through trough mass spectrometric detection. Table 8.1 shows the chromatographic conditions of several HPLC postcolumn methods.

8.3.1.1 Sample Extraction

An important step for any analytical method is the sample extraction. The majority of these postcolumn derivatization HPLC methods apply, for shellfish samples, an extraction protocol based on the 959.08 AOAC Official Method [4] that uses hydrochloric acid with boiling. This leads to partial hydrolysis of certain PSP toxins (C1, C2, and GTX5) into more toxic analogs (GTX2, GTX3, STX).

TABLE 8.1
Summary of the Conditions for the Separation of the PSP Toxins in Several HPLC Postcolumn Methods

Method and Reference	Column Type and Temperature	Mobile Phases	Injection Volume, Detector λs	Postcolumn Reaction System
Sullivan and Wekell [20]	PRP-1, 15 cm × 4.1 mm, packed with 10 µm polystyrene divinyl bencene resin material At 35°C	A: 1.5 mM each C6 and C7 sulfonate, 1.5 mM ammonium phosphate (pH 6.7) B: 25% acetonitrile, 1.5 mM each C6 and C7 sulfonate, 6.25 mM ammonium phosphate (pH 7.0) Flow rate: 1.3 mL/min Gradient conditions	10 µL λ_{Exc}: 340 nm λ_{Em}: 400 nm	Pump A: Oxidant (periodate), 0.5 mL/min Pump B: 0.75 M nitric acid, 0.3 mL/min Reaction coil: 90°C, volume 1 mL
Oshima [27]	RP C_8-bonded silica gel, 15 cm × 4.6 mm	A: tetrabutylammonium phosphate 1mM (pH 5.8 with acetic acid), for C group B: sodium 1-heptanesulfonate 2 mM in 10 mM ammonium phosphate (pH 7.1), for GTX and dc-GTXs groups C: sodium 1-heptanesulfonate 2 mM in 30 mM ammonium phosphate (pH 7.1)-acetonitrile (100+5), for STX, dc-STX and NEO Flow rate: 0.8 ml/min Isocratic conditions	10 µL λ_{Exc}: 330 nm λ_{Em}: 390 nm	Oxidation with 7 mM periodic acid in 50 mM potassium phosphate buffer (pH 9), 0.4 mL/min Acidification with 0.5 M acetic acid, 0.4 mL/min 10 m Teflon tubing (0.5 mm i.d.) at 65°C in a water bath and at 85°C in a dry oven
Franco and Fernández-Vila [26]	RP-18, 12.5 cm × 4 mm, 5 µm	A: 95% 0.5 mM sodium octansulphonate in 10 mM ammonium phosphate (pH 7.2) 5% acetonitrile, for STX, dc-STX and NEO B: 1.5 mM sodium octansulphonate in 10 mM ammonium phosphate (pH 7), for GTX and dc-GTXs C: 2.0 mM tetrabutylammonium sulphate in 10 mM ammonium phosphate (pH 6.5), for C1–4 Flow rate: 0.8 mL/min Isocratic conditions	λ_{Exc}: 330 nm λ_{Em}: 390 nm	Oxidation with 7 mM periodic acid in 50 mM sodium phosphate (pH 9), 0.4 mL/min Acidification with 0.5 M acetic acid, 0.4 mL/min Teflon coil (10 m × 0.5 mm i.d.) at 65°C

Hummert and coworkers [7]	RP-18, 250 cm × 4.6 mm, 5 µm	A: 98.5% 40 mM ammonium phosphate buffer (pH 6.9) with 11 mM octane sulfonic acid (sodium salt); 1.5 % tetrahydrofuran B: 83.5% 50 mM ammonium phosphate buffer (pH 6.9) with 15 % acetonitrile; 1.5 % tetrahydrofuran C: 98.5% 40 mM ammonium phosphate buffer (pH 6.9) with 1.5 % tetrahydrofuran Flow rate: 1 mL/min Gradient conditions	λ_{Exc}: 330 nm λ_{Em}: 390 nm	Oxidation sol.: 40 mL ammonia solution (25%) and 1.14 g periodic acid in 500 mL water, 0.3 mL/min Acidifying solution: 30 mL acetic acid (100%) in 500 mL water, 0.3 mL/min Reaction conditions: 50°C, 1.0 min (10 m coil),
Papageorgiou et al. [30]	A Source 15Q PE 4.6/100 anion exchange column and two source 15S PE 4.6/100 cation-exchange columns, connected in series	A: 20 mM sodium acetate (pH 6.9 with acetic acid) B: 450 mM sodium acetate (pH 6.9 with acetic acid) Flow rate: 0.8 mL/min Gradient conditions	λ_{Exc}: 330 nm λ_{Em}: 390 nm	Oxidation sol.: 5 mM periodic acid, 33 mM ammonium formate and 33 mM potassium dihydrogen phosphate (pH 10 with sodium hydroxide or pH 7.5 with phosphoric acid), 0.4 mL/min Acidifying solution: 5 M acetic acid, 0.4 mL/min Reaction at 65°C
Diener et al. [31]	C18 (2) 100 Å, 250 cm × 4.6 mm	A: 6 mM octanesulfonic acid and heptanesulphonic acid, 40 mM ammonium phosphate and 0.75% tetrahydrofuran (v/v) in water (pH 7 with phosphoric acid) B: 7 mM octanesulfonic acid and heptanesulphonic acid, 48 mM ammonium phosphate 1% tetrahydrofuran (v/v), and 10% acetonitrile (v/v) in water (pH 7 with phosphoric acid) Flow rate: 1 mL/min Gradient conditions	λ_{Exc}: 330 nm λ_{Em}: 395 nm	Oxidation with 10 mM periodic acid and 550 mM ammonia in water, flow rate: 0.3 mL/min Acidification with nitric acid 0.75M in water, flow rate: 0.4 mL/min Reaction unit with Teflon coil, 15 m × 0.3 mm i.d. (1.0 mL), 50°C

As this process can also occur in the human stomach, this extraction can be considered as a reasonable approach to estimate the sample potential toxicity [34]; but this needs to be further studied. Moreover, the extent of the hydrolysis is dependent on the pH during extraction; hence, fine adjustment of the pH of the extract, before and after heating, is needed to achieve reproducible results. However, some authors [22,26,33] employed acetic acid as the extraction solvent. The main advantage of an extraction with acetic acid is the fact that it leaves the toxin profile of the sample practically intact, since hydrolysis of PSP toxins does not occur.

8.3.2 Precolumn Derivatization Methods

Over the last years, considerable progress has been made in developing HPLC methods with precolumn derivatization as an alternative to the mouse bioassay. In 1991, Lawrence and Ménard [35] developed a prechromatographic oxidation method for the determination of PSP toxins in shellfish. The technique followed an extraction step with 0.1 M HCl (based in the 959.08 AOAC Official Method [4]) and involved the oxidation of the toxins with hydrogen peroxide and sodium periodate, before their separation and fluorescence detection. Several changes in the method improved the separation and quantitation of most PSP analogs [36]. However, an important drawback of this method was the inability to distinguish easily between the sulfocarbamoyl and nonsulfocarbamoyl analogs of the *N*-1-hydroxylated toxins (GTX6 from NEO, C3,4 from GTX1,4) [37], which are very different in toxicity. This could lead to an inaccurate evaluation of the shellfish toxicity.

Later, Lawrence and Niedzwiadek [37] modified the method to improve its performance (repeatability, ruggedness, recovery) for routine regulatory purposes. Earlier chromatographic conditions (gradient and flow) were changed to reduce the analysis time. The oxidation reaction conditions were modified to achieve the best compromise possible for all the PSP toxins, since it has been shown that the optimum pH for the oxidation of individual toxins varies considerably [38]. A matrix modifier (blank oyster extract) was added to improve the yield and repeatability for the periodate derivatization of standards and sample extracts. Finally, the performance of the second cleanup with SPE-COOH cartridges, which enables the separation of the sulfocarbamoyl toxins from their carbamoyl counterparts, was reevaluated.

Recently, the method has been AOAC validated through a collaborative trial [39] and adopted as First Action 2005.06 AOAC Official Method [40]. This method involves the prechromatographic oxidation of PSP toxins with hydrogen peroxide and periodate followed by HPLC with fluorescence detection (FLD). Samples are extracted in duplicate with 1% acetic acid solution (first extraction with heating). The acetic acid extract is cleaned up in solid phase extraction (SPE) C18 cartridges. Then, cleaned extracts are derivatized with periodate and peroxide oxidants before HPLC analysis. If the sample contains C1,2, dc-STX, GTX2,3, GTX5, and STX, these toxins can be directly quantified after SPE-C18. Extracts containing GTX1,4, C3,4 (standard not available), NEO, and GTX6 (standard not available) must be further purified with SPE-COOH cartridges, to obtain three fractions with the separated toxins that undergo periodate derivatization and HPLC-FLD analysis. A schematic diagram of the method is given in Figure 8.4. So far, the method has been validated for the determination of STX, NEO, GTX2 and 3 (combined), GTX1 and 4 (combined), dc-STX, GTX-5 (B-1), C1 and C2 (combined) and C3 and C4 (combined) in shellfish (mussels, clams, oysters, and scallops). Figures 8.5 and 8.6 show chromatograms of standards and a mussel sample obtained with this method. Although the method is not validated for dc-GTX2,3 and dc-NEO, these toxins have been included in them to give an indication of their possible determination.

In 2006, an interlaboratory exercise was organized by the Community Reference Laboratory for Marine Biotoxins (CRLMB) to evaluate its "fitness for purpose" for the Official Control of PSP toxins in the EU laboratories [41]. Eighteen European Union (EU) laboratories took part in the study. The participants had to analyze six bivalve mollusks samples with various PSP toxic profiles. The performance of the participant laboratories in the application of the 2005.06 AOAC Official

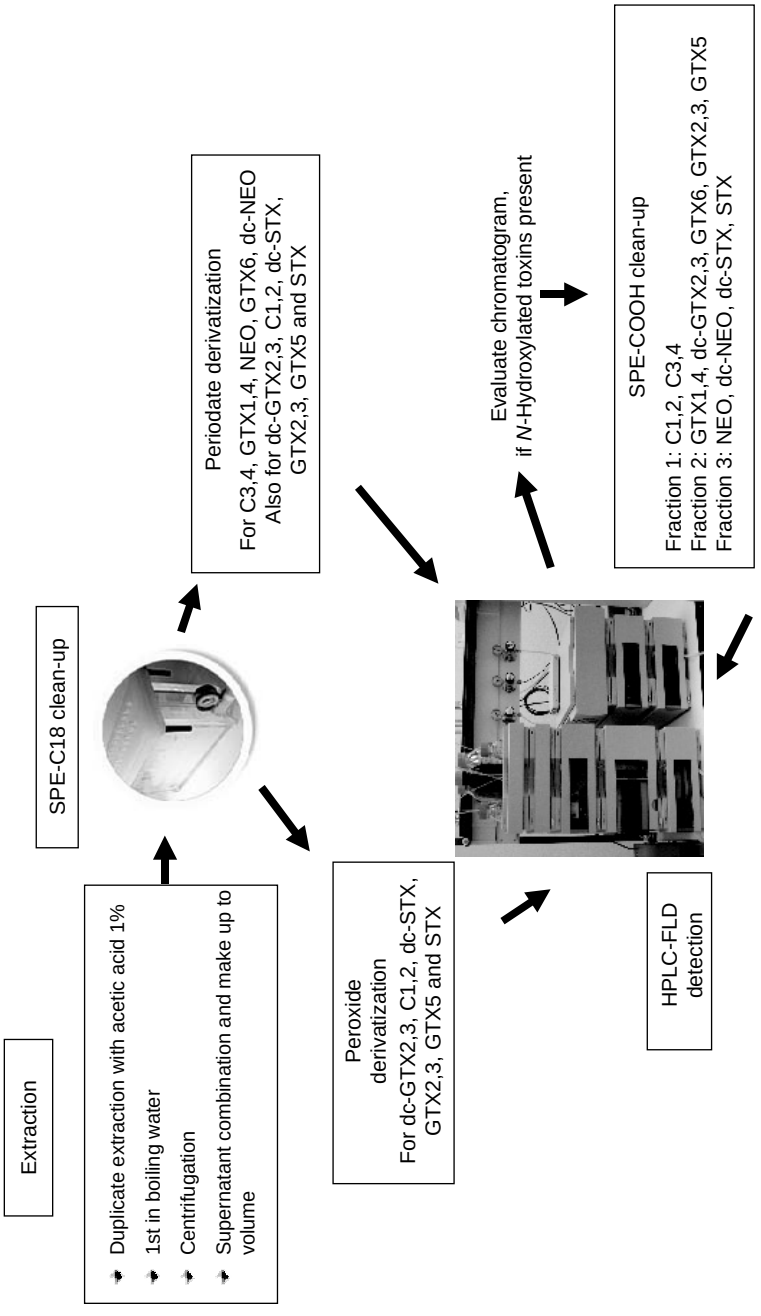

FIGURE 8.4 Schematic diagram of Lawrence et al. (2005) method.

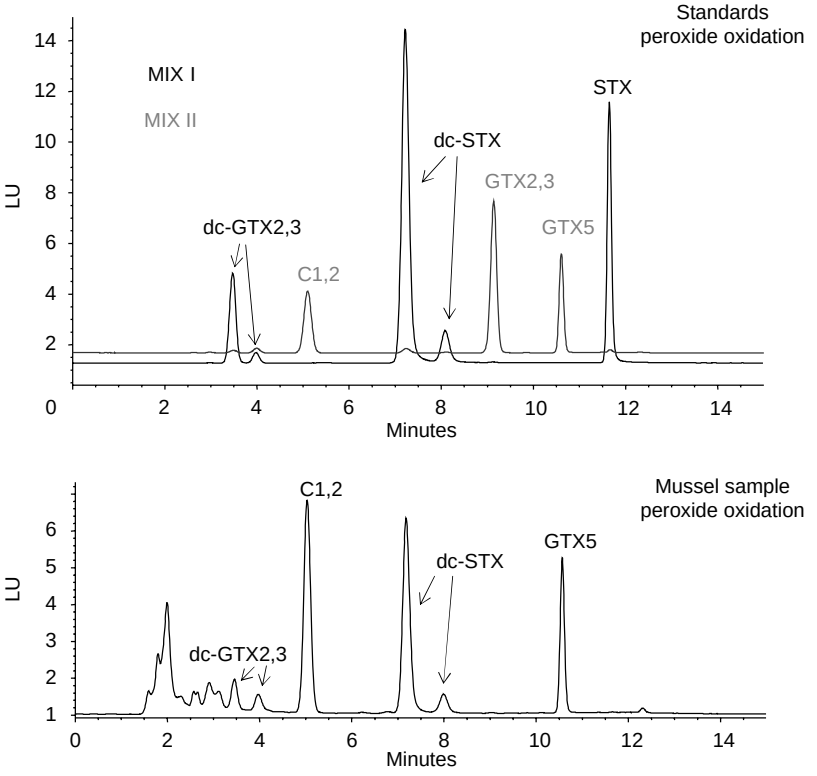

FIGURE 8.5 Chromatographic profile of two standards mixtures and a mussel SPE-C18 cleaned-up extract after peroxide oxidation.

Method was compared with that obtained at the collaborative trial [39]. The results were encouraging for the performance of most laboratories except for sample six. The participants quantified dc-GTX2,3, dc-STX, and GTX5 in this sample, but were not able to quantify GTX-6, C1,2, C3,4, and dc-NEO owing to the lack of standards and coeluting peaks. The performance between laboratories (RSD_R%) for some PSP toxins was similar or better than the one obtained in the Lawrence's collaborative study [39].

Information on drawbacks encountered by participants in the application of this method was also compiled. Some of the problems found are related to all the chemical methods in general and others are inherent to this method.

8.3.2.1 Drawbacks due to the Method Itself

1. The method is not validated for all the PSP toxins (i.e., dc-GTX2,3, dc-NEO); this could be solved by the extension of the method validation to these toxins.
2. Coelution of different peaks during the periodate oxidation (i.e., dc-STX and dc-NEO oxidation peaks; second oxidation peaks of GTX1,4 and dc-GTX2,3; third oxidation peak of GTX1,4 and GTX2,3 peak) can lead to problems in the adequate identification and quantitation of several PSP toxins.
3. The method is time consuming and this could limit its application for handling a large number of samples in a monitoring context.
4. The use of the combined standards mixtures (as indicated in the method, reagents part) can lead to a not-so-accurate quantification owing to the fact that current certified standards of a given PSP toxin have impurities of others. Chromatograms of the standards mix-

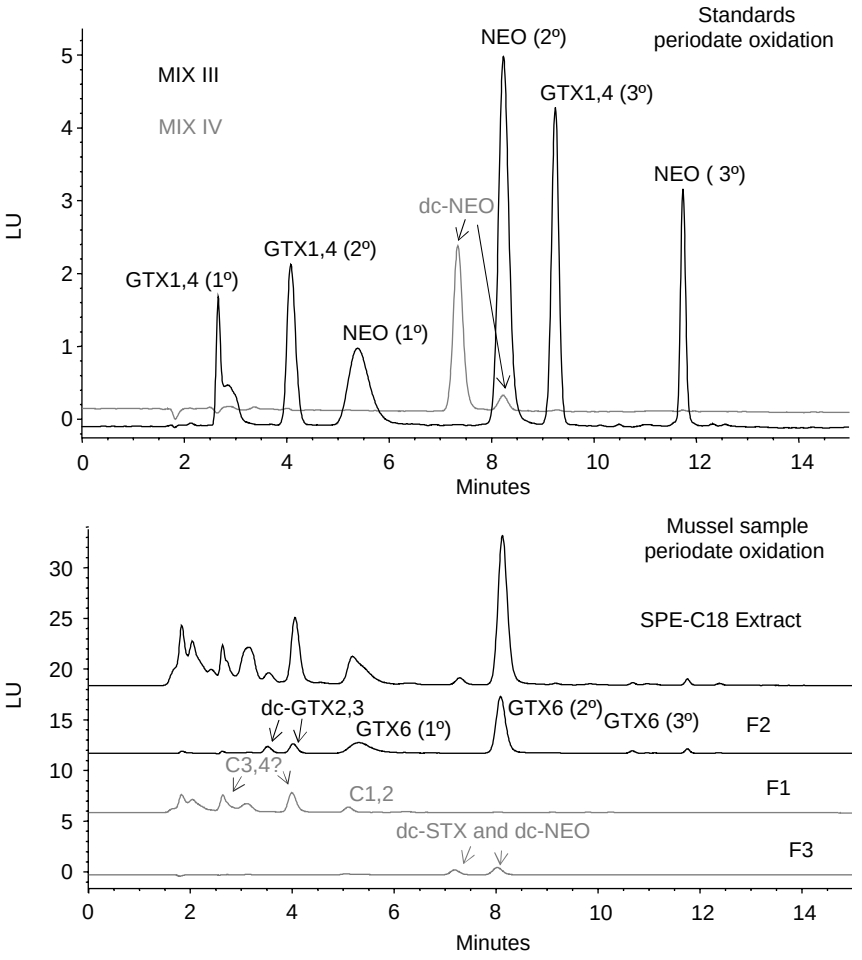

FIGURE 8.6 Chromatographic profile of two standards mixtures and a mussel SPE-C18 and SPE-COOH (three fractions: F1, F2, F3) cleaned-up extract after periodate oxidation.

tures prepared at the CRLMB to avoid this problem are shown in Figures 8.5 and 8.6.

5. In the case of the isomer toxins that coelute but possess different toxicities (i.e., GTX2 and GTX3), there is no internationally agreed criteria with regard to which toxicity factor should be used for calculations of the total sample toxicity.

8.3.2.2 Problems Associated with All Chemical Methods

1. The main problem is the lack of standards (GTX6, C3, C4, dc-GTX1, dc-GTX4) for the correct identification and quantitation of all the PSP toxins. So far, certified reference standards for STX, NEO, dc-STX, dc-NEO, GTX1,4, GTX2,3, GTX5, dc-GTX2,3, and C1,2 are commercially available. Noncertified standards are only available for STX and NEO toxins.
2. Lack of a uniform (internationally agreed) criteria for the application of toxicity factors to convert each toxin concentration into µg eq STX·diHCl/100 g sample.

8.4 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METHODS

Liquid chromatography with mass spectrometric detection (LC-MS), owing to its high sensitivity and specificity, has become an essential research tool in the marine biotoxins field. In the case of PSP

TABLE 8.2
LC Conditions for Analysis of PSP Toxins by HILIC-MS [45]

Column	5 µm Amide-80 (250 mm × 2 mm i.d.)
Column temperature	20°C
Mobile phase	A: water containing 2 mM ammonium formate and 3.6 mM formic acid (pH 3.5)
	B: acetonitrile/water (95:5) containing 2 mM ammonium formate and 3.6 mM formic acid (pH 3.5)
Elution	Isocratic: 65% B
Flow	0.2 mL/min

toxin analysis, several LC-MS methods using electrospray ionization have been developed with the aim of comparing and confirming the results obtained using LC-FLD methods [42–44]. However, these LC-MS methods are based on typical reverse-phase LC methods. Consequently, according to structural characteristics of PSP toxins, ion-pairing reagents are required in the mobile phase to provide sufficient retention of charged PSP toxins. For this reason, these methods are capable of having a poor ionization due to the aqueous mobile phase, as well as suppression of ionization because of the presence of those reagents that improve the separation of the toxins. In 2005, Dell'Aversano et al. [45] reported improvements in PSP toxin analysis using LC-MS by developing a method based on hydrophilic interaction liquid chromatography (HILIC) that is more sensitive than the previous LC-MS methods. This method does not use mobile phase containing ion-pairing reagents, therefore it does not reduce ionization efficiency, and its high content in organic modifier favors the ionization process facilitating the ionization process. To improve the resolution and sensitivity of the analysis, several parameters such as type and percentage of organic modifier, pH, buffer character and concentration, flow rate, and column temperature were tested. In Table 8.2, the LC conditions recommended for HILIC can be observed. The HILIC-MS method is able to determinate simultaneously a wide range of PSP toxins in a single 30 min analysis [45] and its application to the qualitative analysis of plankton and shellfish samples was demonstrated, as well as its ability to detect the presence of new toxin analogues. Figures 8.7 and 8.8 show the chromatograms obtained after analyzing by HILIC-MS *Alexandrium tamarense* and *Mytulis edulis* extracts containing several PSP toxins.

Regarding MS detection, authors described methods performed in positive ion mode using full scan and selected ion monitoring for PSP analysis [42, 43]. The pioneering PSP research in the area of electrospray ionization-mass spectrometry (ESI-MS) detection analysis has been conducted in the group of Quilliam et al. [46, 47]. In their studies, the authors used LC separation before pneumatically assisted ESI. Detection limits as low as 30 pg were obtained for positive ions of several STX analogs [46].

Since then, many other groups have established LC/ESI-MS methods for PSPs with equally good sensitivities, mainly utilizing single or triple-quadrupole mass spectrometers, although some authors have successfully established ESI methods on ion-trap [48], time-of-flight [49], and quadrupole time-of-flight instruments [50]. Despite that, STX and its analogs are well suited to ESI producing abundant fragment ions; only a few mass spectra data of PSP toxins have been reported. In fact, a very limited number of tandem mass spectrometry (MS/MS) analyses of PSP toxins have been reported, and consequently, no detailed information on the dissociation pathways of protonated PSP molecules appears to be available in the scientific literature. Sleno and Volmer [51, 52] reported studies focused on investigating the behavior of the protonated STX and NEO in ESI using different tandem mass spectrometry techniques and reference standard solutions. In these studies, the authors point out that, from a mass spectrometric standpoint, PSP analogs are complicated as they contain a large number of different functional groups within a rather small skeletal structure. For this reason, the product ion mass spectra of the STX and NEO exhibited an unusually rich variety and abundance of species. The authors also indicated that the high degree of similarity between many of the pathways of these toxins would undoubtedly help characterize the chemical structures of other PSPs.

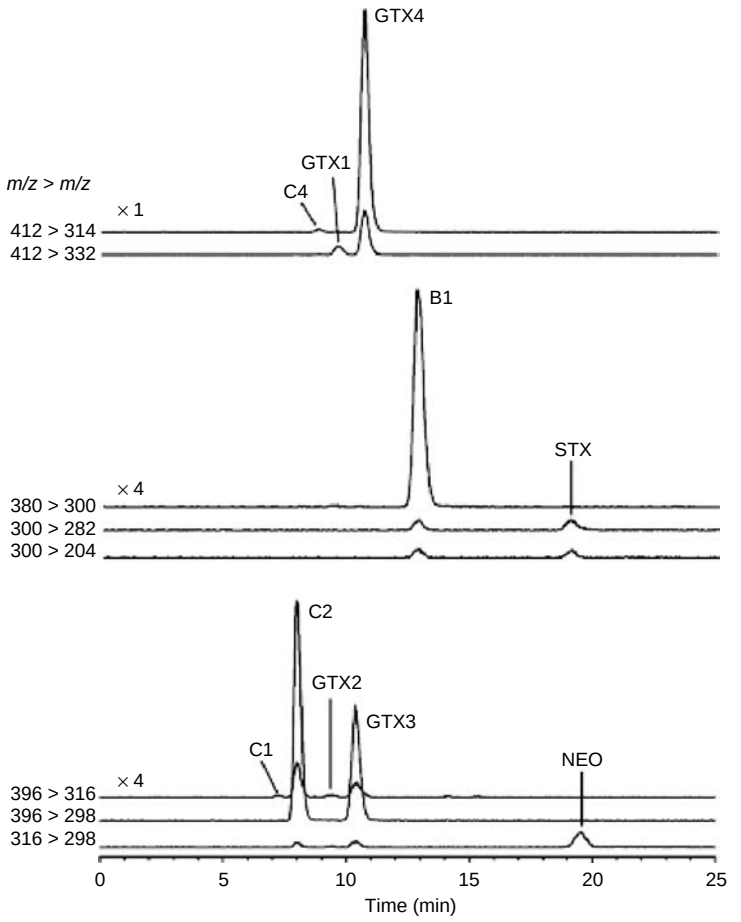

FIGURE 8.7 HILIC-MC analyses of an *Alexandrium tamarense* extract containing various PSP toxins. Experiments were carrried out in SRM mode on API-III+ MS. Separations were carried out on a 5 µm Amide-80 column (250 × 2 mm i.d.), isocratically eluted with 65% B with eluent A being water and B acetonitrile-water (95:5), both containing 2 mM ammonium formate and 3.6 mM formic acid (PH 3.5). Column temperature was 20°C and flow rate 0.2 mL/min. (From Dell'Aversano, C., Hess, P., and Quilliam, MA., *J. Chromatogr. A*, 1081, 190, 2005. With permission.)

In the above-mentioned work, Dell'Aversano et al. [45] also carried out an exhaustive description about the fragmentation pattern of several saxitoxin analogs using tandem mass spectrometry, indicating that for some PSP toxins the mass spectra vary strongly with instrument type. Table 8.3 shows the typical fragment ions monitored for PSP toxin analysis by LC-MS.

8.5 CAPILLARY ELECTROPHORESIS

As a high-resolution separation technique, CE is an alternative method to HPLC for analysis of PSP toxins. Its advantages over other separation techniques include high efficiency, short analysis times, and low sample consumption. CE is based on the different migration of polar substances in an electric field depending on the charge and the size of the molecule. PSP toxins have chemical structures with functional groups that are capable of protonation, so that they produce charged molecules and can be easily analyzed by CE with the exception of C toxin group, which cannot be analyzed by this technique owing to their neutral global charge in acidic media.

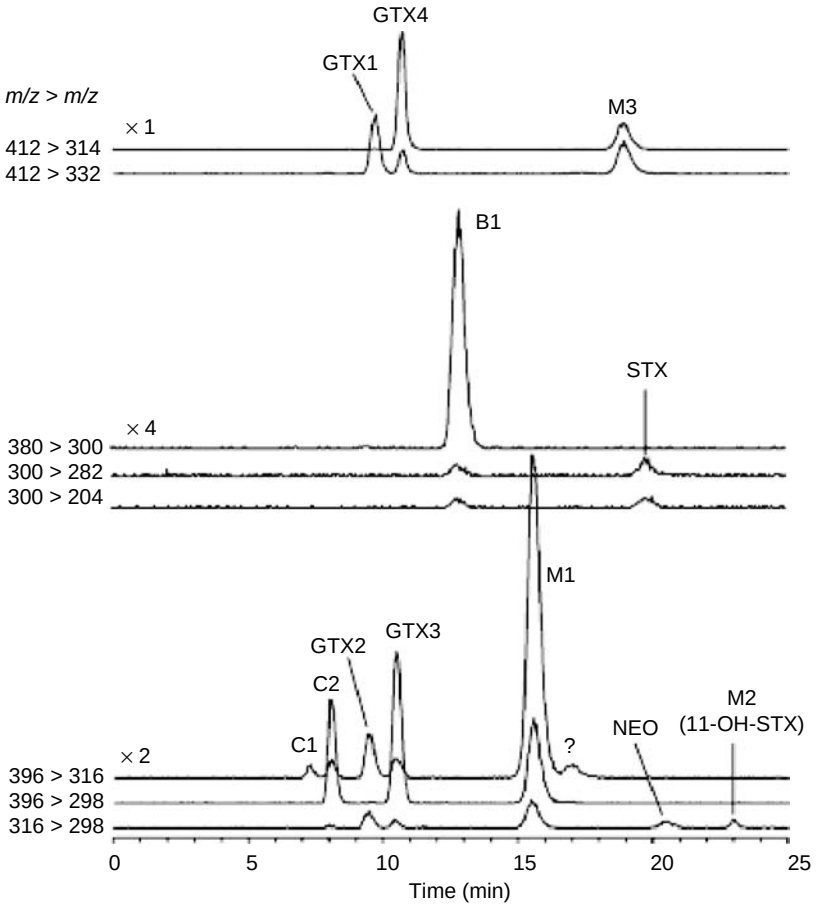

FIGURE 8.8 HILIC-MC analyses of an *Mytilus edulis* extract containing various PSP toxins. Experiments were carrried out in SRM mode on API-III+ MS. Separations were carried out on a 5 μ m Amide-80 column (250 × 2 mm i.d.), isocratically eluted with 65% B with eluent A being water and B acetonitrile-water (95:5), both containing 2 mM ammonium fomate and 3.6 mM formic acid (PH 3.5). Column temperature was 20°C and flow rate 0.2 mL. (From Dell'Aversano, C., Hess, P., and Quilliam, MA., *J. Chromatogr. A*, 1081, 190, 2005. With permission.)

Earlier investigations on CE analysis of PSP toxins were performed by Thibault et al. They developed a CE method with UV detection for the separation and determination of PSP toxins [53]. CE seems an attractive approach for the separation of these toxins; however, not much research has been focused on this area. Consequently, only several references about CE analysis of PSP are available [53–58]. The main drawback of this technique is that CE suffers from low sensitivity owing to the small amount of sample loaded into the capillary. In fact, the handling of the extremely small volumes for injection results in about one magnitude higher limits of detection of the method compared to the HPLC with fluorescence detection.

There are numerous modes of operation in CE. Capillary zone electrophoresis (CZE) is the simplest mode and it was chosen by many authors for PSP toxin analysis. CZE is based on the different migration of solutes in an electric field and toxin separation is performed in narrow-capillaries, which are usually filled only with buffer. Separation occurs because PSP toxins migrate in discrete zones and at different velocities. In this EC mode, selectivity can be readily altered through changes in running buffer pH or by the use of buffer additives.

TABLE 8.3
Characteristic MS Fragmentation Pathways for PSP Toxins

Toxin	Ion	Description	Reference
STX	300	$[M+H]^+$	[2], [4], [10]
	282	$[M+H-H_2O]^+$	
NEO	316	$[M+H]^+$	[2], [4], [10]
	298	$[M+H-H_2O]^+$	
GTX2/3	396	$[M+H]^+$	[2], [4]
	316	$[M+H-SO_3]^+$	
GTX1/4	412	$[M+H]^+$	[4]
	332	$[M+H-SO_3]^+$	
	314	$[M+H-SO_3-H_2O]^+$	
GTX5 (B1)	380	$[M+H]^+$	[4]
	300	$[M+H-SO_3]^+$	
	282	$[M+H-SO_3-H_2O]^+$	
C1/C2	493	$[M+NH_4]^+$	[4]
	476	$[M+H]^+$	
	396	$[M+H-NH_3-SO_3]^+$	
GTX6 (B2)	396	$[M+H]^+$	[4]
	316	$[M+H-SO_3]^+$	
C3/C4	509	$[M+NH_4]^+$	[4]
	492	$[M+H]^+$	
	412	$[M+H-NH_3-SO_3]^+$	
dc-STX	257	$[M+H]^+$	[4]
	239	$[M+H-H_2O]^+$	
	126	N/A	
dc-GTX2	353	$[M+H]^+$	[4]
	273	$[M+H-SO_3]^+$	
	255	$[M+H-SO_3-H_2O]^+$	
dc-GTX3	353	$[M+H]^+$	[4]
	335	$[M+H-H_2O]^+$	
	255	$[M+H-SO_3-H_2O]^+$	
dc-Neo	273	$[M+H]^+$	[4]
	255	$[M+H-H_2O]^+$	
	126	N/A	
dc-GTX1	369	$[M+H]^+$	[4]
	289	$[M+H-SO_3]^+$	
dc-GTX4	369	$[M+H]^+$	[4]
	289	$[M+H-SO_3]^+$	
	271	$[M+H-SO_3-H_2O]^+$	

To improve the sensitivity of the CE system, several approaches of on-column concentration have been developed such as field amplification, sample stacking, and online coupling of capillary isotachophoresis (CITP) with CE. CITP has been selected by some authors as sample concentration technique for the analysis of PSP toxins. In CITP, samples are inserted between leading and terminating electrolytes with higher and lower electrophoretic mobilities, respectively, than the sample compounds. A steady-state configuration is ultimately reached according to the moving boundary principle and all sample zones migrate at the same velocity. The sample concentration in each migrating zone adjusts itself with respect to the concentration of the leading electrolytes.

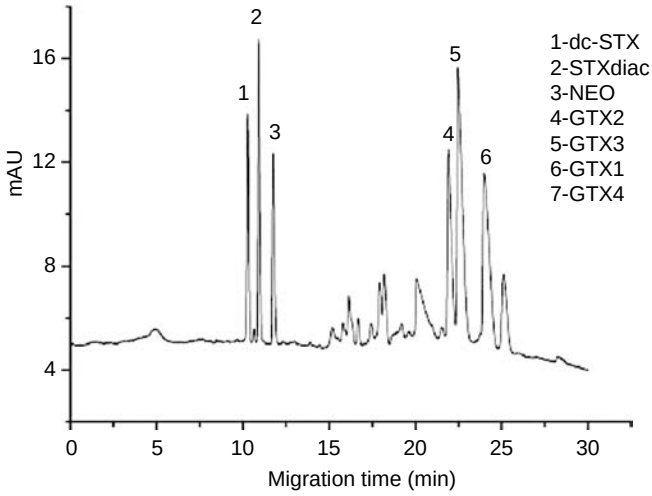

FIGURE 8.9 Electropherogram of seven PSP toxins obtained from CITP/CZE analysis according to Wu et al. [58]. The standard solution used in the experiments contained 9.3 μM dc-STX, 12.0 μM STX, 9.2 μM NEO, 7.4 μM GTX2, 8.9 μM GTX3, 13.2 μM GTX1, and 4.4 μM GTX4. The experimental conditions were 30 mM morpholine buffer (pH 5.3), 14kV separation voltage, 1.2 min duration time in the CITP process and 10 mM formic acid (pH 2.7) as the terminating electrolyte. (From Wu, Y. et al., J. Sep. Sci., 29, 399, 2006. With permission.)

Although CITP has an extremely high stacking power and can produce very sharp bands, the complexity of this procedure deals with the choice of leading and terminating electrolytes [59]. Locke and Thibault [55] performed the analysis of a mixture of eight PSP standards by the application of CITP and discontinuous buffer systems before CZE. They reported a careful choice of leading and terminating electrolytes for the preconcentration step, which has provided an improvement of the concentration detection limit of at least two orders of magnitude over that obtainable using the conventional CZE format.

Buzy et al. [54] described the application of a sample concentration procedure before the analysis of decarbamoyl toxins. The separation conditions developed were found to be entirely compatible with electrospray mass spectrometry, which allowed the analysis of PSP toxins and their decarbamoyl derivates in crude enzyme digests. The products released during the enzymatic digestion were identified using CE combined with tandem mass spectrometry.

In 2006, Wu et al. [58] reported the determination of seven PSP toxins by using CITP/CE with UV detection. They performed the optimization of the separation parameters including duration time and voltage in CITP process, separation voltage, pH and concentration of buffer by using standard stock solutions of STX, dc-STX, NEO, GTX1,4, and GTX2,3. The developed method provided linear response from 1.3 μM to 200 μM for the investigated toxins and the limit of detection (LOD) ranged from 0.1 μM to 0.3 μM. In Figure 8.9, the electropherogram after CITP/CE analysis of a standard mixture containing seven PSP toxins can be observed. This method was applied for determining PSP levels in sample extracts from two algal strains of *Alexandrium tamarense*.

In CE, the most important prerequisite for achieving reproducible separation and high sensitivity is that samples have to be free of electrolytes. Thus, sample cleanup is often required for those samples that normally contain a high concentration of salts.

8.6 ENZYME-LINKED IMMUNOSORBENT ASSAYS

Immunoassay, based on antibodies against saxitoxin, is also used for detection of PSP toxins, and both radioimmunoassay and ELISA have been employed for PSP toxins analysis. Although

radioimmunoassay techniques were developed about the middle of 1980 [60,61], they are rarely used any more. However, several ELISA methods for PSP toxins have been developed using polyclonal antibodies to conjugated saxitoxin [62–66]. The method of Usleber et al. [63] was commercially developed as Ridascreen™ test kit (R-Biopharm, Darmstadt, Germany) and this ELISA kit for saxitoxin is now on the market. The presence of more than 20 STX congeners in varying proportions has posed a problem for all the immunoassays developed for PSP toxins: NEO and GTX1,4 are poorly cross-reactive with the Ridascreen assay. In an intercomparison study with HPLC methods for determining STX in mussels, the Ridascreen ELISA overestimated STX concentration, but underestimated total PSP concentration as compared with the mouse bioassay [67]. However, used as a semiquantitative prescreen for regulatory testing of PSP in mussels and scallops, the ELISA did not yield any false negatives, correctly identified all samples above the regulatory limit of >80 μg STX equivalent/100 g, and detected over 87% of shellfish samples that were below the 40 μg STX equivalent/100 g by mouse bioassay [68–70]. Inami et al. [71] performed the comparison between the Ridascreen kit and the mouse bioassay for saxitoxin analysis in mussels and oysters. They reported that the Ridascreen correlated to the mouse bioassay at 0.849 when used for presence/absence detection, obtaining 2% of false negative and 5% false positive extracts. Recently, Etheridge et al. [72] suggested that, when STX was dominant with some dc-STX and GTX5 present in the samples, there were no cross-reactivity issues with the Ridascreen ELISA, and in this case, the ELISA served as a good indicator of toxicity. However, it is notable that others have found cross-reactivity problems for NEO, GTX1, and GTX4. The authors indicated that overall toxicity determined by this ELISA kit for samples that contain NEO, GTX1, and GTX4 toxins may not be a good estimate.

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9 Paralytic Shellfish Toxins—Pharmacology, and Toxicology Biological Detection Methods

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CONTENTS

9.1 INTRODUCTION

Paralytic shellfish toxins (PSTs) correspond to three main groups of structures: saxitoxins (STX), tetrodotoxins, and μ-conotoxins. These toxins bind to the receptor site 1 of the voltage-dependent sodium channel located in the pore-forming region of the protein, block the influx of sodium ions into excitable cells, interrupt the conduction of action potentials, and impair nerve and muscle excitability. In sufficient amounts these toxins can cause death by respiratory failure, a syndrome known as paralytic shellfish poisoning (PSP). These compounds are toxic at submicromolar concentrations and represent a severe public health risk. By far, most cases of human PSP intoxications are caused by the consumption of shellfish that have accumulated saxitoxins after filtration of toxic marine dinoflagellates. Other activating toxins such as brevetoxins and ciguatoxin also bind to the sodium channel but to different receptor sites (Cestele and Catterall[19]).

The frequency and extension of blooms of PSTs producing dinoflagellates have increased in the last 40 years, and new geographic areas without previous instances of PSP cases have been impacted. Thus, PSTs and several other marine neurotoxins are a current and present hazard to public health (see Ibister and Kiernan[1]). In addition, PSP has presently a significant economic impact in commercial shellfish areas worldwide, including the United States and Canada,[2] Europe,[3] and Chile.[4] Regulatory control of these potentially lethal toxins has relied for approximately 65 years on the mouse bioassay (MBA) developed by Sommer and Meyer[5] as a field test. This rugged bioassay has proved to be a useful regulatory tool to protect public health and shellfish production, in spite of its low sensitivity, semiquantitative nature, false positives, high variability, and increasing ethical concerns about the use of live animals in the test.

In recent years, demand for better methods for phycotoxin management has emerged as a result of increasing public concerns on health risks, action levels, method specificity, and performance. These technical issues have become matters of discussion in international bodies such as the EU Commission[6] and the Codex Alimentarius.[79] As a result, new analytical and functional approaches for phycotoxins have been developed.[7,8] In particular, efforts have been undertaken to design *in vitro* methods alternative to the current MBA for paralytic shellfish poison.[9]

The electrical excitability of cells in all chordates and many invertebrate species is based on the voltage-dependent entry of Na^+ ions into cells through Na channels. This essential process that maintains cellular functionality and viability has been extensively studied.[10,11] However, it is still an open problem how to reduce into practice a simple design that measures directly how sodium currents, as end point signal, report PSP blocking effects (and/or NSP excitatory actions). The same applies to other end points such as cell viability (cell based assays), changes in intracellular fluorescence, displacement of a radiolabeled ligand (RBA), chemical coupling to organic sensors, or antibody recognition.

Functional methods (see Rossini,[12] 2005; Hungerford,[7,8] Suárez-Isla and Vélez[13] for reviews) have not been properly validated to be applied in regulatory work. This review focuses on recent studies, critically analyzing their advantages and limitations, and assays, examining the requirements they have to fulfill to be accepted as methods alternative to the MBA. Analytical and immunological techniques are reviewed in Chapter 10 and are not discussed here. At the time of writing, a new HPLC-FD method for the quantification of saxitoxins (Lawrence et. al.[14]) was approved by the Association of Official Analytical Chemists (AOAC) as an official method to replace the MBA.

It will be clear as a conclusion of this review that there is no shortage of research methods but lack of certified reference materials, QC/QA protocol application, and pending proficiency studies hinder the transformation of good ideas into official methods for practical regulatory work. As stated by Natajaran et al.,[15] "the need continues to exist for a high-throughput functional toxin detection system, which could detect and identify unknown/unexpected toxic chemicals in continuous long term experiments in field conditions" (see references therein).

9.2 SODIUM CHANNEL MOLECULAR TOXICOLOGY: RECENT TRENDS

Sodium channel molecular pharmacology and toxicology is a well established area of ion channel biophysics.[16–20] In recent years the exciting discovery of new genes and sodium channel isoforms[21,22] involved in new physiological functions in nociceptive pathways[23] and providing an extraordinary example of adaptive evolution[24] have been reported. Venkatesh et al.[25] investigated the genetic basis of TTX resistance in pufferfishes by comparing the sodium channels from two pufferfishes (*Takifugu rubripes* [fugu] and *Tetraodon nigroviridis*) and the TTX-sensitive zebrafish. It was found that (a) all three fishes contained duplicate copies of Nav1.4 channels (Nav1.4a and Nav1.4b), (b) several substitutions were found in the TTX binding outer vestibule of the two pufferfish channels. Electrophysiological studies showed that the nonaromatic residue (Asn in fugu and Cys in *Tetraodon*) in domain I of Nav1.4a channels conferred TTX resistance. The Glu-to-Asp mutation in domain II of Tetraodon channel Nav1.4b was found to be similar to that in the saxitoxin- and TTX-resistant Na^+ channels of softshell clams.[26] In conclusion, besides helping to deter predators, TTX resistance enables pufferfishes to selectively feed on TTX-bearing organisms.

In an intriguing paper, Lewellyn[27] presented evidence that STX binds to targets different from the customary voltage-gated sodium channel. Significantly high-affinity binding of STX has been found for calcium and potassium channels, neuronal nitric oxide synthase, STX metabolizing enzymes and two circulatory fluid proteins, namely, a transferrin-like family of proteins and a unique protein found in the blood of pufferfish. These results should be taken into account when explaining other toxicological effects of this toxin group and could help design new functional assays.

9.3 OLD AND NEW METHODS—ASSAYS USING LIVE ANIMALS

The mouse bioassay (MBA) was developed 70 years ago by Sommer and Meyer[5] as part of their pioneering work that related the dinoflagellate *Alexandrium catenella* (Gonyaulax) as a causative agent of PSP (see Wekell et al.[28]). At the time, the identity of the paralyzing toxic compounds was not known and the MBA remained for several years as a "field test" to help health officials manage toxic outbreaks in Alaska and British Columbia.[29,30] Only after a purified saxitoxin dihydrochloride was made available in 1950 was it possible to calibrate the assay, and the AOAC accepted the new version of the MBA as an official method.[31,32] Reasons for the sustained application of the MBA lie in its simplicity of application, relatively low cost, and availability of test protocols to calibrate mice strains to compare operator performance and to perform repeatability and reproducibility studies (National Shellfish Sanitation Program, USA[33]).

The MBA in its current version[9,34] has encountered criticisms during its 50 years of application worldwide, though infrequently. A discussion on the future of the MBA, the risks involved in the use of new technologies, and regulatory acceptance was held at the HABTech Workshop 2003 in New Zealand.[80] As the number of new toxins and other hazards have increased the cost of shellfish testing considerably, it was agreed that the long-term goal should be to eliminate the use of animals, but that a realistic medium-term objective was only to reduce their number. The issue of MBA variability was analyzed by Parks et al.[35] using a CD-1 mouse strain and by Oshima et al.[36] using ddY mice. High salts present in the acidic extracts can decrease apparent toxicity.[37,38] In contrast, high levels of zinc can produce deaths with neurological symptoms[39] that can be interpreted as caused by PSP. Another important variable to consider is the difficulty in the mouse test to assure optimal pH to generate an extract that displays maximal toxicity. It is known that complete hydrolysis of *N*-sulfocarbamoyl saxitoxin derivatives (less potent analogues) is pH dependent and that the AOAC-recommended extraction procedure with hydrochloric acid does not result in extracts that display maximal potency. One of the pending issues regarding revision of the MBA is that in the approved Lawrence HPLC-FD method,[14] the acidic extraction is carried out with acetic acid and not HCl. In addition, binding of saxitoxins to the receptor site is also pH dependent.[40] Thus, the interferences of high salts, Zn, and pH might be explained by the specific modification of STX binding to its receptor.

A group of eight French laboratories[41] were found to be proficient in their use of the AOAC MBA on natural and spiked shellfish samples. However, the same study indicated some limitations in their performance. For example, low recoveries for all levels of toxicity, good relative standard deviations within laboratory (repeatability; 5.4%–9.8%), and rather large interlaboratory variations (reproducibility; 7.8%–39.6%) were observed, being higher for higher toxicity levels. This demonstrates (a) the large variability inherent to the MBA and (b) the need for rigorous interlaboratory proficiency studies adhering to a single protocol and conditions.

Ethical criticism prompted Holtrop et al.[42] to explore the use of concurrent anesthesia to improve animal welfare. Recalibration of the death time versus [STX] curves was necessary and a high variability and severe underestimation of toxicity (50%) by the usual MBA procedure without anesthesia were observed if the original Sommer table was applied.

From the analytical point of view, variability in not the only critical aspect of the MBA but the fact that the minimum amount of saxitoxins that elicits deaths (ca. 0.4 mg STX equivalent/kg) is very near the current regulatory level of 0.8 mg/kg. For example, when naturally incurred samples near 0.4 mg STX equivalent/kg level were tested concurrently with the MBA and an electrophysiological assay,[43] the MBA detected PSTs in only 50% of the cases[44](see Section 9.6).

A serious challenge to MBA (both for PSTs and diarrhetic shellfish toxins, DSTs) has come from the Bundesinstitut fuer Risikoverwertung (BfR, Germany). In a position paper,[45] BfR claims that the MBA is not suitable as a method of reference for DSTs, as stated in the European Commission Decision 2002/225/EC[6] of March 15, 2002. This decision set rules regarding the maximum levels and the methods of analysis of marine biotoxins in bivalve molluscs, echinoderms, tunicates, and

marine gastropods. The decision allows for the use of alternative *in vitro* methods, such as physicochemical, functional, or immunological methods, as substitutes of the MBA (for DSTs toxins), provided that the new methods are validated and an internationally accepted protocol is in place. Article 5 explicitly states that in case of discrepancies, the MBA shall be the definitive reference method. BfR has recently asked EFSA to examine whether the MBA deserves such proposed status, including in this request the MBA for PSTs.

The use of insects as an alternative to the MBA has been explored by some authors. An insect bioassay designed for the detection and quantification of saxitoxins in shellfish samples has been designed and evaluated as an alternative to the MBA.[45a] The assay uses the desert locust, *Schistocerca gregaria* L., a species that has been well studied, is inexpensive, widely available, and that does not pose ethical concerns. This bioassay detects saxitoxins in the range of regulatory importance but has a high rate of false positives. The bioassay has to be refined to decrease its variability and to better control critical points that severely affect its performance. Similar performance defects were mentioned in previous attempts to develop an insect bioassay.[46–48] In conclusion, it seems the MBA is not going to be completely replaced, but it should be possible to drastically reduce the number of test animals, distinguishing between monitoring and statutory testing.

9.4 CELL-BASED ASSAYS FOR PARALYTIC SHELLFISH TOXINS

Functional assays for toxins are based on their molecular interaction with a specific receptor binding site. If the toxin interferes with the normal physiological function of the receptor, toxicity can be measured using as end point the response of a cellular or *in vitro* system before and after toxin addition. As related compounds within a group of toxins will interact with the same receptor site (e.g., saxitoxins with site 1, alfa subunit of the Na channel), a complex mixture of toxins could be detected simultaneously. If the combined effects add linearly and are a simple function of affinities and molar ratios (but cf. Llewellyn[49]), the measured signal will be related quantitatively to toxin(s) potency.[12]

9.4.1 Cytotoxicity Tests

The original format of a cytotoxicity test for neurotoxins was put forward by Kogure et al.[50] to detect saxitoxins and tetrodotoxins. The end point was the direct observation of the percent of cells swelling and dying. Neuroblastoma cells were pretreated with veratridine to open sodium channels and the effect was potentiated by the addition of ouabain to inhibit the Na, K- ATPase. Under these conditions, cells can no longer maintain the asymmetric ion distribution; they accumulate sodium ions and water and swell. The addition of sodium channel blockers, saxitoxins or tetrodotoxins, inhibit this effect and preserve cell survival.[51–54] The effect can be correlated with the concentration of blocking toxins and can also be used with sodium channel–activating toxins, such as brevetoxins and ciguatoxins.[53,55]

End point measurement has been facilitated by including dyes such as crystal violet[56] and the mitochondrial dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl H-tetrazolium bromide).[53] Okomura et al.[57] (2005) made two modifications to the original method reported by Manger et al.,[54] using maitotoxin to reduce the incubation time from 6 h to 15 min and WST-8, a dehydrogenase detecting water-soluble tetrazolium salt to determine target cell viability, simplifying the previous procedure.[52]

9.4.2 Fluorescent Voltage-Dependent Dyes

Cell-based assays utilizing voltage-sensitive dyes are promising as they could be adapted to high throughput formats (Louzao et al.[58–60]). A membrane potential assay[61] based on synaptoneurosomes prepared from mouse brain was evaluated further for its capability for quantitation of saxitoxin-like activity in shellfish tissues and to zooplankton samples. Also, a functional pharmacologically based assay for the brevetoxin group of sodium channel activators was developed[62] using synaptoneurosomes isolated from the brains of CD1 mice. The authors conclude that the assay can detect the depolarizing effect of brevetoxin congeners, PbTx-2 and PbTx-3. Recent studies indicate that efforts

continue to obtain a robust *in vitro* method for PSTs. Mohan et al.[63] have used a NG108-15 neuroblastoma/glioma hybrid cell line to explore the possibility of identifying toxins on the basis of their effect on the shape of intracellularly or extracellularly detected action potentials using a computer model. This cell line has been frequently used for toxin detection and pharmaceutical screening. To demonstrate the applicability of the method for toxin detection and discrimination, the effects of tetrodotoxin (a sodium channel blocker) and tefluthrin (a pyrethroid that is a sodium channel opener) were studied. Authors describe this study as a first step to establish a complex model of NG108-15 cells for quantitative toxin detection based on action potential shape analysis.

9.5 RECEPTOR-BASED ASSAYS

Previous studies (Vélez et al.[43]) indicated a good correlation between receptor-based assay (RBA) results and mouse i.p. toxicity ($R = .97$; $N = 41$). In routine work performed between 1995 and 1997, the practical detection limit was 0.04 µg STX eq./100 g, three orders of magnitude lower than the MBA, providing quantitative data below the detection level of the MBA and thus early warning measurements. Typically, 20–40 samples plus the standard curve, three internal positive controls and 1 negative control could be run per work shift using a conventional liquid scintillation counter.

The woldwide unavailability of tritiated saxitoxin was solved temporarily in 2004, thanks to collaborative efforts among the U.S. Food and Drug Administration, the International Atomic Energy Agency, and the National Oceanographic and Atmospheric Administration. A relatively stable ^{3}H STX is now distributed worldwide by IAEA and is being used in collaborative trials of the PSTs receptor binding assay (RBA). Single laboratory validation of the RBA using new radiolabeled saxitoxins were presented at the Marine and Freshwater Toxins Analysis: First Joint Symposium and AOAC Task Force Meeting in Baiona, Spain, in April 2005. The limit of quantitation of the microplate format assay[64] was found to be 1.2 µg STX equivalent/100 g shellfish (regulatory limit 80 µg/100 g), with an overall repeatability of 17.7% for shellfish extracts run by one analyst on 5 independent days, and a correlation $r = .98$ with the mouse bioassay.

In a parallel study,[65] naturally contaminated shellfish extracts obtained during recent *A. catenella* blooms in Chile were concurrently analyzed by MBA, electrophysiological assay, and RBA using a conventional scintillation counter.[43] A membrane preparation from chick cerebellums was used and the steady state binding characteristics of two batches of tritiated STX provided by two suppliers were evaluated. The RBA protocol was standardized and within-lab performance characteristics including calibration curve limits, precision, and accuracy were established using a conventional scintillation counter. Active concentration of radioactive STX in different batches was measured independently by the electrophysiological assay. The limit of quantitation of the RBA using a conventional liquid scintillation counter was 1.5 µg STX equivalent/100 g shellfish.

Llewelly et al.[66] compared the MBA for PSP toxins with four methods, including high performance liquid chromatography (HPLC) analysis, a commercial cell culture test, and RBAs performed with rat brain sodium channels and saxifilin. It was found that HPLC analysis had good correlation with RBAs and MBA, but HPLC estimated more toxicity than the MBA and RBAs underestimated toxicity as compared with MBA.

Usup et al.[67] characterized the binding properties of six saxitoxin congeners using the displacement assay against 3H- STX and a rat brain membrane preparation rich in sodium channels. EC_{50} values ranged from 4.38 nM for STX to 142 nM for GTX5. Binding affinities correlated well with mouse toxicities (Oshima[68]) in the order STX>GTX1/4>neoSTX>GTX2/3>dcSTX>GTX5. The authors concluded that the rat brain sodium channel RBA could yield a good total toxicity value for STX congeners. However, in this study competition experiments were carried out only with pure and single STX congeners. In this respect, Llewellyn[49] has presented a compelling theoretical work on the combined effects or binary and tertiary mixtures of STX congeners of different toxicities that has been validated in 3H-STX displacement experiments with the rat brain Na channel and a saxiphilin isolated from a xanthid crab. It was found that a highly toxic congener in a mixture can dominate the overall toxicity value in a nonlinear manner. Thus, the assumption that overall toxicity

correlates linearly with specific toxicities and molar ratios may fail for mixtures with a single high potency congener, a finding that has implications for practical testing with the RBA.

In summary, results of preliminary single laboratory validation studies confirmed the utility of the RBA as a screening tool to analyze shellfish for PSP before the application of the MBA, diminishing operational costs, the number of test animals, and providing early warning information to health authorities. However, several critical issues have to be considered for application of the RBA in the future as a complementary tool for regulatory work. In the first place, the sustainable availability of a highly purified reagent has to be assured. Current availability of ^{3}H-STX is supported by international technical cooperation grants. In the near future, the position of the industry toward radiolabeled versus luminescent probes (charge transfer) or surface plasmon resonance-based methods has to be taken into account.

9.6 ELECTROPHYSIOLOGICAL ASSAYS

An interesting approach was reported by Cheun et al.[69] These authors developed a tissue biosensor for STX and TTX that consisted of a Na^+ electrode covered with a frog bladder membrane integrated within a flow cell. Active Na^+ transport that takes place from the internal to the external face was found to be TTX and STX sensitive. This procedure allowed the detection of PSP toxins well below the detection limit of the mouse bioassay but no quality control data was provided in the paper.

Velez et al.[43] reported a very sensitive and functional assay based on the electrophysiological effect (Na channel blockade) of saxitoxins on sodium currents. The assay is directly related to the mechanism of mammalian toxicity and is based on cultured, patch-clamped whole cells (HEK 293, rat skeletal muscle cells) expressing the voltage-gated Na channel. The procedure provides a far more sensitive (three orders of magnitude) alternative to the MBA. In the examination of shellfish extracts the method correlated very well with mouse bioassay ($R = .95$, $N = 30$). Current recordings could be recorded from a single patch for 0.5–2 h, and variations between cells do not compromise assay precision (less than 9% standard error). Since the electrophysiological assay uses cultured cells, it avoids the ethical problems associated with the MBA and does not require the use of radioactive saxitoxin.

The electrophysiological assay for STXs was applied to determine the limit of detection of the mouse bioassay.[44] Naturally intoxicated shellfish samples obtained during a recent PSP outbreak in the island of Chiloé (southern Chile; January–May 2002) were utilized ($N = 157$). All samples higher than 60 μg STX/100 g were detected by mouse bioassay. In contrast, samples between 40 μg and 60 μg STX eq/100 g had a chance of 80.0% of being detected, a percent that decreased to only a 52.6% for the range 30–40 ug/100 g and to 17.2% for 20–30 μg/100 g. This suggested that the operational detection limit for the mouse bioassay should be set at 40 μg STX eq./100 g. It was recommended that for regulatory decisions and in view of the uncertainty, values lower than 40 μg/100 g as determined by mouse bioassay, should be reported as "below the detection limit."

Microelectrode array (MEAs) recordings of cardiac action potentials have been proposed by Natajaran et al.[15] as a high throughput method to evaluate toxicity of the pyrethroids; a-Cypermethrin, Tetramethrin, and Tefluthrin. Confluent monolayers of cardiac myocytes were cultured on MEAs composed of 60 substrate-integrated electrodes. Spontaneous activity of these beating cells produced extracellular field potentials in the range of 100 μV to nearly 1200 μV with a beating frequency of 0.5–4 Hz. Pyrethroids primarily act by causing after depolarizations that prolong the action potential duration in neuronal cells.[70] Intracellular electrophysiological experiments indicated that pyrethroids bind to a specific site on the sodium channels,[71] which leads to an increase in the amplitude of the slowly inactivating Na current[72] and a slowing down of both the activation and inactivation of the sodium channel gating.[73] However, the effects of pyrethroids on cardiac cells have been less extensively studied. It has been observed that some pyrethroids prolonged the duration of action potentials and after depolarizations in isolated guinea pig myocytes in a dose-dependent manner.[74,75] In Najataran's study[15] it was found that all of the tested pyrethroids reduced beating frequency and amplitude. These findings are in general agreement

with previous results and confirm the feasibility of using MEAs in conjunction with primary or line cell cultures as a relatively high throughput toxin detection method. The authors claim sensitivity of this toxin detection method to be comparable to earlier patch clamp studies.

9.7 METHODS TESTED TO BE USED IN REGULATORY WORK

Implementation of new methods in regulatory work faces several critical difficulties. Among them, lack of certified reference materials, QC/QA protocol application and pending validation studies hinder the transformation of good ideas into official methods for practical regulatory work. For example, Inami et al.[76] recently compared two immunoassays and a 5 h neuroblastoma assay with the MBA for presence/absence detection of saxitoxins in shellfish. The study concluded that a reduction in the number of mouse bioassays in the state of California could be achieved, provided that the alternative assays were applied as a screening tool.

In fact, any method has advantages and limitations and the results obtained have to be examined with caution. This is specially the case with cell-based assays. An interesting example is represented by a series of papers on the controversial issue of PSTs production by marine bacteria associated to toxic dinoflagellates. Martins et al.[77] carefully designed a series of experiments and controls to ascertain whether soluble products of bacterial metabolism present in cell-free extracts, corresponded to saxitoxin isoforms. They used a mouse neuroblastoma assay (NMB) for bacteria[51,78] and confirmed that the assay was responsive to growth media used as controls for supernatants and extracts. In addition, a postderivatization HPLC-FD method[68] applied to the extracts showed chromatographic peaks that did not correspond to saxitoxin isoforms. These findings strongly suggest that the NMB assays and HPLC results have to be examined with great caution. The mouse neuroblastoma assay has been used in a number of research papers and their conclusions could now be subject of new interpretations. Thus, it is not surprising that the MNB method continues to be used mainly as a research method.

A transition phase could be envisaged by researches, regulators, and stakeholders in which a set of minimal validation requirements for screening/early warning methods are proposed and agreed on, whereas customary full requirements continue to be enforced for official methods for statutory analyses.

9.8 THE FUTURE

High throughput assay technologies for ion channel drug discovery could be applied for the detection and quantitation of PSP toxins. Automated patch clamp platforms have become commercially available (e.g., PatchXpress® and IonWorks® from Molecular Devices) that in theory could process hundreds of samples per day. Although expensive in the short term, these new machines could offset initial investment if used in extensive PSP monitoring programs. Besides costs, current shortcomings are (a) the quality of voltage control for Na currents that have very rapid onset and inactivation kinetics, and (b) the need for official method validation.

It is clear from the literature review that "the need continues to exist for a high throughput functional toxin detection system, which could detect and identify unknown or unexpected toxic chemicals in continuous long term experiments in field conditions."[15] Microelectrode array recordings may show some promise in some specific fields as they are relatively more rugged, simpler and cheaper to implement than automated patch clamp devices. However, in addition to international validation studies, cell type selection and automated data analysis capabilities of multiple signals will be critical.

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Part V

Diarrheic Episodes: Phosphatase Inhibitors

10 Chemistry, Metabolism, and Chemical Analysis of Okadaic Acid Group Toxins

Paul McNabb

CONTENTS

10.1 INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is caused by a group of polyether toxins based on okadaic acid (OA) **1**. Several analogs of OA are known to occur, including dinophysistoxin-1 (DTX-1) **2**, dinophysistoxin-2 (DTX-2) **3,** and a group of esterified forms of these toxins. Collectively, the group is known as OA group toxins. In the context of this review, DSP does not include other toxins, such as yessotoxin and pectenotoxin that have in the past been labeled (incorrectly) as DSP toxins. Codex has recently asked for clarification on a number of issues relating to marine toxins and an expert review has recommended removing reference to shellfish poisoning symptoms when naming toxin groups (Joint FAO/WHO/IOC ad hoc Expert Consultation, 2004). The OA group found in shellfish is derived from microscopic algae of two genera, *Dinophysis,* and *Prorocentrum.* They exert toxic effects in humans through bioaccumulation in shellfish which are consumed. The OA group toxins

are toxic due to potent protein phosphatase (PP) inhibition. Members of the group are generally described as either "free," meaning unesterified (i.e., OA, DTX-1, or DTX-2) or "esterified." The esterified toxins are formed either within algae (as diol esters and DTX-4, 5, 6) **5, 6, 7** or within shellfish (DTX-3) **4**. It is likely that esterification is a means of detoxifying the free toxin for the organisms that produce it and for the shellfish that ingest and absorb or excrete it.

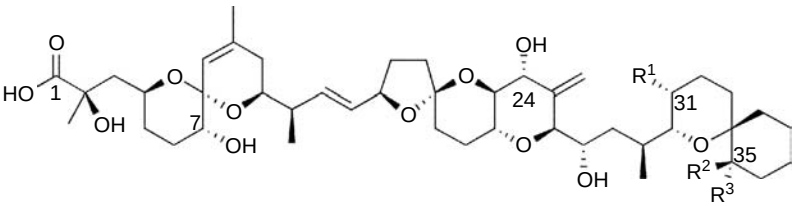
This review summarizes the chemical nature of OA group toxins especially as it relates to toxicity. A brief summary of the chemical nature of PP binding is given. The metabolism of OA group toxins is discussed with particular reference to the biotransformations that occur in shellfish. The chapter concludes with a look at analysis of OA group toxins and some example protocols are included for convenience.

10.2 CHEMISTY AND MODE OF ACTION

The isolation and structural elucidation of OA and DTX-1 was first reported in 1982 (Murata et al., 1982). OA had earlier been identified and was first isolated from the marine sponge *Halichonrira okadaii* (Tachibana et al., 1981), from which OA derives its name. DTX-2 was isolated from toxic mussels in 1992 (Hu et al., 1992a) (see Figure 10.1). Reports of the parent toxins were followed by numerous reports of esterified derivatives from shellfish (Yasumoto et al., 1985; Marr et al., 1992) and algae (Yasumoto et al., 1987; Hu et al., 1992b; Miles et al., 2004; Suzuki et al., 2004) (see Figure 10.2).

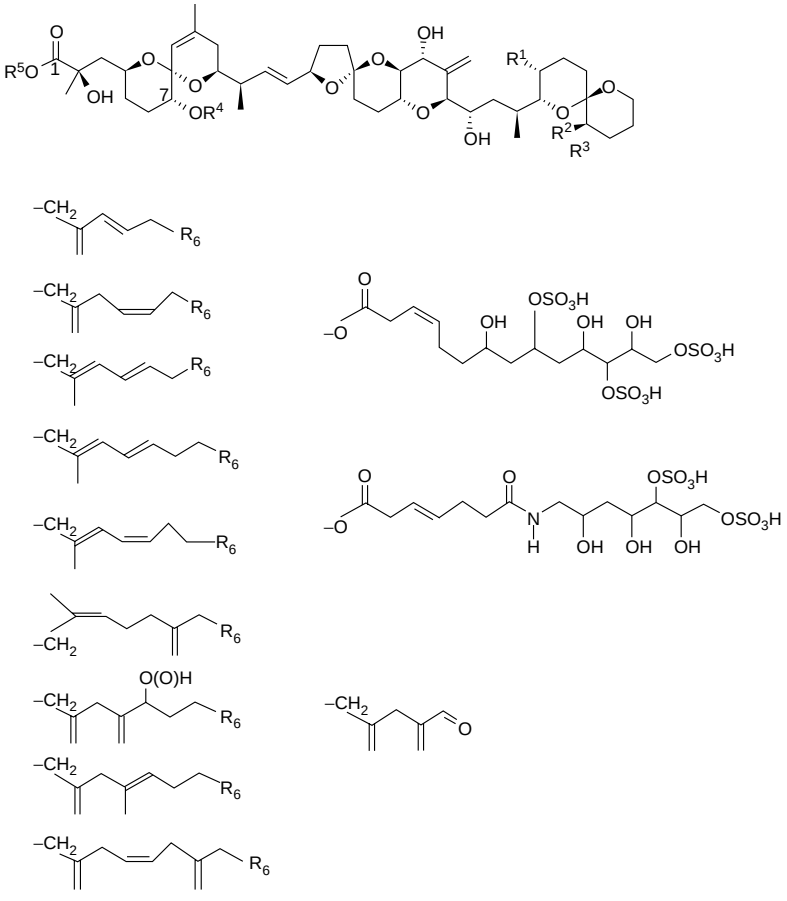
OA is a polyether compound containing a 38 carbon backbone. There are 17 chiral centers within the molecule and 3 spiroketal moieties. Of the many functional groups, the C-1 carboxyl terminus and the C-7 hydroxyl group are both commonly modified by esterification. The two known natural analogs of OA are 35(*R*)-methyl OA (DTX-1) and 35(*S*)-methyl 31-desmethyl OA (DTX-2). The stereochemistry at C-35 has recently been confirmed as *R* for DTX-1 (Sasaki et al., 1998) and *S* for DTX-2 (Larsen et al., 2007). This variation in the stereochemistry may explain why DTX-1 and DTX-2 are each known to co-occur with OA but rarely with each other. It has been hypothesized (Larsen et al., 2007) that a specific methylation enzyme is responsible for adding a methyl group at C-35 and that it will add either an equatorial (DTX-1) or an axial methyl (DTX-2). Despite the complicated nature of the task, three full syntheses of OA have been completed (Ichikawa et al., 1987; Ley et al., 1998; Urbanek et al., 1998) and these are worthy of note.

It is widely accepted that DSP is caused by the inhibition of serine/threonine PPs (for comprehensive review, see Dounay and Forsyth, 2002). In mammalian cells protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are the most common phosphatases; however, OA also inhibits a range of other phosphatases including PP3 and PP4. OA, DTX-1, and DTX-2 are potent inhibitors of both PP1 and PP2A, with over a thousand times more affinity for PP2A than PP1 enzymes (Takai et al., 1992). Table 10.1 shows the relative affinity of OA, DTX-1, and DTX-2 for a range of



		R^1	R^2	R^3
1	Okadaci acid	CH_3	H	H
2	DTX-1	CH_3	CH_3	H
3	DTX-2	H	H	CH_3

FIGURE 10.1 The structure of the key OA group toxins. (Courtesy of Dr Chris Miles.)



		R^1	R^2	R^3	R^4	R^5	R^6
1	Okadaic acid	CH_3	H	H	H	H	—
2	DTX-1	CH_3	CH_3	H	H	H	—
3	DTX-2	H	H	CH_3	H	H	—
4	DTX-3	CH_3	CH_3	H	Acyl	H	—
5	Diol esters	CH_3	CH_3	H	H	A	OH
6	Sulphated DTX-4/5	CH_3	CH_3	H	H	A	B
7	DTX-6	CH_3	CH_3	H	H	C	—

FIGURE 10.2 The structure of esterified OA group toxins. 4–5 are shown as DTX-1 analogues, however the corresponding OA or DTX-2 esters are also known or proposed. (Courtesy of Drs Chris Miles and Michael Quilliam.)

TABLE 10.1
Comparison of PP2A Binding for the Okadiate Group, Relative K_i or IC_{50}, Values Greater than 1 Indicate Binding Affinity Higher than OA

Study	PP2A Source	OA/OA	OA/DTX-1	OA/DTX2
Takai et al., (1992) (K_i)	Rabbit	1	1.6	—
Rivas et al., (2000) (K_i)	Molluscan	1	4.2	—
Aune et al., (2007) (IC_{50})	Human	1	—	0.5
Mountfort et al., (2001) (IC_{50})	Human	1	0.63	—

PP2A enzymes. Experiments have shown that the relative PP2A binding and mouse IP toxicity for OA and DTX-2 is the same (Aune et al., 2007). As the relationship between PP2A binding and toxicity is very strong, PP2A binding is the preferred method to investigate OA group structure–activity relationships.

X-ray crystallographic studies of OA have shown that OA has a secondary cyclic confirmation in which the C-1 carboxyl terminus is hydrogen bound to the C-24 hydroxyl (Tachibana et al., 1981). The known OA analogs (DTX-1 and DTX-2) have modifications at C-31 and C-35 and are likely to assume the same cyclic conformation; therefore DTX-1 and DTX-2 will exhibit similar PP binding affinity. The results of toxicity and PP2A binding comparisons between OA and DTX-1 or DTX-2 confirm this. In addition, there is likely to be some error in the exact quantification of the DTXs as OA is currently the only toxin available as a certified reference material. The free toxins are currently regarded as of equivalent toxicity within mammalian systems for food regulatory purposes and can be regarded as equivalent for most other purposes.

When bound to PP1 (Maynes et al., 2001) and PP2A (Xing et al., 2006), OA retains its cyclic conformation indicating that modifications to the backbone between C-1 and C-24 are likely to affect the PP binding and, therefore, toxicity. Indeed, studies of OA analogs (Takai et al., 1992) have shown that modifications to the hydroxyl groups in the C-1 to C-24 circular region do significantly reduce PP binding affinity, with the notable exception of the C-7 deoxy analog. The lower PP affinity of these analogs is most likely due to disruptions to hydrogen bonds, leading to destabilization of the cyclic conformation of the molecule that in turn disrupts the PP binding. Comparison between PP1 and PP2A shows that a hydrophobic cage that accommodates the hydrophobic end of OA in PP2A is absent in PP1. This provides a rational basis for the higher affinity of OA for PP2A (Xing et al., 2006).

Esterification at the C-1 and C-7 positions of OA, DTX-1, and DTX-2 markedly decrease the binding of the toxins to PP2A. This provides a plausible reason for the existence of the C-1 and 7-*O*-acyl esters in natural systems as these low toxicity forms may protect the host in someway from the PP activity of the free toxin. The 7-*O*-acyl esters have been found to bind less than 3000 times as effectively as the equivalent free toxin and modification to the C-1 terminus (methylation or decaboxylation) totally destroys binding. It is noteworthy that the C-7 deoxy analog does retain significant activity and therefore modification at this site (e.g., esterification) may not interrupt the cyclic conformation but binding may be affected by steric interactions depending on the nature of the side chain. Indeed, the fact that the C-7 hydroxyl is free from intramolecular interactions might be the reason this position is frequently subjected to esterification. The potential for hydrolysis of these 7-*O* acyl esters *in vivo* is known (Garcia et al., 2005) and, therefore, determination of the ester content is important when testing samples for DSP; however, it is also important to distinguish between the toxicity derived from PP inhibition by the free toxin and the potential for toxicity that is found with the esterified toxins.

The macrocylic secondary structure of OA (and DTX-1/2) arising from its primary structure determines the toxicity of OA group toxins. Recent clarification of the stereochemistry at C-35 is important but does not change the conclusion that OA, DTX-1, and DTX-2 can be regarded as equivalent in terms of their structure–activity relationships. Although the ester forms of OA group toxins are common in nature, they are not toxic as they have no PP2A binding affinity; however, they may become toxic if the free toxin is released through hydrolysis of the ester bond. The importance of analyzing the ester forms is discussed shortly.

10.3 BIOSYNTHESIS AND METABOLISM BY SHELLFISH

The esterified OA group toxins can be classified into two groups. DTX-4 and DTX-5 compounds are C-1 esters that have long polyhydroxylated and sulfated aliphatic side chains ester bound to a shorter "diol" linkage that is in turn esterified to the carboxyl group of OA (DTX-1 or DTX-2). 7-*O*-acyl esters, collectively known as DTX-3, have long-chain fatty acids (C14-C22) bound to the

C-7 hydroxyl (7-*O*-acylation). The C-1 esters are formed within algae and the 7-*O*-acyl esters are formed within shellfish. There are many reports of not fully characterized variations to this general scheme and for most purposes, it can be accepted that when an OA diol ester has been reported, a DTX-1 and DTX-2 ester might also exist. For the purpose of simplicity, many, but not all, the elucidated OA group toxin structures are shown in Figure 10.2.

10.3.1 Biosynthesis Environmental Fate

The biosynthetic pathways that lead to the formation of OA group toxins almost certainly involve poyketide synthases; however, the details of the biosynthetic pathway have not been fully elucidated (Daranas et al., 2004). The evidence from isolation of OA group toxins using algal culture (*Prorocentrum lima*) suggest that the primary biosynthetic products are water-soluble polysulfated esters (DTX-4 and DTX-5) (Hu et al., 1995a,b). DTX-4 makes up approximately 80% of the OA group toxins found within cultured *P. lima* cells (Quilliam et al., 1996), with the remaining being free toxin. These polysulfated OA group toxins are subjected to partial hydrolysis by intracellular and extracellular enzymes (half life ca. 10 min) to produce diol esters (Windust et al., 2000). DTX-4 is, however, stable in sterile solution. Algal samples that are not carefully extracted may appear to contain only free toxins and/or diol esters, but careful pretreatment by heating to destroy esterase activity can facilitate the extraction of intact DTX-4 or DTX-5 (Quilliam et al., 1996). Conversion from the sulfated to the diol esters is supported by the fact many new compounds have first been isolated as diol esters and subsequently isolated as a polysulfate esters (Cruz et al., 2006).

The isolation of diol esters DTX-4, DTX-5, and DTX-6, has primarily been accomplished from cultures of *Prorocentrum* spp. (Yasumoto et al., 1987; Hu et al., 1992b; Quilliam et al., 1996; Suarez-Gomez et al., 2001; Suarez-Gomez et al., 2005) and a wide range of side chains and analogs have been identified (Figure 10.2). The hydrolysis of the diol ester to free OA appears to occur at a much slower rate (half life ca. 2 days, Windust et al., 2000). Recent evidence from wild harvests of *Dinophysis* spp. (Miles et al., 2004; Suzuki et al., 2004) suggests that similar pathways exist in this species. Two diol esters have been identified but as yet, no polysulfated DTX-4 type compound has been isolated.

Although many detailed studies have been completed on cultured *Prorocentrum,* there are few references to the relative amounts of extracellular toxins. We know that OA group toxins originate in algal cells as sulfated analogues and the levels of free toxins within the cells is very low (Quilliam et al., 1996). The esterified toxin is processed by esterases, then exported from the cell as the free toxin or the diol ester. The diol ester toxin can more easily cross cell membranes and may be an important intermediate in OA transfer (Windust et al., 1997). Further processing of the diol ester by cultures of diatoms has been demonstrated to involve oxidation of the diol side chain (Windust et al., 2000), and oxidized products have also been detected in *Prorocentrum* cultures (Suarez-Gomez et al., 2005). The only study of natural populations has been of *Dinophysis acuminata* in New Zealand (MacKenzie et al., 2004). The results of OA and DTX-1 analysis of cell free water filtrate and the particulate (cellular) fraction showed that about half the toxins were found in the extracellular filtrate. In the New Zealand study, the cellular fraction was not heat treated and we can only guess that a large portion of the OA and DTX-1 found may have originally been present within the cells as a sulfated analog. These processes are summarized in Figure 10.3.

The biological function of the exported toxins is not known. The most common hypothesis to explain the presence of OA group toxins within algal cells is that they form a chemical defense against predation or that the toxin confers an advantage in the competition for resources (Wright and Cembella, 1998). The finding that large proportions of the toxin are released into the surrounding seawater mean that future research into the environmental interactions and fate of the toxins is important.

10.3.2 Shellfish Uptake and Metabolism

One of the primary reasons to study OA group toxins is that they cause human illness through shellfish poisoning events and, therefore, the fate of the OA group toxins within shellfish is of

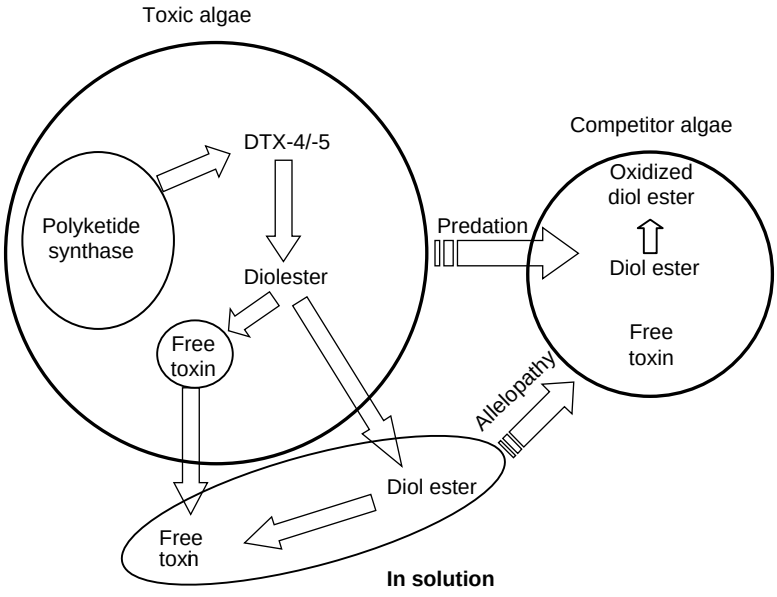


FIGURE 10.3 Proposed pathways for OA group toxin metabolism within algae. Enzymatic processes are shown by block arrows and striped arrows represent algal interactions.

intense interest. The fate of the algal OA group toxins (diol esters, DTX-4, and DTX-5) within shellfish has not been extensively studied and only diol esters have been observed in shellfish contaminated with OA group toxins. It is likely that they may only be short lived and found in low concentrations within shellfish. Scallops fed *P. lima* were found to contain free toxins and diol esters in similar proportions to the algae (Bauder et al., 1996); however in Bauder study no C-7 ester analysis of the shellfish was completed and it is possible that the proportion of diol esters was minor compared to the untested 7-*O*-acyl esters. When a purified diol ester was incubated with a crude extract from shellfish (*Perna* spp.), rapid hydrolysis to free toxin was observed (Miles et al., 2006). This confirmed the long-standing assumption that rapid hydrolysis to free toxin occurs within the shellfish and that the shellfish then esterifies the free toxins to the observed 7-*O*-acyl esters (Yasumoto et al., 1985; Marr et al., 1992). Shellfish that are fed algae that contains only free DTX-1 have been shown to convert the free toxin rapidly into C-7 esterifed toxins (Suzuki et al., 1999). We know that free OA group toxins cause DSP but the C-7 esterified toxins that are known to exist in shellfish may also be hydrolyzed to release free toxin and cause DSP (Garcia et al., 2005). The C-7 esterified toxins, therefore, need to be considered during analysis. It is noteworthy that traditional mouse bioassay methods (not considered in this review) used to protect shellfish consumers from DSP do not detect DTX-3. DTX-3 is a slow-acting toxin with a higher mouse intraperitoneal (IP) LD_{50}, most likely owing to the fact the DTX-3 must be hydrolyzed to free toxin before exerting its effect; however DTX-3 has been shown to possess almost equal potential to cause DSP and should not be excluded from any assessment of DSP risk (Yanagi et al., 1989).

Analysis of a wide range of shellfish species reveals that the fatty acid component of the 7-*O*-acyl esters is highly conserved across shellfish species (Table 10.2). The most abundant ester is always the C-7 palmitoly ester, being close to 50% of the total C-7 ester component. Vale identified fatty acid esters (not confirmed as DTX-3 type) within algal samples and the potential for these to occur, especially within *Dinophysis* samples, warrants further investigation, as does the possibility of C-1 (neutral) esters in shellfish.

In 2002, we completed a review of the proportions of free toxin and esterified toxin found in shellfish, as reported in the literature and from New Zealand shellfish monitoring (McNabb and

TABLE 10.2
A Combination of 11 Sets of LC-MS Data for OA, DTX-1, and DTX-2 Esters in Mussels, Clams, Cockles, and Scallops from Portugal, Ireland, Japan, and New Zealand

Acyl Group	Mean (%)	Standard Deviation (%)
C14:0	13	7
C16:0	50	9
C16:1	11	5
C18:1	5	2
C18:2	5	4
C18:3	2	1
C20:5	6	2
C22:6	11	7

Note: Suzuki et al., 1999 and Unpublished, Vale 2006a, McNabb Unpublished.

Holland, 2002). In general, *Mytilus* spp. (blue mussels) tended to have the lowest proportion of esterified toxin of any shellfish species tested and it is rare to find more than half the OA group toxin content of blue mussels esterifed, whereas, for most other species of shellfish, it is common to find 90% or more of the OA group toxins as esters. Limited time series data for *Mytilus galloprovinciallis* in New Zealand suggest that the esterified portion is highest as toxins accumulate in the shellfish early in a bloom cycle. We hypothesized that the esterification pathways within *Mytilus* spp. became saturated leading to a lag in the build up of free toxin. This mechanism also explained the relatively low proportion of esters in this species. High initial levels of esterifed toxins were also recorded recently in Portugal (Vale, 2006b) from the clam *Donax trunculus*, although in this case the esterified to free toxin ratio was around ten. Vale also showed that the elimination of esterified toxins was faster than that of free toxins. The rate for the breakdown of DTX-4 and diol esters by lysed algal cells is very rapid (Quilliam et al., 1996) and the rate of formation of 7-*O*-acyl esters also proceeds quickly (Suzuki et al., 1999, 2005); therefore an elimination mechanism that involves rapid C-7 esterification followed by further metabolic breakdown of OA group toxins within the shellfish may be separate from another slower, but possibly related, mechanism that removes free toxin. The initial rapid build up of esterified toxins may be due to delayed upregulation of enzymes that further process the esterified toxin and eliminate it. It should also be remembered that only 2–12% of the toxins are absorbed by shellfish (Suzuki et al., 2005a) and most are immediately excreted. This proposed scheme is illustrated in Figure 10.4.

10.4 ANALYSIS

Marine algal toxins have been documented to cause human poisoning for thousands of years and in an attempt to prevent these illnesses, various local traditions have been developed. There is also evidence that some marine mammals have sophisticated methods to avoid poisoning and can detect toxic shellfish before they consume them (Kvitek et al., 1991). Throughout the twentieth century, methods of laboratory analysis were developed in order to protect humans from the harmful effects of the OA group toxins. The tests were often on the basis of sacrificial animal bioassay (usually mice) and initially developed without knowledge of the chemical nature of the toxicity. Under these challenging conditions, some surprisingly effective tests have emerged; however in the case of DSP toxicity, the lipophilic nature of the toxic component has resulted in the common use of a number of related (but not identical) bioassays that are subject to numerous interferences. Interferences range from common free fatty acids (Suzuki et al., 1996) to other algal toxins such as yessotoxin (Murata et al., 1987).

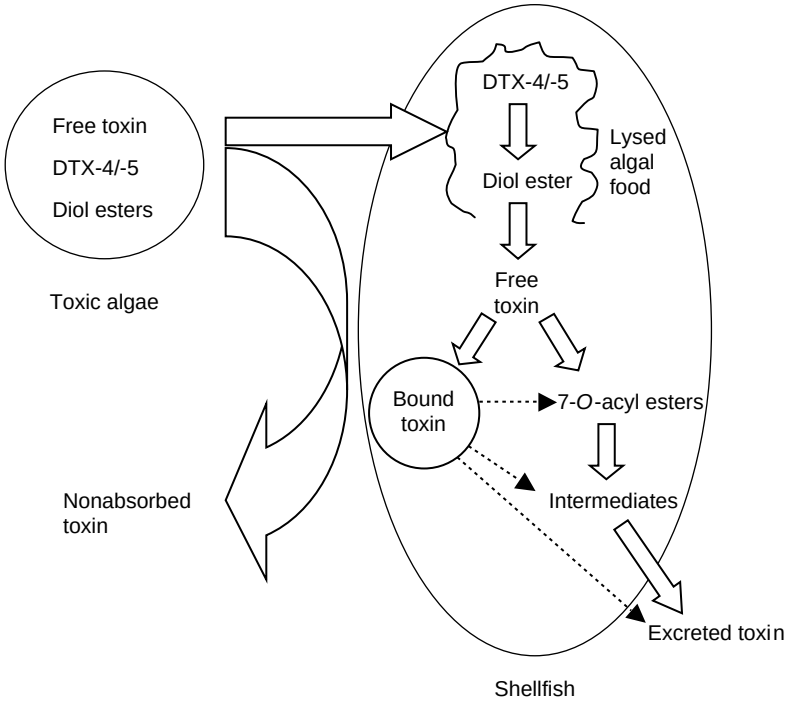


FIGURE 10.4 Proposed pathways for OA group toxin metabolism with shellfish. The block arrows represent relatively rapid processes whereas the dashed arrows are proposed slow processes. Intermediate metabolites have not been identified.

Because it is only relatively recently that the chemical nature of shellfish poisons has become known, new tests that can chemically detect the toxins have also only recently been developed. The analysis of OA group toxins usually involves determination of both the free and esterified toxin, or the hydrolysis of the esterified toxins back to free toxins, and the analysis of total toxin content. When hydrolysis is completed, both free toxin and free plus esterified ("total") toxin can be determined. In this case, the proportion of esterified toxin can be calculated by subtracting the free toxin from the total toxin result.

10.4.1 Extraction

Extraction of the sample should be efficient (i.e., give a high recovery of OA group toxins) and simple. The resulting extract must be compatible with the chosen analysis method.

10.4.1.1 Water Samples

Water samples containing algal material that requires analysis for OA group toxins, require special treatment to stop degradation of the sulfated analogs found within algal cells.

A boiled extraction (Quilliam et al., 1996) will retain the OA group toxins in a natural state and is required for the analysis of sulfated esters (e.g., DTX-4).

1. Centrifuge a suitable volume of culture (e.g., 50 mL of a dense culture) and decant the supernatant.
2. Retain this culture media supernatant for further analysis as required.
3. Resuspend the cellular pellet in 0.5 mL 50 mM TrisHCl pH 7.4 and immerse the tube in boiling water for 3 min.

4. Add 2 mL of 80% methanol and sonicate.
5. Repeat the centrifugation, extraction (1 mL methanol), and sonication two times.
6. Combine the methanolic supernatants and make to 5 mL.
7. Filter with a 0.45 μm filter before analysis.

Various procedures to convert the sulfated esters to diol esters have been reported (Quilliam et al., 1996); however a base hydrolysis for the determination of total OA group toxins in samples is recommended (McNabb et al., 2005).

1. To 1 mL of final methanolic extract, add 125 μL of 2.5 M NaOH, seal in a vial, and heat to 76°C for 40 min.
2. After heating, immediately cool the extract, and neutralize with 125 μL of 2.5 M acetic acid; check if the pH is neutral using pH paper.
3. 0.45 μm filter before analysis.
4. Include a 1.25 dilution factor in all final calculations.

For extraction from culture media or seawater, a solid phase extraction (SPE) is recommended (MacKenzie et al., 2004). Filtered seawater (retain cellular material for analysis as required) can be passed directly through a hydrophobic SPE resin (e.g., Waters Oasis, Phenomonex StrataX, etc.) and eluted with 100% methanol. The methanol extract may be evaporated to dryness and resuspended as required. C18 SPE cartridges will also perform well but are more prone to blockage if a large volume of sample is being treated.

10.4.1.2 Shellfish Samples

Shellfish samples for the analysis of OA group toxins are recommended to be analyzed as an unhydrolyzed extract to determine the free OA group toxin component and as hydrolyzed extract to determine the total OA group toxin component. Care is needed during the preparation of extracts for the testing of C-7 esterified (DTX-3) toxins to ensure that degradation of polyunsaturated side chains is avoided. Either the whole shellfish or the excised digestive gland (d.g.) may be tested. For the OA group, toxins up to 30% (Vale, 2006b) have been reported outside the d.g. and, therefore, testing of the d.g. only may be justified is some cases.

Extraction of homogenized shellfish tissue with 90% methanol provides excellent recoveries of a range of OA group and other toxins (Goto et al., 2001). This method has been extensively validated (McNabb et al., 2005). In summary,

1. To 2 g of shellfish sample in a 50 mL tube add 18 mL of 90% methanol.
2. Homogenize with a dispersion tool (UltraTurrex or similar) at high speed for 1 min.
3. Centrifuge the resultant slurry and remove aliquots of the methanolic extract as required.

Further processing of the methanolic extract before analysis may be required and hydrolysis is highly recommended.

Some applications may require additional cleanup and the Goto method provides an excellent example of a solvent partition with chloroform and then a silica SPE cleanup; however it should be noted that during the hexane cleanup, the nonpolar C-7 acyl esters of OA group toxins will partition into hexane, and hydrolysis must be performed on crude methanolic extracts.

We routinely perform a hexane cleanup and hydrolysis as follows:

1. Hexane partition: add 2 mL of the methanolic extract to 5 mL of hexane and shake.
2. Allow phases to separate.
3. Remove the hexane.
4. Filter with a 0.45 μm filter or ultracentrifuge the methanolic extract before analysis.
5. Hydrolysis: To 1 mL of methanolic extract (prehexane partition) add 125 μL of 2.5 M NaOH seal in a vial and heat to 76°C for 40 min.

6. After heating immediately, cool the extract and neutralize with 125 μL of 2.5 M acetic acid, and check if the pH is neutral using pH paper.
7. Filter with a 0.45 μm filter before analysis.
8. Include a 1.25 dilution factor in all final calculations.

Variations to the protocols mentioned are common and the most common is use of 80% methanol rather than 90% (Stobo et al., 2005). We have found (McNabb et al., 2005; Holland et al., 2006) that the recoveries of C-7 acyl esters and azaspiracids are lower when extracting with 80% methanol.

The careful preparation of shellfish for the complete profiling of esters has also been published (Suzuki et al., 1999; Vale 2006a). The esters can be partitioned into hexane, chloroform (Goto et al., 2001), or dichloromethane (Vale, 2006a) from the methanol extract. The Goto method acidifies the methanolic extract (5 mL plus 2 mL 0.5% acetic acid) and the Vale method dilutes the methanol extract with water (2 mL methanol extract: 1 mL water) before this. Both methods then employ a silica SPE step to cleanup the extract for analysis.

10.4.1.3 Other Methods

Immunoaffinity columns (IAC) have been used to purify free OA group toxins from shellfish (Puech et al., 1999). Preparation of methanolic extract is followed by evaporation and resuspension in a buffer compatible with the IAC. This protocol would be amenable to the addition of a hydrolysis step before evaporation and resuspension in IAC buffer. Alternatively, production of IAC from antibodies that select a range of OA group toxins including esters (Lawrence et al., 1998) would yield a broader range of OA group toxins.

10.4.2 Analysis

Principally, there are four main modes of detection for the specific analysis of OA group toxins: enzyme-linked immunosorbent assays (ELISA), PP binding assays, and high performance liquid chromatography (HPLC) with fluorometric or MS detection.

10.4.2.1 Enzyme-Linked Immunosorbent Assay

ELISA test kits are not widely available and some information on potential sources has been included in Appendix. None of these kits have undergone interlaboratory trials and this information should be requested when considering a purchase. In addition, suppliers of kits should be able to specify the cross-reactivity of the antibody to OA analogs. Rather than review the development of these kits when other more complete reviews exist (this edition Chapter 11). I simply mention them as potentially relatively simple and convenient methods to analyse OA group toxins. The application of the immunoaffinity enzymes used on these kits for extraction has been discussed.

10.4.2.2 Protein Phosphatase Binding

Protein phosphatase assays have been covered elsewhere in detail (this edition Chapter 11); however, PP assay is worth noting as a quick, sensitive, and effective way to detect OA group toxins without the need for specialized equipment. A quick reference in Appendix to potential sources of commercial kits has been included. At this time, no results have been published for interlaboratory trials with these kits and this information should be requested when considering a purchase.

10.4.2.3 High Performance Liquid Chromatography-Fluorescence

HPLC-florescence (HPLC-FL) requires the derivatization of the OA group toxins to florescent analogs. This is most commonly accomplished by precolumn reaction of sample extracts with 9-anthryldiazomethane (ADAM). This is not the only derivatizing agent to have been used and

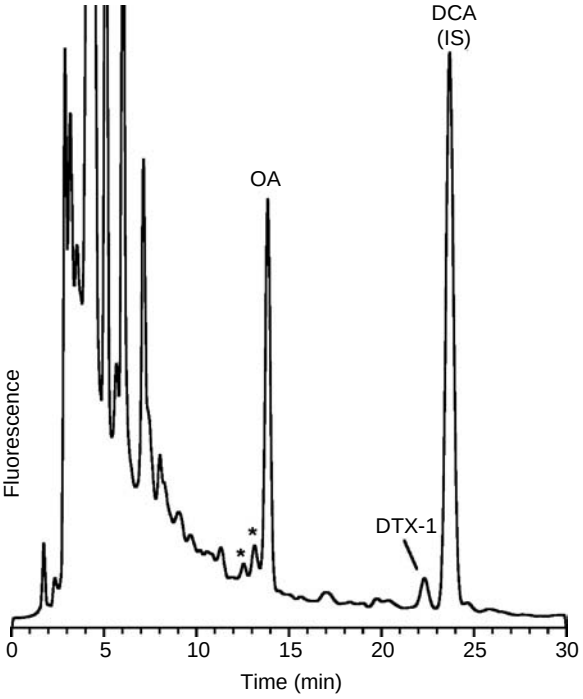


FIGURE 10.5 HPLC analysis of CRM-DSP-MUS after derivatization with ADAM and cleanup by silica solid phase extraction. An internal standard (IS) deoxycholic acid (DCA) is used and the peaks are marked. LC conditions: 250 mm × 4.6 mm Keystone betabasic C-8 (5 μm) column, 80% acetonitrile mobile phase (1.0 mL/min). Fluorescence detection (excitation λ 254 nm, emission λ 412 nm). Data is smoothed. (Courtesy of Dr Michael Quilliam).

alternative as well as details on conditions for use, have been reviewed elsewhere (James et al., 2000).

Figure 10.5 shows a typical chromatogram from use of the ADAM reagent according to the method of Quilliam (Quilliam 1995; Quilliam et al., 1998). The full method protocol is available elsewhere (Quilliam, 2003). Many of the derivatizing reagents including ADAM are unstable and challenging to use. They require storage in the dark at low temperatures (–70°C) and dilute solutions must be used immediately. The HPLC-FL test is subject to numerous interferences from degradation products (e.g., from ADAM, in James et al., 2000) or from the shellfish matrix. The most notable interference is PTX-2 seco acid (Norgueiras et al., 2003) that is commonly found to co-occur with OA group toxins in *Dinophysis* contaminated shellfish (MacKenzie et al., 2002).

The HPLC-FL methods are generally only suitable for the analysis of free OA group toxins; however, a method for the direct analysis of 7-*O*-acyl esters has been developed (James et al., 2000), but is complex in comparison to liquid chromatography-mass spectrometry (LC-MS) methods.

10.4.2.4 Liquid Chromatography-Mass Spectrometry

Most improved or new methods for OA group toxins that have been reported in the last few years involve the use of HPLC-mass spectrometry (LC-MS). One reason that LC-MS methods have been so popular is the fact that a single LC-MS run can determine all the lipophilic toxins currently required by most official agencies, including OA group toxins. The topic of general toxin methods has been reviewed elsewhere (Quilliam, 2003). LC-MS has also become popular as electrospray ionization (ESI) sources have become more robust and more LC-MS systems are now in

use Worldwide; therefore, most researchers and many routine laboratories have access to a LC-MS. LC-MS is also capable of performing a wide range of experiments, from fully quantitative analysis in multireaction monitoring (MRM) mode, to qualitative full scan experiments of unknown or interesting samples. The adapability, relative robustness, and quantity of data that modern automated LC-MS systems deliver have made the need for HPLC-FL systems almost redundant.

The lower concentration limit of HPLC-FL methods for OA group is generally 10 pg of toxin. In practice, the method is limited by a huge range of interferences and the actual limit of detection for the method applied to shellfish is 0.1 mg/kg (Quilliam, 1995); howver, in our lab we have tested thousands of samples for OA group toxins by LC-MS to 0.01 mg/kg without any sophisticated cleanup or pre-concentration (McNabb et al., 2005).

LC-MS systems are dependant upon two key processes: ionization and the type of ion processing. All LC-MS systems utilize atmospheric chemical ionization (API) and ESI has dominated the analysis of OA group toxins. This topic has been reviewed elsewhere (Cole, 2000). The two most common types of mass spectrometers used for OA group toxins (and marine toxins in general) are quadrupole mass spectrometers and ion trap mass spectrometers. There are frequent reports of methods that utilize single quadrupole instruments (Stobo et al., 2005; Suzuki et al., 2005b; Vale 2006a,b) or triple quadrupole instruments (Puente et al., 2004a,b; McNabb et al., 2005). There are also references to ion trap instruments being applied to OA group toxins (Marcaillou et al., 2005). The development of LC-MS systems is continuing and triple quadrupole instruments that include a linear ion trap have now become available. Figure 10.6 shows some examples of LC-MS data generated by a triple quadrupole instrument.

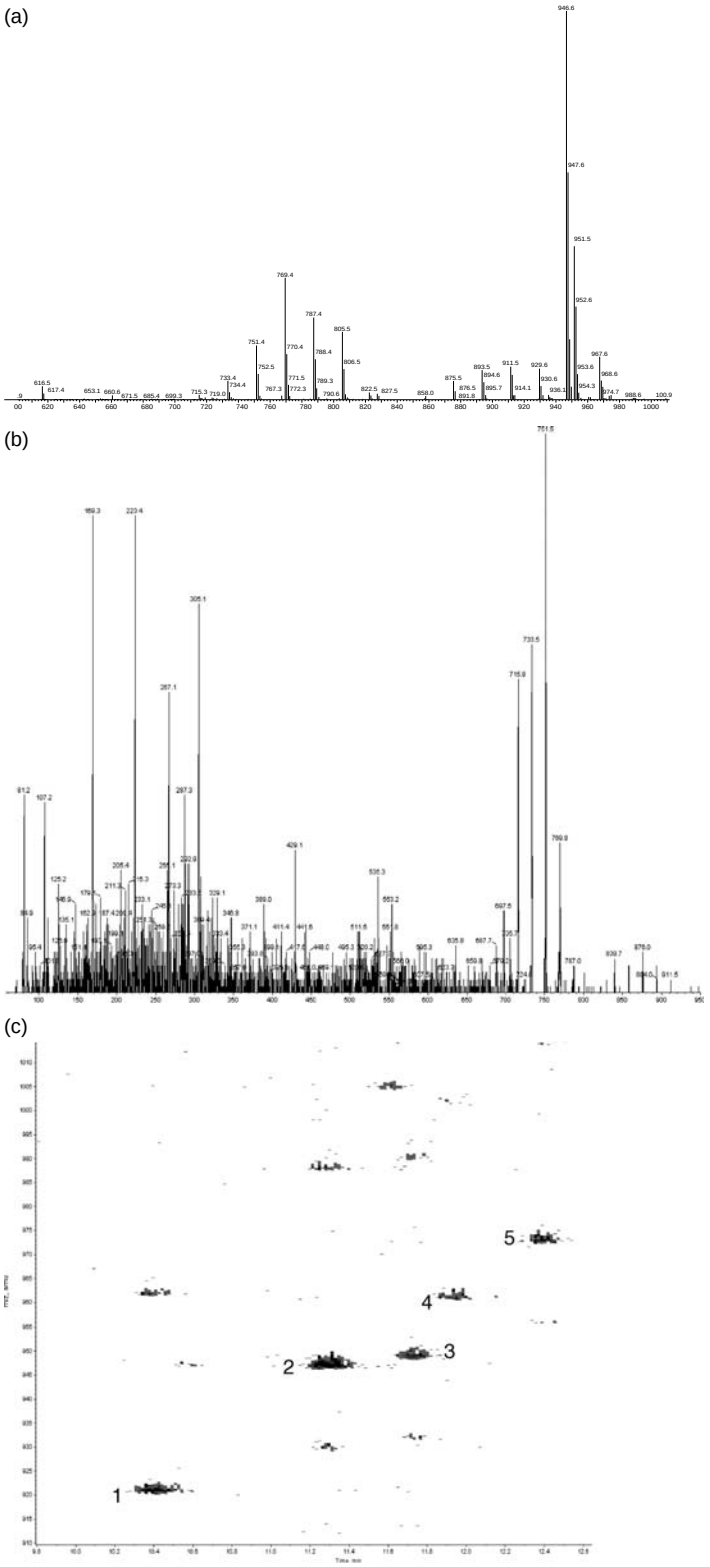
10.4.2.5 High Performance Liquid Chromatography Systems

LC-MS methods are limited to mobile phase and column combinations that are compatible with electrospray ion sources. The mobile phase must be volatile and this usually means a combination of water:acetonitrile:methanol. The range of buffers and modifiers is also limited to volatiles, such as formic and acetic acid, or ammonium salts. Although most electrospray sources can accept flow rates of 1.0 mL/min, a flow rate of 0.2 mL/min or less is preferred; therefore, LCMS methods for OA group toxins are usually reverse phase methods using 2 mm ID columns. Literature reports for the HPLC separation of OA group toxins before LC-MS include the following:

Free toxins: C8 (50 × 2.1 mm) separation by gradient elution with acetonitrile containing 5 mM ammonium acetate (pH 6.8) (Stobo et al., 2005). C8 (50 × 2.1 mm) isocratic separation with 50% acetonitrile containing 2 mM ammonium formate and 50 mM formic acid (Stobo et al., 2005). C8 (50 × 2 mm) isocratic separation with 87% acetonitrile containing 0.05% acetic acid (Vale, 2006a). We employ the following conditions in a multitoxin screen (McNabb et al., 2005):

1. *LC solvents:* A—1 + 9 v/v acetonitrile and water. B—9 + 1 v/v acetonitrile and water. C—33 mM ammonium hydroxide and 500 mM formic acid in water. All solvent mixtures filtered (0.45 μm).

FIGURE 10.6 Example LC-MS data generated from *Prorocentrum lima* culture using an API 4000 LC-MS/MS system in positive electrospray mode. (a) Parent ion scan of the C8:2 OA diol ester showing a parent ammoniated ion (*m/z* 946.6) and typical in-source fragmentation. (b) Product ion mass spectra of the 946.6 parent ion showing *m/z* 751 is abundant. (c) Intensity plot of precursor scan data for the *m/z* 751 product ion. The *x*-axis is time, the *y*-axis is *m/z* and the intensity of the signal is shown by the density of each "spot." 1 = *m/z* 920, ammoniated C6 OA diol ester, 2 = *m/z* 946, ammoniated C8:2 OA diol ester, 3 = *m/z* 948, ammoniated C8:1 OA diol ester, 4 = *m/z* 960, ammoniated C9:2 OA diol ester, 5 = *m/z* 972, ammoniated C10:3 OA diol ester. (Courtesy of Andy Selwood.)


(a)
(b)
(c)
1
2
3
4
5

2. *Column:* Luna C18(2) 5 µm 150 × 2 mm column (Phenomenex, CA) operated at 30°C, 0.2 mL/min.
3. *Injection volume:* 10 µL.
4. *Gradient elution:* A constant buffer composition of 3.3 mM ammonium formate/46 mM formic acid achieved by mixing 10% LC solvent C with all gradient mixtures. Initial conditions are 85% LC solvent A and 5% LC solvent B. A linear gradient to 5% A and 85% B is run from 2 to 13 min and maintained until 25 min when re-equilibration begins.

Diol esters: C8 (150 × 2 mm) isocratic separation with 55% acetonitrile containing 1 mM ammonium acetate pH 7.0 (Windust et al., 2000). HPLC-UV detection at 238 nm is also possible owing to the diene function common in many diol esters.

DTX-4: C8 (150 × 2 mm) separation by gradient elution with acetonitrile containing 1 mM ammonium acetate (Quilliam et al., 1996).

DTX-3: C18 (150 × 2 mm) isocratic separation with 100% methanol containing 1 mM ammonium acetate (Suzuki et al., 1999).

10.4.2.6 Quantitative Liquid Chromatography-Mass Spectrometry

For the quantitative analysis of OA group toxins, either an ion trap or a quadrupole instrument is used. All LC-MS instruments are capable of generating and detecting a molecular ion for the OA group toxins. Using negative polarity ESI (ESI-), the free OA group toxins generate deprotonated *molecular ions* ($[M\text{-}H]^-$) of *m/z* 803.5 (OA, DTX-2) and 817.5 (DTX-1). Using positive polity ESI (ESI+), the free OA group toxins will usually generate protonated, ammoniated, and sodiated molecular ions ($[M+H]^+$, $[M+NH4]^+$, $[M+Na]^+$) of *m/z* 805.5, 822.5, 827.5 (OA, DTX-2) 819.5, 836.5, and 841.5 (DTX-1). A full list of OA group toxins and *m/z*s is available in Appendix. Ion trap and triple quadrupole instruments are also capable of more specific analysis by multiple stage LC-MS. To do this the MS selects a molecular ion (as above) and performs a fragmentation reaction to generate *product ions*; the product ions are specific to the target compound (OA group toxin). This type of MS process is usually abbreviated to MS/MS and includes MRM. Ion trap instruments can continue to react on the product ions to perform MS^n where n can be 3–10.

Although ion trap mass spectrometers can be used for quantitative analysis, they do not perform as well as quadrupole instruments (Soler et al., 2005). Triple quadrupole instruments provide the best quantitative results, especially in MRM mode and they generally have better linearity, higher precision, and less matrix interference than ion trap instruments.

10.4.2.7 Qualitative Analysis

In addition to ion trap and quadrupole mass spectrometers, OA group toxins can be analyzed on other types of MS analyzers. For example, time of flight (ToF) mass spectrometers provide accurate mass data and have been used to isolate and characterize new compounds (Suzuki et al., 2004). However, it is more common for ion trap (Cruz et al., 2006) and quadrupole instruments (Vale, 2006a) to be used for OA group toxin qualitative analysis. Various MS experiments can be used to gain information about the composition of samples and some examples are given in Figure 10.6.

Other chemical analysis methods have been developed and used. Capillary electrophoresis (CE) has been applied to the analysis of OA group toxins. The method of Bouaicha (Bouaicha et al., 1997) using micellular electrokinetic capillary chromatography has been adopted by others (Li et al., 2001). In general, the use of CE has been limited by the poor sensitivity of CE ultraviolet (UV) detectors for OA group toxins. The coupling of CE and MS may see more investigations of this technique in future (de la Iglesia et al., 2006). Other techniques that may be used in the future are

solid phase micro extraction and nanospray LC-MS (Walles et al., 2005); however, it seems likely that most of the development will come in the area of improved LC-MS methods as LCMS system become faster and more sensitive (McMillan et al., 2006). Thin layer chromatographic methods and gas chromatographic methods have been developed but are not reviewed here, as their usefulness in light of LC-MS development is minimal.

10.4.2.8 Quality Control

For the result of a quantitative analysis to be valid, it should be derived from a validated method that includes suitable quality control checks. A test method must be calibrated using the highest quality calibration standards available. If a commercial test kit is being used, the supplier should be able to provide a technical report summarizing the kit's performance. Ideally, validated methods should undergo both single-lab validation and interlaboratory study before use. The analytical protocol should include control points for assessment of the quality of analytical results. These might include recovery, linear calibration, minimum response, and duplicate sample data. The process of setting up a new analytical method occurs in three stages: method implementation (i.e., get it running), method validation, and ongoing QA/QC.

For the analysis of OA group toxins it is recommended that the primary calibrant is a certified standard and that the analysis (whatever the method) has been shown to give acceptable results for a certified reference material (see Appendix, Table A.1). In addition, fortified samples should show acceptable recoveries and the analysis should be calibrated over the range of expected results. Challenges with accurate quantitation from LC-MS often arise due to variation in ionization and lower than expected results due to suppression as well as higher than expected results from enhancement of ionization are common. Fortification of a known blank sample matrix can be useful to define the problem, and remedies include changing the LC elution method and servicing (cleaning) the mass spectrometer.

APPENDIX

Commercial Sources of ELISA Kits

ERFA biotech (www.erfabiotech.com) advertise an ELISA kit and OA antibodies; however, at the time of writing all email was returned to the author with an error.

Sceti (Japan) market the DSP-check ELISA kit; however, they could not supply any information on DTX-1 or DTX-2 cross-reactivity (www.sceti.co.jp).

CER laboratory of hormonology (Belgium). E.F.2. ELISA kit claims to have 50% cross-reactivity to DTX-1 and DTX-2.

Commercial Sources of PP2A

1. Toxiline-DSP is an assay using fluorescence detection. The method is suitable for shellfish species such as mussels, clams, oysters, and scallops. The kit is supplied with all the reagents needed for the assay and allows running up to 26 samples at a time. It has a shelf-life of 3–6 months and is available for purchase. For information or to place orders, please contact Elena Domínguez at Elena@zeu-inmunotec.com or phone +34 976531533- Ext 101.
2. Biosense (Norway) intend to release an OA group toxin detection kit based on PP2A competitive displacement. In February 2007, Biosense were optimizing a recombinant PP2A to ensure stability and high affinity to toxins, and did not foresee a pilot assay before 2008. For more information, please refer to www.biosense.com.

TABLE A.1
List of Ions *(m/z)* to Be Monitored in the LC-MS Analysis of OA Group Toxins

	Positive Ion Mode		Negative Ion Mode	
Toxin	$[M+NH_4]^+$	$[M+H]^+$	$[M-H]^-$	$[M-H]^{-2/3}$
OA,DTX-2	822.5	805.5	803.5	
DTX-1	836.5	819.5	817.5	
Shellfish toxins				
C14:0-OA,-DTX-2	1032.7	1015.7	1013.7	
C16:1-OA,-DTX-2	1058.7	1041.7	1039.7	
C16:0-OA,-DTX-2	1060.7	1043.7	1041.7	
C18:1-OA, DTX-2	1086.7	1069.7	1067.7	
C20:5-OA-DTX-2	1106.7	1089.7	1087.7	
C22:6-OA,-DTX-2	1132.7	1115.7	1113.7	
C14:0-DTX-1	1046.7	1029.7	1027.7	
C16:1-DTX-1	1072.7	1055.7		
			1053.7	
C16:0-DTX-1	1074.7	1057.7	1055.7	
C18:1-DTX-1	1100.7	1083.7	1081.7	
C20:5-DTX-1	1120.7	1103.7	1101.7	
C22:6-DTX-1	1146.7	1129.7	1127.7	
Algal toxins				
Diol C7:2-OA,-DTX-2	932.6	915.6		
Diol C8:2-OA,-DTX-2	946.6	929.6		
Diol C9:2-OA,-DTX-2	960.6	943.6		
Diol C10:3-OA,-DTX-2	972.6	955.6		
Diol C7:2-DTX-1	946.6	929.6		
Diol C8:2-DTX-1	960.6	943.6		
Diol C9:2-DTX-1	974.6	957.6		
Diol C10:2-DTX-1	986.6	969.6		
DTX-4			1471.6	735.3/489.9
DTX-5a			1391.6	695.3
DTX-5b			1405.6	702.3

Prepared with the assistance of Dr Michael Quilliam.

Sources of Calibration Standards

Numerous sources of okadaic acid exist and some companies offer OA methyl ester. DTX-1 is only available from Wako Chemicals (www.e-reagent.com). The only source of certified reference material and certified calibration solutions is the National Research Council (Canada) Certified Reference Materials Programme (CRMP). More information is available online at: http://www.imb.nrc.ca/crmp/natural/index_e.php. Or email crm.imb@nrc-cnrc.gc.ca.

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11 Pharmacology and Toxicology of Diarrheic Shellfish Toxins

A. Tubaro, S. Sosa, A. Bornancin, and J. Hungerford

CONTENTS

11.1 BACKGROUND

Diarrheic shellfish poisoning (DSP) is a specific type of food poisoning due to the ingestion of filter-feeding bivalves contaminated with a specific suite of toxins. The first reported cases of DSP were in the Netherlands in the 1960s; subsequently, similar outbreaks occurred in Japan in the late 1970s.[1] It is now known that the problem is worldwide. Although, like all shellfish intoxications, the illness is named for the observable symptoms, this review will show that the implicated toxins can impact multiple and critical biological processes due to their effect on a central control mechanism in living systems, that of protein phosphorylation and dephosphorylation.

The main symptom of DSP, after which the poisoning is named, is represented by diarrhea, often associated with other gastrointestinal distresses such as nausea, vomiting and abdominal cramps. Usually, these symptoms occur within 30 min to a few hours after contaminated shellfish consumption. In general, no hospitalization of the patients is necessary and, usually, there is a complete recovery within 3 days without any pharmacological treatment. In fact, no specific antidote is available for diarrheic toxins, to date. The treatment, if necessary, is only supportive to replace fluid and electrolyte losses. Although no lethality is attributed to the diarrheic episodes, they represent a worldwide problem to both public health and bivalve production and marketing.[2]

The diarrheic shellfish toxins are heat-stable and lipophilic polyether compounds such as okadaic acid (OA) and related analogues (for the chemical structures of the toxins, see Chapter 12). Okadaic acid was first isolated from the sponges *Halichondria okadai* and *H. melanodocia*,[3] while the causative organisms of this toxin and its derivatives were identified as dinoflagellates species belonging to the planktonic *Dinophysis* and the benthic *Prorocentrum* genus.[4] The first species responsible for shellfish contamination by diarrheic toxin in Japan was *Dinophysis fortii*.[2] Among the *Dinophysis* genus, other species were recorded to be involved in DSP such as *D. acuminata*, *D. acuta*, *D. caudata*, *D. norvegica*, *D. mitra*, *D. rotundata*, *D. sacculus*, *D. tripos*, and *D. truncu-lus*.[5–7] These dinoflagellates are widely distributed, but do not always form red tides; for instance, 200 cells of *D. fortii*/l induce contamination of mussels and scallops that can provoke human toxicity. There are differences in toxic potential among regionally different species, which cause great fluctuations in toxin levels of a given species. For instance, in Japan *D. fortii* produces mainly dinophysistoxin-1 (DTX-1), while in Europe OA represents the main diarrheic toxin produced by this species. Furthermore, qualitative and quantitative seasonal variations in toxin production have also been reported in the same area by the same species.[8]

Other toxins of this group are dinophysistoxins (DTXs), namely dinophysistoxin-1 (DTX-1, 35-methyl OA), dinophysistoxin-2 (DTX-2, 31-demethyl-35-methyl OA), and dinophysistoxin-3 (DTX-3). Originally, the term DTX-3 indicated a group of compounds in which saturated or unsaturated C_{14}–C_{18} fatty acid groups are attached at the 7-hydroxy function of DTX-1,[4] but it is now known that OA or DTX-2 can also be acylated.[9] DTX-3 toxins are metabolic products of shellfish metabolism rather than compounds synthesized by the microalgae.[10] Furthermore, a wide array of OA analogues, where the carboxyl group of OA is conjugated to several different unsaturated C_7–C_9 diols to form allylic "diol esters," was isolated from *Prorocentrum lima* and *P. maculosum* cultures. Some of these diol esters are further conjugated to a polar side chain, and the relevant compounds represent water-soluble derivatives of OA.[9] Two of these compounds, DTX-4 and DTX-5, seem to be a form for storing and excreting the toxin from the dinoflagellate cells.[11] Subsequently, a variety of DTX-4 and DTX-5 analogues have been detected in various *Prorocentrum* species. These compounds can be hydrolyzed, chemically or enzymatically, to their parent diarrheic toxins, ultimately sharing with them the same toxicity/mechanism of action.[9] From a culture of a particular strain of *P. lima*, another derivative of OA was isolated. The structure was elucidated showing a C_1 ester derivative of OA, called DTX-6.[12] For more details on the chemistry of the diarrheic toxins, see Chapter 12.

Diarrheic toxins are accumulated by several bivalves, including mussels, scallops, cockles, oysters, clams, and crabs,[7,13] which filter the seawater containing the toxic phytoplankton and cause human poisoning after their ingestion. Analyses for this group of toxins represent a key aspect of toxin-monitoring programs all over the world. The most used detection method for DSP is the mouse bioassay, in which the survival time of mice treated with a suspension of a shellfish extract by intraperitoneal injection, usually over a 24-h observation period, is recorded.[14] Analysis for diarrheic toxins is often complicated by the presence of other lipophilic toxins that can cause false positives in animal bioassays. To evaluate the actual DSP risk, confirmatory tests are used.[15] Among these, the most important is the protein phosphatase 2A inhibition assay, an *in vitro* assay based on the mechanism of action of OA. Also, chemical and immunoenzymatic methods have been developed for DSP detection, including fluorescence-derivatization high performance liquid chromatography (HPLC), capillary electrophoresis (CE), liquid chromatography-mass spectrometry techniques (LC-MS), and enzyme-linked immunosorbent assays (ELISA).[9,16]

Diarrheic toxins are reported worldwide and, although Japan and Europe are the most impacted, diarrheic contaminations occur also in North and South America, Australia, Indonesia, and New Zealand.[6] In particular, in European areas, in spring and early summer or in autumn, toxic dinoflagellates of *Dinophysis* genus are often found.[17]

For regulatory purposes, a limit of 0.16 mg of OA equivalents per kg of shellfish edible parts, has been established in many countries.[15]

11.2 MECHANISM OF ACTION

Okadaic acid and its analogues DTX-1 and DTX-2 are specific inhibitors of serine/threonine protein phosphatases 1 and 2A, two enzymes involved in the regulation of many cellular processes by modulation of proteins phosphorylation/dephosphorylation degree.[18,19]

11.2.1 Protein Serine/Threonine Phosphatases

Phosphorylation is a posttranslational modification of proteins important for the regulation of many cellular functions, such as control of cell growth and death, differentiation, metabolism, and signal transduction.[19] Usually, protein kinases add phosphate residues on the amino acids serine, threonine, or tyrosine, generating *O*-phosphomonoesters, whereas protein phosphatases remove these phosphate groups. Thus, the phosphorylation level of a protein is reversibly regulated by the activity of the relevant protein kinase(s) and phosphatases(s). The protein phosphatases (PP) family is represented by serine/threonine-specific and tyrosine-specific enzymes, according to the dephosphorylated amino acid residues. Seven groups of serine/threonine protein phosphatases have been identified on the basis of their catalytic domain and biochemical properties. Originally, two main groups were classified as type 1 (PP1) and type 2 (PP2) phosphatases. Type-1 protein phosphatases dephosphorylate the β-subunit of phosphorylase kinase and are potently inhibited by two proteins (endogenous inhibitor-1 and -2), whereas type-2 protein phosphatases preferentially dephosphorylate the α-subunit of phosphorylase kinase and are insensitive to inhibitor-1 and -2. The PP2 type is further divided in three distinct subtypes, considering the ionic requirements for their activity: PP2A, which is not dependent on metal ions for its activity; PP2B, which is Ca^{2+}-calmodulin-dependent for the activity and PP2C, which requires Mg^{2+} or Mn^{2+} ions for its activity. Further studies revealed that PP1 and PP2A are present as holoenzymes in the cells, with the catalytic subunit ($PP2A_C$) associated with one or more regulatory proteins responsible for targeting the catalytic core of a specific substrate and/or cellular location. Various isoforms of the catalytic and regulatory subunits of these enzymes have been identified. Thus, each enzyme can be found in multiple forms characterized by different patterns of expression, specificity, and sensitivity to inhibitors. More recently, additional isoforms of phosphatases (PP3-PP7) have been discovered.[19,20]

PP1 and PP2A represent the most relevant serine/threonine phosphatases in mammalian cells, accounting for over 90% of all serine/threonine phosphatase activity and hydrolyzing a large number of substrates.[19] PP1 is involved in the regulation of cell functions, such as varied as glycogen metabolism, synaptic plasticity, cell cycle regulation, and smooth muscle contraction. PP2A is also implicated in the control of several cellular events, such as metabolism, apoptosis, cell cycle control, DNA replication, gene transcription, protein translation, and cell transformation.[21] PP4 is involved in the regulation of microtubule assemblage, spindle assembly and centrosome maturation during cell division.[22] It has been recently shown that PP4 plays an important proapoptotic role in T lymphocytes.[23,24] PP5 is involved in the regulation of multiple cellular functions as DNA repair, apoptosis and circadian clock in mammals, although its precise role in the cellular activities has still to be clarified.[25-27] PP6 seems to play a role in regulating inflammatory signaling *in vivo*, being regarded as a possible novel pharmacological target in the therapy of inflammatory diseases and inflammation-associated tumors.[28]

11.2.2 Inhibition of Serine/Threonine Protein Phosphatases by DSP Toxins

Okadaic acid (OA) is a potent inhibitor of the purified catalytic subunits of PP1 and PP2A, being about two orders of magnitude more potent against the latter with respect to the former. PP4 showed sensitivity to OA comparable to that of PP2A, while sensitivity of PP2B, PP3, PP5, and PP6 to the toxin is lower than that of PP4. In contrast, OA is inactive against PP2C and PP7.[19,29–33] The variable amino acid composition in the β12–β13 loop, a region close to the catalytic site of the enzyme, accounts in part for the different sensitivity of phosphatases to OA.[32,34]

Studies on the nonmembrane-spanning type 1 tyrosine phosphatase revealed no significant inhibition of the enzyme by OA class compounds at concentrations up to 0.01 mM.[35] In previous reports, OA was also shown to be inactive as inhibitor of any protein tyrosine phosphatase, confirming its selectivity for serine/threonine phosphatases.[36]

Various authors reported that, in cell-free systems, OA selectively inhibits PP2A and to a lower extent PP1, with IC_{50} values in the ranges of 0.02–0.2 nM and 10–100 nM, respectively. The IC_{50}-reported values vary over a wide concentration range, depending on variances in experimental conditions, such as the purity degree of the enzyme preparation, its concentration, differences in the substrate used, temperature, and duration of the enzyme reaction.[37] Using cellular systems, total concentrations of OA higher than those active in cell-free assays are required to obtain significant inhibition of phosphatase activity.[38] Also, DTX-1 and DTX-2 inhibit PPs, whereas DTX-3 is inactive against these enzymes if not hydrolyzed. Comparisons of the inhibition potencies of OA and its congeners have been reported. One study revealed that OA is slightly more potent than DTX-1 against PP1 (IC_{50} = 19 nM and 55 nM, respectively) and PP2A (IC_{50} = 0.2 nM and 0.6 nM, respectively).[39] Similarly, Honkanen et al.[40] found that DTX-1 less active than OA against PP2A, whereas other studies showed that DTX-1 more active than OA in inhibiting PP2A.[41,42] Furthermore, Aune et al.[43] revealed that the potency of DTX-2 was slightly lower than that of OA against PP2A (IC_{50} = 5.94 ng/mL and 2.81 ng/mL, respectively). It should be kept in mind that the enzyme sources, substrates, etc., can vary between these studies, and so the most conservative conclusion is that the inhibition potencies of OA and these DTXs vary by less than an order of magnitude, whereas DTX-3 is inactive.

Serine/threonine protein phosphatases are inhibited also by other natural toxins, such as cyclic peptides (microcystins and nodularins), terpenoids (cantharidin and thyrsiferyl 23-acetate), polyketides (calyculin A and tautomycin), and other compounds, such as fostriecin.[19,32,44] A comparative study revealed the following order of potency for some compounds: microcystin-LR > calyculin A > tautomycin > okadaic acid for PP1, and okadaic acid > microcystin-LR > calyculin A > tautomycin for PP2A. Calyculin A, microcystin-LR and tautomycin are inhibitors of both PP1 and PP2A, with IC_{50} values ranging from 0.1 nM to 0.7 nM.[35] Okadaic acid, microcystin-LR, calyculin A, tautomycin, cantharidin, and fostriecin inhibit also PP4 with an IC_{50} of 0.1 nM, 0.15 nM, 0.2 nM, 0.4 nM, 50 nM, and 3 nM, respectively,[24,30,32] whereas protein phosphatase 5 is inhibited only by okadaic acid (IC_{50} < 3 nM) and microcystin-LR (IC_{50} >1 nM).[32,45]

11.2.3 Structure–Activity Relationships

The main studies on structure–activity relationships for OA have been carried out using various toxin analogues and by evaluating their affinity for PP1 and PP2A and/or inhibition of these enzymes.[19,42,46,47] Structural modification studies with OA indicated that the carboxylic group of carbon-1 (C-1) is essential for inhibition of protein phosphatase activity, since its removal or esterification determines the loss of activity. In addition, the hydroxyl groups throughout the structure at C-2, C-7, C-24, and C-27 seem to play important roles in the interaction of the toxin with its target, since their modification caused a significant decrease of protein phosphatase inhibition.[39,48] In particular, methoxylation of all the hydroxyl groups of OA or DTX-1 impaired the activity of the parent molecules.

Other changes of OA molecule can partially influence its PPs inhibitory activity or its affinity for the enzymes. Considering the C-1→C-24 fragment of OA, hydroxylation and reduction of carbons in C-14 and C-15 positions lead to a compound (glycol OA) still possessing an acidic function, but with an activity approximately half that of OA. This suggests that the spatial position of the carboxyl group of OA is also important for activity. The reduction of the double bond between C-14 and C-15 to give 14,15-dihydro-OA increased the K_i values for the interaction with PP2A 30-fold and 2-fold, respectively. In contrast, this causes less effect on the affinity for PP1. The acylation of the hydroxyl group at carbon C-7 with a saturated fatty acid yielding 7-*O*-palmitoyl-OA resulted in a >3000-fold

increase in the K_i value but with lesser effects if the esterification involved a polyunsaturated fatty acid, as in 7-*O*-docosahexaenoyl-OA. Also, the 24-hydroxyl group plays an important role in the interaction with the PPs as highlighted by an OA derivative possessing the epimerized hydroxyl group at this position, which was less active than OA by 100 orders of magnitude.[19,42,47]

Concerning the C-25→C-38 fragment of OA, oxidation of the hydroxyl group at carbon C-27 causes the most marked reduction of the affinity to the phosphatases, increasing the K_i values 40-fold toward PP1 and 230-fold toward PP2A. Anyway, in contrast with methoxylation of all four hydroxyl groups, this oxidation did not completely abolish the inhibitory effect against protein phosphatases.[47]

Other changes of OA structure, such as the addition of a methyl group on its G-ring, as in DTX-1, or the substitution of the double bond in the B-ring at the 9–10 carbon position with an episulphide (acanthifolicin), do not appreciably modify the activity.[19]

Mutagenesis studies on PP1 and PP2A catalytic subunits suggested that the β12–β13 loop of these enzymes is critical for recognition by OA. This region also seems to confer to OA the higher selectivity towards PP2A, with respect to PP1. The most energetically favorable structure observed for OA-PP1 complex showed a potential hydrogen bond between the hydroxyl groups at carbon C-24 and C-27, and Arg-221. Other important hydrophobic interactions with the β12–β13 loop were supposed: this domain presents the amino acidic residues Phe-276 and Tyr-134, which interact with the methyl group at carbon C-13 and the methyl group at carbon C-29, respectively. Studies on PP2A revealed a possible interaction between C-10 and Tyr-263 of PP2A and the hydroxyl group at carbon C-7 and Arg-264. The latter contact may explain the selectivity observed with PP2A in this region. Other possible interactions related to those found with PP1 are the hydroxyl groups at carbons C-24 and C-27 with Arg-210, and the methyl group at carbon C-29 with Tyr-123.[19]

The crystal structure of OA is characterized by a circular conformation that allows the interaction between its carboxylic group and the hydroxyl at carbon C-24. This spatial conformation exposes the methyl groups of OA at carbons C-10 and C-13 at the surface of the molecule, allowing an interaction with the protein phosphatase β12–β13 loop.[19,49] The whole discussion reported above is based on the currently accepted structure of okadaic acid, although the definitive structural conformation has been recently questioned, requiring possible re-evaluation of the sites of interaction of OA with the phosphatase molecules.

11.2.4 Biological Effects due to Protein Phosphatases Inhibition

Inhibition of protein phosphatases by OA class compounds leads to a rapid increase of phosphorylated proteins in cells. This effect seems to be responsible for the diarrhea and the degenerative changes in absorptive epithelium of small intestine induced by these toxins. In fact, the increased level of phosphorylated cytoskeletal proteins modifies the junctional elements between the intestinal epithelial cells controlling the permeability to solutes, which, in turn, results in passive loss of fluids.[1,50–52] For more details about the diarrheic mechanism, see the *in vitro* activities section.

PP1 and PP2A phosphatases have been shown to control key signal transduction mechanisms involved in cell death, cell cycle progression, tumor promotion, and other cellular processes such as metabolism, muscle contraction, and exocytosis.[19] Thus, inhibition of PPs can influence these phenomena, provoking many and varied biological effects, the most relevant being the tumor promotion demonstrated for the OA-class compounds. These effects will be described in more detail in the next section.

11.3 *IN VITRO* ACTIVITIES

Cellular responses to the OA family of DSP toxins can differ enormously, and sometimes yield opposite effects, depending on the concentration of the toxin, the length of exposure to the toxin, and the cell type. In general, an acute exposure of cells to moderate or high concentrations of OA

leads to their death, while a repeated exposure to low toxin concentrations leads to proliferation of cells and tumor promotion.[19,53] The overall effect of OA and its analogues at the cellular level depends also on the efflux of the toxin from cells, which can vary between different cell lines, and on the concentration of the different protein phosphatases within the cell. For instance, a complete inhibition of protein phosphatase 2A in the cells can be achieved with OA concentrations that may also affect other phosphatases.[20]

11.3.1 Cytotoxicity and Apoptosis

The first biological activity reported for OA was its antiproliferative activity in human epidermoid carcinoma KB cells and the cytotoxic effect in murine leukemia P388 and L1210 cell lines.[3] Further studies on OA toward L1210 cells confirmed its cytotoxic effect, revealing an IC_{50} value (concentration inhibiting cells growth by 50%) of 0.13 μg/mL after 48 h incubation. Moreover, the toxic effect of OA was moderately decreased by 7-*O* esterification with fatty acids (IC_{50} = 0.78–1.5 μg/mL).[54] Cytotoxicity of OA was compared also to that of DTX-1 and DTX-2 in different cell lines. Using human larynx HEp-2 and colon Caco-2 carcinoma cells, DTX-1 (IC_{50} = 15 and 26 ng/mL, respectively) was shown to be more potent than OA (IC_{50} = 26 and ≥160 ng/mL, respectively), after 24 h incubation. Moreover, Hep-2 cells were more sensitive to both these compounds than Caco-2 cells.[55] The toxic effect of OA compared to that of DTX-2 was evaluated using primary cultures of rat cerebellar neurons. After 24 h incubation, OA induced neurodegeneration and cell death with a potency about fourfold higher than that of DTX-2 (IC_{50} ≈ 2 and 8 nM, corresponding to about 1.6 ng/mL and 6.4 ng/mL, respectively). A cytotoxic effect of DTX-2 was observed also towards astrocytes. This effect required significantly higher concentrations of DTX-2 (IC_{50} ≈ 50 nM, corresponding to about 40 ng/mL), although cell damage appeared much earlier (after 7 h exposure) than in neurons (at least 20 h exposure).[56]

The toxic effects of OA and its analogues were determined also in other primary cell cultures and stabilized cells lines to investigate, at a cellular level, the biochemical mechanisms involved in their activity, as well as to develop cytotoxicity assays for detecting these toxins in contaminated mussels.[19,55–59] In general, a cytotoxic activity was observed at concentrations above 10 nM, with morphological and functional changes and cell death that occurs mainly by apoptosis rather than by necrotic phenomena.[60]

Apoptotic changes in the cells involve both nuclear and cytoplasmic structures as well as extracellular matrix components, and exhibit characteristic morphologic features consisting mainly in plasma membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation.[61]

Landmark morphological changes typical of apoptosis were frequently observed in cells exposed to OA, DTX-1, or DTX-2. They include altered cell shapes (rounded or flattened cells), detachment, loss of microvilli and focal adhesion structure, shedding of cell contents through surface bleb formation, appearance of cytoplasmic vacuoles, redistribution and compacting of cytoplasmic organelles, chromatin condensation, hyperconvolution of the nuclear membrane, and nuclear fragmentation. These changes occur within minutes (or sometimes hours) after exposure to the toxin, depending on the toxin concentration and the cell type.[50,55,56,62–70]

These morphological changes, accompanied by the detachment of monolayer cultured cells from the substratum are due at least in part to perturbations of cytoskeletal elements (microtubules, microfilaments, and intermediate filaments) and of extracellular matrix components induced by OA class compounds. In particular, by altering the phosphorylation level of key cytoskeletal proteins (i.e., F-actin) as well as of integrins and of the scaffolding proteins (i.e., talin and vinculin), the toxins alter the morphology of the cells and impair their attachment to the extracellular substrate. The loss of cells anchorage, in turn, leads to a particular apoptosis of cells via a process named anoikis.[70–76]

Phosphorylation and dephosphorylation processes are key events influencing almost every single step in signaling cascades within cells, and can be regulated by the serine/threonine protein phosphatases. Indeed, the phosphorylation state of proteins involved in apoptosis, such as membrane

receptors, structural proteins, Bcl-2 proteins, caspases, transcription factors, kinases and other molecules, regulates their activity, leading to cell survival or death. Therefore, OA and its analogues can induce apoptosis through protein phosphatase inhibition,[77–79] and this is supported further by the finding that OA tetraacetate (an OA derivative without inhibitory activity of protein phosphatases) does not provoke apoptosis.[78,80] In general, these toxins act independently of new protein synthesis,[81] through mechanisms leading to apoptosis via mitochondrial-dependent and -independent pathways. In particular, OA was shown to induce cytoskeletal disruption in apoptotic cells. F-actin depolymerization associated to the signal transduction pathways leading to apoptosis was described in human neuroblastoma BE(2)-M17 cells, together with an impairment of integrins and anchorage to extracellular matrix.[73–76] Cellular detachment, cytoskeleton disorganization, and focal adhesion loss were observed as early morphologic events also in other cellular types, such as human neuroblastoma SH-EP cells, rat small intestinal cells (IEC-6), and renal tumor ERC-18 cells.[50,66,70]

Apoptosis-related cellular modification, induced by OA, such as mitochondrial transmembrane potential decrease, cytochrome *c* release from mitochondrial intermembrane space to the cytosol, activation of multiple caspases isoforms, decreased levels of monomeric Bcl-2 and Bax proteins, phosphorylation of Bcl-2, inhibition of protein synthesis and DNA fragmentation has been described in a large variety of cell lines.[19,64,66,69,70,75,77,81–91] Finally, phosphorylation of histones, but not changes in their quantity, was reported to be an early step in DNA fragmentation, as observed in thymocytes apoptosis induced by OA.[92,93]

The mechanisms of OA-induced apoptosis involve also the expression of specific genes, such as the tumor suppressor gene p53, encoding for protein p53 regulating the expression of Bcl-2 family proteins, and Fas receptor. In this respect, OA can lead to increase phosphorylation of p53 protein and its transcriptional activity, inducing apoptosis. Other transcription factors, such as nuclear factor NF-κB, can be activated by OA through their increased phosphorylation level, leading to increased expression of proteins involved in apoptosis. The expression of pro- and anti-apoptotic proteins can be influenced by OA also stabilizing or destabilizing the relevant mRNA molecules at the post-transcriptional level.[77,82,83,94–100] Apoptosis induced by OA and its derivatives is associated with changes in the activity of other proteins and in the expression of specific genes, some of which are also involved in cell cycle control.[19]

11.3.2 Control of Cell Cycle, Cell Proliferation, and Differentiation

Okadaic acid (OA) and related compounds can influence the cell cycle, affecting the phosphorylation level of proteins involved in the control of cell proliferation. In this context, a diversity of response to OA, sometimes opposite, was observed among varying cell types and under different experimental conditions. On one hand, OA was shown to stimulate DNA synthesis, promote cell division, and prevent apoptosis. On the other hand, the toxin was found to reverse the phenotype or inhibit their transformation, arrest cell division, and induce apoptosis.[101]

The eukaryotic cell cycle is tightly controlled by regulatory mechanisms that ensure faithful replication of the genome before cell division. These regulatory mechanisms, named cell-cycle checkpoints, are based, to a large extent, on the reversible phosphorylation of enzymes on serine/threonine residues, and the major control sites are G1/S and G2/M phases.[19,20,102]

These toxins can influence the cell cycle by a complex series of direct and indirect actions on a variety of molecular targets by inhibiting protein phosphatases, hence altering the phosphorylation state of proteins involved in the control of the cell cycle. Exposure of mammalian cells to OA leads to hyperphosphorylation and activation of cyclin-dependent kinase 1-cyclin B complex (CDK1-cyclin B, called also M-phase-promoting factor, MPF; or Cdc2-cyclin B), which leads the cells to G2/M transition and to a mitosis-like state, characterized by a premature chromosome condensation and break of the nuclear lamina. This event seems to depend mainly on the inhibition of protein phosphatase 2A, which is necessary to maintain the complex CDK1-cyclin B in its inactive form.[19,20] Nevertheless, inhibition of other phosphatases, such as protein phosphatases 4 and 5, could mediate,

at least in part, this effect.[19] Other examples of cellular proteins whose phosphorylation is increased by OA exposure are the retinoblastoma protein (pRb), histones, and vimentin, in which the phosphorylation degree is strictly related to the stimulatory effect of these toxins on cell-cycle progression toward mitosis.[79]

Consequent to protein phosphatases inhibition, OA and its derivatives can also maintain the phosphorylation state of serine/threonine residues of a variety of protein kinases, among which are mitogen-activated protein kinases (MAPKs). An MAPK cascade involves different kinases (Raf-1, MEK, ERK, and Rsk) stimulated by OA class compounds, which play a central role in mediating cell cycle reentry and activation of different transcription factors downstream from cell-surface receptors. The duration of the kinase cascade activation is critical for the cells direction towards growth (transient activation) or differentiation (prolonged activation), and sometimes the activation is associated to apoptotic events.[19,79] The MAPKs pathway also increases the transcription of genes required for cellular proliferation, such as the proto-oncogenes *c-jun* and *c-fos*.[102] OA also induces the expression of cell-cycle regulatory proteins, such as cyclin A, cyclin B, CDK1, and pRb, with an effect that can be either stimulatory or inhibitory, depending on its concentration and time of cells exposure to the toxin. Furthermore, OA induces the expression of the WAF1/CIP1 gene, encoding for the protein-inhibiting cyclins D, E, and A dependent kinases, thereby blocking the cells in G1 phase.[79,84,102]

By preventing dephosphorylation of specific proteins, OA can stimulate DNA synthesis, promote mitosis, and exert proliferative effects.[79,103–106] A complete induction of cells to enter in mitosis was observed for a mixture of DTX-1, 27-*O*-acetyl DTX-1, and 27-*O*-acetyl AO: 100 ng/mL of the mixture inhibited G2 checkpoint, promoting G2/M transition.[107] In turn, preventing a timely dephosphorylation of proteins would eventually block the chain of events underlying cell division, leading to the arrest of mitosis and cell death.[79] In this respect, evaluation of the effect of OA on a human colon cell line (Caco-2) revealed reduction of cell viability, fragmentation of DNA and cell cycle arrest, an increased number of S-phase cells, and a decreased G0/G1 phase cells.[108] These opposite events can depend on the types of cells and experimental conditions, the concentration of the toxin, and the time of exposure to the compound. In general, it has been observed that long-term exposure to low OA concentrations makes the cells resistant to this toxin.[84]

Among these other impacts, OA can influence the cell cycle affecting the microtubule dynamic and the spindle formation during the cell division.[19,109]

11.3.3 Mutagenic and Genotoxic Activity

Studies on genotoxicity of DSP toxins gave conflicting results. In particular, no mutagenic activity was observed for OA using *Salmonella typhimurium* strains, with or without metabolic activation.[110] In contrast, genotoxic effects were observed in various eukaryotic cell lines *in vitro*.[1,111] OA (10–15 ng/mL, corresponding to 12.4–18.6 nM) was mutagenic in Chinese hamster lung cells without metabolic activation, with a potency comparable to that of one of the strongest known mutagens, 2-amino-*N*6-hydroxyadenine. It was also hypothesized that the mutagenic action was due to changes in the phosphorylation level of proteins involved in DNA replication or repair rather than to OA-DNA adduct formation.[1,110] On the other hand, DNA adducts were observed in BHK21 C13 fibroblasts and HESV keratinocytes after 24 h exposure to OA at concentration ranges of 1–5 nM and 0.1–5 nM, respectively. At 1 nM, the number of adducts in BHK21 C13 cells was the highest (13 adducts), and it decreased with increasing concentration of the toxin (3 adducts at 5 nM). In HESV cells, the number of adducts increased between 0.1 nM and 0.5 nM and decreased in a concentration-dependent manner from 1 nM to 5 nM. Moreover, ten adducts were similar in the two strains, while nine were specific of BHK21 C13 cells, and five of HESV ones.[111] Genotoxicity of OA was observed also in the monkey kidney epithelial Vero cells, where the toxin (7.5 ng/mL, corresponding to 9.3 nM) induced hypermethylation of the bases of DNA,[112] and in the human colorectal adenocarcinoma cell line Caco-2, where the concentration of 15 ng/mL (corresponding to 18.6 nM) increased the level of methylation and oxidation of the bases in DNA.[113] Furthermore, low

concentrations of OA (0.75–7.5 ng/mL, corresponding to 0.93–9.3 nM) caused CG to AT transversion mutations and gene deregulation in Caco-2 cells.[114] Studies on OA genotoxicity in Chinese hamster ovary cells (CHO-K1) and human lymphocytes revealed also an aneugenic potential for this compound, which induced chromosome loss and micronuclei formation at nanomolar concentrations.[115,116] Moreover, aneuploidy induced by OA in CHO-K1 cells seems to depend on the inhibition of the chromosome attachment to the mitotic spindle and centrosome amplification. Aneugenic properties of OA were also observed in human colorectal Caco-2 cells, with micronuclei formation, mitotic arrest, and polyploidy, as well as in mouse oocytes.[117,118] Transient exposure of mouse oocytes to OA (10–1000 ng/mL, corresponding to 12.4–1.242 nM) during meiosis I led to premature homolog and sister chromatid separation and a concentration-dependent increase of the frequency of aneuploid mature oocytes.[118]

11.3.4 Tumor Promotion and Cell Transformation

The tumor promotion activity of OA and DTX-1 was first observed *in vivo* on mouse skin, initiated with 7,12-dimethylbenz[a]anthracene (DMBA), and subsequently in rat glandular stomach, initiated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).[44] The *in vivo* tumor-promoting activity of OA and DTX-1 was confirmed also in *in vitro* cell cultures. In particular, exposure of mouse embryonic fibroblasts BALB/3T3 to the initiator 3-methylcholanthrene for 72 h, followed by treatments for 2 weeks with OA (12.4 nM or 24.8 nM) or DTX-1 (3.66 nM) led to transformation of cells. However, OA tetramethyl ether (34.7 nM) did not affect initiated cells transformation. Moreover, OA failed to induce transformation of cells without 3-methylcholanthrene pretreatment and did not show initiating activity when cells were treated first with the toxin and then with the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA).[119,120] The tumor promotion activity of OA was subsequently confirmed by an *in vitro* assay using co-cultures of keratinocyte cell line 308 derived from mouse skin, initiated with DMBA and confluent normal primary keratinocytes. In this assay, the potency of the toxin was more than 60-fold higher than the tumor promoter TPA since 2.48 nM OA induced as many transformed foci as TPA at the concentration of 162 nM.[121] The tumor promotion by OA was studied in other cell lines, such as T51B rat liver cells initiated by MNNG,[106] mouse embryonic fibroblast C3H/10T1/2 cells transfected with a plasmid containing a full-length bovine papilloma virus DNA,[122] and v-Ha-*ras* oncogene transfected BALB/c 3T3B cells (Bhas 42 cells).[123] In contrast to its promoting activity, OA can also act as antipromoter or as a transformation revertant, depending on its concentration, timing, and the different *in vitro* experimental model systems.[44,79]

The tumor-promoting activity of OA class compounds is due to their inhibitory activity of protein phosphatases, which increases phosphorylation/activation of various proteins involved in the signal transduction pathways regulating the cell cycle and cell transformation.[19,20,96,124,125] Different effects of OA leading to transformation of cells were reported, such as induction of mitosis,[103,105] cycle progression,[104] as well as cell proliferation genes and proto-oncogenes expression.[124,126] It has been demonstrated that OA induces the release and expression of tumor necrosis factor-α (TNF-α) and other cytokines,[127–129] phosphorylation of the tumor suppressors p53 and retinoblastoma pRb proteins,[96,130] and activation and induction of the nuclear transcription factors, such as activator protein-1 (AP-1) and NF-κB.[19,98,131]

In different cell types, OA was shown to induce the release or expression of TNF-α, one of the key cytokines involved in tumor promotion by changes in phosphorylation/dephosphorylation of proteins, activating transcription factors and increasing genes expression.[125,128,132] OA also induces hyperphosphorylation of the tumor suppressor p53 protein, a transcription factor involved in the regulation of the cell cycle and coordinating a complex network of proteins protecting cells from malignant transformation. In normal conditions, p53 is regulated at low levels by its targeted degradation and suppression of its transcriptional activity. The latter is controlled by co-regulators and by oligomeric state and posttranslational modifications of p53 itself, such as phosphorylation.

By inhibiting protein phosphatases, OA and analogues increase the phosphorylation level of p53, with the possibility to induce aberrant mitoses and cellular transformation.[19,79,106,133,134]

Several growth-regulatory genes are activated by OA, the most prominent being the proto-oncogenes *c-fos* and *c-jun*, whose products interact forming the heterodimeric transcription factor AP-1, involved in the regulation of cell growth and differentiation, and playing a central role in tumor promotion. Moreover, *in vitro* experiments showed that OA increases the phosphorylation level of AP-1, enhancing its transcriptional activity, inducing aberrant cell proliferation and maintaining a malignant phenotype.[19,124,135,136]

The tumor-promoting activity of OA can be also partially related to other actions at cellular level, such as oxidation of different substrates, methylation of DNA bases, and inhibitory mechanisms of gap intracellular communication, which is believed to be of importance in the promotion stage of carcinogenesis.[114] An additional factor hypothesized to contribute to the tumor promotion by OA is amplification of genes conferring multidrug resistance to cells.[137]

11.3.5 Diarrheogenic Activity

Diarrheic shellfish poisoning (DSP) toxins provoke dose-dependent diarrhea, both in humans and in rodents.[138,139] First reports suggested that the diarrheic effect of OA and its derivatives is related to their inhibitory activity on protein phosphatases, which results in phosphorylation of proteins controlling the electrolytes secretion by intestinal and cytoskeletal proteins influencing the permeability of intestinal epithelium.[1] Cytoskeletal elements affected by OA were shown to be actin filaments that control the integrity and function of tight junctions between epithelial intestinal cells.[1,50,52] This effect was observed in a nontransformed cell line derived from rat small intestine (IEC-6 cells) by fluorescence and scanning electron microscopy. Micromolar concentrations of OA induced a time- and concentration-dependent cell retraction and rounding due to the reorganization of the F-actin network. Within 30 min of exposure to OA, marginalization of F-actin molecules was observed and, prolonging the time of exposure to the toxin up to 90 min, also showed a marked disruption of stress fiber organization. Although the vimentin pattern and microtubular apparatus were not altered within 60 min exposure to OA, a clear reorganization of these cytoskeletal elements (collapse of vimentin filaments around the nucleus and formation of a net-like structure of microtubules) was detectable after 90 min of exposure to the toxin. No alteration of plasma membrane permeability and transmembrane potential was detected in OA-treated cells by flow cytometric analysis.[50,140] Further *in vitro* studies on cultured human intestinal epithelial cells (T_{84} and Caco-2) revealed that OA decreased transepithelial electrical resistance in a polarized way, an index of the increased permeability across the paracellular pathway, governed by tight intercellular junctions.[51,52] Moreover, OA was shown to increase transepithelial permeability of Caco-2 cells monolayers to a fluorescent compound without damaging the cell membrane or inducing lactate dehydrogenase release from cells, as index of their damage or death. This effect was suggested to be a consequence of alterations of the cytoskeletal structure that lead to tight junctions opening and to an increased transepithelial permeability.[52] Contrary to this, the toxin does not directly stimulate cellular Cl^- secretion but attenuates the ions secretion by secretagogues. Therefore, the diarrhea induction by OA can be attributed to the paracellular permeability of intestinal epithelium rather than to a stimulation of Cl^- secretion by intestinal cells.[51] It has also been suggested that OA can influence the nutrient, ionic, and water absorption across the small intestinal cells by altering the transport system.[33]

11.3.6 Other Activities

Okadaic acid (OA) class compounds were shown to exert other biological effects, such as on metabolism, muscle contraction, secretion, and biosynthesis of different molecules. Considering the effects on the metabolism, *in vitro* studies revealed that OA can influence the metabolism of glucose and that of lipids.[19,33,38] Glucose metabolism can be affected by the toxin through inhibition of glycogen

synthesis and increasing glycogenolysis. These effects had been attributed mainly to inhibition of protein phosphatase 1, a phosphatase playing an important role in the control of glycogen metabolism.[19,33,141] Moreover, OA can influence the transport of glucose into the cells, exerting an insulin-like effect, but, in particular conditions, it can also decrease insulin-stimulated uptake of glucose. In particular, OA was shown to induce insulin secretion, as observed after short treatment of beta cells or permeabilized rat pancreatic Langerhans islets with the toxin: OA promoted entry of Ca^{2+} ions with the consequent insulin exocytosis, probably through hyperphosphorylation and activation of voltage-gated L-type Ca^{2+} channels.[142] OA was also shown to exert an insulin-like effect in different cells, such as adipocytes of rats, mice, and humans, and mouse skeletal muscle, since it increased glucose uptake. The effect is mediated mainly by translocation of a specific glucose transporter from intracellular vesicles to the cell surface.[143–147]

Opposing effects of OA on insulin exocytosis, glucose uptake, and its metabolism are also reported. In particular, preincubation of mouse or rats pancreatic Langerhans islets or beta cells with OA-inhibited insulin release induced by glucose, by decreasing both the magnitude and efficacy of the Ca^{2+} signal in β cells.[148,149] Other studies revealed that pretreatment of adipocytes with OA inhibited insulin-induced increase of the rate of glucose transport and glycogen synthesis. This action had been attributed mainly to an inhibition of the hormone-induced redistribution of glucose transporter to the plasma membrane. Furthermore, in rat adipocytes and in rat and guinea pig hepatocytes, the glycogen synthesis and glucose uptake induced by insulin were inhibited by low concentrations of OA, which did not affect these events in the absence of the hormone.[19,33,146,150,151] OA stimulates also basal lipolysis in adipocytes and inhibits fatty acids synthesis induced by insulin.[19,152]

Studies on muscle contraction revealed that OA increases the contraction of smooth muscle fibers and provokes a long-lasting contraction of smooth muscle isolated from human arteries.[153] Muscle contraction is triggered by phosphorylation of myosin light chain and relaxation occurs when this protein is dephosphorylated by a phosphatase (myosin light chain phosphatase), consisting of the catalytic subunit of protein phosphatase 1 and of two regulatory subunits. OA effect seems to be due mainly to inhibition of this protein phosphatase and consequently to inhibition of dephosphorylation of myosin light chain.[19,154] Other studies revealed that OA exerts inhibitory effects on some smooth muscle preparations, which suggest that although myosin light chain phosphorylation is required for the contraction, it may not be sufficient for its mainteinance.[19] Finally, OA was reported to influence smooth muscle contraction also by influencing intracellular Ca^{2+} levels, and K^+ channel activity.[19]

OA can influence the synthesis and secretion of several molecules by different cell types. An example is represented by mast cells and basophils, immune effector cells playing a pivotal role in allergic and inflammatory responses. An *in vitro* study revealed that OA inhibits immunoglobulin-E-mediated release of histamine from human mast cells and basophils in a concentration-dependent way, whereas two structural analogues of the toxin, okadaol, and okadaone, known to be less active than the parent molecule as inhibitors of protein phosphatases, were less active than OA as inhibitors of histamine secretion.[155] Other *in vitro* studies on rat mast cells or basophilic leukemia cells revealed that OA inhibited their immunological activation and degranulation, but it had no effect on mast cell activation triggered by other artificial secretagogues. On the other hand, following treatment of unstimulated mast cells with OA for 24 h, a concentration-dependent production of newly formed mediators, such as interleukin-6 was observed. OA effect on mast cell degranulation and their *ex novo* synthesis of mediators seems to be related to its inhibition of protein phosphatase 2A, which also plays a crucial role in the biology of these cells.[38]

11.4 *IN VIVO* TOXICITY

11.4.1 Experimental Toxicity

Studies on the toxic effects of DSP toxins in animals were performed mainly after acute toxin administration, and only few studies after repeated OA administration are available.

TABLE 11.1
Lethality of DSP Toxins after Single Intraperitoneal Injection in Mice

Toxin	Lethality (μg/kg)	Animals	Observation Time	Reference
OA	LD_{50} = 192	No information	No information	[3]
	LD_{50} = 210	HLA:(SW)BR female mice, 16–22 g	No information	[156]
	LD_{50} = 225	CD-1 female mice, 18–20 g	24 h	[157]
	LD_{50} = 204	CD-1 female mice, 19–22.5 g	24 h	[43]
DTX-1	MLD = 160	Mice, 17–20 g	24 h	[1]
DTX-2	LD_{50} = 352	CD-1 female mice, 19–22.5 g	24 h	[43]
DTX-3	MLD = 500	Mice, 17–20 g	24 h	[1]
7-*O*-palmitoyl OA	MLD = 100	ddY male mice 16–20 g	24 h	[54]
7-*O*-linoleoyl OA	MLD = 100	ddY male mice 16–20 g	24 h	[54]

LD_{50} = median lethal dose; MLD = minimum lethal dose.

11.4.1.1 Acute Administration

Studies after *acute intraperitoneal injection* (i.p.) of OA in adult mice revealed that the LD_{50} (median lethal dose) values for this toxin ranged from 192 to 225 μg/kg (Table 11.1).[3,43,156,157] Recently, a LD_{50} value of 352 μg/kg was determined in mice for DTX-2, estimating for this compound a relative toxicity of 0.6 as compared to that of OA.[43] The minimum lethal dose of OA, DTX-1, and DTX-3 in adult mice was reported to be 200, 160, and 500 μg/kg, respectively.[1] Furthermore, the semisynthetic OA derivatives 7-*O*-palmitoyl and 7-*O*-linoleoyl OA revealed a minimal lethal dose in adult mice of 100 μg/kg[54] (Table 11.1). Anyway, some of these studies did not detail the number of mice injected, their strain, their gender, their age, or the range of the administered doses.

Pathological changes induced by OA, DTX-1, and DTX-3, intraperitoneally injected in mice and rats, were evaluated by morphological examinations. A study in suckling mice (7–10 g) was carried out by Terao et al. for DTX-1 (50–500 μg/kg).[158] Within 15 min after administration, the toxin provoked distension of the duodenum and upper portion of the small intestine, with accumulation of mucoid, but not bloody fluid. Morphological changes induced by 50 μg/kg of DTX-1 showed a slight congestion during the first 2 h after administration, while higher doses led to more severe congestion of the villous and submucosal vessels within 1 h. At histological level, marked edema in the lamina propria of villi and intracellular vacuolization of mucosal epithelium were observed with 300 μg DTX-1/kg. At the ultrastructural level, three consecutive stages of the intestinal villi injuries were described: extravasation of villi vessels, degeneration of the absorptive epithelium, and desquamation of the degenerated epithelium from the villous surface. Throughout the three stages, only slight changes in Lieberkuehn glands cells were observed, indicating a protection of the crypts from the toxic action of DTX-1.[158]

In another study, OA or DTX-1 (375 μg/kg) induced marked intestinal injuries, while only slight changes at this level were caused by the same dose of DTX-3. In particular, OA and DTX-1 provoked marked and progressive disruption of the absorptive epithelium of villi in the duodenum and upper portion of jejunum, which led to desquamation of epithelial cells from the lamina propria and to intestinal erosions.[159,160] Similar morphological changes in mice were observed after injection of OA at doses ranging from 100 μg/kg to 400 μg/kg, with an incidence related to the administered dose of the toxin.[157] On the other hand, the effect of DTX-3 at the intestinal level was limited to a dilation of the cysternae of the Golgi apparatus and to the appearance of vesicles in the cytoplasm of the absorptive epithelium.[159,160] Intestinal injuries provoked by OA were not permanent and, after injection of 200 μg/kg, tissue recovery in living mice began within 2 h of the toxin administration, to be complete in 24 h.[160]

Mice and rats injected with OA, DTX-1, or DTX-3, at doses ranging from 100 μg/kg to 400 μg/kg, also showed damages in the liver, with vacuolization and/or necrosis of hepatocytes.[157,159] Hepatotoxicity seems to be more pronounced for DTX-3, which caused severe degeneration and necrosis of the hepatocytes in the midzone of the liver. Moreover, at ultrastructural level, an unusual proliferation of rough and smooth endoplasmic reticulum, membrane-whorls, non-fatty vacuoles, ribosomes, and fat droplets in the cytoplasm of hepatocytes in the midzone of liver lobules were observed.[159]

Lethality of DSP toxins after *oral administration* seems to be much lower than that observed after their intraperitoneal injection. After intragastric administration of OA to adult mice, the LD_{50} ranged between 1 mg/kg and 2 mg/kg,[157] and administration of the toxin to rats (1 mg/kg) caused the death in 10% of the treated animals.[161] Oral administration of DTX-1 to adult mice provoked lethality at the doses of 300 μg/kg and 400 μg/kg (2/4 and 3/4 mice within 6 h, respectively), and at a lower dose (100 μg/kg), 1/5 mice died 30 h after treatment.[162]

DSP toxins were orally administered to suckling mice (4–5 days old) to evaluate their diarrheic effects. OA and DTX-1 induced intestinal fluid accumulation at 0.1 mouse units (MU)/mouse, while DTX-3 was effective already at 0.05 MU/mouse (1 MU is the minimum dose necessary to kill at least 2 out of 3 mice in 24 h and corresponds to 4 μg for OA and 3.2 for DTX-1).[138] In other studies, the diarrheic potency of 7-*O*-acyl esters of OA in suckling mice was similar to that of OA.[54,162]

In adult mice (18–26 g) or rats (100–200 g), oral administration of OA (0.13–4 mg/kg), DTX-1 (0.75 mg/kg) or DTX-3 (0.75 mg/kg) was shown to induce fluid accumulation in the intestinal lumen and, at 0.75 mg/kg or more, also diarrhea. Morphological signs of toxicity induced by these toxins were similar to those observed after intraperitoneal injection of OA or DTX-1. These signs were observed also after oral administration of DTX-3, which did not seriously affect intestinal tissues when administered intraperitoneally.[159,163] In general, within 1 h after the toxin's administration, the absorptive cells lining of the villi were disrupted and desquamated from the lamina propria, while cells located in the crypts of Lieberkuehn were unaltered for 24 h. The degenerated enterocytes and villi debris were subsequently absorbed, and mucosal regeneration began within 2 h, being completed after 24–48 h, both in mice and rats.[159–161,163] Minimal morphological changes were reported also at colon level in rats administered with OA, where regenerative changes observed after 24 and 48 h (e.g., fewer goblet cells and more distinct nuclei in the absorptive cells) suggested that some colonic damage had occurred.[163] On the other hand, Yuasa et al.[164] observed edema or acute inflammation of the squamous mucosa of the esophagus and forestomach, and erosion of the fundic mucosa of mice after acute oral administration of OA (2–10 μg/mouse of 6 weeks age), but no significant morphological changes were noted in other parts of the gastrointestinal tract. Similarly, no morphological changes were evidenced after single oral administration of DTX-1 (2–10 μg/mouse). However, these doses of the two toxins increased cell proliferation in several parts of the gastrointestinal tract in a dose-dependent way, as shown by the enhancement of bromodeoxyuridin (BrdU)-labeling indices. In particular, OA increased cell proliferation in all parts of the gastrointestinal tract of mice, except in pyloric mucosa, 18 h after the toxin administration. Administration of DTX-1 was followed by two peaks of cell proliferation at 18 h and 36 h in the esophagus, ileum, and colon. Moreover, cell proliferation in forestomach, fundus, pylorus, and jejunum continuously increased from 6 h after administration. A significant proliferation was observed also in the skin 30 h after OA and DTX-1 administration, whereas no effect on the liver or kidney was evident within 36 h observation period.[164] Le Hégarat et al.[117] evaluating the genotoxicity of OA and the induction of cell proliferation after single oral administration in mice (435–610 μg/kg), observed an increase of micronucleated and mitotic gut cells 24 h after the treatment with 525 μg OA/kg body weight. In a second experiment, administering doses ranging from 115 to 1341 μg OA/kg, no increases in micronucleus frequency were observed 24, 36, or 48 h after the administration of 115 and 230 μg OA/kg, but an increase of mitotic index was detected 36 h after the gavage with 115 μg OA/kg. Doses of OA higher than 230 μg/kg were lethal to the mice within few hours after gavage.[117]

In addition, single oral administration of OA or DTX-1 to rats (1–50 μg/rat of 8 weeks age) dose-dependently increased cell proliferation in the digestive tract of animals. The proliferative

effect at gastrointestinal level and skin of OA and DTX-1, after single oral administration, suggested that these toxins might have been promoting potential for carcinogenesis.[164]

Other studies revealed that single oral administration of OA and its derivatives affect the stomach and liver. In particular, DTX-1 (0.75 mg/kg) and DTX-3 (0.75 mg/kg) provoked the degeneration of surface cells of gastric mucosa in mice and rats.[159] Similarly, forestomach alterations were observed after OA administration to mice (1 mg/kg), evidenced as vacuolar degeneration of epithelium, associated with acute inflammation of submucosa, and hyperplasic reactions of the keratinized epithelium.[157] Some studies reported the hepatotoxic effects induced by intragastric administration of these toxins, even though the results are sometimes contradictory, probably due to differences of the administered doses and gender, age, and animal species used in the different studies. In particular, Terao et al.[158] observed a marked accumulation of fat droplets and necrotic foci in the midzonal and periportal regions of the hepatic lobule of mice treated with DTX-3 (0.75 mg/kg), but not in mice administered with the same dose of OA or DTX-1. Similarly, OA administration to rats (1–4 mg/kg) did not provoke any detectable liver injury.[161] On the other hand, a study in mice receiving 1 or 2 mg OA/kg revealed liver damages as degenerative changes of hepatocytes, confirmed by a marked raise in the plasma transaminase levels.[157] Intragastric administration of OA to mice (115 and 230 μg/kg) was also reported to induce apoptotic DNA strand breaks in ileum, liver, and kidney.[117]

Berven et al.[161] described the toxic effects induced by OA after *intravenous injection* in rats (100–200 g). The toxin (50–500 μg/kg) had little effect on intestinal function, but caused liver damages. At doses of 0.2 μg/kg or more, rats became inactive, cyanotic, and died within few hours. Autopsy and histological examination revealed a pronounced congestion of blood in the liver and a dissolution of hepatic bile canalicular actin sheaths, but neither the microtubular nor the keratin network of hepatocytes was affected by the toxin. Similarly, administration of OA did not induce apoptotic DNA fragmentation in hepatocytes.

Injection of OA or its derivatives in ligated intestinal loops of rats and mice was performed to evaluate the induction of hypersecretion by these toxins, related to their diarrheic effects. Injection of OA in rat intestinal loops (0.5–5 μg/loop) induced strong and rapid fluid secretion, with peak values within 2 h. Light microscopic changes were observed within 15 min after injection of the toxin: enterocytes at the top of the villi became swollen and subsequently detached from the basal membrane. After 90 min, most of the enterocytes at the upper part of the villi shed into the lumen, and large parts of the flattened villi were covered by goblet cells, which were not affected by the toxin. The OA effect was dose-dependent since the lower doses (<3 μg) affected only the top of the villi, whereas a higher dose (5 μg) induced a collapse of the total villi architecture.[165,166] Furthermore, a study in mouse intestinal loops model showed that the fluid-accumulating potency of OA was reduced by esterification of its 7-hydroxy group with a fatty acid, contrary to the observation in suckling mice.[54]

Direct OA *microinjection into the dorsal hippocampus* of rats (220–260 g) was performed to evaluate its effects in brain. OA (55–300 ng) provoked a dose-dependent neurodegeneration in the CA1 subfield, dentate gyrus, and/or hilus, associated with increased serine phosphorylation of some hippocampal proteins. These damages were apparent as early as 3 h after injection of 300 ng OA, being more evident and dose-related after 24 h. After OA (300 ng) injection, hyperexcitability of rats and epileptiform electroencephalogram (EEG) discharges were also observed till 4–6 h. In contrast, DTX-1 injection (300 ng) provoked EEG discharges with a longer latency and lower frequency than those registered in OA-treated rats, but it did not induce any neuronal damage.[167,168] Neuronal and glial damages, as well as hyperphosphorylated neurofilament proteins, were observed also after OA microinjection in the rat basal nucleus.[169]

11.4.1.2 Repeated Administration

Tubaro et al.[170] evaluated the toxic effects of OA after daily repeated oral administration to mice, over a period of 7 days. OA (1 mg/kg/day) induced diarrhea within 30 min after each administration, loss

of body weight, reduced food consumption, and the death of 2/5 mice at the fifth day of treatment. The toxic effects were observed in different organs, but mainly in the forestomach, which showed ulcerated and/or hyperplasic mucosa as well as acute or subacute inflammation of submucosa. Since the forestomach is typical of rodents and its lesions are probably "species-specific," these effects of OA are not predictive for toxicity in humans. The toxin also induced liver alterations, revealed as atrophic signs of hepatocytes and by the increase of plasmatic levels of transaminases. Nevertheless, liver alterations were less pronounced than those observed after acute oral administration,[157] probably as a consequence of activation of regenerative mechanisms and/or improvement of detoxifying functions of the liver, stimulated by the repeated exposure to the toxin. Other histological changes were observed histologically in lymphoid organs, pancreas, and fatty tissues as atrophic signs consequent probably to the body weight loss of mice. At the ultrastructural level, a package of rounded mitochondria and alterations of fibers in cardiac muscle cells were observed. No apoptotic changes were observed at the cardiac level. Although small intestine alterations were caused by single administration of OA, no degenerative lesions in the small intestine were observed by light microscopy after repeated toxin treatment. This might be the consequence of activation of mechanisms of resistance to OA at the intestinal level and/or to activation of regenerative processes such as those reported for the intestinal mucosa after single toxin administration.[170]

Repeated oral administration and topical skin application of OA or DTX-1 were carried out to evaluate the *tumor-promoting activity* of these toxins in rats or mice. Two-stage experiments revealed tumor promotion properties of OA and DTX-1 on mouse skin and in the rat glandular stomach.[44,171,172] Tumor promotion was evaluated in the glandular stomach of rats initiated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in a two-stage carcinogenesis experiment. OA in drinking water (10 mg/rat/day, from weeks 9–55 of the experiment, and 20 mg/rat/day, from weeks 56–72), significantly increased the development of neoplastic changes (adenomatous hyperplasias and adenocarcinomas) in the glandular stomach.[172] Furthermore, 1 week after initiation with a single skin application of 7,12-dimethylbenz[α]anthracene (DMBA) to mouse skin, OA or DTX-1 (1 μg) was applied twice a week, for 30 weeks. High percentages of tumor-bearing mice and high average numbers of tumors (carcinomas and papillomas) per mice were observed.[44,171]

In other studies, OA was repeatedly administered to rats as chronic infusion into lateral brain ventricles for up to 4 months (70 ng/day). The toxin was shown to induce severe memory impairment in treated animals, changes in gray and white matter areas typical for Alzheimer disease, and apoptotic cell death.[173,174]

11.4.2 Human Toxicity

Diarrheic shellfish poisoning (DSP) cases were reported in different areas of the world, and the distribution of the poisoning involved mainly Japan, Europe, Chile, Thailand, Canada, Australia, and New Zealand.[6] Already in 1990, about 15,000 DSP cases had been recorded worldwide, mainly in Europe and Japan.[139] The DSP incidence, or at least the presence of diarrheic toxins, appears to be increasing, probably also because of increasing awareness about the disease and better surveillance programs.

Symptoms of DSP are mainly diarrhea, nausea, vomiting, and abdominal pain. Table 11.2 shows the rates of various symptoms of poisoning, which occur a short time after the ingestion of contaminated shellfish: about 70% of the patients develop symptoms within 4 h.[139] Although the intoxication can be highly debilitating for some days, no human mortalities from DSP were reported, so far. Recovery is usually complete within 1–3 days, also without any pharmacological treatment. No antidote for DSP is available so far. The treatment of the diarrheic poisoning is supportive with regard to short-term diarrhea, which causes fluid and electrolyte losses. In general, hospitalization is not necessary, as the lost fluid and electrolytes can usually be replaced orally.

Other diarrheic illnesses associated with shellfish consumption, such as those caused by bacterial or viral contamination, should be ruled out on the basis of anamnesis of the patients.[1] Shellfish con-

TABLE 11.2
Incidence of the Main Symptoms of Diarrheic Shellfish Poisoning

Symptoms	Incidence (%)
Diarrhea	99
Nausea	80
Abdominal pain	55
Vomiting	53

taminated with azaspiracids can also provoke diarrheic symptoms similar to those induced by OA. Since, no antidote is presently available for azaspiracids as for OA, differential diagnosis between the two poisonings is irrelevant for the management of patients. Unusual intoxication symptoms, resulting in some hospitalizations, were reported after eating wild rock mussels harvested in the Galician Rias, in the northwestern Atlantic coast of Spain: the case history was mainly neurotoxic, although some cases of diarrhea also were referred. The unusual intoxication, characterized by atypical clinical signs, was due to the simultaneous occurrence of DSP and PSP (Paralytic Shellfish Poisoning) toxins in the ingested mussels.[175]

A lowest observable adverse effect level (LOAEL) was estimated to be 1.2–1.6 μg/kg, on the basis of human poisoning in Japan (eight people from three families, aged 10–68). In another study from Norway, 38 of 70 adults were affected at levels ranging from 1.0 to 1.5 μg/kg. From these data, a provisional acute reference dose (RfD)* of 0.33 μg OA equivalents/kg (based on the LOAEL of 1.0 mg OA/kg and a safety factor of 3) was established (for details, see Reference 15).

Tumor-promoting activity and genotoxic effects are experimentally well documented for OA and DTXs, suggesting human health risks for (sub)chronic exposure to low levels of these compounds. Unfortunately, chronic studies in animals are not available for OA or for DTXs. Due to the insufficient chronic data available, no Tolerable Daily Intake was established by the Joint FAO/WHO/IOC ad hoc Expert Consultation on Biotoxins in Molluscan Bivalves.[15]

An epidemiological study on the potential long-term effects on human gastrointestinal system after exposure to DSP toxins, based on monitoring data, is available. Although only a limited amount of information can be obtained, due to potential bias, an association between contaminated shellfish from French coasts and colon cancers in the inhabitants (mostly among men) of the studied areas was reported.[176]

11.5 TOXICOKINETICS

Data on the toxicokinetics of diarrheic shellfish toxins are limited, and few experimental studies in animals are reported for OA. For humans, the available data derive from only one case report on DTX-3-contaminated mussels.

Distribution of radiolabeled [^{3}H]OA was evaluated after intraperitoneal injection in mice (14 μCi/0.2 mL in saline solution). Most of the radioactivity (33%) was detected in the content of the gastrointestinal tract 3 h after administration and 5% was found 19 h later. Moreover, 27% and 16% of the radioactivity was measured in the liver 3 and 19 h after injection, respectively.

* The acute RfD is an estimated amount of the substance in food, usually expressed on a body-weight basis, that can be ingested in a period of 24 h or less without appreciable health risk to the consumer, on the basis of all known facts at the time of evaluation.

These findings suggested that, following intraperitoneal administration, OA is excreted through hepatobiliary circulation.[44,177]

After acute *oral administration* of radiolabeled [^{3}H]OA by gastric intubation in mice (14 μCi/0.2 ml in sesame oil), most of the radioactivity (>77%) was found in the contents of the gastrointestinal tract 3 h after administration, and 19 h later it decreased to 4%, while 30% was found in feces. After 3 and 19 h after intubation, 1% of the radioactivity was detected in the liver. Thus, most of [^{3}H]OA remained in the intestinal tissue.[44,177]

Matias et al.[178] evaluated the distribution of OA in organs and biological fluids of mice following single oral administration of non-lethal doses of [^{3}H]OA or unlabeled toxin, after gastric intubation. Twenty-four hours after OA administration (50 μg/kg, corresponding to a mild OA oral intoxication dose, which does not cause diarrhea or other signs of toxicity), the toxin was found in all tissues, mainly as unmetabolized compound. The highest toxin concentrations were detected in intestine tissues and contents as well as in urine and feces, the overall amount in the other organs being much lower. Results indicated an enterohepatic cycle of OA with an accumulation within the intestinal tract, which can be considered the main site of its action after oral exposure. A higher oral nonlethal dose (90 μg/kg) induced diarrhea from 8 h on, which did not wash out the toxin. After 24 h, OA was rather accumulated in intestinal tissues and content at high levels, indicating a slow elimination of OA, while distribution in blood, urine, and feces was of the same extent, probably because of hypovolemia caused by diarrhea, which did not allow a normal blood circulation, urine elimination, and enterohepatic cycle.

A study by Ito et al.[179] revealed that oral administration of a wider dose range of OA (75–250 μg/kg) in mice induced secretion of fluids at the intestinal level, which were re-absorbed without diarrhea development. The toxin was absorbed very quickly from the small intestine, mainly from jejunum, and reached the liver within 5 min, as shown by an immunostaining method. OA was accumulated at intestinal and hepatic levels and was systemically distributed, being detected in lungs, heart, and kidneys. It was still detected after 2 weeks in the liver and blood vessels, whereas excretion from kidneys and intestine began 5 min after administration, and excretion through feces continued for 4 weeks. Another immunohistological study after acute oral administration of OA in mice (115 and 230 μg/kg) revealed the presence of low amounts of the toxin in the liver 24 and 48 h later, respectively. The toxin was detected in the duodenum and in the ileum, where it was localized in the mucosal secretions of the goblet cells. However, the toxin was not detected in the colon sections at any dose after the treatment.[117]

In conclusion, after acute oral administration in mice, OA seems to be distributed following this rank order: intestinal content > urine > feces > intestine tissue > lung > liver > stomach > kidney > blood.[15]

After oral administration of [^{3}H]OA to pregnant mice at day 11 of gestation (50 μg/kg), the toxin was found to cross the placental barrier to the fetus, where the percentage of OA was higher than that detected in the liver or kidneys of the mother.[180]

After *intramuscular injection* of [^{3}H]OA to mice (25 μg/kg), the toxin was detected in bile and intestine contents after 1 h, and it showed a pattern of elimination involving biliary excretion and enterohepatic circulation.[181]

A massive intoxication episode in humans due to consumption of shellfish contaminated with a 7-*O*-acyl-derivative of DTX-1 (DTX-3) gave evidence for its metabolic transformation into DTX-1. In fact, while the only DSP toxin detected in mussels was DTX-3, in the patient fecal samples, the only DSP toxin found was DTX-1, indicating that metabolism could occur in the human gastrointestinal system.[182]

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Part VI

Lipophyllic Toxins: Nonphosphatase Inhibitors

12 Planktonic Dinoflagellates That Contain Polyether Toxins of the Old "DSP Complex"

Beatriz Reguera and Gemita Pizarro

CONTENTS

12.1 INTRODUCTION

Three groups of polyether toxins—okadaic acid and its derivatives (OA, OAs, okadaiates), yessotoxins (YTXs), and pectenotoxins (PTXs)—are traditionally included in the diarrhoetic shellfish poisoning (DSP) toxin complex [1,2], because they often co-occur and their toxins are coextracted in the same lipophilic fraction from plankton samples and shellfish. Further, the three groups are detected together by mouse bioassays (MBAs) that provide a measure of bulk lipophilic shellfish toxin (LST) content. Nowadays, it is known that these three groups of toxins have different biological effects, that only the OAs are diarrhoegenic, and that they can be analyzed with distinct analytical methods, facts that recently led European health authorities [3] to regulate them separately. Till date, it has been shown that the sources of OAs are mainly planktonic marine dinoflagellates of the genus *Dinophysis* (order Dinophysiales)—also the only known producers of PTXs—and to a much lesser extent, epibenthic dinoflagellates of the genus *Prorocentrum* (order Prorocentrales), and that the sources of YTXs are the planktonic dinoflagellates—*Protoceratium reticulatum* (= *Gonyaulax grindleyi*) and *Lingulodinium* (= *Gonyaulax*) *polyedrum*—that belong to the order Gonyaulacales. A fourth group of polyether toxins, the azaspiracids (AZP), was recently described, also from the common lipophilic extracts, and the culprit organism was found to be the large heterotrophic dinoflagellate *Protoperidinium crassipes* (order Peridiniales). The production of different groups of polyether toxins by the same species, and the co-occurrence in the plankton of species from different taxonomic orders that produce different lipophilic toxins, explain past uncertainties in the identification of the causative agents of toxic outbreaks, and discrepancies observed in results obtained with different toxin-detection methods.

There are few reports in which the presence of OAs in bivalves can be unambiguously associated with epibenthic *Prorocentrum* spp. [4], that may require resuspension by high turbulence and/or storms to become available to filter-feeders in significant amounts, but we cannot ignore the possibility that they pose a serious risk of intoxication for minor exploitations of gastropods and sea urchins that browse on seaweeds where they are attached. This chapter is mainly focused on planktonic dinoflagellate species of the genus *Dinophysis*, producers of OAs and PTXs, as they are by far the most important cause of prolonged closures of shellfish harvesting due to detection of lipophilic toxins above regulatory levels in Europe, Central and South America, Japan, and New Zealand. We also mention the scanty information available on the producers of YTXs and AZP. The co-occurrence of different dinoflagellate species, which produce OAs, YTXs, PTXs, and AZP, is not uncommon in summer-autumn phytoplankton assemblages in temperate seas, leading to the accumulation of complex LST profiles, such as those described in New Zealand shellfish resources [5].

12.2 HISTORICAL ANTECEDENTS

In 1955, a shellfish poisoning outbreak owing to ingestion of cockles (*Cardium edule*) in Óbidos Lagoon (Portugal) was associated with a bloom of *Prorocentrum micans* [6]. Nevertheless, the clinical description of the syndrome (lips tingling, numbness of limbs, paraplegia) corresponded with that of paralytic shellfish poisoning (PSP). In 1961, a toxic gastrointestinal (vomiting, diarrhea) outbreak associated with consumption of mussels was reported from the Netherlands, but the causative agent was unknown [7]. Later in 1970, more than 100 people suffered severe gastrointestinal disorders after eating mussels, *Aulacomya ater*, from the Reloncavi Estuary in the southern Chilean province of Los Lagos [8,9]. Hermosilla [10] was probably the first to associate this event with a dense bloom of *Dinophysis* (*D. acuta*), but his observations were not reported internationally until 1991. Kat [11] revised reports of diarrhetic shellfish outbreaks in the Netherlands in the 1960s and 1970s and rejected the possibility that either faecal contamination or allergies could explain the syndrome. Observations of mussel stomach contents, with dominance of planktonic *Prorocentrum* spp. (*P. micans* and *P. minimum*), and to a much lesser extent of *Dinophysis acuminata*, led Kat to assume that *P. micans* and *P. minimum* were the causative agents of the intoxications. Nevertheless, using rats as bioassay organisms, she was unable to reproduce the toxic effect of wild mussels in those fed

with *P. micans* and *P. minimum* cultures. Kat concluded that the natural populations of *Prorocentrum* might have had associated bacteria, absent in laboratory cultures, that conferred their toxigenic capabilities on them. Those were prescient interpretations at the time—nowadays we know that there are bacteria–dinoflagellate associations that may enhance dinoflagellate toxin production, and bacterial strains with diarrhoegenic potential—but as in the case of Pinto and Silva [6], the main mistake was to assume that dominant dinoflagellates in the plankton and in the mussels' digestive tracts had to be responsible for toxicity. These historical confusions, together with the fact that *Prorocentrum lima* [12] and a large list of other epibenthic *Prorocentrum* spp. [13] do produce diarrhoegenic toxins, explain why even today some experts still include the planktonic species *P. micans* and *P. minimum* in their lists of potential producers of diarrhoegenic toxins.

It was not until the late 1970s that a new syndrome, DSP, was described. Until then, DSP events were probably often mistaken for gastroenteritis of bacterial origin. Severe gastrointestinal outbreaks occurred, in 1976 and 1977, among consumers of mussels (*Mytilus edulis*) and scallops (*Patinopecten yessoensis*) in the prefectures of Miyagi and Aomori (NE Tohoku, Japan). It was a fortunate coincidence that an eminent Japanese chemist, Prof. Takeshi Yasumoto, himself suffered the consequences of these outbreaks. His natural curiosity drove him to investigate the causes of intoxication after eating cooked bivalves, and finally to isolate two fat-soluble thermostable toxins, and to implement a MBA that allowed quantification of this kind of toxicity [14,15]. Two years later, Yasumoto's group identified the dinoflagellate *D. fortii* as the causative agent of the intoxications by means of analyses of different size-selected plankton concentrates with increasing percentages of this species [16]. OA, a polyether previously isolated and described from the sponge *Halichondria okadai* [17], was finally identified by Murata et al. [18] as the main bioactive compound responsible for DSP.

After these findings, retrospective analyses of phytoplankton monitoring data and several new severe intoxications affecting thousands of people in different European countries allowed experts to confirm the association of these events with different *Dinophysis* spp. (*D. acuminata*, *D. acuta*, *D. norvegica*) in NW Spain [19], the Netherlands [20], Norway [21,22], western Sweden [23], and Brittany [24]. New outbreaks were also reported from Thailand [25] associated with the occurrence of *D. caudata*. The important fact established was that other dinoflagellate species (i.e., *Prorocentrum* spp., *Ceratium* spp.) could be dominant components of the phytoplankton assemblage at the time of these outbreaks, but that a few thousand cells per liter of *Dinophysis* spp., or even a few hundreds, co-occurring with 10^5–10^6 cell/L of other phytoplankton species, were enough to render shellfish toxic to consumers. Species of *Dinophysis* became target organisms in new phytoplankton monitoring programs established in the 1980s. New phytoplankton sampling and counting protocols were established so as to be able to detect *Dinophysis* spp. in the water column, even at very low concentrations ($<10^2$ cell/L), and alert health and fisheries authorities of the imminent arrival of toxins to shellfish resources [26].

In the mid 1980s, the list of polyether toxins associated with *Dinophysis* spp. events started to grow. Besides the dinophysistoxins (DTX1, DTX3), derivatives of OA, two new groups of polyether toxins were described: the PTXs [1] and the YTXs [2]. Both of the new groups were obtained from lipophilic extracts of *Patinopecten yessoensis*, one of the species involved in the Japanese outbreaks 10 years earlier, by the same extraction procedure as that for OA, and could be detected in the standard MBA applied for control of DSP toxins in shellfish. Consequently, PTXs and YTXs, together with OAs, were all included in the DSP toxin complex. Progress in determination of toxin profiles in single dinoflagellate species was hindered by the lack of established cultures of *Dinophysis* spp. As an alternative, Lee et al. [27] developed a highly sensitive high performance liquid chromatography method that allowed chemical analyses of samples composed of several hundreds of individually picked cells of *Dinophysis*. These first results showed that OA and/or DTX1 were the main toxin components in *Dinophysis* spp., that only *D. fortii* (Japanese strains) was found to contain PTXs, and that there were large differences in toxin content per cell, even within the same species and locality [12]. The origin of YTXs remained unknown. Early analyses of lipophilic toxins in European mussels by HPLC led to the conclusion that OA was the main toxin of concern at the time of *Dinophysis*

occurrences [28], and it became a common practice to equate results from MBA with those of HPLC analyses of OA alone [29]. Nevertheless, discrepancies between the two toxin determination methods in the 1990s led experts to suspect the presence of other DSP toxins in mussels. The lack of commercial standards other than OA delayed resolution of these uncertainties. A new OA derivative, DTX2, was finally reported in Irish mussels [30], and its presence further confirmed in Galician [31, 32] and Portuguese [33, 34] mussels and plankton hauls rich in *D. acuta*, and in picked cells of *D. acuta* from Ireland [35] and Galicia [36].

In 1995, intoxications with typical DSP symptoms detected in the Netherlands after consumption of mussels that contained only traces of OA and DTX2, led to the report of an unknown toxic agent, "K-toxin," in mussels from Killary Harbor (Western Ireland), and the subsequent search for and description of a new group of lipophilic toxins, the azaspiracids [37]. Recently, large armored heterotrophic dinoflagellates of the genus *Protoperidinium*, known to feed on planktonic microalgae, were identified as the causative agent of azaspiracid shellfish poisoning (AZP) in samples of picked specimen from natural populations [38].

A new step forward was the identification of two unexpected armored gonyaulacoid dinoflagellates, *G. grindleyi* (= *P. reticulatum*) and *L. polyedrum* (= *G. polyedra*) as the producers of YTXs. Highly toxic (according to MBA results) green mussels (*Perna viridis*) exposed to a bloom of *D. acuta* and *P. reticulatum* in New Zealand in 1996, revealed only trace amounts of OA and derivates by HPLC analyses and enzyme-linked immunosorbent assays (ELISA). Chemical analyses of plankton concentrates and cultures of *P. reticulatum* showed that the latter was the source of YTX derivates [39, 40]. The same year, two new YTX analogs, homoYTX and 45-OH homo YTX, were described in mussels exposed to a quasi-monoalgal bloom of *L. polyedra* in the Adriatic Sea [41]. The production of YTXs by this species in culture was demonstrated a few years later [42]. Very recently, and following detection of YTXs from an unknown source in New Zealand shellfish resources, cultured isolates of *G. spinifera* were identified as the probable source of the toxins [43]. It cannot be ruled out that the list of toxigenic *Gonyaulax* spp. will increase in the near future, as new species from different parts of the world are isolated and tested.

Till the end of the 1990s, little attention was paid to PTXs, considered then to be a problem restricted to *D. fortii* proliferations in Japan. Nevertheless, improved analytical methods in recent years by liquid chromatography-mass spectrometry (LC-MS) applied to analyses of plankton hauls rich in *Dinophysis* spp. and of shellfish [44, 45] led to the suspicion, confirmed with numerous new reports, of a widespread presence of PTXs in *Dinophysis* spp.

12.3 THE UNAMBIGUOUS IDENTIFICATION OF TOXIGENIC MICROALGAE

The conventional procedure for unambiguous identification of the causative agent of a toxic outbreak requires the application of rigorous stepwise protocols that include taxonomic classification of the suspected organism, and characterization of toxins in (i) the contaminated shellfish, (ii) the planktonic populations to which the shellfish was exposed to, and (iii) phytoplankton cultures established after isolation of the suspected causative agent from the plankton [46]. This was the procedure followed to identify *L. polyedra* and *P. reticulatum* as YTX producers [39,42]. Knowledge of the causative agents in other parts of the world may constitute a useful starting point, but does not always work, as species with strains shown to be very toxic in one region may have weakly toxic or nontoxic strains elsewhere. In the case of *Dinophysis* spp., owing to the lack of established monoalgal cultures, the alternative has been either to pick hundreds of specimen from natural populations by microcapillarity or to analyze plankton hauls (or even better plankton size fractions) rich in the target species of *Dinophysis*. Picking and transferring hundreds of *Dinophysis* cells through

drops of sterile seawater is a time-consuming task, especially when the cells are smothered by dense concentrations of diatoms, but recent technological improvements now allow analyses with smaller and smaller samples, that range from one (with the most sophisticated LC-MS systems) to a few dozen cells of *Dinophysis* or other potentially toxic dinoflagellate species [47]. Single cell picking was also the method applied for the identification of *P. crassipes* as an AZP source in Ireland [38]. The advantage of this technique is that it allows estimates of field values of toxin content per cell that may be quite different from those observed in mass cultures.

12.4 GLOBAL DISTRIBUTION

12.4.1 Distribution of *Dinophysis* Spp.

Dinophysis is a cosmopolitan genus, and problems associated with their proliferation can emerge in practically any shellfish cultivation area where the cells and their toxins are sought with appropriate methods. Till date, 13 species of *Dinophysis*, that is, all species that have been isolated and analyzed, have been reported to contain OA derivatives and/or PTXs. Obviously, these 13 species are the most abundant members of the genus, with more coastal/neritic distributions, and the most likely to have significant impacts on shellfisheries. Many other *Dinophysis* spp. reported in coastal areas, are present in such low concentrations (<10 cell/L) that their analyses become an impossible task. Table 12.1 lists *Dinophysis* spp. confirmed to contain lipophilic toxins, their global distribution, and their profile and toxin content. Figure 12.1 shows areas of the world where the presence of lipophilic toxins in shellfish has been related to the occurrence of *Dinophysis* spp. It is not by chance that the reported distribution of toxigenic *Dinophysis* spp. mirrors that of areas with intense shellfish exploitation and/or exports. Thus, aquaculture installations, and especially the implementation of strict regulations for seafood safety, act like the canary in the mine, making evident the presence of species that have always been there.

Each region has at least one, and often several problem species of *Dinophysis* that are the main curriers of toxins affecting shellfish resources. The various species of *Dinophysis* in a particular locality can occur at different seasons and, when they co-occur, their individual contributions to the overall toxin content detected in shellfish samples is difficult to determine unless species-specific analyses of picked cells are carried out. *D. acuminata*, the most cosmopolitan *Dinophysis* species, can be found from the tropics to the Greenland Sea. Other *Dinophysis* spp. have a much narrower latitudinal distribution: *D. miles* is restricted to the tropics, and *D. norvegica* to cold-temperate waters of the northern hemisphere. *D. sacculus* has only been reported in Europe, especially in Mediterranean coastal waters. From the viewpoint of the magnitude of their impacts, the main problem species are *D. acuminata* + *D. acuta* in temperate waters (with *D. norvegica* in cold-temperate waters and the Baltic Sea); *D. fortii* (with *D. sacculus* in the Mediterranean Sea) in warm-temperate seas, and a *D. caudata* + *D. tripos* + *D. miles* assemblage in tropical and subtropical waters. Nevertheless, it would be too simplistic to assign a range of temperature and salinity to each species on a global scale, as the same species may have regional varieties or strains that grow well under quite different thermohaline conditions. Thus, *D. acuta* on the Atlantic coast of Iberia is considered a stenohaline and stenothermic species, with cell maxima at temperatures of 15–17ºC and salinities close to 35.5 psu [48–50], whereas the same species grows well at much lower temperatures and salinities in Chilean fjords [10]. Another factor to consider is if the problem species is a permanent resident in coastal waters of a region, or a seasonal visitor brought there by warmer/colder ocean currents.

The Intergovernmental Oceanographic Commission (IOC) has compiled references of *Dinophysis* spp. records associated with DSP toxin detection in their Taxonomic Reference List of Toxic Algae [13]. Some recent additional reports can be found in Akselman et al. [51] for South America and in the IOC network on Harmful Algae in North Africa [52].

TABLE 12.1
List of *Dinophysis* Species Proved to Contain Toxins, Their Distributions, Their Toxin Profile and Their Toxin Contents Estimated from Picked Cells and Net-Haul Concentrates

Species	Distribution	Toxins (Range) (pg/cell) Picked Cells	References	Toxins (Range) (pg/cell) Concentrates	References
D. acuminata	Cold and warm temperate	OA (nd-23) DTX1 (nd-60) PTX2 (0.9–180) PTX12 (0.2–1.6)	[36,119,157, 166,167] [119,157,166, 168] [119,168] [119]	OA (tr-170) DTX1 (0.1–2.4) PTX2 (2.4–25.8) PTX2SA (0.1–2.7) PTX11 (0.1–2.1) PTX11SA tr	[12,116, 152,169, 170,171] [170] [170] [170] [170]
D. acuta	Temperate, cold-temperate	OA (nd-85) DTX1 (nd-7) DTX2 (1–78) PTX2 (0.2–32.3) PTX2SA (nd-8.2) 7epiPTX2SA (nd-4.6) PTX12 (6.3–10.5)	[12,36,119,157, 172,173,174] [12,119,157] [36,172,173,174] [119,130,173, 174] [119,173,174] [173,174] [119]	OA (tr-60) DTX1 (nd-0.1) DTX2 (nd-80) PTX2 (nd-107.5) PTX2SA (nd-6.5) 7epiPTX2S nd DTX3 11.5 PTX11 (4.7–80) PTX11SA (nd-0.7) PTX1i 47 PTX1iSA 0.5 OA esters (nd-10.2)	[5,35,36,95,170, 175,176,177] [35,36,160, 170,177] [5,95,129, 160,170, 176,177] [5,95,129, 160,170,177] [177] [5] [170,178] [5,170] [5] [5] [95,170]
D. caudata	Warm temperate to tropical	OA (nd-56.5) DTX1 (nd-53.9) PTX2 (100–127.4)	[36,179] [179] [130]	PTX2SA, 7epiPTX2SA	[180]
D. fortii	Cold-temperate to tropical	OA (nd-57.7) DTX1 (nd-191.5) PTX2 42.5	[12,166] [12,166] [12]	OA nd DTX1 (nd-252) PTX2 182	[181] [181,182] [183]
D. infundibulus	temperate	PTX2	[184]		
D. miles	tropical	OA (5.7–25.0) DTX1(nd-10.7)	[179] [179]		
D. mitra	Warm temperate to tropical	DTX1 10.0	[12]		
D. norvegica	Cold waters	OA (nd-0.8) DTX1 2.5–14 PTX2 (0.3–1.7) PTX12 (0.1–0.8)	[12,119] [12] [119] [119]	OA (32.6 + 5.2)	[152]
D. rotundata	Cold to warm waters	OA (nd-101) PTX2 0.8 PTX2 (0.4–1.3)	[12,119] [119] [119]	OA nd	[152]
D. sacculus	Southern Europe, Mediterranean Sea	OA (12.9–29.6)	[126]*	OA (tr-16.2)	[126,185,186]
D. tripos	Warm temperate to tropical	DTX1 36.0	[12]		

nd = not detected; tr = traces; * = probably a mixture of *D. acuminata* and *D. sacculus.*

FIGURE 12.1 Distribution of reported presence of okadaiates and/or pectenotoxins in shellfish associated with the occurrence of *Dinophysis* spp.

12.4.2 Distribution of *Lingulodinium polyedra*, *Protoceratium reticulatum*, and *Protoperidinium crassipes*

P. reticulatum is widely distributed in temperate and subtropical waters but rarely forms water-discoloring blooms [53]. It has been confirmed as the source of YTXs in shellfish in New Zealand [40], the Adriatic Sea [54], and Norway [55,56]. *L. polyedra* is a well-known red tide-forming species in California, North and South African upwelling systems [57], and in the Adriatic Sea. It has been related to YTXs in shellfish in the Adriatic Sea [58,59], the Galician Rías Altas [60], and on the Mediterranean coasts of Morocco [61].

P. crassipes is so far the only dinoflagellate unambiguously identified as an AZP source in Ireland [38]. It is a frequently recorded but rarely abundant species in temperate-cold temperate seas. AZP toxins, whether related or not to this dinoflagellate, seem to be widespread in European Atlantic coastal waters [38,62].

12.5 MORPHOLOGY AND TAXONOMY

12.5.1 *Dinophysis* Spp.

Dinophysis (order Dinophysiales) is one of the largest genera of dinoflagellates, comprising over 200 species [63]. Cells are heterokont, that is, the two flagella arise laterally; (one beats longitudinally, the other transversally) and thecate, with three regions: epitheca (or epicone), cingulum (or girdle), and hypotheca (or hypocone). Figure 12.2 shows a typical *Dinophysis* cell that can be divided into two lateral halves rather like a miniature bivalve. The armor or theca is composed of 18 plates, but the two large (left and right) hypothecal plates, ornamented with areola of different patterns, comprise most of the body. Cells are very compressed laterally, and normally lie on their left or their right hand side in fixed samples. The *cingulum* is short and delimited by an anterior and a posterior funnel-like membrane or wing that may be supported by ribs. The epitheca can be high and

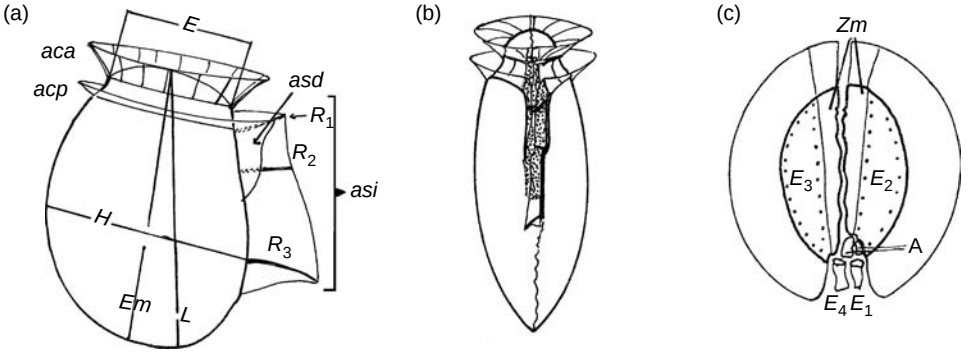


FIGURE 12.2 Structure of *Dinophysis* cell. (a) Right side view; *aca, acp* anterior and posterior cingular lists; *E* depth of the epitheca; *H* depth of the hypotheca; *L* length; *Em* central axis; *asd, asi* right and left sulcal lists; R_1, R_2, R_3 the three ribs; (b) ventral view. Shaded area corresponds to s*ulcus*; (c) apical view; E_1–E_4 epithecal plates; *A* apical plates; *Zm* megacytic zone. (Modified from Balech 2002 [64].)

visible, or low and indistinguishable in lateral view. The *sulcus*, difficult to see in lateral view, occupies a good part of the ventral side, and has a right sulcal wing—small and difficult to distinguish—and a left sulcal wing—prominent and supported by three strong ribs. Originally, species with the epitheca showing above the cingular list in lateral view were classified as *Phalacroma*, and there is still controversy about the splitting or fusion of these two genera. Routinely, species of *Dinophysis* are distinguished by their size and contours. Important diagnostic features to consider in their classification are (i) size, shape, and proportions of the cell contour; (ii) areolation of the two large hypothecal plates; (iii) morphology of the left sulcal list; and (iv) presence or absence of chloroplasts [65, 66]. Figure 12.3 show micrographs of some of the most common *Dinophysis* spp. associated with DSP outbreaks. Large *Dinophysis* spp., such as *D. caudata* and *D. tripos*, can measure more than 100 µm in length, whereas most of other species fall into a 30–70 µm range.

Dinophysis spp. exhibit high variability in their morphology (size and shape of their large hypothecal plates) and their cellular contents. Through the growing season of *D. acuminata*, the most ubiquitous and persistent species of the genus in temperate coastal waters, cells can appear thin and quite empty, or swollen with digestive vacuoles. The size composition of a population can range from homogeneous to a bimodal distribution of large and small cells, or even to a complex mixture of small, intermediate, and large cells. This variability constitutes a source of uncertainty, and is a nuisance in routine identification and counting carried out in monitoring centers. A large part of the size and shape complexity within a given locality can be explained by complex polymorphic life cycles [67], where large cells can go through a reductionary division and produce two dimorphic offspring (with dissimilar halves) that further lead to the formation of small gamete-like cells, traditionally classified as if they were different species. Thus, each *Dinophysis* species has two extreme morphotypes: a large form that corresponds to the vegetative cell and a small form traditionally classified as a separate species that is a stage of the sexual cycle. Examples of these pairs of large/small cells are *D. acuminata*/*D. skagii*; *D. acuta*/*D. dens,* and *D. caudata*/*D. diegensis*. Nevertheless, a *continuum* of intermediate forms between the large and the small extremes can be found at certain stages of a bloom. Cyst-like forms have been reported in natural populations of *D. acuta, D. caudatea*, and *D. tripos*. These forms are very rare, represent a very small part of the population (<0.1%), and are suspected to play a minor role in the populations dynamics of *Dinophysis* spp. [68]. Swollen, deformed cells full of digestive vacuoles are the result of heterotrophic [69] or mixotrophic feeding [70–72].

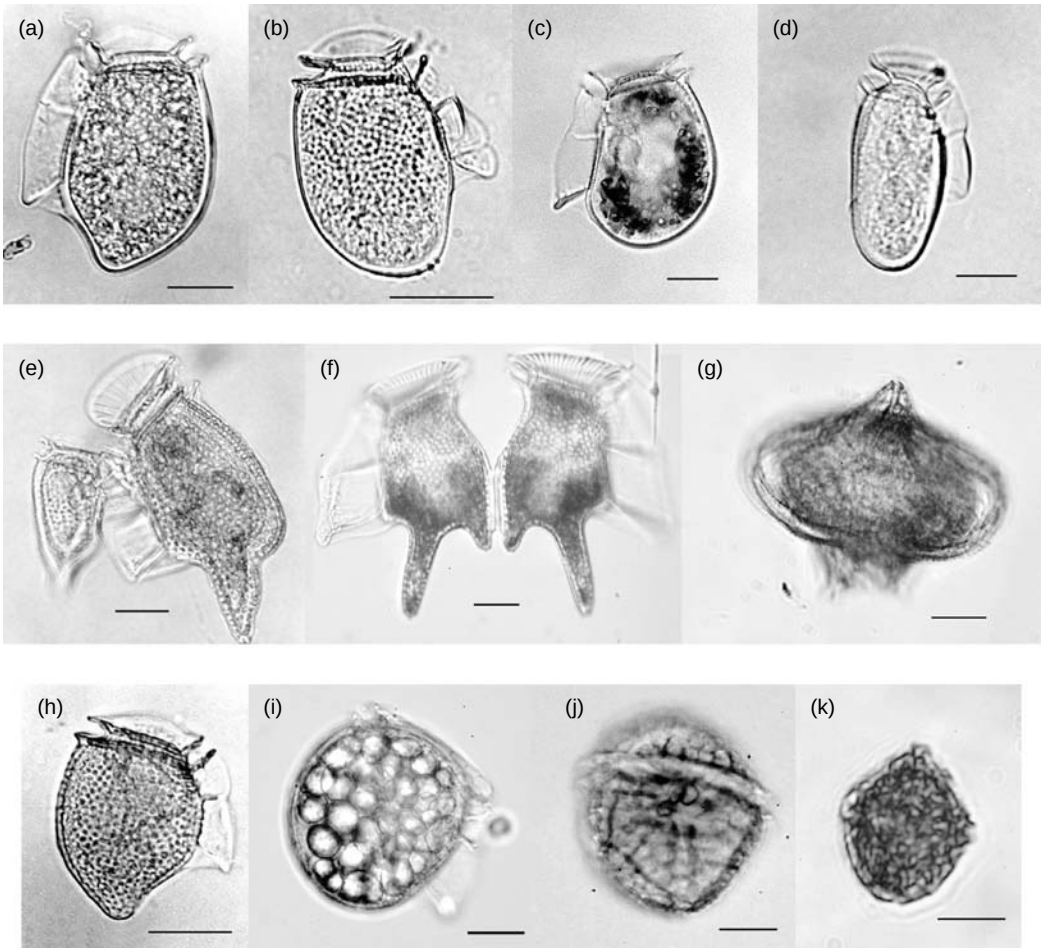


FIGURE 12.3 Micrographs of toxin-producing microalgae. (a) *Dinophysis acuta*, (b) *Dinophysis acuminate*, (c) *Dinophysis fortii*, (d) *Dinophysis sacculus*, (e) *Dinophysis caudata* conjugated with a small *D. diegensis*-like cell, (f) pair of *Dinophysis tripos*, (g) *Protoperidinium crassipes*, (h) *Dinophysis norvegic*, (i) *Dinophysis rotundata* full of digestive vacuoles, (j) *Protoceratim reticulatum*, (k) *Lingulodinium polyedra* live cultured cell from CCVIEO culture collection. Scale bar, 20 μm in all frames.

Variability in the cellular contents is very obvious when live *Dinophysis* cells are examined by epifluorescence microscopy. The first notable difference between phototrophic *Dinophysis* spp. and other dinoflagellate genera is that they fluoresce orange. This is because like small (5–15 μm) cryptophyte flagellates, cyanobacteria, and the ciliate *Mesodinium rubrum* (=*Myrionecta rubra*), they have reddish accessory pigments called phycoerythrins. *Dinophysis* cells can exhibit an intense orange color throughout all or part of the cell (preferentially in the peripheral part), or even have parts that fluoresce with a different color. These peculiar fluorescence characteristics led to a study of their ultrastructure and description of their peculiar plastids with double membranes, distinct from the conventional peridinin-containing plastids of other dinoflagellates [73]. In recent years, DNA analyses of the plastids of phototrophic *Dinophysis* spp. have shown that their sequences coincide with those of living cryptophytes. An exception has been found in *D. mitra*, a species that contains plastids of haptophyte origin [74,75], but there is still controversy over whether the plastids are constitutive

(acquired from endosymbiosis in the past) or kleptoplastids derived from their prey [72,74,76,77]. Resolution of this question should come from studies of the ultrastructure of *Dinophysis* plastids and those of their prey.

12.5.2 *Lingulodinium polyedrum*, *Protoceratium reticulatum*, and *Protoperidinium crassipes*

Both *L. polyedrum* (= *G. polyedra*) and *P. reticulatum* (=*G. grindleyi*) belong to the order Gonyaulacales, the same order in which most PSP species (*Alexandium* spp., *Pyrodinium* spp.) are included. Within this order, they are small-medium-sized (15–35 μm) members of the gonyaulacoids, characterized by the displacement of their cingulum; the thecal plates have species-specific tabulation formulae that are used for their taxonomic classification [53]. The epithet *polyedrum* refers to the regular polyhedral shape of *Lingulodinium*, whereas that of *reticulatum* refers to the coarse reticulated structure of the thecal plates of *Protoceratium*. Both species are photosynthetic and can be grown in standard culture medium for dinoflagellates [42]. Nevertheless, under certain physiological and environmental conditions, both species can feed mixotrophically [78,79].

P. crassipes, Order Peridiniales, is a large (80–100 μm) heterotrophic species. Specimens are slightly wider than longer, with wall of the epitheca and the hypotheca markedly concave [80]. Like other *Protoperidinium* species, *P. crassipes* is a pallium-feeder, that is, extends a spider web-like structure or pseudopodium called a *pallium* to trap prey (other diatoms and dinoflagellates); digestion is extracellular [81]. Successful cultures of *P. crassipes* on *L. polyedra* have been described [82].

12.6 ECOLOGY

Until the discovery of *D. fortii* as the agent of DSP outbreaks [16], little attention was paid to *Dinophysis* spp. with the exception of some keen taxonomists interested in exotic phytoplanktonic organisms. One reason may be that *Dinophysis* species are rarely abundant members of the microplankton (20–200 μm) community; that is, they do not reach high densities (10^6 cell/L) and form "red tides" that discolor the sea; they cause toxic outbreaks at concentrations of $1–5\times10^2–10^4$ cell/L, often a small fraction (1–5%) of the total phytoplankton assemblage. Another common characteristic of *Dinophysis* spp. is their tendency to aggregate in thin layers of the water column. Thus, their scarcity and patchiness renders sampling a cumbersome task, and tracking maximum population densities can be a matter of luck—sampling the right depth at the right moment—or of carefully planned sampling with very high vertical resolution by means of advanced instrumentation [83,84]. Exceptionally, there have been reports of "red waters" of *Dinophysis* in Chile [8], Scotland [85], Canada [86], Norway [87], and India [88]. Rather than originating from intense growth, these red tides of *Dinophysis* seem to have been the result of physically driven accumulation (winds, convergences) combined with upward vertical migration of the cells that allowed them to remain near the sea surface despite down welling. This kind of physical–biological interaction is quite common among flagellates with good swimming capacity [89,90], and not exclusive to *Dinophysis* spp.

Maestrini [91] provides a comprehensive review of the bloom dynamics and ecology of *Dinophysis* spp., with a worldwide compilation of different scenarios of *Dinophysis* spp. development. The review describes how the same *Dinophysis* spp. is able to form blooms by different mechanisms in a given locality. The main weakness of many of these examples is the lack of information on *in situ* division rates (μ), a key parameter needed to decide whether increased numbers result from active division or rather from physically driven accumulation. Maestrini [91] identifies important gaps in knowledge that include (i) life cycle description and overwintering strategies; (ii) nutritional sources; and (iii) the passive or active character of cell aggregation. Progress achieved since that review is summarized below.

12.6.1 Bloom Dynamics, Life Cycle and Overwintering Strategies

Variability in the distribution of *Dinophysis* spp. has to be considered on different time scales ranging from daily (circadian rhythms), to seasonal and interannual. Daily migration, from 10 m at night to 3–5 m at midday has been reported for *D. acuta* [92] and *D. acuminata* [93], but the same species may not perform any vertical migration at certain phases of population growth [94,95]. In contrast, *D. norvegica* in the Baltic Sea seems to be always detected in deeper waters in the pycnocline region in the Baltic [96,97] perhaps to avoid the extremely low-salinity waters of the upper layer. Each *Dinophysis* spp. predominant in a locality exhibits its species-specific seasonality, and when several species co-occur, they usually occupy different water masses, as is usually the case with the pair *D. acuminata/D. acuta* in Western Iberia [49,98]. The onset of salinity and/or temperature-driven stratification (density gradients) in the water column as an apparent prerequisite for the initiation of numerical increase associated with *in situ* growth has been confirmed in different locations with improved vertical resolution sampling. The optimum gradients for *Dinophysis* development may be established under different scenarios, such as late winter and heavy runoff in the case of *D.* cf *acuminata* blooms in Greek waters [99], moderate upwelling and increased insolation in spring for *D. acuminata* blooms in the Galician Rías [100], or late summer temperature-driven stratification combined with moderate upwelling for *D. acuta* blooms in Iberian waters [50,98]. Nevertheless, very high concentrations may be reached in a matter of days owing to wind-induced cross-shelf and/or along-shore transport of shelf populations into coastal waters, as is the case with autumn blooms of *D. acuta* in the Galician Rías [101]. In places where two species of *Dinophysis* cause problems, multiannual changes in the expansion or contraction of their populations, and hypothetical climate change-driven replacement of one species by the other may be observed. In such cases, time series analyses may provide insight into the autoecology of each species, and improve long-term prediction capabilities.

In situ growth rate estimates for *Dinophysis* spp., with maximum values around one doubling per day ($\mu \approx 0.7$/d) [102–104], have shown that cell division occurs at the onset of light, but timing varies between species. There has been considerable progress on the description of the life cycle of *Dinophysis* spp. [67,68,105,106]. The formation of small cells from normal vegetative cells is a reversible process that endows *Dinophysis* with physiological plasticity in the face of varying environmental conditions. An important question is to decide whether the *inoculum* results from the aggregation of scattered overwintering cells or from residual populations located in retention areas, such as eddies or gyres. Some new results off the Loire Estuary (Brittany), supported by hydrodynamic models, suggests that overwintering populations of *D. acuminata* may be gathered in retention areas or "incubators" on the shelf, that can be transported to coastal areas [107]. The scarcity of cysts in natural populations of *Dinophysis* and the permanent presence of planozygotes during the growth season of *D. acuminata* [108] support the hypothesis that planozygotes, rather than permanent cysts, play a major role in the initiation of blooms [68].

12.6.2 Nutritional Sources

During the last 15 years, considerable efforts have been invested to determine the nutritional sources of phototropic species of *Dinophysis*, and attempts have been made to grow them in all sorts of enriched media, with or without additions of small prey organisms [91]. In the luckiest cases, picked cells, incubated in cell culture plates, underwent four or at most six divisions, and when transferred to fresh medium, small cells started to appear and the cultures did not progress in a conventional way [109–111]. A key observation by Hansen [69] was that the heterotrophic *Dinophysis* (*Phalacroma*) *rotundata* can feed on the ciliate *Tiarina fusus* after piercing its lorica with a feeding peduncle and sucking its contents, a feeding mechanism known as "myzocytosis" [112]. Later, remains of ciliates were found in the digestive vacuoles of *D. acuminata*, *D. norvegica*, and *D. fortii* [70,71], thus providing evidence of the mixotrophic behavior of phototrophic *Dinophysis* spp., but nobody had

seen in nature what the potential prey of *Dinophysis* might be. More recently, Setälä et al. (2005) showed that *D. acuminata* populations located in deep (>70 m) waters in the Baltic Sea exhibited poor photosynthetic activity compared with those from subsurface water layers. In the same region, Gisselson et al. (2002) [113] found that *D. norvegica* cells aggregated in the thermocline at 15–20 m depth had a division rate (up to 0.4/d) that could not be supported with the observed ^{14}C uptake rates. These observations and the presence of digestive vacuoles in 2–22% of the population led them to suggest that the thermocline may provide *D. norvegica* cells with their suitable prey.

Advances in molecular biology allowed further advances in the understanding of *Dinophysis* nutritional behavior. Janson [77] found that portions of the ribosomal DNA that code the plastids of *D. acuminata* are identical to those with the same function for plastids of the cryptophyte *Teleaulax amphioxeia*. Attention was diverted to a potential cryptophyte prey, but nobody managed to grow *Dinophysis* on them. Takahashi et al. [114] confirmed the cryptophyte-like sequence of the plastids of several DSP toxin-producing *Dinophysis* spp., and even developed genus-specific molecular probes that, as a very innovative early warning system, could be bound to cryptophytes with a plastid sequence like that of *Dinophysis* spp. plastids, and detect the prey before the buildup of *Dinophysis* populations. Recent results from Park et al. [72] have shown that *D. acuminata* can exhibit similar feeding behavior (myzocytosis) as the heterotrophic *D. rotundata*. Dense yields ($>10^6$ cell/L) of *D. acuminata* were obtained in cultures in which the ciliate *M. rubrum*—fed with a cryptophyte, *Teleaulax* spp.—was provided as prey for the dinoflagellate. Division rates ($\mu = 0.9$/d) were much higher than values previously obtained in unsuccessful short-term experiments, and were higher than maximum values estimated *in situ* [104]. This may be because Park et al. [72] used continuous illumination in their cultures, and division in the field is triggered by the onset of light. These multispecific cultures are quite complex, and require more maintenance efforts than conventional monoalgal cultures. Nevertheless, the results of Park et al. [72] constitute a real breakthrough that opens new avenues for research on *Dinophysis* spp., so far hampered by the lack of established cultures. From the ecological point of view, these results raise lots of new questions: Is *Mesodinium* the main (or the only) prey in natural populations of phototrophic *Dinophysis* spp.? Can *Dinophysis* survive on other nutritional sources when *Mesodinium* is not available? Do *Dinophysis* perform photosynthesis with plastids stolen from *Mesodinium*, or does the ciliate act only as an exogenous nutritional source?

12.6.3 Cell Aggregations in Discontinuity Layers

Maestrini [91] poses the question, do dense vertically patchy populations of *Dinophysis* result from active or passive concentration, from growth, or from a combination of both mechanisms? Reguera et al. [115] observed that estimates of *in situ* division rates at the depth of the *Dinophysis* cell maxima can be almost double those estimated from integrated water column samples. This observation suggests that cells aggregated in the density discontinuity layer are the most active. Nevertheless, observation of *D. norvegica* populations during an experiment in the Baltic [96] showed that cells aggregated in the pycnocline were not dividing at all. More observations on different scenarios and species, and at different phases of population growth, will be needed to resolve this question, but most field vertical distributions of *Dinophysis* spp. show that, whether as a result of active (swimming, division) or passive (physically driven accumulation) processes, discontinuity layers seem to provide a signal for aggregation. These layers may also constitute an optimum niche regarding decreased turbulence, higher densities of dissolved organic matter and of potential prey organisms, and a reference point to enhance reproduction, release of growth factors and other biological processes of an otherwise very dispersed organism [84].

12.7 TOXIN PROFILE AND TOXIN CONTENT (CELL TOXIN QUOTA)

The toxin profile is defined here as the relative contribution (percentage) of different toxins to the overall toxin content or cell toxin quota of a species or a strain. Toxin content or cell toxin quota is

a quantitative term that refers to the amount of toxin accumulated per cell. The toxigenic capability of a dinoflagellate bloom depends on its cell concentration, but most importantly on the toxin profile and cell toxin quota, and on the ratio of toxigenic cells to the total particulate matter filtered by the bivalves [116].

12.7.1 Influence of Collection and Extraction Procedures on Toxin Profiles and Toxin Content Estimates

The pioneer work of Lee et al. [12, 27] provided a sound analytical method to determine toxin profiles and cell toxin quota in different species of *Dinophysis* that had never been established in culture. The main inconvenience of the method was the time-consuming task of picking out individual cells, and to avoid that, estimates from multispecific plankton net-hauls were often carried out as an alternative. In the latter, the total amount of toxins determined from a fixed volume of a plankton haul or concentrate is divided by the number of *Dinophysis* cells present in that volume. Table 12.1 shows estimates of toxin content per cell from individually picked cells of *Dinophysis*, the only unambiguous way to ascribe a toxin profile and toxin content to a single species of *Dinophysis*. Table 12.1 also shows estimates from either bulk *Dinophysis*-rich plankton hauls or size-selected plankton concentrates, both comprised of multispecific plankton populations. Estimates from multispecific populations have several sources of error: (i) it is assumed that all extracted toxins derive exclusively from cells of *Dinophysis*; (ii) the method does not allow estimates of species-specific contributions to the overall cell toxin quota when several species of *Dinophysis* co-occur; and (iii) possibilities of disruption of cells and enzymatic transformations of toxins during the concentration procedure are ignored. With respect to the first assumption, the complex trophic relationships that exist within the same size classes in the pelagic food web [117], where other heterotrophic dinoflagellates or microzooplankton may feed on *Dinophysis* spp. and act as vectors of toxins [118], need to be considered. As an example, Miles et al. [119] found OA (up to 8.8 pg/cell) and PTX2 (up to 41.2 pg/cell) in picked cells of heterotrophic *Protoperidinium* spp. (*P. depressum*, *P. divergens*, *P. crassipes*). In such cases, the cell toxin quota of *Dinophysis* would be overestimated. An attempt to separate species-specific contributions in samples in which various *Dinophysis* spp. co-occurred was made by Blanco et al. [31] by means of backward-propagation multiple regression analysis. In that case the assumption was that, for each subset of data analyzed, each species of *Dinophysis* had a constant cell toxin quota. In conclusion, only estimates of toxin profiles and cell toxin quota from net-hauls with overwhelming dominance of single target species can provide reliable results; analyses of accompanying species should also be reported to reject the possibility of contributions from heterotrophic dinoflagellates, and to provide information on the accompanying assemblage of planktonic material.

New findings since the mid-1990s force us to be even more cautious (or skeptical) in our interpretations of past estimates of toxin profiles and cell toxin quota. A key observation on cultures of epibenthic *Prorocentrum* spp. was that a large proportion of the OA-related toxins can be stored in the cells as the less toxigenic sulfated esters (mainly DTX4 and DTX5); these are hydrolized to diol esters and to their acidic forms (more toxigenic) during extraction procedures by enzymes liberated from the cell lyzosomes [120,121]. Thus, to describe the real toxin profiles of the cells, a previous boiling (baine-marie) of the cells to inactivate their enzymes before toxin extraction would be required. Recent studies, with specifically designed extraction procedures, suggest that sulfated compounds of OA are also important in *Dinophysis* spp. [5,122]. More recently, Johansen and Rundberget [123] have claimed that cell concentration procedures (pumps, nets, etc.) may exacerbate the release of toxins to the medium by intact cells.

The occurrence of sulfated forms of OA is not a constant feature in cultures of epibenthic *Prorocentrum* spp. The absence of sulfated forms was confirmed in cultures of *P. lima* (strain PL2V) [124]. In contrast, large amounts of DTX5c-like sulfated toxins were found in cultures of *P. belizeanum* (strain PBMA01) grown under the same experimental conditions and with the

application of identical toxin extraction procedures [125]. These results led the last authors to the hypothesis that diol-esters are intermediate forms, derived from OA, which may give way to sulfated compounds, as final products, following an elongation of the side chain of OA.

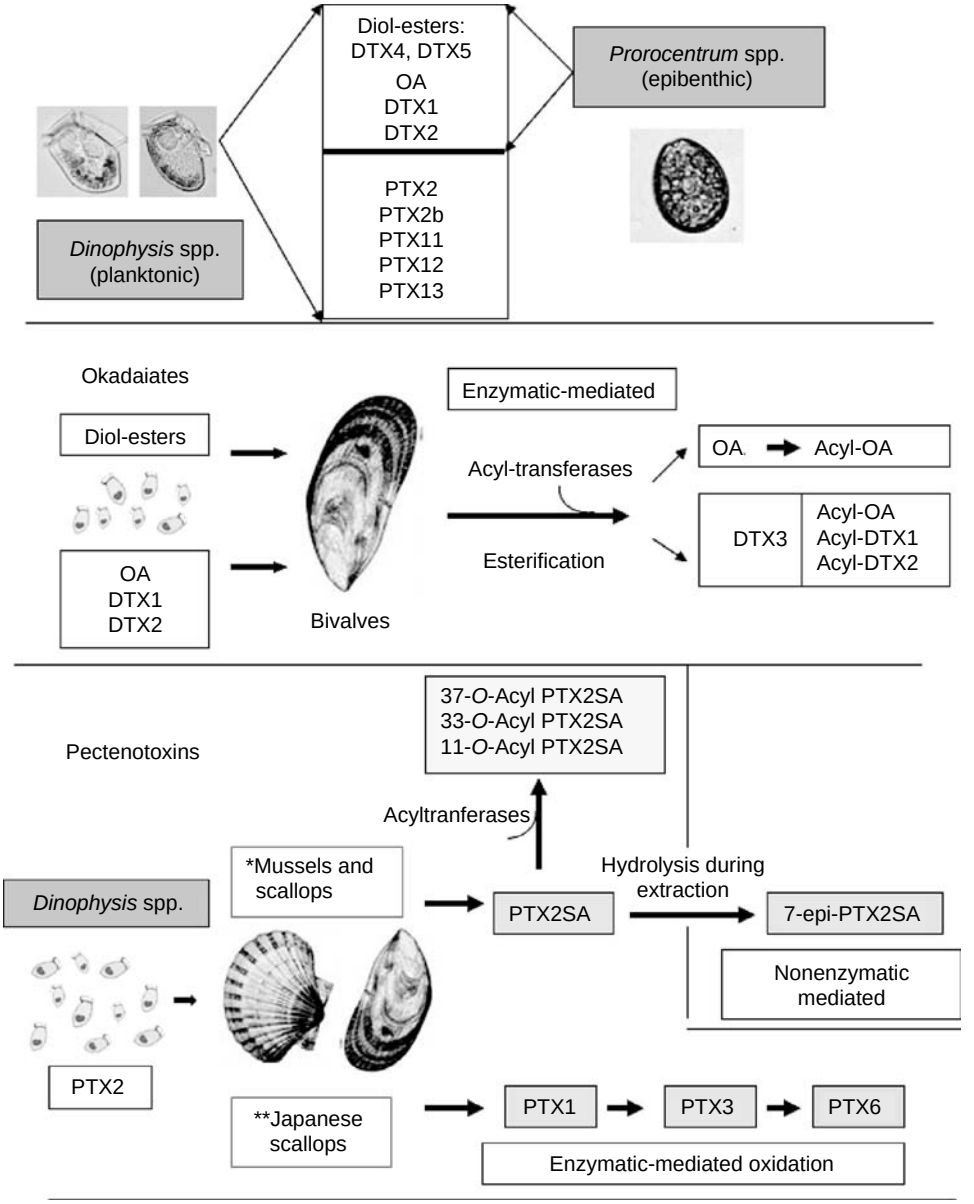
Discrepancies have been found, both qualitative and quantitative, in estimates of toxicity from cells collected during the same event, depending on the collection procedure. Masselin et al. [126] found that cellular toxin content values were up to one order of magnitude higher in analyses of picked cells as compared with those obtained from plankton concentrates. Similar results were described by Blanco et al. [31], who found that discrepancies were reduced, as the plankton concentrates were more and more diluted. Here, it may be that the accompanying assemblages in bulk plankton hauls interfere with the analyses, but more recently, Pizarro et al. [127] estimated a higher cell toxin quota in net-haul specimen than in picked cells. PTX2SA, a derivative of PTX2 from enzymatic conversion in shellfish [128] has been found in plankton hauls rich in *D. acuta and D. caudata*, but not in picked cells of the latter species [129,130]. These results suggest that PTX2SA in plankton concentrates results from enzymatic conversions after cell breakage, or from secretions of accompanying plankton species; such a conversion has been demonstrated in *Thalassiosira weissflogii* cultures [131]. One advantage of toxin estimates from dense plankton concentrates is that they allow identification of toxins that represent a very small percentage of the total profile, and that could easily be overlooked in small picked-cell samples. Nevertheless, it will not be possible to confirm if rare toxins in a bulk plankton extract correspond to a suspected toxin producer, or are the result of bioconversion by the accompanying material. Figure 12.4 shows a summary of possible transformations of the OAs and PTXs that result either from enzyme-mediated processes or are handling artifacts.

In conclusion, data on toxin profiles and cell toxin quota of *Dinophysis* have to be interpreted with caution and careful readings of the collection, extraction, and analytical procedures used. Extreme care with handling procedures is necessary with plankton concentrates; they should be filtered and deep-frozen as soon as possible, or diluted to keep the cells in better condition if processing has to wait for transportation to the laboratory. Frequently, only OA and DTX1 were sought in chromatographic analyses in the past, either because they were the only toxins suspected to be present in the cells, or because there were no certified standards available to explore the presence of other toxins. In other cases, chromatographic conditions may have been optimized for the detection of only one group of toxins (i.e., PTXs) in detriment to optimal detection of other groups (OAs).

12.7.2 Variability in Toxin Profiles of *Dinophysis* Spp.

It has been proposed that the toxin profile of a given strain of a toxigenic species is genetically determined, and only minor changes in this profile should be expected in response to environmental conditions [132]. Within *Dinophysis* spp., an intriguing question is whether the toxins found in heterotrophic species, such as *D. rotundata*, are synthesized *de novo* or are derived from their prey. Unfortunately, toxins have never been analyzed during experiments on *D. rotundata* feeding on *T. fusus* [69], a planktonic ciliate reported to feed itself on *L. polyedra* and other harmful algal species in laboratory cultures [133]. The same point can be made about the heterotrophic azaspiracid-producer *P. crassipes* that has been cultured using *L. polyedra* as prey [82].

Recent results obtained with LC-MS analyses show that any species of *Dinophysis* may have either OAs or PTXs or both, but with very different proportions between species, and between strains of the same species from different regions. Table 12.1 shows that differences in toxin profiles between strains are just as marked as those between different species. Some clear-cut features from the available results, are summarized in Table 12.1. *D. acuta* strains from northwest Spain, Portugal, and western Ireland usually have OA and DTX2 as okadaiate-components of their profile, whereas strains of the same species from Sweden and Norway have OA and DTX1; *D. acuminata* is the main OA producer in Iberian and French coastal waters and DTX2 and PTXs have never been found (so far) in single cell isolates of *D. acuminata*, nor in plankton extracts or mussels exposed to blooms



* *Mytilus edulis, M. galloprovincialis, Perna canaliculus, Pecten novaezelandiae*
** *Patinopecten yessoensis*

FIGURE 12.4 Simplified diagram of possible enzymatic- and nonenzymatic-mediated conversions of okadaiates and pectenotoxins from microalgae to shellfish.

of this species in Iberian waters, while in strains from Norway and New Zealand PTXs are predominant, and are the only toxins detected in strains from Northern Chilean waters. The toxin profile of Japanese strains of *D. fortii*—the first *Dinophysis* spp. reported to contain PTXs—is dominated by DTX1 and PTX2.

Another important issue is to establish whether there are non-toxic strains of given species of *Dinophysis* spp. proved to be toxigenic in other parts of the world. A good example is that of *Dinophysis* on the eastern coasts of the United States. During 2 years of monitoring in Narragansett

Bay, only one shellfish sample was reported to have DSP toxins associated with *Dinophysis* [133] and DSP outbreaks are not considered a problem there. During an exceptional bloom of *D. acuminata* in Chesapeake Bay in 2002 [135,136], HPLC analyses were used to explore the presence of OA only, and values below regulatory levels were detected; shellfish species tested during this bloom were oysters (*Crassostraea virginica*). However, oysters in Japan [137] and in Galicia (F. Arévalo, pers. com.) exhibit very low DSP levels, or none at all, when other bivalves nearby reach high toxin concentrations. Furthermore, in cases when PTXs are the dominant components of the toxin profile during *Dinophysis* spp. blooms, and the commercially exploited bivalves analyzed are species with high conversion rates of PTX2 to PTX2SA, routine monitoring analyses by MBA will reflect a practical absence of LFTs. High PTXs levels have been found in recent LC-MS analyses of picked cells of *D. caudata* from NW Spain [130]. This species had previously been considered harmless on the basis of its very low OA content as determined by HPLC and by phosphate inhibition assays [36, 138]. The dominance of PTXs in toxin profiles of *D. acuminata* strains from western Japanese coastal waters may also explain why DSP outbreaks there are not a major problem. In summary, only in those cases where the presence of both OAs and PTXs have been explored with appropriate methods can we be certain that a species of *Dinophysis* lacks toxins.

To establish whether differences in toxin profiles are due to genetic variability between strains of the same species, DNA analyses are required. Nevertheless, polymerase chain reaction (PCR) amplification of ribosomal DNA subunits and internal transcribed spacers of *Dinophysis* spp. have shown very little variability in this part of the genome commonly used to separate species and strains of other microalgal species [139–142]; sequences of plastid DNA show no differences among the phototrophic *Dinophysis* spp. tested, or between these and the plastid sequences of some small (8–15 μm) cryptophytes of the genus *Teleaulax* [74]. As a result, DNA probes able to separate *Dinophysis* at the species level and to find differences between strains of the same species are still on a developmental stage.

Field data on toxin profiles of *Dinophysis* spp. are few and scattered in space and time. Assumptions of a constant toxin profile have been made on the basis of a small number of samples, mainly collected at the time of *Dinophysis* spp. maxima. However, even if the hypothesis of a genetically determined (constant) toxin profile is accepted, this may apply separately to each group of toxins: different environmental conditions may enhance the production of OAs and PTXs in different ways, and lead to different OAs/PTXs ratios. An intriguing question is why PTXs predominate in some strains of *Dinophysis*, but are scarce or absent in other strains of the same species where OA and DTXs are the dominant components of the toxin profile. There is biosynthetic evidence that most polyether toxins are produced through polyketide pathways in which acetate is the common building block [143]. Thus, although OAs and PTXs have different chemical structures and different biological effects, both groups may be synthesized from a common precursor. If that is the case, different sets of environmental conditions, different physiological conditions, or different phases of the population, in conjunction with the genetic characteristics of different species or strains, may trigger processes leading to formation (and predominance) of one group of toxins or the other.

12.7.3 Variability in Cellular Toxin Content

There is no experimental data on toxin production rates in cultures of *Dinophysis* spp.; the only information available is on toxin content per cell. Both terms are confused, and observations of high toxin content per cell are often misinterpreted as cells with a high toxin production rate. It is important to remember that toxins are secondary metabolites, and that the toxin content (accumulation) per cell results from a balance between toxin production rate, toxin excretion, and cellular division (that dilutes the toxin produced by the mother cell between two offspring). Imbalances between these processes may lead to very low accumulation rates of toxins (if either division rates or toxin release rates are high), or high accumulation rates (if division stops and toxin production continues). Cell size should always be taken into account in relation to variability in toxin content. The balance

between growth, stress, and toxin production was discussed by Flynn and Flynn [144], but toxin release has not so far been considered in toxin production and accumulation budgets.

At present, possible sources of variability of toxin content per cell in *Dinophysis* are inferred from observations of natural populations or by extrapolation of results obtained with cultures of epibenthic dinoflagellates (i.e., *P. lima*) used as a model organism. MacKenzie et al. [145], with "Solid Phase Absorption Toxin Tracking" (SPATT) resins, that is, resins able to adsorb *in situ* lipophilic toxins from the water, have shown that toxins are continuously released by *Dinophysis* cells. This observation is not surprising, since secretion of sulfated forms of OA and of YTXs has been observed in monoalgal cultures of *P. lima* [146] and *P. reticulatum* [42,147,148] respectively. Nevertheless, neither in the cultures of *P. lima* and *P. reticulatum*, nor in natural populations of *Dinophysis*, is it clear if the released toxins come from old leaky cells, from broken or dead individuals, from active secretion by healthy cells, or from a combination of all these sources. In the field, additional sources to consider are excretions—rich in *Dinophysis* and/or their toxins—from the zooplankton [149] and from shellfish (faeces, pseudofaeces) [97] in which the original toxin profiles of the dinoflagellate may already be transformed.

Pan et al. [150] found that in cultures of *Prorocentrum* spp. toxin production stops in the dark, and Zhou and Fritz [151] showed that OA is mainly localized in the peripheral chloroplasts. These observations led to the conclusion that toxin production is light-mediated. Nevertheless, high estimates of OAs per cell were obtained in a few isolated analyses of the heterotrophic *D. rotundata* [152]. In cultures of *Prorocentrum hoffmannianum*, toxin production rates are similar to growth rates, with maximum values observed during the exponential phase [153,154], but other studies show that production continues in the stationary phase after division has ceased [155,156]. Increased toxin content per cell in incubations of picked cells of *D. acuta* and *D. acuminata* was interpreted as a response to nitrogen and/or phosphorus limitation [157], but a similar increase was observed in cells of *D. acuta* maintained a few days in untreated seawater from the same location where they were collected [36].

Some new field experiments may improve our understanding of the relationship between growth and toxin content in *Dinophysis* spp. In Sweden, large differences in the intensity and frequency of DSP outbreaks in two contiguous fjords puzzled experts in the area for many years [158]. During a systematic search for OA content per cell in the two areas, Lindahl et al. [159] found a negative correlation between *Dinophysis* (*D. acuminata* + *D. acuta*) cell density and toxin content. Maximum values (17 pg OA/cell) were found in low-density populations of the outer Gullmar Fjord, and minimum values in high-density populations in the pycnocline in the semienclosed Koljö Fjord. In contrast, Pizarro et al. [160] found increasing values of toxin per cell in a population of *D. acuta*, in the Galician Rías, that reached its peak concentration in November as a result of physical transport [100]; in this instance, values of toxin per cell were negatively correlated with estimated *in situ* division rates, and positively correlated with cell density. It is possible that in Lindahl et al. [159] study the high-density populations of *Dinophysis* located in the pycnocline in the semienclosed Koljö Fjord resulted from a high division rate of cells aggregated at their optimal depth, whereas the low-density population in the Gullmar Fjord had a lower division rate and a higher accumulation of toxin per cell as a result of an imbalance between growth and toxin production. Nevertheless, PTXs were not considered in this study, and may have made an important contribution to the toxin profile.

This discussion makes it clear that to improve interpretation of field observations of toxin content per cell, they should preferably be accompanied by other biological observations (cell size, growth rate, cell vacuolation, phase of the population) and environmental conditions. The mixotrophic feeding behavior of *Dinophysis* spp. has been pointed to as a possible explanation of the high variability of toxin content per cell, but a range of 25 times has been observed in YTXs content in conventional cultures *P. reticulatum* [147,148]. Nevertheless, the definitive answer on the causes of intraspecific differences in toxin content per cell should await improvements in genetic studies to assess intraspecific genetic variability in *Dinophysis* spp.

12.8 RELIANCE ON TRIGGERING LEVELS OF *DINOPHYSIS* AND OTHER LIPOPHILIC TOXIN PRODUCERS

Cell concentrations of potentially toxic planktonic microalgae have often been used as a guide to trigger intensification or even initiation of toxin analyses in shellfish growing areas [161,162]. To rely on cell counts as an early warning for the presence of phycotoxins in shellfish may prove useful in the case of toxigenic species that (i) are very seasonal, (ii) their *inoculum* population develops *in situ* or in easy-to-track offshore waters that are advected to the shellfish grounds, and (iii) need to reach high cell numbers to cause harmful effects. Obviously, to decide if these criteria are met requires a sound knowledge of the annual succession and dynamics of local plankton populations in response to environmental forcing (runoff, winds, tidal regime, etc.). Nevertheless, the use of cell concentrations as a trigger to initiate analyses of toxins in shellfish cannot in general be considered good practice to protect public health and to regulate shellfish marketing. This is especially so in the case of toxic microalgae of the genus *Dinophysis*, the main cause of bivalve harvesting closures on the Atlantic coasts of Europe, that (i) are always present in the plankton; (ii) often constitute a small percentage of the total phytoplankton population, and (iii) can render shellfish unsuitable for human consumption at very low cell concentrations ($1–2 \times 10^2$ cell/L).

In the previous sections, enough reasons have been provided that serve against the routine application of "triggering levels" in monitoring systems. These and some additional reasons can be summarized in four main issues.

12.8.1 Spatial and Temporal Variability of Cell Distributions

Distributions of *Dinophysis* spp. and other swimming dinoflagellates are extremely variable in time and space. Patchiness results from physical–biological interactions, such as aggregation of dinoflagellate cells in the pycnocline, persistence of swimming organisms in convergent hydrographic cells, and transient retention features. Further, physical advective processes, such as those observed during wind reversal, downwelling events, and so forth can cause the accumulation of high concentrations of toxigenic cells on time scales of 1–2 days, that is, shorter than the frequency of routine phytoplankton sampling or toxin monitoring. Large differences (over one order of magnitude) in concentrations can be observed throughout a daily cycle owing to circadian rhythms in vertical migration, and to the concentration and dispersion effect of tides (Figure 12.5).

There is possibly no monitoring program in the world with a sufficient coverage of stations (spatial) and frequency (time) of sampling to provide, based only on cell counts, an "operational" forecast of the distribution of potentially toxic cells that can become accessible to bivalve feeders on the following days and render them unsuitable for human consumption at concentrations of 10^2–10^3 cell/L. Nevertheless, these limitations can be overcome if the cell distributions are complemented

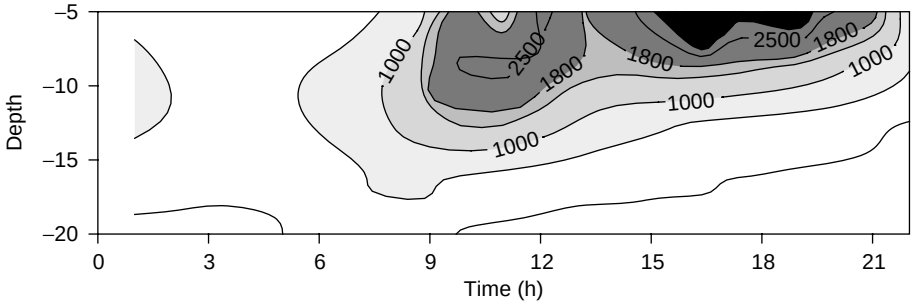


FIGURE 12.5 Vertical distribution of *Dinophysis acuminata* during a 24 h sampling in a Galician ría. (Modified from [115,122])

with a sound knowledge of local hydrodynamics and experience gained from previous toxic events in the same locality.

12.8.2 Variability of Toxin Content per Cell

Available data shows high variability (up to 1–2 two orders of magnitude) in toxin content per cell (Table 12.1), while toxin profiles in a given locality seems to be more stable features. Data are still scattered, and a more systematic monitoring on toxin profile content per cell related to intrinsic and environmental factors is needed to improve knowledge and reach predictive capabilities on this topic.

12.8.3 Absolute Cell Concentrations Can Be Meaningless if Unrelated to Concentrations of Other Accompanying Organisms

Since the late 1980s, Sampayo et al. [163] observed an inverse relationship between mussel toxin levels and total phytoplankton concentrations at similar concentrations of *Dinophysis* spp., that is, toxin content in shellfish depended on the ratio between toxic phytoplankton cells and the total phytoplankton population. Further studies of Blanco et al. [31,164] showed the importance of intrinsic factors linked to bivalve physiology, and developed kinetic models that take environmental conditions (temperature, salinity, water column stability) into account, and the quantity and quality of food (chlorophyll concentration, seston) available to the bivalves. Concentrations of toxigenic cells and toxin content per cell are important parameters in these kinetic models of intoxication and detoxification. Obviously, in waters low in particulate organic matter, filter-feeders need to filter larger volumes to fulfill their nutritional needs. Blanco et al. [164] introduced a new parameter, "toxic quality" of food, by analogy with the term "food quality" commonly employed in assimilation models for bivalves. Dahl and Johannessen [165] recommended the use of the ratio between *D. acuta* concentration and the chlorophyll content as a better index to predict DSP events associated with this species in Norwegian coastal waters.

12.8.4 Toxicity in Absence of Toxigenic Cells

After severe outbreaks caused by growth of toxigenic microalgae in late autumn—early winter, toxins in shellfish above regulatory levels may be observed long after the source organisms have disappeared from the water column. In these cases, the end of harvesting closures depends more on the onset of spring bloom diatoms—that will provide abundant non-toxic food to shellfish—than on the reported concentrations of toxic dinoflagellates in the water.

12.9 CONCLUSIONS AND PERSPECTIVES

Several *Dinophysis* species are the main worldwide sources of OAs and PTXs. YTXs produced by *L. polyedra*, *P. reticulatum*, and *G. spinifera* are a more recently identified problem. Widespread application of LC-MS analyses will lead to identification of a much larger list of causative species. AZP toxins, so far associated with *P. crassipes*, seem to be a European problem, but their presence has not been properly explored elsewhere. Bloom dynamics, toxin profiles, and toxin content per cell need to be studied on regional and local scales. Some key issues that require further research and/or improved procedures and tools are (a) genetic (inter- and intraspecific) variability of *Dinophysis* spp.; (b) identification of inoculum populations and other behavioral processes; (c) toxin production studies in cultures of *Dinophysis* and *Protoperidinium* spp.; and (d) improved determination of "hidden" toxins, such as diol esters of OA, in microalgal extracts.

Genetic studies of *Dinophysis* spp. will require sequencing of unexplored parts of their genomes that show higher variability between species and strains. Field studies, focused on target species,

will certainly benefit from operational oceanography approaches. Recent findings on feeding behavior of *D. acuminata* open many new possibilities to explore, in combination with improved genetic studies, and the causes of different toxin profiles produced by the same species in different parts of the world.

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Part VIA

Yessotoxins

13 Chemistry, Metabolism, and Chemical Analysis

Patrizia Ciminiello and Ernesto Fattorusso

CONTENTS

13.1 INTRODUCTION

Yessotoxins (YTXs) represent a group of polycyclic ether compounds, structurally closely related to brevetoxins and ciguatoxins. Several reviews on this topic have been previously published. Most of them report on YTXs within a wider survey including other biotoxins;[1–6] a review focused on the occurrence, detection, chemistry, and pharmacology of YTXs and structural analogs has been recently published.[7]

The parent compound of this class of marine toxins is yessotoxin (YTX, **1**), whose structure was established first[8] and which gave the basic framework to elucidate the structures of the other YTXs.

YTXs were originally included among the toxins responsible for diarrhetic shellfish poisoning (DSP), mainly because they are extracted together with the DSP toxins, okadaic acid (OA) and the dinophysistoxins (DTXs), when they co-occur in contaminated shellfish, as has sometimes been noticed. YTXs, however, are nondiarrheagenic and, compared to OA, show a much lower (four orders of magnitude) potency for the inhibition of protein phosphatase 2A.[9] For these reasons it was proposed that YTX (and all of its analogs) should not be included in the list of DSP toxins. The few toxicological studies carried out on YTX revealed that it is more toxic than DSP toxins when intraperitoneally injected, since the dose inducing 50% of mice lethality is very low (100 μg/kg);[2] on the contrary its oral toxicity is weaker, as deduced by considering that the oral dose of 1mg/kg—which is ten times the lethal dose by intraperitoneal injection—does not kill the mice.[9,10] Thus, the European Food Safety Authorities now consider that YTXs are no longer part of the DSP toxin complex and have set a higher control limit for YTXs (1 mg/kg) than OA and its analogs,[11] to eliminate long-term shellfish bans in the market. However, it has been demonstrated that YTX has adverse pharmacological effects on cellular Ca^{2+} regulation[12] and

phosphodiesterase coordination.[13] Therefore, a careful monitoring of this toxin is needed to protect the health of seafood consumers.

13.2 IDENTIFICATION AND STRUCTURES OF YESSOTOXINS

Until now more than 30 YTXs have been identified in contaminated shellfish and/or in cultures of dinoflagellates, which are considered their producer organisms.

All the YTXs isolated so far are reported here, specifying the organism in which they were first found. Figures 13.1 and 13.2 report the chemical structures of all the YTXs that were first identified in shellfish, while Figures 13.3 through 13.6 report those of YTXs exclusively found in algal cultures.

13.2.1 Yessotoxins from Shellfish

The main structural features of yessotoxin (YTX, **1**) (Figure 13.1) consist of a ladder-shaped polycyclic ether skeleton, an unsaturated terminal side chain of nine carbons, and two sulfate groups. It was first isolated from scallops, *Patinopecten yessoensis*, that were implicated in a DSP episode in Japan.[8] The planar structure of YTX was elucidated by means of 2D nuclear magnetic resonance (NMR) techniques,[8] and confirmed by fast atom bombardment mass spectrometry/mass spectrometry (FAB MS/MS) experiments;[14] the stereochemical details were successively determined by assigning the relative and then the absolute stereochemistry.[15,16] YTX has subsequently been isolated from shellfish collected along the coasts of several different countries such as Norway,[17] Chile,[18] New Zealand,[18] Italy,[19] suggesting the spread of this toxin worldwide.

In 1996 Satake et al. reported the isolation of the first two analogs of YTX, 45-hydroxyyessotoxin (45-hydroxyYTX, **2**) and 45,46,47-trinoryessotoxin (norYTX, **3**) (Figure 13.1) from toxic scallops; their structures comprising the relative stereochemistry were assigned mainly through NMR studies.[20] However, the C-45 configuration in **2** remained undetermined until 2000 when it was assigned through the modified Mosher method.[21]

Homoyessotoxin (homoYTX, **4**) and 45-hydroxyhomoyessotoxin (45-hydroxyhomoYTX, **5**) (Figure 13.1) were first reported in 1997 from the digestive glands of the mussels *Mytilus galloprovincialis* collected from a site on the Emilia Romagna coast of Italy.[22]

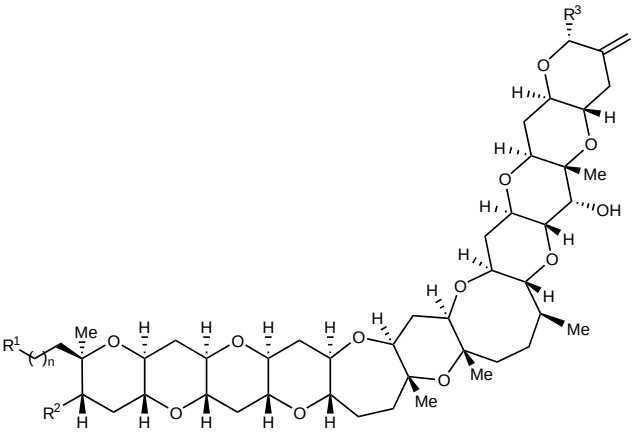
In 1998, the isolation of adriatoxin (ATX, **6**, Figure 13.2) along with YTX (**1**), 45-hydroxyYTX (**2**), and homoYTX (**4**) (Figure 13.1) from the hepatopancreas of mussels collected in the northern Adriatic sea was reported.[23] The structure of the new YTX analogue, including the absolute stereochemistry, was determined on the basis of negative FAB-MS and NMR spectral data. The main differences between the structure of YTX and ATX were shown to be in the eastern part of the molecule where the absence of the side chain as well as of an ether ring and the presence of a further sulfate group were observed.

1-Desulfoyessotoxin (dsYTX, **7**, Figure 13.1) was first reported in 1998 from the digestive glands of mussels growing in the Sognefjord, Norway.[24]

Two new analogs of YTX , carboxyyessotoxin (carboxyYTX, **8**)[25] and carboxyhomoyessotoxin (carboxyhomoYTX, **9**)[26] (Figure 13.1), were reported in 2000 from the digestive glands of mussels collected in July 1997 and autumn 1998, respectively, from the Emilia Romagna coast of Italy. Their structures were determined by MS and mono- and bi-dimensional ^{1}H NMR experiments.

Noroxohomoyessotoxin (noroxohomoYTX, **10**, Figure 13.1), a new addition to the class of YTXs, was reported in 2001.[27] It was isolated from the digestive glands of mussels collected in 1998 from the northern Adriatic coast of Italy. The polycyclic skeletal structure was mainly assigned on the basis of homonuclear 2D NMR spectral data obtained from correlation spectroscopy (COSY) and HOmonuclear HArtmann HAhn (HOHAHA) experiments; negative ion FAB MS/MS provided further valuable information to confirm the structure.

The following year the structurally strictly related noroxoyessotoxin (noroxoYTX, **11**, Figure 13.1) was reported.[28] Its presence in the crude extract of toxic mussels was highlighted through



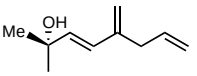
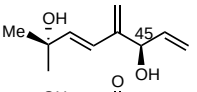
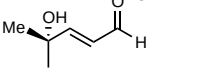
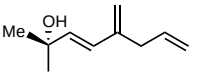
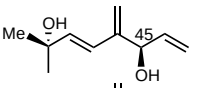
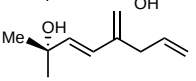
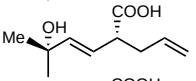
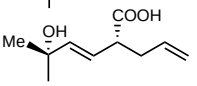
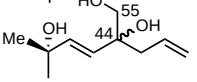
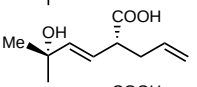
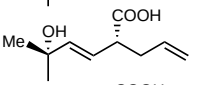
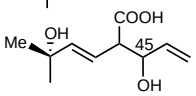
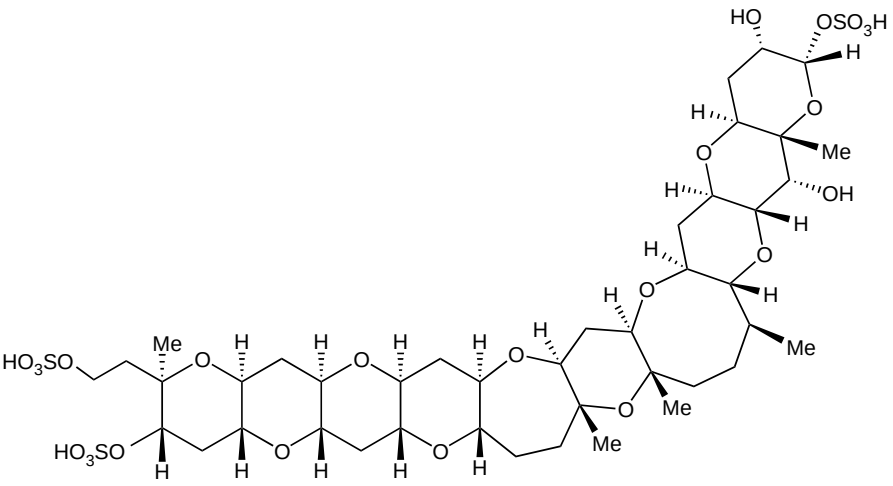
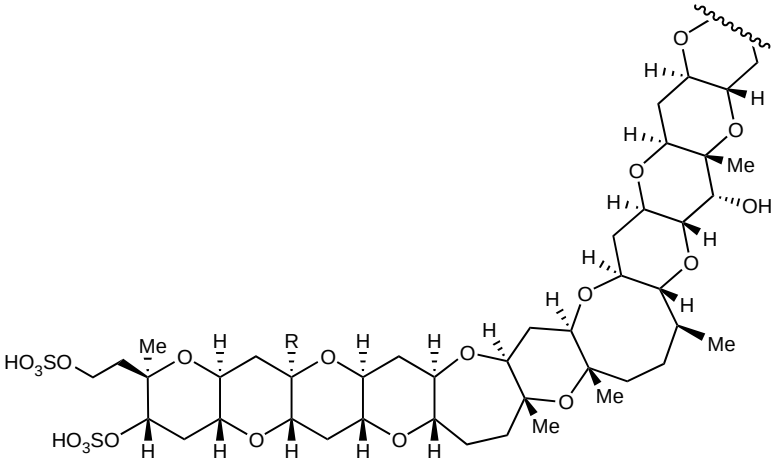
		R^1	R^2	R^3	n
1	Yessotoxin (YTX)	OSO_3H	OSO_3H		1
2	45-Hydroxyyessotoxin (45-hydroxyYTX)	OSO_3H	OSO_3H		1
3	45,46,47-Trinoryyessotoxin (NorYTX)	OSO_3H	OSO_3H		1
4	Homoyessotoxin (homoYTX)	OSO_3H	OSO_3H		2
5	45-Hydroxyhomoyessotoxin (45-OHhomoYTX)	OSO_3H	OSO_3H		2
7	1-Desulfoyessotoxin (1-dsYTX)	OH	OSO_3H		1
8	Carboxyyessotoxin (carboxyYTX)	OSO_3H	OSO_3H		1
9	Carboxyhomoyessotoxin(carboxyhomoYTX)	OSO_3H	OSO_3H		2
10	Noroxohomoyessotoxin (NoroxohomoYTX)	OSO_3H	OSO_3H		2
11	Noroxoyessotoxin (NoroxoYTX)	OSO_3H	OSO_3H		1
12	44,55-Dihydroxyyessotoxin	OSO_3H	OSO_3H		1
13	1-Desulfocarboxyhomoyessotoxin	OH	OSO_3H		2
14	4-Desulfocarboxyhomoyessotoxin	OSO_3H	OH		2
15	45-Hydroxycarboxyyessotoxin	OSO_3H	OSO_3H		1

FIGURE 13.1 Structures of yessotoxins, shown in their sulfonic acid forms, which were first reported from shellfish. The configuration at C-45 in **5** has been assigned by analogy with **2**, as reported by Morohashi et al.[21]



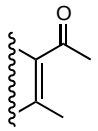
Adriatoxin (ATX)

FIGURE 13.2 Structure of adriatoxin.

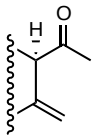


R

16 Yessotoxin-enone (YTX-enone) H



17 40-epi-Noroxoyessotoxin (40-epi-noroxoYTX) H



35 9-Me-Yessotoxin-enone (9-Me-YTX-enone) CH_3

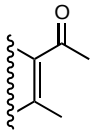
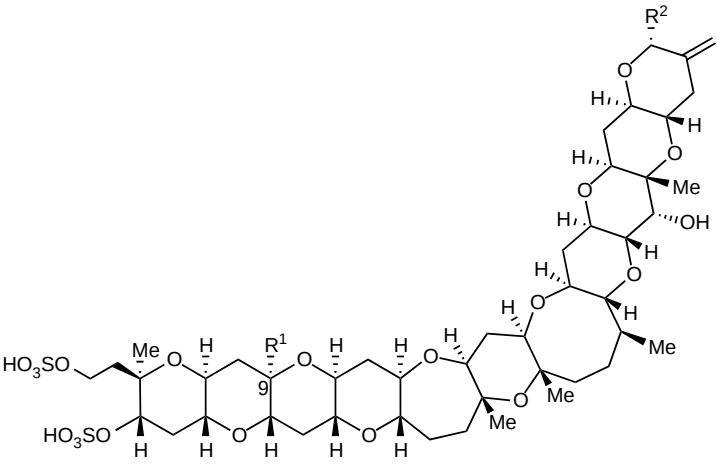


FIGURE 13.3 Structures of yessotoxin methyl ketone analogs from *P. reticulatum*. The structures are shown in their sulfonic acid forms.



		R1	R2
18	41a-Homoyessotoxin	H	
19	9-Me-41a-Homoyessotoxin	CH_3	
24	44,55-Dihydroxy-41a-homoyessotoxin	H	
25	44,55-Dihydroxy-9-methyl-41a-homoyessotoxin	CH_3	
26	Trihydroxyamide of 41a-homoyessotoxin	H	
27	Trihydroxyamide of 9-Me-41a-homoyessotoxin	CH_3	
33	45-Hydroxy-46,47-dinoryessotoxin	H	
34	44-Oxo-45,46,47-trinoryessotoxin	H	

FIGURE 13.4 Structures of yessotoxins, shown in their sulfonic acid forms, which have been only found in *P. reticulatum*.

high performance liquid chromatography coupled with electrospray ion trap MS analysis. The structure was proposed on the basis of LC-MS/MS experiments.

In 2005, LC–MS^3 analysis of extracts of naturally contaminated mussels collected along the coasts of countries belonging to three different continents, Norway (*M. edulis*), Canada (*M. edulis*), and New Zealand (*Perna canaliculus*), revealed the presence of 44,55-dihydroxyyessotoxin

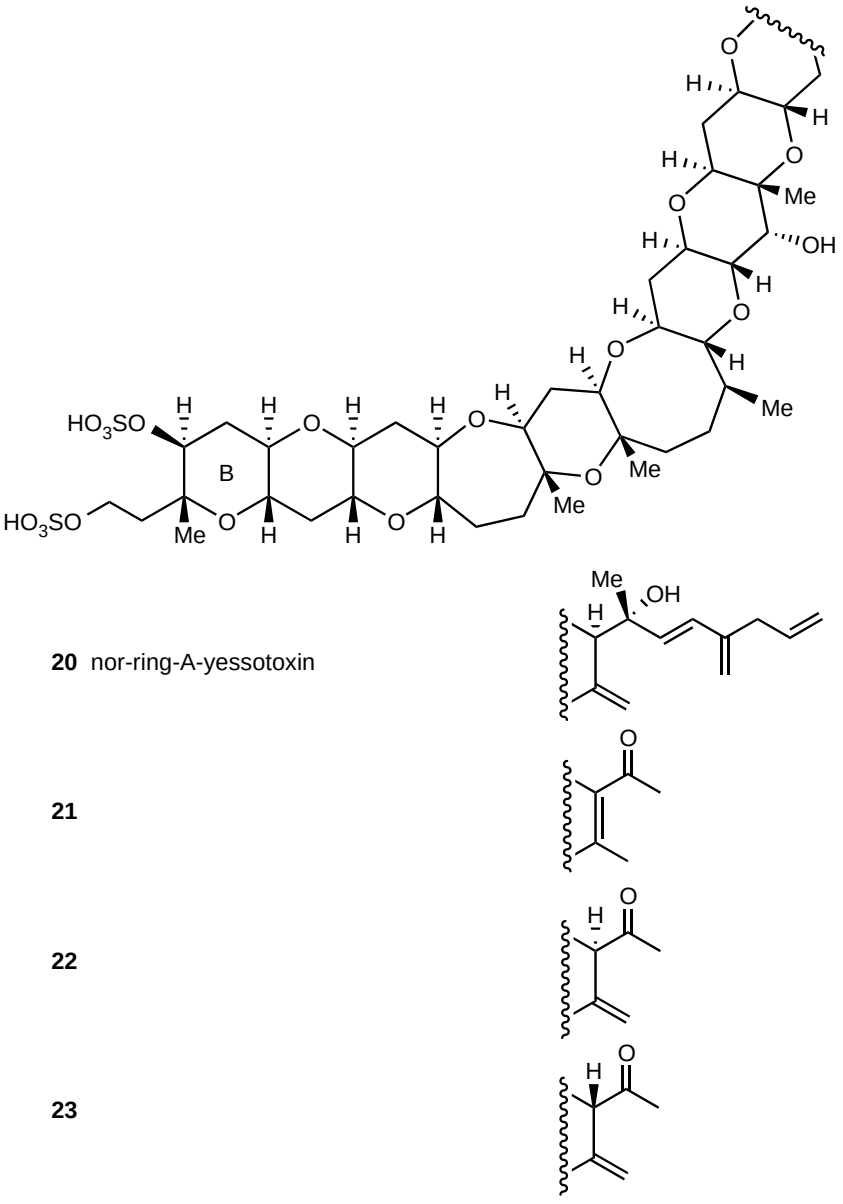
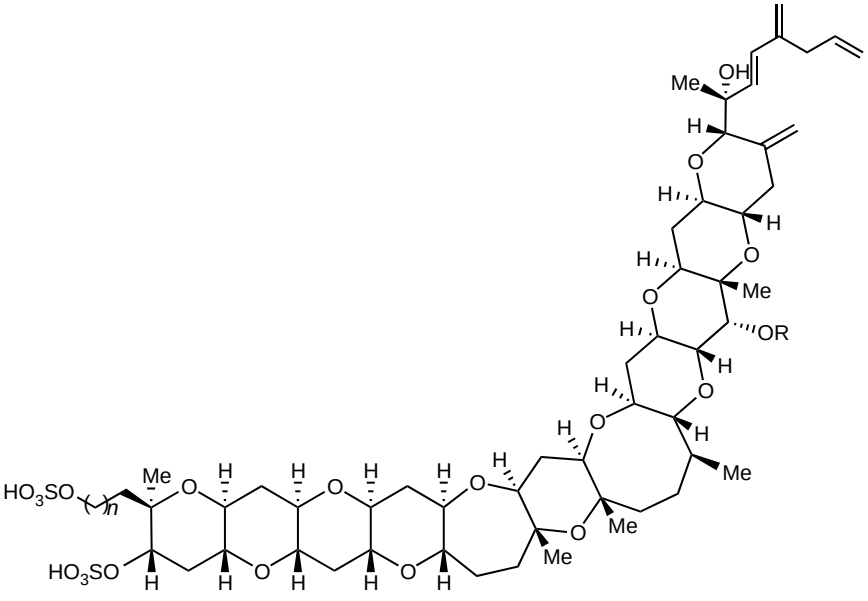


FIGURE 13.5 Structures of nor-ring A-yessotoxin and its heptanor-41-oxo-analogs.

(44,55-dihydroxyYTX, **12**, Figure 13.1).[29] Similar analysis of extracts from *Pe. canaliculus* and *M. galloprovincialis* that had been fed with a culture of *Protoceratium reticulatum* strain also revealed the presence of **12**.

Very recently, two new desulfated yessotoxin analogs (1-desulfocarboxyhomoYTX, **13** and 4-desulfocarboxyhomoYTX, **14**) (Figure 13.1) were isolated from a toxic batch of adriatic mussels collected in October 2004. Their stereostructure was elucidated through extensive NMR and MS-based analysis.[30]

The last YTX-analog from shellfish was found as a metabolic product in Norwegian blue mussels, *M. edulis*. Its structure, 45-hydroxycarboxyyessotoxin (45-hydroxycarboxyYTX, **15**, Figure 13.1), was tentatively assigned on the basis of LC-MS evidence.[31]



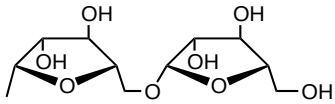
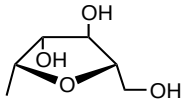
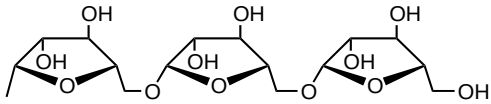
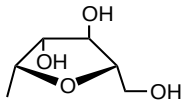
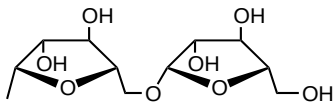
		R	*n*
28	Diarabinoside-homoyessotoxin		2
29	Monoarabinoside-homoyessotoxin		2
30	Triarabinoside-homoyessotoxin		2
31	Monoarabinoside-yessotoxin		1
32	Diarabinoside-yessotoxin		1

FIGURE 13.6 Structures of yessotoxins arabinosides from *P. reticulatum*.

13.2.2 Yessotoxins from Algal Cultures

The first report on the presence of yessotoxin (YTX, **1**, Figure 13.1) in microalgal cultures dates from 1997, when it was isolated from cultured cells of a New Zealand strain of the marine dinoflagellate *Pr. reticulatum* and unambiguously identified by high performance liquid chromatography, and ^{1}H NMR and MS data.[32] This result was the first to confirm toxigenicity of this algal species and demonstrate to it as one of the biogenetic origins of YTX found in shellfish.

The production of YTX or its derivatives by cultures of *Pr. reticulatum* was later also observed in a strain collected in Japan[33] and in Adriatic Sea.[34]

The production of YTXs by *Pr. reticulatum*, however, differs from strain to strain; whereas some cultures produce specific YTXs, others were shown to be not toxic.[33] A similar behavior was observed in many other toxigenic dinoflagellates such as *Dinophysis* spp.[35] and *Alexandrium* spp.,[36] producer organisms of DSP and PSP toxins, respectively.

LC–MS analyses carried out on a cultured strain of *Pr. reticulatum* collected along Cesenatico coasts (Emilia Romagna, Italy) in January 2002 led to the identification of YTX (**1**) together with homoYTX (**4**), 45-hydroxyYTX (**2**), carboxyYTX (**8**), and noroxoYTX (**11**) (Figure 13.1).[37]

Paz et al., by examining cultures of different *Pr. reticulatum* strains through both liquid chromatography coupled to fluorometric detection and liquid chromatography-mass spectrometry (LC-MS), confirmed the presence of YTX (**1**), which was also detected for the first time in *Lingulodinium polyedrum* cultures, although about 10 times less than in the cultured strains of *Pr. reticulatum*.[38] YTX was mainly present in the cells, but also, to a lesser extent, dissolved in the culture medium. When comparing toxin production at different stages of culture growth, larger amounts of toxins were observed in the cellular fraction and in the culture medium at the last stage of the culture in both species.

L. polyedrum was also initially considered producer organism of homoYTX (**4**) and 45-hydroxyhomoYTX (**5**), since shellfish collected from a site on the Emilia Romagna coast of Italy during a bloom of this alga were shown to contain homoYTX and 45-hydroxyhomoYTX.[22] However, the production of YTXs of the homoseries from *L. polyedrum* has not been supported by studies on its cultures.[39]

The 1,3-enone isomer (**16,** Figure 13.3) of noroxoYTX (**11**, Figure 13.1) was isolated from extracts of *Pr. reticulatum* during large-scale production of YTX.[40] It was found that **11** readily isomerizes to **16** in the presence of dilute ammonia and evidence for the existence of 40-epi-**11** (**17**, Figure 13.3) that also isomerizes to **16** was also reported.[40]

Three new YTX derivatives, 41a-homoyessotoxin (**18**, Figure 13.4), 9-methyl-41a-homoyessotoxin (**19**, Figure 13.4), and nor-ring A-yessotoxin (**20**, Figure 13.5), were reported from a New Zealand culture of *Pr. reticulatum*, together with evidence, based only on LC-MS data, for the presence of three new heptanor-41-oxo-analogs of **20** (**21–23**, Figure 13.5).[41]

In 2005 Finch et al. reported the isolation of 44,55-dihydroxyyessotoxin (**12**, Figure 13.1) from New Zealand and Norway *Pr. reticulatum* cultures; it was identified through NMR and MS experiments.[29] In addition, LC–MS analysis revealed the presence of compounds tentatively identified as (44-*R*,*S*)-44,55-dihydroxy-41a-homoyessotoxin (**24**) and (44-*R*,*S*)-44,55-dihydroxy-9-methyl-41a-homoyessotoxin (**25**), respectively (Figure 13.4).

During the course of a large-scale purification of YTX from extracts of a culture of *Pr. reticulatum* carried out by Miles et al., fractions enriched in some of YTX analogs were obtained. From these enriched extracts two new analogs of YTX were isolated (Figure 13.4); they were identified as trihydroxylated amides of 41a-homoyessotoxin (**26**) and 9-methyl-41a-homoyessotoxin (**27**), respectively, by ^{1}H and ^{13}C NMR spectroscopy and LC-MS^3 analyses.[42] The assigned structures were further confirmed by microscale chemical conversions combined with LC-MS analyses. The authors claimed that **26** and **27** could be biosynthesized from the corresponding 46-hydroxy-47a-carboxyYTXs. Compounds with masses corresponding to such analogs were detected by LC-MS^3 in extracts of the alga,[42] but their identity remains to be determined.

In the course of a search for antitumor compounds in dinoflagellates, the culture broths of two strains of *Pr.* cf. *reticulatum* showed extremely potent cytotoxicity against human tumor cell lines. The four equally active compounds, named protoceratins I II, III, and IV, were purified and their structures were studied.[43] The major compound (protoceratin I) was proved to be identical to homoYTX (**4**). The remaining bioactive compounds were determined to be the diarabinoside, 32-*O*-[β-L-arabinofuranosyl-(5′→ 1″)-β-L-arabinofuranosyl]-homoyessotoxin (**28**) together with minor quantities of the mono- (**29**) and tri-arabinoside (**30**) (Figure 13.6). This was the first report of glycosides of dinoflagellate polyethers. Shortly after, cultures of *Pr. reticulatum* were shown to produce the 32-monoarabinoside of yessotoxin, G-YTXA (**31**, Figure 13.6).[44]

Successively, Miles et al., during a large-scale isolation of YTX, confirmed the presence of **31** in extracts of *Pr. reticulatum* and in addition isolated 32-O-[β-L- arabinofuranosyl-(5′→ 1″)-β-L-arabinofuranosyl]-yessotoxin (**32**, Figure 13.6),[45] whose structure was confirmed by comparison of its NMR data with those established for its 1-homoYTX homologue (**28**).[43]

Very recently, Miles et al. during a large-scale purification of 44,55-dihydroxyyessotoxin (**12**, Figure 13.1) obtained fractions enriched in previously unidentified YTX analogs. Careful analysis of these fractions by HPLC-UV, LC–MS^3, and NMR spectroscopy, revealed the identities of some of these analogs as 45-hydroxy-46,47-dinoryessotoxin (**33**, Figure 13.4), 44-oxo-45,46,47-trinoryessotoxin (**34**, Figure 13.4), and 9-methyl-42,43,44,45,46,47,55-heptanor-38-en-41-oxoyessotoxin (9-Me-YTX-enone, **35**) (Figure 13.3).[46] The HPLC-UV and LC–MS^3 data indicated the presence of large numbers of additional YTX.[46,47]

In 2006, *Gonyaulax spinifera,* previously considered a non-YTX producer, was isolated from the seawater containing contaminated Greenshell™ mussels (*Pe. canaliculus*) in New Zealand; its cells grown in culture were shown to produce YTXs identified by ELISA analyses.[48] The algal identification was based on the type description and cyst form. DNA sequence data for *G. spinifera* differed from the morphologically similar *G. membranacea, G. digitale, and G. cf. spinifera.* Surprisingly, *Pr. reticulatum* isolated from the same site as *G. spinifera* 3 weeks later was shown to be not a YTX-producer by LC-MS and ELISA analyses.[48]

13.3 YESSOTOXINS IN SHELLFISH AND ALGAL CULTURES: QUANTITATIVE ASPECTS AND METABOLISM

Since 1997, when YTX was first isolated from Japanese scallops, more than 30 of its analogs have been identified in toxic shellfish and/or algal cultures. However, some of them represent the major compounds of the YTX-fraction of the biological extract, whereas others are only very minor constituents.

A profile of the relative amounts of the various YTXs both in contaminated shellfish and in the producer organisms is essential to define two important issues: (a) individuation of the YTXs that play a major role in the shellfish toxicity; (b) metabolism of the accumulated toxins within the shellfish. These two topics are strictly related to the impact of YTXs contamination on public health and seafood resources.

As for the YTXs profile in the producing organisms, the data on the relative amounts are not so numerous, and all refer to cultures of *Pr. reticulatum.* About the other two microalgae that were reported to produce YTXs, cultures of *L. polyedrum* were shown to contain YTX,[38] while *Gonyaulax spinifera* cultures were reported to produce unspecified YTXs identified by ELISA analyses.[48]

The first YTXs profile dates from 2003,[37] when the YTXs content reported in Table 13.1 was shown for a culture of an Adriatic *Pr. reticulatum* strain.

Further data on the relative amounts of YTXs in cultures of *Pr. reticulatum* can be deduced from several recent papers of Miles et al. reporting the isolation of a number of analogs of YTX.[40–42,45–47] The authors claimed that all the isolated YTXs were present in minute amounts in cultures of *Pr. reticulatum*, where the dominant component was always YTX.

TABLE 13.1
Profile of YTXs[a] Measured by LC-MS in a Culture of an Adriatic *P. reticulatum* Strain[37]

YTX (1)	homoYTX (4)	45-HydroxyYTX (2)	CarboxyYTX (8)	NoroxoYTX (11)
93.5	0.7	1.6	3.0	1.2

[a] Percentages referred to the total of identified YTXs. The estimated ratios of analogs should be considered as tentative in the absence of quantitative reference standards and data on recoveries.

Recently, eight strains of *Pr. reticulatum*, isolated from the Mutsu bay (Japan), were cultured in the laboratory, and analyzed by fluorometric high pressure liquid chromatography and LC/MS methods for YTX production and composition.[49] All strains tested were confirmed to produce YTX, even if the toxin amount differed from strain to strain. None of them was shown to produce known YTX analogs.

In conclusion, the data reported in the literature concur that YTX is by far the major toxin produced by *Pr. reticulatum.* HomoYTX and a great number of other YTX analogs were sometimes shown to be present in the cultures, but in much lesser quantities.

However, two reports conflict with the above statement. Konishi et al., by searching for antitumor compounds in dinoflagellates, found that culture broths of two strains of *Pr.* cf. *reticulatum* contained homoYTX as the major principle and minor quantities of three homoYTX arabinosides.[43] Interestingly, the authors claimed that their isolates did not produce YTX.

Souto et al. reported that an acetonic extract of *Pr. reticulatum* cells contained a monoarabinoside of yessotoxin in larger amounts than YTX (2.90 mg vs 0.59 mg).[44]

A very recent paper of Suzuki et al.[50] provides further evidence that YTX is in most cases the major toxin produced by *Pr. reticulatum* with occasional variations of the toxin profile. The authors examined several strains of *Pr. reticulatum* collected from various shellfish producing areas in Japan. Out of the 11 analyzed cultured isolates, only one turned out to be not a YTXs producer. Among the remaining ten strains, one was shown to produce only homoYTX, six of them to produce only YTX, whereas three were shown to be producers, along with YTX, of some YTX analogues (trinorYTX, trinor-homoYTX, and noroxoYTX enone). Interestingly, in two of the latter strains, even if the most dominant toxin was always YTX, the total amount of analogues was considerably high, representing about 50% of the whole production of YTXs.

In the contaminated shellfish, the YTX profile dramatically changes. YTX (or homoYTX) is no more the dominant toxin; by way of example, Table 13.2 reports the percentages of YTXs present in naturally contamined *M. galloprovincialis* collected at different time in Adriatic sea.

Table 13.3 reports profiles of some YTX analogs (percentages), namely, YTX (**1**), 45-hydroxy YTX (**2**), carboxyYTX (**8**), and 44,55-dihydroxyYTX (**12**) measured by LC-MS in three species of naturally contaminated mussels (*Pe. canaliculus, M. galloprovincialis, M. edulis*).[29] Other YTX analogs were also detected as minor constituents in some samples, but were not included in the analysis.

Very recently, a surprising result was obtained by examining a toxic batch of Adriatic mussels; they were shown to contain two desulfocarboxyYTXs (**13**, **14**, Figure 13.1) and no detectable amounts of other YTXs.[30]

All the above data highlight a striking difference between the YTX profiles of algae and those of contaminated shellfish. YTX represents the dominant toxin in algal cultures; there are, however,

TABLE 13.2
Profile of YTXs[a] in *Mytilus galloprovincialis* Collected at Different Times in Adriatic Sea

YTX (1)	Homo YTX (4)	45-Hydroxy YTX (2)	45-Hydroxy homo YTX (5)	Carboxy YTX (8)	Carboxy homo YTX (9)	Noroxo YTX (10)	Noroxo homoYTX (11)	ATX (6)	References
7	40	27	—	—	—	—	—	27	Ciminiello et al.[23]
22	29	18	—	22	—	—	—	9	Ciminiello et al.[25,26]
—	41	—	17	—	25	—	17	—	Ciminiello et al.[27]
32	—	23	1	23	2	17	—	—	Ciminiello et al.[28]

[a] Percentages referred to the total of identified YTXs. The estimated ratios of analogs should be considered as tentative in the absence of quantitative reference standards and data on recoveries.

TABLE 13.3
Profiles of Yessotoxin Analogs[a] Measured by LC-MS in Three Species of Naturally Contaminated Mussels from New Zealand, Norway, and Canada[29]

Species	Location	YTX, 1	45-Hydroxy YTX, 2	Carboxy YTX, 8	44,55-Dihydroxy YTX, 12
P. canaliculus	Westport, SI, New Zealand	66	14[b]	12	8
P. canaliculus	Westport, SI, New Zealand	86	6[b]	3	4
P. canaliculus	Marlborough Sounds, NZ	90	6[b]	3	1
M. galloprovincialis	Marlborough Sounds, NZ	32	19	40	9
M. edulis	Newfoundland, Canada	64	13	19	4
M. edulis	Hardangerfijord, Norway	77	16	4	3
M. edulis	Sognefjord, Norway	68	17	—	15

[a] Percentages referred to the total of identified YTXs. The estimated ratios of analogs should be considered as tentative in the absence of quantitative reference standards and data on recoveries.
[b] Includes several other hydroxyyessotoxins in addition to 45-hydroxyYTX.

Source: Adapted from Finch, S.C., Wilkins, A.L., Hawkes, A.D., Jensen, D.J., MacKenzie, A.L., Beuzenberg, V., Quilliam, M.A. et al., *Toxicon*, 46, 160, 2005. With permission.

two reports in which the major toxin of *Pr. reticulatum* cultures is homoYTX.[43,50] This conflicting results, which are indirectly confirmed by the toxin profiles in some contaminated shellfish,[22,26,27] can be attributed to a genetic variability of the producer species inducing a slight modification of the genes encoding the polyketide synthetase (PKS) involved in the YTX biosynthesis.

The toxin profiles in contaminated shellfish clearly point to a structural modification of the absorbed YTX or homoYTX within the mollusk. It is to be noted, however, that, on the basis of the results reported in Table 13.3, the extent of the YTX metabolism within the mollusk greatly depends on the species of the host. Anyway, some YTX analogs, which are reported as very minor constituents of the algal extracts, are present in large quantities in contaminated shellfish: 45-hydroxyYTX (**2**), carboxyYTX (**8**), noroxoYTX (**11**), and/or their homoanalogs.

A recent article by Aasen et al. contributes information on this important matter.[31] The aim of the work was to confirm that *Pr. reticulatum* was responsible for YTX contamination of Norwegian blue mussels (*M. edulis*), and to study the subsequent metabolism and depuration of YTX and its analogs in blue mussels over a year by analysis with LC-MS and ELISA. Regular sampling of algae and mussels indicated that the occurrence of *Pr. reticulatum* in water led to contamination of blue mussels with YTXs. After absorption from the alga, YTX (**1**) was rapidly oxidized to 45-hydroxyYTX (**2**) and more slowly to carboxyYTX (**8**) (and possibly also 45-hydroxycarboxyYTX) within blue mussels (Figure 13.7). The depuration rate for carboxyYTX was considerably slower than for YTX and 45-hydroxyYTX (Figure 13.8). Comparison of ELISA and LC-MS data revealed the presence of significant levels of as-yet unidentified YTX analogs in the mussels.

More recently, in the frame of a study on the kinetics of DSP toxins in scallop *Pa. yessoensis*, the oxidative conversion of YTX to 45-hydroxyYTX within the shellfish has been confirmed;[51] YTX (6 μg) was administered through syringe to scallop and its distribution in the hepatopancreas, adductor muscle, and combined other tissues was analyzed by LC/MS. Toxins exclusively remained in the hepatopancreas irrespective of the injection site. When injected to hepatopancreas, YTX was partially metabolized to 45-hydroxyYTX. Such a change was insignificant when YTX was injected in the adductor muscle. Table 13.4 reports the toxin content (μg/g) in hepatopancreas after injection of the toxin to the hepatopancreas.

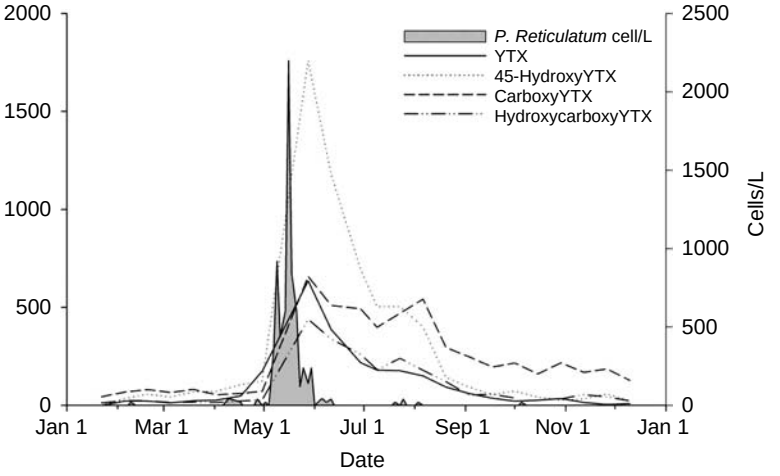


FIGURE 13.7 Concentrations of YTX, 45-hydroxyYTX, carboxyYTX, and hydroxycarboxyYTX (mg/kg mussel meat) by LC-MS in blue mussels, and counts of *P. reticulatum* cells in Fløvedigen Bay for 2001. (From Aasen, J., Samdal, I.A., Miles, C.O., Dahl, E., Briggs, L.R., and Aune, T., *Toxicon*, 45, 265, 2005. With permission.)

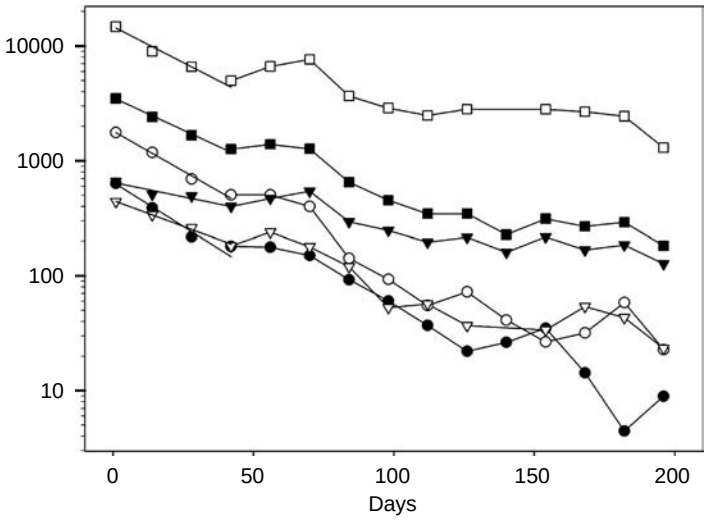


FIGURE 13.8 Depuration of yessotoxins from blue mussels at Fløvedigen Bay. Concentrations of YTX (δ), 45-hydroxyYTX (σ), carboxyYTX (.), 45-hydroxycarboxyYTX (.), the sum of YTX+45-hydroxyYTX+carboxyYTX+hydroxycarboxyYTX by LC-MS (φ), and of total YTXs by ELISA (η), versus time (days) after the peak of the contamination. Linear least-square fits for the first four measurements are shown. (From Aasen, J., Samdal, I.A., Miles, C.O., Dahl, E., Briggs, L.R., and Aune, T., *Toxicon*, 45, 265, 2005. With permission.)

It appears intriguing that the major YTX (or homoYTX) analogs (45-hydroxy, carboxy, noroxo), found in contaminated shellfish and supposed to be formed by oxidation of YTX, are also present, even if in much lesser quantities, in algal cultures. This could be explained assuming that both the alga and the shellfish share a similar potential for YTX biooxidation. Another possibility is that the biooxidation within the shellfish is performed, at least in part, by the alga and/or by its symbiont(s). Anyway, an exhaustive elucidation of this topic will be gained only when the biogenesis of YTX will be fully clarified including the possible role of microorganisms associated to the alga.

TABLE 13.4
Toxin Content (μg/g) in Hepatopancreas after Injection of the Toxin to the Hepatopancreas[a,b]

Hours	YTX (1)	45-HydroxyYTX (2) (Metabolite)
1	0.26 ± 0.14	0.11 ± 0.04
6	0.20 ± 0.17	0.08 ± 0.07
24	0.06 ± 0.06	0.02 ± 0.01
48	0.16 ± 0.06	0.07 ± 0.01
72	0.04 ± 0.02	0.02 ± 0.01

[a] 6 μg of YTX were injected.
[b] Toxins were not detected, or negligible, in muscle and other combined tissues (mantle, gill, gonad)

Source: Adapted from Suzuky, T., Igarashi, T., Ichimi, K., Watai, M., Suzuki, M., Ogiso, E., and Yasumoto, T., *Fish. Sci.*, 71, 948, 2005. With permission.

To the best of our knowledge, at present there is only one report on the biosynthesis of YTX dealing with the incorporation of labeled acetic acid and methionine in YTX produced by *Pr. reticulatum* cultures.[52]

A further important issue, possibly related to the YTX metabolism in shellfish, is the presence of desulfoYTXs in contaminated shellfish, whose presence has never been reported in algal cultures. The first report on the presence of these compounds in shellfish appeared in 1998, when 1-desulfoyessotoxin (**7**) was isolated from the digestive glands of mussels growing in the Sognefjord, Norway;[24] from 11 kg of the digestive glands, 100 μg of 1-desulfoYTX (**7**), 1.4 mg of YTX (**1**), and 1.7 mg of 45-hydroxyYTX (**2**) were obtained.

Very recently, two other desulfocompounds, 1- (**13**) and 4-desulfocarboxyhomoYTX (**14**), were isolated from Adriatic mussels.[30] These compounds were present in the CH_2Cl_2-soluble fraction from shellfish extraction. Interestingly, only this fraction resulted positive to the mouse bioassay; in particular, the BuOH-soluble fraction, where YTXs are mainly concentrated, was negative, excluding the presence of remarkable amounts of the YTXs commonly found in contaminated shellfish. Despite their small structural innovation, the new desulfoYTXs are not recovered in the YTX-layer but in the DSP one, when extracted according to the official EU protocol.[11] The finding of desulfoYTXs in shellfish, where, as for compounds **13** and **14**, they represent the dominant toxins, poses an intriguing question about their origin: do these YTX analogs derive from an unidentified dinoflagellate or are they products from YTX or homoYTX metabolism by microorganisms associated to microalga and/or shellfish?

In conclusion, all the data reported in the literature indicate that, in addition to the parent compound (YTX or homoYTX), several derivatives co-occur in the contaminated shellfish in quantities comparable and sometimes in larger amounts. This raises issues about the toxicity control. YTX is much less toxic by oral ingestion when compared to the intraperitoneal administration; therefore the European Union has recently raised the allowance level in shellfish.[11] Unfortunately, data on the toxicity by oral ingestion for most YTX analogs are lacking; this appears particularly critical for some compounds, such as desulfoYTXs, where the lack of a sulfate group decreases their hydrophilicity in comparison to YTXs. So the biomembranes permeability and, consequently, the toxicity level by oral ingestion could be greatly affected. An additional issue related to the presence of desulfoYTXs in contaminated shellfish comes from two toxicological studies. In 1990, Terao et al.[10] found that totally desulfated YTX, differently from YTX, did not affect health, but caused severe fatty degeneration

and intracellular necrosis in the liver and pancreas. Finally, a recent report [53] provided evidence that desulfated YTX interacts with transmembrane helix domains.

13.4 CHEMICAL SYNTHESIS OF YESSOTOXINS

The impressive molecular architecture and biological activities have made YTX and its analogs challenging synthetic targets. Efforts toward the synthesis of polycyclic ether frameworks of YTX and ATX have been reported mainly by Japanese groups.[54–64] The obtained results represent a good prerequisite for the total synthesis of YTX that presumably will be accomplished in the near future.

13.5 INFLUENCE OF ENVIROMENTAL CONDITIONS ON YTXs PRODUCTION BY *PROTOCERATIUM RETICULATUM*

Environmental conditions are believed to be important factors for toxins production in algae. However, the results obtained until now on this topic for some toxic microalgae show a strong difference of the effect of environmental factors on the total amount of toxins produced among the different algal classes and even among different strains.

Even after the European Union regulatory limit for YTX was raised to 1 mg/kg of YTX equivalents,[11] bivalves are often shown to contain so high levels of YTXs as to result in farm closure for long periods. These events are not always related to high cell numbers of *Pr. reticulatum*, and therefore, it appears essential to investigate the environmental conditions, which prompt not only cell growth, but also the high toxin production and accumulation.

Only a few studies of this kind were performed and all had as object the YTXs producer organism *Pr. reticulatum.* Seamer investigated the effects of nutrient, light, salinity and temperature on YTX production by using strains of *Pr. reticulatum* from New Zealand.[65] Recently, Paz et al. studied the influence of temperature, irradiance, and salinity on a *Pr. reticulatum* strain from Spain.[66] These studies that were carried out on algae of different geographic origin gave in some cases contradictory results: Seamer[65] found that the best growth occurs at light intensity of 45 μmol m^{-2} s^{-1}, whereas Paz et al.[66] observed higher growth and YTX production in the range 50 μmol to 90 μmol m^{-2} s^{-1}; it is to be noted, however, that conflicting responses in terms of toxicity related to salinity and temperature variations among different strains were also reported for other microalgae such as *Alexandrium* species.[67–69] Mitrovic et al. evaluated the effects of selenium, iron, and cobalt addition on growth as well as on YTX production[70] and the persistence of YTX under light and dark conditions.[71] The authors found that selenium and iron supplementation increased cell growth rates, while cobalt had no effect; as for the production of YTX, only selenium supplementation was shown to provide an increasing effect.[70] Regarding the influence of the light on YTX persistence, it was reported that, after the cells of *Pr. reticulatum* were killed, YTX in seawater did not persist for long under light conditions, whereas, under dark conditions, YTX persisted longer with approximately 10% remaining after 18 days.[71]

A report of field monitoring of *Pr. reticulatum* and related YTXs (YTX and 45-hydroxyYTX) contamination in scallops in Okkirai Bay, northern Japan, has been recently reported.[72]

Hydrological parameters were monitored, and it was shown that *Pr. reticulatum* flourished under low salinity (30.59–32.60) and occurred at highest density in the surface layer (0–5 m depth) where effects from rainfall were greatest. In addition, dinoflagellate density increase and decrease were well correlated with inflows of oceanic water into the bay. YTX and 45-hydroxyYTX concentrations in scallops reached maximum levels 2 weeks after the maximum cell density of *Pr. reticulatum*, and high levels of the toxin continued for a month. Low levels of the toxin were detected even during periods when cells were not observed.[72]

Very recently, a study on the effect of nutrient limitation, salinity, and temperature on growth and YTX content in cultures of Adriatic *Pr. reticulatum* strains was carried out.[73] Particular attention was paid to measure the difference between the YTX amount retained in cells and that released in

the growth medium, since this aspect could highly affect toxicity of natural population. LC-MS analyses were carried out to determine YTX production as well as the difference between the YTX amount retained in cells and that released in growth medium. The toxin content was determined in cells collected at the stationary phase, since both toxin production and release were found to be higher in this growth stage than in the exponential phase. The obtained results showed that in *Pr. reticulatum* cultures maximum growth rate and high cell concentrations were obtained under balanced nutrient conditions, at a temperature of 20°C and at low salinity values (22–27 psu). Maximum toxin yield per liter was reached by cells cultured under replete nutrient conditions, at 20°C and at 32 psu, which, in the used strains, were confirmed as the best conditions for large-scale production of YTX. The effect of 26°C temperature is remarkable in that, although the cells appear greatly impaired in division ability, YTX synthesis is highly enhanced. The environmental implications of these physiological behaviors highlight that farmed shellfish can become less toxic in colder waters and at lower salinity values.

13.6 CHEMICAL METHODS FOR IDENTIFICATION AND DETERMINATION OF YTXs

The mouse bioassay currently represents the basic tool for most YTX monitoring program. The most commonly used assay method is the mouse bioassay developed by the Japanese Ministry of Health and Welfare.[74,75] Although this bioassay is applied worldwide, there are large differences in the performance (toxicity criterion; animal death; no consensus on appropriate observation time) among different countries, resulting in differences in specificity and detectability. A major problem is the fact that in the mouse bioassay all DSP components are likely to be detected, including those DSP toxins, which do not cause diarrhea (PTXs and YTXs) and have an unknown toxicity for humans. Other unknown toxin groups exhibiting ichthyotoxic and hemolytic properties may also cause mortality of mice in this bioassay. Therefore, major disadvantages of this assay are the lack of specificity (no differentiation between the various components of DSP toxins), subjectivity of death time of the animals, and the maintaining and killing of laboratory animals.

The problems observed with the original mouse bioassay of Yasumoto et al. (1978)[74] have led to several modifications.[76–78] As in the case of YTXs, the difference in their dangerousness compared with that of the DSP toxins is substantial. OA is considered a most harmful compound because of its potent tumor promoting activity,[79] whereas YTX , whose toxicology still presents many blind spots, is of significantly lower oral toxicity.[80] Nonetheless, YTX and OA show an almost equal lethal potency when tested through the mouse bioassay. On account of the relative harmfulness to public health, the European Union has recently established different allowance levels in shellfish for DSP-toxins and YTXs (16 μg of OA and 100 μg of YTX in 100 g of mollusk, respectively).[11]

Consequently, a new official procedure for analyzing seafood that would be capable of separating YTXs and DSP-toxins in distinct layers (Figure 13.9) was set up.[11] In fact, the old official scheme did not allow any separation between YTXs and DSP-toxins; therefore the mouse bioassay—the reference method in Europe to detect toxicity in seafood—could not lead to any confident assessment of the toxin(s) involved. The new method, however, presents some weak points; in particular, based on the difference of solubility between YTXs and OA, it fails when the extract to be analyzed contains less hydrophilic YTXs such as their desulfo derivatives, which are being recovered in the OA-containing fraction.

The application of the mouse bioassay as a method of reference for the detection of marine biotoxins is criticized in scientific terms as well as in terms of animal protection legislation. The conflict with the animal welfare legislation is high since the test inflicts considerable suffering to the animals and death of the animals is the methodical end point. The scientific literature describes the mouse bioassay as “impossible to validate.” The test results show great variability between laboratories. Gender, weight, and strain of the mice influence the outcome of the test. It presents low sensitivity (too many false negative findings), variable results between whole body and

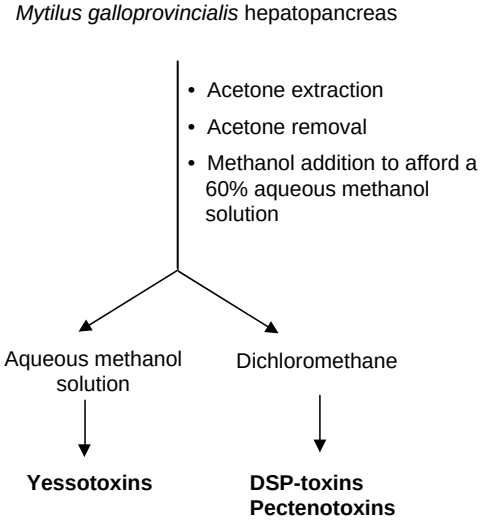


FIGURE 13.9 Extraction protocol for toxic shellfish currently in force in the European Community.[11] Such experimental procedure allows to separate YTXs and DSP-toxins in different layers on the basis of their relative lipophily.

hepatopancreas extracts,[2,81] and low discrimination (too many false positive findings)[82] as compared with chromatographic methods (i.e., LC/MS).

On the basis of the scientific literature, the use of the mouse bioassay as a method of reference for marine biotoxins is no longer considered appropriate.[83] Relevant international expert publications have reported the reliability of *in vitro* detection methods, which can be used as alternatives to animal experiments. Also, the European Commission has recognized the needs of the analytical community to develop methods alternative to animal testing.[84]

On the other hand, a number of factors hamper the development and validation of analytical methodologies alternative to the biological bioassays. A serious problem is that accurate and readily available calibration standards are required for each of the various toxins. The lack of pure analytical standards and reference materials has always been—and still is—the major drawback both in shellfish toxin research and monitoring. Moreover, only limited progress has been achieved in the synthesis of these compounds.

Additional difficulties that inhibit the development of alternative methods are represented by the complexity and variability of marine biological material, the presence of multiple toxins in samples, and the biotransformation in shellfish of the original toxins to metabolites. In addition, each year the situation becomes more complicated with the discovery of new toxin analogs and even new toxin classes. The number of toxins from sea is remarkably increasing, despite the problems dealing with their identification. Such a study consists in a first step involving the isolation of the molecules, generally present in minute amounts, from very complex biological extracts, through a series of chromatographic separations guided by biological tests. During this phase of the investigation most of the precious material is inevitably lost during the purification procedure. The successive step is the structure determination; the recently developed spectral methods, mainly multidimensional NMR techniques, applied to structural studies currently enables to hit the final target even if, as very frequently it happens, the isolated toxin is not very pure and, in addition, is available in submilligram amounts.

In recent years a number of biochemical and chemical detection methods alternative to mouse bioassay have been developed.[85] Although the biochemical assays are excellent for screening out

negative samples, it is generally recognized that positive results should still be confirmed by chemical methods that have the potential for sensitive, precise, and fully automated quantitation of known toxins, as well as confirmation of identity.

13.6.1 Liquid Chromatography

Liquid chromatography has proven to be a valuable instrumental analytical tool for toxins owing to its capability to analyze polar, nonvolatile compounds. It is easily automated and provides excellent quantitative precision. However, problems in applying the technique exist—above all the lack, for most toxins, of analytical standards as well as of a chromophore for sensitive UV or fluorescence detection.

To overcome the latter difficulty, chemical modification of toxins is required in order to improve their detectability. A sensitive fluorometric liquid chromatography method for specific detection of YTXs has been developed. It provides for derivatization of each toxin with an auxiliary dienophile reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) for fluorescence labeling followed by HPLC analysis.[18]

The derivatization of YTX with DMEQ-TAD results in the formation of two C-42 epimers in a ratio approximately 3:1 (Figure 13.10) and, thus, in the presence of two peaks in the chromatogram of the adducts (Figure 13.11). The method is free from interference, and is also able to resolve chromatographically YTX (**1**), 45-hydroxy-YTX (**2**), and 45,46,47-trinorYTX (**3**), but does not allow separation between YTX and homoYTX (**4**). In addition, for the application of this method, the presence of a conjugated diene functionality in the side chain of YTX-like compounds is a prerequisite (Figure 13.10). Thus, the method is not reliable for detection of those derivatives lacking a conjugated diene functionality in the molecule, such as some YTXs, which often occupy a prominent place in the toxin profile of contaminated shellfish: noroxoYTX (**11**), noroxohomoYTX (**10**), adriatoxin (**6**) as well as carboxyYTX (**8**) and carboxyhomoYTX (**9**), and the desulfoderivatives (**13**, **14**). In conclusion, the current state is the lack of a derivatization method for the direct and combined determination of all YTX-like compounds. In addition, the derivatization of the YTXs reactive to DMEQ-TAD is very labor-intensive because of the multiple cleanup steps required; in addition low levels of toxins are difficult to quantify in a reliable way.

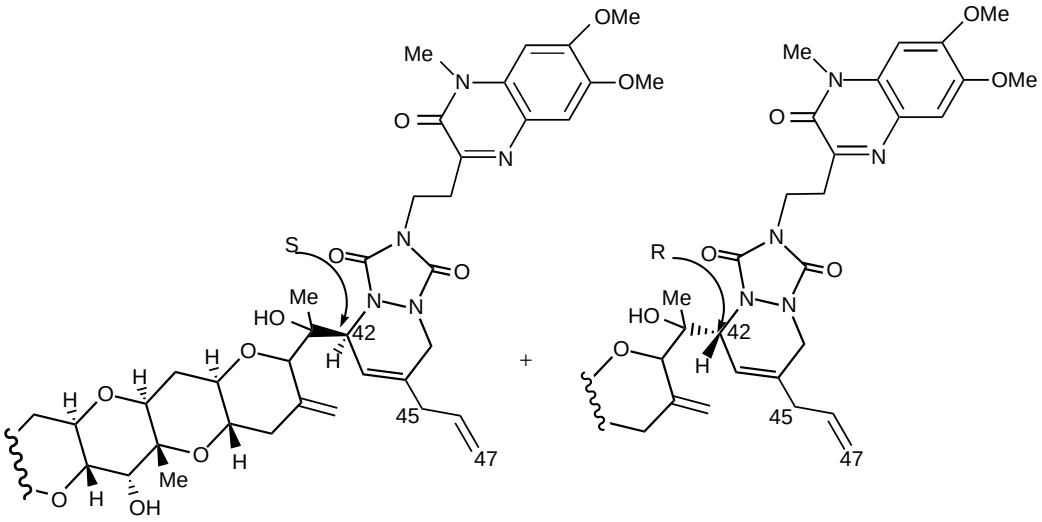


FIGURE 13.10 Structure of the two C-42 epimers from derivatization of yessotoxin with DMEQ-TAD.

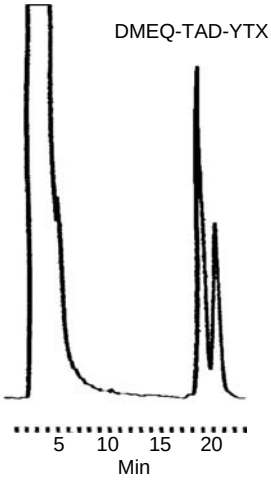


FIGURE 13.11 Chromatogram of DMEQ-TAD adducts of standard YTX.

13.6.2 Liquid Chromatography-Mass Spectrometry

The combination of liquid chromatography and mass spectrometry (LC-MS; LC-MS/MS) represents the most powerful tool for detection and quantitation of marine biotoxins. LC-MS combines the power of chromatography to separate for complex mixtures with the sensitivity and high information content of MS. It is an universal, selective, and highly sensitive tool, which provides information on the molecular formula as well as on structural details through tandem MS experiments, thus allowing not only the confirmation of toxin identities, but also the detection of new toxins. An invaluable advantage of this approach is the possibility of detecting intact, underivatized toxins and related compounds in relatively crude extracts of both shellfish and plankton samples. Therefore, LC-MS and LC-MS/MS methods are rapidly becoming the methods of choice for the determination of toxins in shellfish and phytoplankton at trace levels and are particularly suitable for studies on the toxin production by microalgae as well as on the toxin metabolism in shellfish.

The adopted ionization system plays a key role for a proper implementation of this technique, since it affects the nature, the number, and the abundance of the ions that appear in the spectrum.

Ion spray ionization (ISI), an atmospheric pressure ionization (API) method for MS,[86] was shown to be highly sensitive for polar compounds such as marine biotoxins.[87] This is a soft ionization technique that eludes the difficulties of ionization of nonvolatile and/or thermally labile analytes and gives abundant molecular-related ions with negligible fragmentation.

The most common analyzers used in combination with ISI are ion trap and single and triple quadrupoles. With these instruments, the sensitivity can be increased by using LC-MS with selective-ion monitoring (SIM) experiments, which allow the monitoring of ions of fixed *m/z* values. This technique can be usefully employed to detect molecular ions in very complex mixtures. However, it does not provide structural information or confirmatory fragment ions. In any case, information about the structure of the selected ions can be obtained on an ion trap or triple quadrupole instrument, by inducing fragmentation of the molecule. This can be achieved by using collision-induced dissociation (CID), usually by performing a tandem mass spectrometry (MS/MS), in which ions of a given *m/z* value, the precursor ions, are selected and fragmented with a collision gas.

A particularly useful MS experiment for sensitive and unambiguous determination of the analyte is the multiple reaction monitoring experiment (MRM). In this approach, precursor ions with a given *m/z* value are isolated and fragmented. Among the generated fragment ions, just a few ions with a given *m/z* value are selected and transmitted to the detector. Thus, only the most informative precursor/fragment ions transitions for each analyte can be analyzed. The experiment allows a high degree

of specificity and sensitivity, since it enables the correlation of diagnostic fragment ions to a parent ion with a specific molecular weight. This rules out the ambiguity that can be raised by the presence of molecules with the same molecular weight of the analyte and similar retention time, but different structure in a matrix.

Over the past few years, the LC-MS and LC-MS/MS techniques have been successfully applied to the quantification and identification of YTXs. Several drafts for methods have already been published, which would facilitate immediate replacement of the mouse bioassay. However, Decision 2002/225/EC (Annex)[11] requires validation of alternative methods by use of reference materials for all the individual compounds specified. So, the lack of pure reference samples of most YTXs prevents the substitution of the mouse bioassay as the routine or reference method for years to come.

The first LC-MS method for identification of YTX in shellfish was proposed in 1998 by Draisci et al.[88] The method, requiring preliminary studies by flow injection analysis (FIA)-MS and MS/MS on a pure standard of YTX, was based on the use of an ionspray interface. The most abundant molecular-related ion observed was the $[M-H]^-$ ion at *m/z* 1141 that was selected as precursor ion for MS/MS experiments. The resulting MS/MS spectra showed fragment ions similar to those produced by FAB MS/MS.

Coupling of LC and MS system was then carried out, by using an LC reversed phase microcolumn and an isocratic elution with a binary mobile phase of acetonitrile-4 mM ammonium acetate, 80:20 (v/v). The method was applied to the analysis of a mixture of YTX and OA, monitoring through selected ion monitoring (SIM) experiments the molecule-related ions at *m/z* 1141 $[M-H]^-$ and *m/z* 803 $[M-H]^-$, respectively. A MRM (Multiple Reaction Monitoring) LC-MS/MS analysis was then implemented by considering the transitions precursor/fragment ions of 1141/1061 and 803/255 for confirmatory YTX and OA analyses, respectively.[88]

In a further research an LC-MS method based on SIM experiments was proposed for the simultaneous determination of YTX, and several DSP toxins in the same chromatographic run.[89]

A more comprehensive and selective LC-MS method for the analysis of YTXs was developed in 2002,[90] on the basis of the analytical procedure adopted by Quilliam et al. for the detection of other lipophylic toxins, such as OA, DTXs, and PTXs.[91] Unlike the method developed by Quilliam et al., for DSP using a triple quadrupole MS system as the LC detector, the method was based on the coupling of HPLC to an electrospray-ion trap-mass spectrometer. Compared to a triple quadrupole mass spectrometer, the ion trap has the advantage that full-scan spectra can be recorded without any loss of sensitivity.[92] Owing to the storage function of the trap, the sensitivity can be increased by collecting ions over a long period, thus resulting in detection limits in the full-scan mode that are 1–2 orders of magnitude lower than the quadrupole systems.[93] The proposed method represented the first method that allowed the direct and univocal detection of the major YTXs isolated until then, namely, YTX (**1**), homoYTX (**4**), 45-hydroxyYTX (**2**), 45-hydroxyhomo YTX (**5**), carboxyYTX (**8**), carboxyhomoYTX (**9**), and noroxohomoYTX (**10**), together with OA, with a high degree of sensitivity and specificity. Toxins with different molecular weight were monitored by extracted ion chromatograms (XIC) of the $[M–H]^-$ ions, thus allowing their identification even if, in some cases, they were chromatographically unresolved. The identity of each peak was confirmed by MS/MS experiments, which allow to unambiguously identify all the known YTXs based not only on the loss of SO_3 from the $[M-H]^-$ ion, but also on the fragmentation of the backbone skeleton of each derivative. In fact, the transition precursor/fragment ions of $[M-H]^-/[M-H-SO_3]^-$ corresponding to the loss of a sulfate group is a common feature to all disulfated YTXs. Owing to the existence of many possible YTX analogs, some of which having the same molecular weight, the only loss of sulfate is not sufficient for structure identification; thus, further transitions were selected. The fragmentations occur by losing neutral species and fragment ions derive from the "western part" of the molecule in which a negative charge is localized by the presence of the sulfate ester.[14]

The developed technique, which allows determination of the toxins in a single chromatographic run of 25 min with a detection limit for YTX of 70 pg., seems to be appropriate for unambiguous identification of all YTXs and represented the first step toward their quantitative determination; it

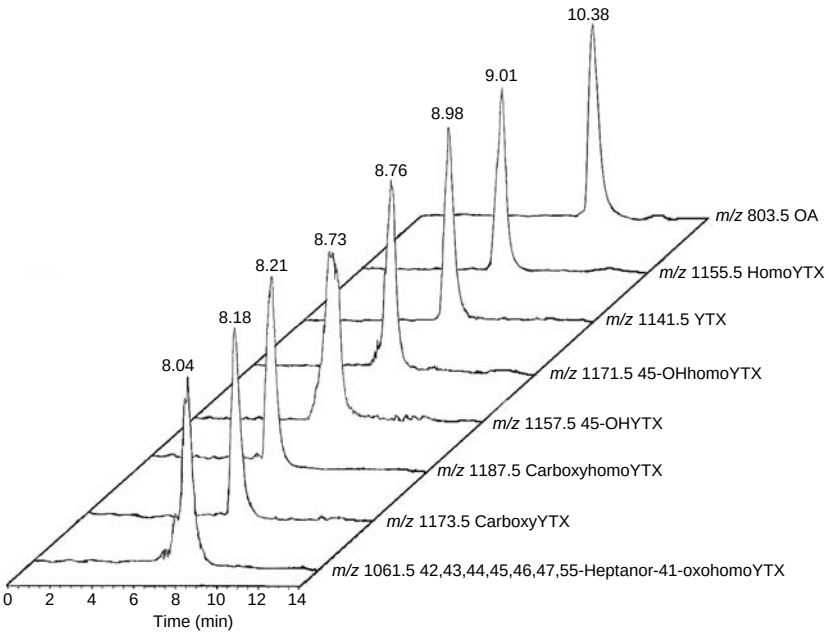


FIGURE 13.12 LC-ESI (negative ion)-ion trap-MS analysis of a wide range of yessotoxins in a blend of mussel tissue extract added of OA standard solution. Selected monitoring of yessotoxins with different molecular masses was carried out by extracted ion chromatograms (XIC) of the $[M\text{-}H]^-$ ions. (From Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, S., and Poletti, R., *J. Chromatogr. A*, 968, 61, 2002. With permission.)

has been successfully applied to the analysis of both toxic mussels and algal cultures.[90] Figure 13.12 reports the chromatogram of a toxic mussel tissue extract added to OA standard solution.

Another LC-MS method based on the use of an electrospray-ion trap-mass spectrometer was proposed by Fernandez-Amandi et al.[94] for the analysis only of YTX (**1**) and 45-hydroxyYTX (**2**). For the separation of the two toxins, a C_{16} amide column instead of the C_8 reversed phase used in the previously described method[90] was employed.

In 2003, Aasen et al.[95] reported a further method for the analysis of phycotoxins based on the LC-MS method set up by Quilliam[91]. The method, using a X-terra MS C18 column with gradient elution, was applied to the analysis of YTX (**1**), 45-hydroxyYTX (**2**), and carboxyYTX (**8**).[31,95]

A study on quantitative determination by LC-MS of some representative DSP toxins together with YTX and 45-hydroxyYTX, was performed by Goto et al.[96] In this study, shellfish tissues were spiked with ten toxins: OA, dinophysistoxin-1 (DTX1), 7-*O*-palmitoylOA, 7-*O*-palmitoyl DTX1, pectenotoxin-1 (PTX1), pectenotoxin-2 (PTX2), pectenotoxin-2 seco acid (PTX2SA), pectenotoxin-6 (PTX6), YTX, and 45-hydroxyYTX. Various columns and mobile phases were tested in order to achieve best separation and quantitative recoveries. Mass spectrometric measurements were performed with a triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) source and an electrospray ionization (ESI) interface. Selected ion monitoring (SIM) for YTX (**1**) and 45-hydroxyYTX (**2**) were performed on their $[M\text{-}2H\text{+}Na]^-$ ions at *m/z* 1163 and 1179, respectively. However, recoveries of these two toxins, in contrast with the data for the other DSP toxins tested, were very low (69–70%). The authors claimed that this was probably due to the use of strong buffers, such as phosphate buffers, in the LC-MS, which caused broadening of the peaks, thus resulting in relatively low recoveries. Detection limits in the muscle and digestive glands were 40 ng/g and 80 ng/g, respectively.

Ito and Tsukada proposed a method to correct for peak suppression owing to matrix effects.[97] The ionization efficiency of API is, in fact, greatly affected by coeluting compounds contained in a

matrix,[98–102] that may cause ion suppression of the analytes, thus resulting in poor reproducibility and accuracy. The matrix effect is especially dependent on the sample purity and degree of chromatographic separation of the analytes from the matrix component on the analytical columns. An evaluation of the matrix effect was performed in order to quantify OA, DTX1, PTX6, and YTX in scallops.[98] When LC-MS was applied to the analysis of scallop extracts, large signal suppressions were observed owing to coelution substances from the column. To correct peak suppression due to matrix effects, the standard addition method was applied. The obtained results showed that the observed large signal suppressions were due to coeluting substances from the column. The method, which is based on the addition of a known quantity of standard solutions of toxins to the extract and the collection of mass spectra with and without the standards, requires at least two LC-MS run per analysis. However, the quantitative results by this method were in good agreement with the theoretical values.

Nanoliquid chromatography and mass spectral fragmentation patterns for YTX in a high resolution orthogonal hybrid quadrupole time-of-flight mass spectrometer (QqTOF) have been recently reported.[103] In addition to the accuracy, resolution, and rapid full-scan capability of TOF technology, the system combines the superior mass selection of a quadrupole MS offering the possibility of performing MS/MS acquisitions to obtain product ion spectra with accurate mass, which is sometimes necessary to differentiate between structural isomers.

One of the advantages of using nanoLC is the low limits of detection, which allow the analysis of toxins in a small number of cells. This is due to the higher concentration of analyte in a small solvent volume, which is almost completely evaporated at the MS interface. The fragmentation of YTX was studied in negative mode using nanoelectrospray (nanoESI) QqTOF MS. The full-scan mass spectrum of YTX revealed the presence of three molecule-related ions, $[M\text{-}H]^-$, $[M\text{-}2H]^{2-}$, and $[M\text{-}2H+Na]^-$. The MS/MS spectrum was obtained by choosing $[M\text{-}2H]^{2-}$ as precursor ion (*m/z* 570). The negative CID MS/MS spectrum from this parent ion of YTX and the proposed fragmentation processes are reported in Figure 13.13. This method, whose detection limit is ca. 0.5 pg, is very sensitive and selective, and allows single cell analysis.

Very recently, Stobo et al. proposed a multiple LC-MS method that allows the detection of YTX together with most of the DSP toxins.[104] The method was set up on a triple quadrupole spectrometer. Chromatographic separation of multiple lipophilic toxins was achieved by using a base deactivated silica C8 column eluted with a 5 mM ammonium acetate-acetonitrile mobile phase under gradient conditions. The method was applied to the analysis of shellfish and was validated for the quantitative detection of OA, YTX, PTX-2, and Azaspiracid-1 (AZA-1).

Suzuki et al. report a hybrid triple quadrupole/linear ion trap MS/MS technique.[50] The triple quadrupole-linear ion trap LC/MS combines the scan speed and sensitivity of ion trap MS while retaining the selective scanning modes of triple quadrupole MS. The ability to perform both types of MS on a single instrument reduces the time required for analysis while eliminating the chance of errors due to analyzing different samples in multiple MS runs. The method, coupled with LC separation, was demonstrated to be usefully applied to the structure elucidation of YTX analogues.[45]

Till date, only one multitoxin LC-MS study has been subjected to a full single-laboratory validation and a limited interlaboratory study.[105] Tissue homogenates were extracted with 90% aqueous methanol and the centrifuged extracts cleaned up by partitioning with hexane to remove nonpolar lipids such as triglycerides, which could affect the robustness of the method through source contamination or slow elution suppressing the responses of toxins. The LC-MS method used reversed phase gradient elution with an acidic buffer, an ESI with positive and negative ion switching and a triple quadrupole MS/MS detection. MRM experiments were used for determination of lypophilic toxins as well as amnesic shellfish poisoning (ASP) toxins in a single multiresidue analysis. The method was found to be quantitative for six toxins, whose reference standards were available: azaspiracid-1 (AZA-1), domoic acid (DA), Gymnodimine (GYM), OA, PTX-2, and YTX. Relative response factors were used to estimate the concentrations of other toxins, including homoYTX (**4**), 45-hydroxyYTX (**2**), and carboxyYTX (**8**). The method validation was carried out with whole tissues from greenshell mussels (*Pe. canaliculus)*, Pacific oyster (*Crassostrea gigas)*, New Zealand

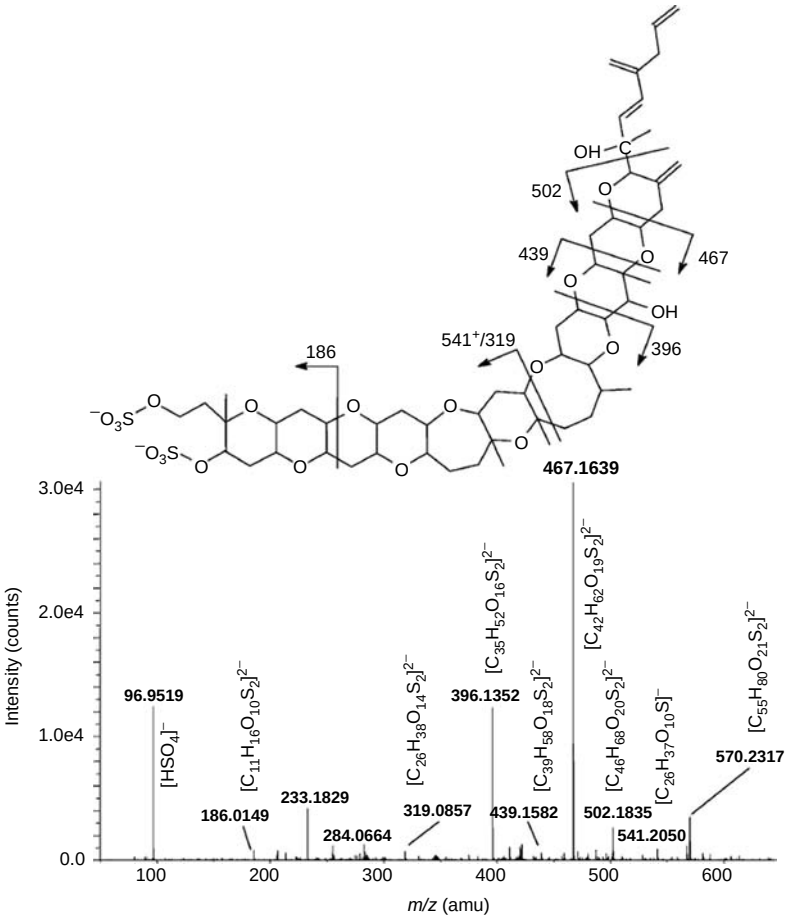


FIGURE 13.13 Negative CID/MS/MS spectrum from the $[M\text{-}2H]^{2-}$ ion, *m/z* 570, of yessotoxin and the proposed fragmentation processes. (From Cañás, I.R., Hamilton, B., Fernandez-Amandi, M., Furey, A., and James, K.J., *J. Chromatogr. A*, 1056, 253, 2004. With permission.)

cockle (*Austrovenus stutchburyi)*, and scallop *(Pecten novaezelandiae)* roe. The extensive within-laboratory validation of the method was supplemented by comparative studies on toxins extractability and stability and a small interlaboratory study. Steaming shellfish before tissue homogenization caused a twofold decrease in measured YTX, although levels of the other toxins were similar to those in fresh homogenate. Mean recoveries were very good, generally >90% for all the toxins, except for YTX where they failed below 80%.

The within-laboratory validation and interlaboratory study reported above afforded valuable data; however, further interlaboratory studies are needed to reach the acceptance of LC-MS method as official screening method.

13.6.3 Capillary Electrophoresis

Capillary electrophoresis (CE) has been recently applied as an alternative to liquid chromatographic methods for the analysis of algal toxins.[106,107] The separation in this case is based on the different mobilities of these compounds in an electric field depending on the charge and the size of the molecules. Recently, the optimization of analytical methodologies was carried out for diverse marine toxins including some polyether toxins, such as YTXs.[108] Several electrophoretic parameters have been optimized for achieving a complete separation of compounds with similar structures.

Different SPE procedures have been applied for the extraction and concentration of the toxins, and the influence of the biological matrices where they are involved has also been studied.

The method was applied for the analysis of greenshell mussels naturally contaminated with YTX. CE resulted in a promising alternative for the analysis of YTX, even though improvements in the sensitivity of the technique are still required.

13.7 CONCLUSIONS

There is no doubt as to the remarkable potential of the LC-MS technique for the analysis of biotoxins; it represents a serious alternative to the mouse bioassay or, at least, a supplementary analytical tool for tracing an extensive toxin profile of contaminated shellfish. The current rapid developments in LC-MS methodology look promising for a close attainment of these objects; however, further improvement and interlaboratory studies will be necessary. In addition, a serious problem, which does not allow, at present, this technique from becoming a generally accepted tool in regulatory analysis is the nonavailability of pure analytical standards and reference materials for most YTXs.

Another important aspect to be considered is the high capital cost of the instrumentation required for the analysis. Additional problems are associated with acquiring the expertise to run and maintain such instruments.

However, it is to be considered that the very low cost per sample for LC-MS helps lessen the economic charges. Compared to other analytical methods based on complicated cleanup and derivatization procedures, minimal sample preparation is required in the case of LC-MS. In addition, the analysis can be very rapid and can be totally automated.

In addition, together with rapid detection in several complex matrices of all the known YTXs at part-per-billion levels, the LC-MS methods allow to highlight the possible presence of coextracted known toxins belonging to other classes.

Finally, through LC-MS/MS studies, new analogs can be successfully investigated whenever great structural analogies occur between toxins under investigation and known compounds.

In conclusion, although a large number of valuable chemical methods for the analysis of YTXs have been developed, none has yet been validated. National and international organizations such as the International Union of Pure and Applied Chemistry, the Association of Official Analytical Chemists International, the European Committee for Standardization, have undertaken collaborative studies to provide standard and official methods based on some of the above screening and/or investigation procedures in the near future.

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14 Pharmacology and Mechanism of Action: Biological Detection

Amparo Alfonso and Carmen Alfonso

CONTENTS

The marine toxin yessotoxin (YTX) and its analogs are polyether compounds produced by the planktonic algae, *Proteceratium reticulatum* and *Lingulodinium polyedrum*, and originally isolated from *Patinopecten yessoensis* [1]. The presence of these toxin groups has been discovered owing to their high acute toxicity in mice after intraperitoneal (i.p.) injection of lipophilic extracts. However, much less toxicity has been reported after oral administration [2–4] and no reports about human intoxications caused by YTXs have been published. In the last 10 years, many studies have been done to know YTXs action; however, several problems appeared due to the large number of analog produced by algae, their metabolism in the shellfish that produce new analogs/metabolites, and the absence of toxin standards. In addition, the YTX pharmacokinetic parameters have not been determined. After i.p. or oral administration, some YTX effects were found in brain, heart, liver, pancreas, or thymus, which point to absorption and body distribution; however, no data about that or about metabolism and elimination are available. Moreover, the YTX amount used to report all those data is in a wide range.

14.1 MECHANISM OF ACTION

Different studies have been done to know the YTX molecular target including results after short and long incubation periods. Even though some of these studies have contradictory results, depending on concentration and toxins purity, most of them point to the same mechanism of action in different activation steps.

14.1.1 Ion Channels, Second Messengers, and Enzymes

The membrane ion channels are the molecular targets for several toxins that induce fast effects in cells. The chemical structure of YTX [5–7] resembles those of brevetoxins and ciguatoxins, with more than ten contiguous ether rings. These two groups of toxins are fast and potent activators of voltage-gated sodium channels, therefore some interaction of YTX with cellular ion channels could be expected. However, YTX did not interact with sodium channels and did not induce any competitive displacement of brevetoxins from site five of sodium channels [8].

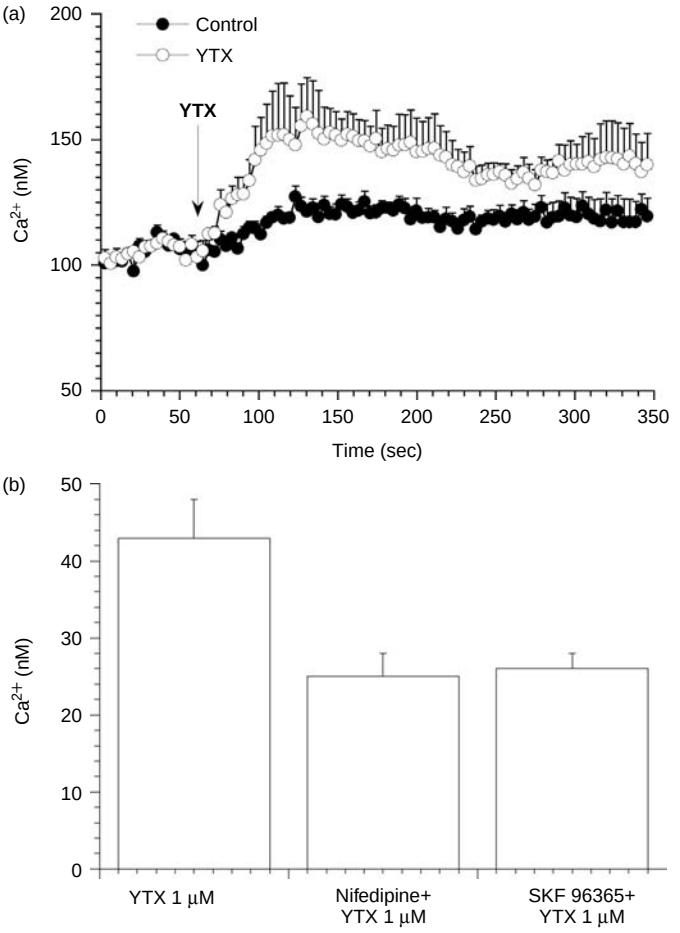

FIGURE 14.1 Effects of YTX on cytosolic calcium levels in human lymphocytes.

Cytosolic calcium and calcium channels are also early steps in cellular activation. As is shown in Figure 14.1, YTX induced a small increase (40 nM) in cytosolic calcium levels in human lymphocytes. This increase was due to the activation of calcium influx through nifedipine and SKF 96365-sensitive channels. In contrast, YTX inhibited capacitative calcium entry activated by thapsigargin or by cell preincubation in a calcium free medium [9]. Maitotoxin is a potent, water-soluble, marine phycotoxin associated with ciguatera food poisoning that induces a high and massive calcium influx in many cell types. In human lymphocytes the maitotoxin calcium influx was increased to 25%–30%, in the presence of YTX. However, since the two calcium influxes were blocked by different drugs, it seems that these toxins do not share the same calcium channel [10]. In summary, since YTX effect in calcium homeostasis was significant but small, this signal should be secondary but necessary to other toxin actions.

YTXs often coexist with diarrhetic shellfish toxins (DSP) but their effects are different. YTX did not induce diarrhea or intestinal fluid accumulation in adult mice after i.p. or oral administration [3, 4]. DSP toxins are specific and potent inhibitors of Ser/Thr protein phosphatases PP1 and PP2A. These enzymes play a critical role in phosphorylation/dephosphorylation processes within eukaryotic cells. YTX also inhibited phosphatases but with a very low potency, lower than four orders of magnitude than DSP toxins [3]. In addition, the cytotoxicity of both toxin groups was compared and YTX was less toxic, by three orders of magnitude [3]. Therefore, it was concluded that YTX mechanism of action was not mediated by these enzymes inhibition.

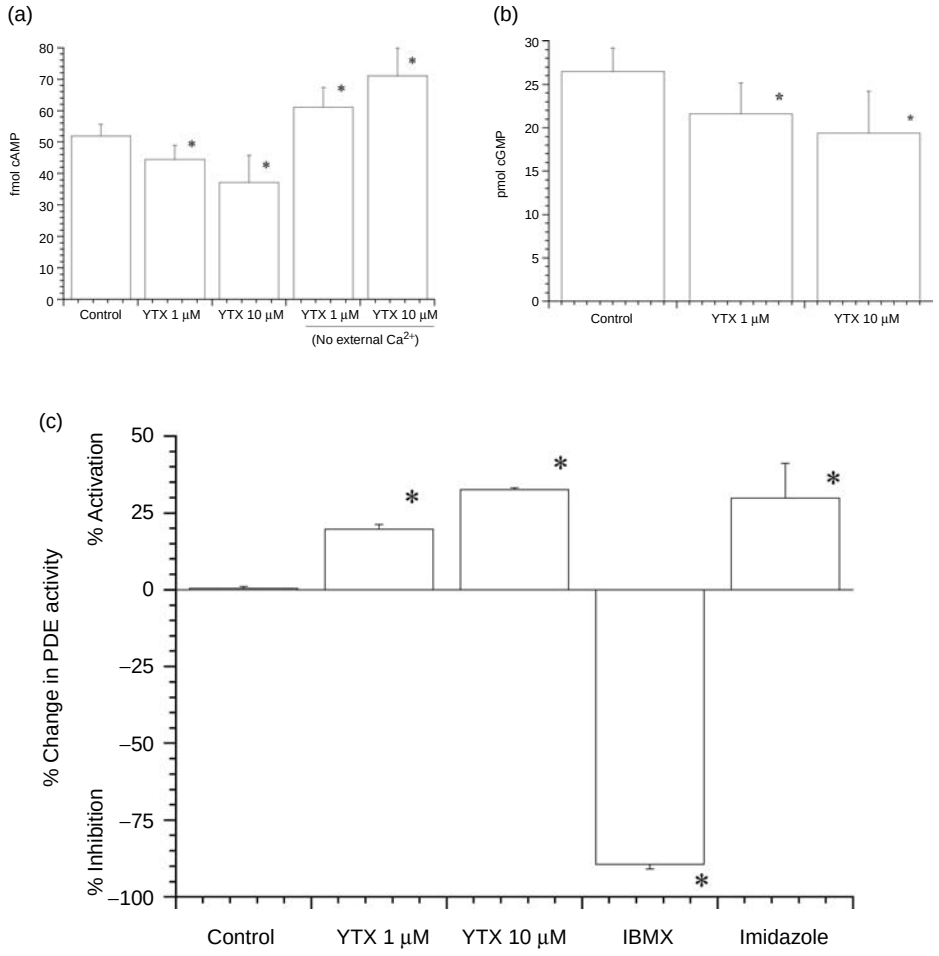

FIGURE 14.2 Effect of YTX over cAMP (a) and cGMP (b) levels, and changes in PDEs activity (c) in the presence of YTX in human lymphocytes.

The cyclic nucleotides adenosine 3′–5′cyclic monophosphate (cAMP) and guanine 3′–5′cyclic monophosphate (cGMP) are second messengers related with early activation pathways in intracellular signaling. Cells regulate cyclic nucleotide levels by a balance between adenylyl cyclases (synthesis) and phosphodiesterases (PDEs) (hydrolysis). When cAMP levels were studied in the presence of YTX, an important calcium-dependent decrease was observed (Figure 14.2a). The decrease in cAMP levels in the presence of YTX was observed even after the modulation of adenylyl cyclase. However, cAMP-YTX effect was modified when PDEs inhibitors were present [11]. From all these data and the observation of a decrease in cGMP levels (Figure 14.2b), an effect of YTX over PDEs activity was hypothesized. This effect was further studied and compared with other enzyme modulators previously described. As is shown in Figure 14.2c, YTX induced an increase in PDEs activity; this effect is dependent on the toxin concentration. The same result was observed in the presence of imidazole, a PDEs activator, whereas the contrary effect was observed in the presence of 3-isobutyl-methylxanthine (IBMX), a PDEs inhibitor. Therefore, these results point to PDEs as a cellular target for YTX and to calcium as an important key factor for YTX effect [11]. The interaction between these enzymes and YTX was probed by immobilizing PDEs in a biosensor surface. When different concentrations of toxin were added over the immobilized YTX a typical association curve indicative of interaction was observed, (Figure 14.3). In these conditions, after

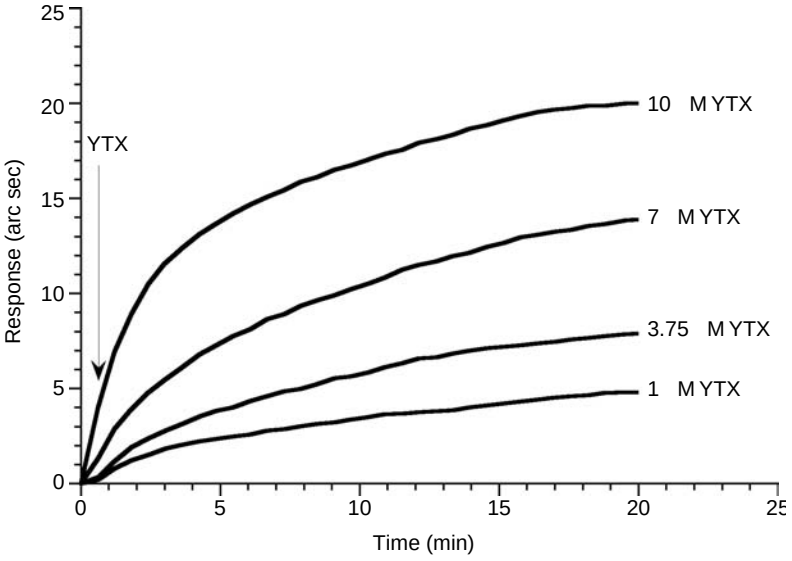

FIGURE 14.3 PDEs-YTX association curves. Typical association curves after addition of different amounts of YTX over immobilized PDEs in a resonant mirror biosensor surface.

adding different toxin concentrations, the value of the kinetic equilibrium dissociation constant K_D was calculated. The K_D value for YTX-PDEs association obtained was 3.74×10^{-6} M YTX. This value is in the range of K_D for associations between active biological species [12]. This effect was dependent on toxin structure involving C9 terminal chain, since K_D for hydroxy-YTX was three times higher than K_D for YTX and in the case of carboxy-YTX, this value was eight times higher [13]. These results point to a structure–selectivity association of YTX-PDEs, and agree with the different lethality power observed with some YTX analogs [14]. The interaction PDEs-YTX was also confirmed by measuring changes in fluorescence polarization of an enzyme–dye conjugate in the presence of YTX [15].

There are 11 different PDE families (PDE1-11) with different substrate specificity, affinity, sensitivity to inhibitors, and tissue localization. In addition, there are exonucleases such as 5′-nucleotide phosphodiesterase (PDE I) and 3′-nucleotide phosphodiesterase (PDE II) that hydrolyze phosphodiester bounds. These enzymes have several known functions in nucleic acids. By using all these enzymes in a sensor surface and by measuring changes in fluorescence polarization it was concluded that YTX binds to cyclic nucleotide PDE1, with a calcium-dependent effect, PDE3, and PDE4, and shows high affinity by exonuclease PDE I [15,16].

After 24 h incubation, YTX increased interleukin-2 production in human lymphocytes [11]. This increase in interleukin production is functionally related to the decrease in cAMP levels, since it has been described that the cellular function is inhibited by agents that increase this second messenger levels in human lymphocytes [17]. In addition, after i.p. YTX injection, the thymus and the immune system were affected and also some inflammatory response was reported [18]. On the other hand, YTX affects the mast cells' response [19], and other cells involved in the immune system. PDEs modulation is often used to regulate the activity of a number of inflammatory cell types, and several drugs used in the asthma therapy interfere within this pathway. Therefore, all these results on immune cells are very interesting and again point to PDEs as the YTX target. In this sense, it has been reported that YTX is a potent inhibitor of fungi and yeast growth comparable with anphotericin B [3]. Imidazole is also a potent inhibitor fungicide drug and, as it has been shown, with the same effect than YTX as PDEs activator.

In summary and from all these evidences, PDEs are pointed as an intracellular target for YTX.

14.1.2 Others

Since the YTX molecule has a hydrophobic polyether skeleton, some hemolytic activity could be expected. However, the toxin did not induce hemolysis [3]. Even an important hemolytic property was described to desulfated YTX by interacting with alfa-helix membrane peptides [20].

Few papers have been published about the effect of YTX in marine bivalves. The toxin is mainly localized in immunocytes and in the digestive gland of mussels [21]. It was described as an increase in mussel phagocytic immunocytes under control conditions after YTX addition but no effect was reported under stress conditions [22]. In this shellfish, YTX and analogs suffered extensive metabolism and had a half-life of 20–24 days [23]. YTX induced cell shape changes in mussel inmmunocytes. The toxin is not able to activate these cells but enhances the activating response of other activators as peptides. This effect is induced through the involvement of both extracellular calcium and cAMP [24]. Therefore, even in mammalian cells the second messengers, calcium and cAMP, are key steps for YTX effect.

14.1.3 Cell Effects (Cytoskeleton, Cell-to-Cell Adhesion and Apoptosis)

It was reported that YTX induces cell detachment from culture dishes [3]. However, after 1 h incubation no effects over F-actin levels were detected in the presence of YTX in neuroblastoma BE(2)-M17 cells [25]. The same absence of effect in F-actin was described when the toxin was studied in fresh enterocytes of rabbits after 4 h incubation [26].

On the other hand, it has been described that first damages due to YTX were induced in lysosomal vesicles, and then a progressive depolymerization of actin microfilaments happened, either in invertebrate, insect fat body IPLB-LdFB, or vertebrate, mouse fibroblasts NIH3T3 cell lines [27].

E-cadherin is a large family of proteins responsible for calcium-dependent cell-to-cell adhesion that mediates in the aggregation-dependent cell survival. A decrease in E-cadherin expression is associated with the tumor expansion in epithelial cells, but in some cases this protein plays a role in survival and apoptosis suppression of other carcinoma cells [28]. After 20 h incubation of the human breast cancer cells MCF-7 in the presence of YTX, an accumulation of a 100 kDa fragment of E-cadherin without a parallel loss of the intact protein was described [29]. The collapse of E-cadherin system happened after 2–5 days of YTX-treatment (subnanomolar range) either in MCF-7 cells or in other tumoral epithelial cellular model as Caco-2 [30]. This effect was related with toxin structure involving the C9 terminal chain. YTX was the most potent analog to induce accumulation of 100 kDa fragment of E-cadherin, then 45-hydroxyhomoyessotoxin showed some activity, and then carboxyyessotoxin [31]. An increase in the polarity of the C9 chain, rather than an increase in size, is related with the decrease in affinity of YTX by the E-cadherin. Surprisingly, as it was mentioned before, the same structure–selectivity relationship and decrease in potency depending on C9 chain was observed in the association PDEs-YTX and YTX analogs [13].

It has been recently shown that YTX did not induce any effect in E-cadherin system *in vivo* [32]. These results confirm the loss of morphological changes the toxin induced in any internal organ after several days of oral treatment, which has been described earlier. Before these last results and from E-cadherin data, YTX was proposed to facilitate tumor spreading and metastasis formation [30]; however, now affirmation of this hypothesis should be reviewed. In addition, an effect over E-cadherin levels was recently reported after azaspiracids exposition, suggesting that both toxins share their mechanism of action [33]. However, while human azaspiracids intoxications had been reported, YTX intoxications have never been described, and the data available about azaspiracid effect show a very different effect over cAMP, cytosolic calcium, intracellular pH, and cytoskeleton structure [34–39].

Apoptosis, or programmed cell death, is a complex process characterized by a variety of morphological and biochemical changes in the cells. Different processes such as changes in mitochondrial membrane potential, caspases activation, or changes in total nucleic acids content are associated to apoptosis. YTX showed different effects as apoptotic drug to several tumoral cellular lines. In the neuroblastoma cell line BE(2)-M17, YTX induced apoptotic events with lower potency

than okadaic acid. YTX induced small changes in mitochondrial membrane potential and nucleic acids content after 48 h incubation. However, the toxin increased caspase-3 activity and annexin-V binding [40]. These effects were found at concentrations between 0.1 and 1 μM. YTX activated human cervix carcinoma HeLa S_3 cells death after 48–96 h of incubation in the subnanomolar range. This effect was mediated by activation of caspases-3 and -7 and caused loss of intact poly(ADP-ribose)-polymerase [41]. Apoptotic effects of YTX were also described in myoblast L6 and DC3H1 cell lines from rat and mouse after 72 h incubation in the presence of 100 nM YTX; these effects were associated with an activation of caspase-3 and caspase-9, while DNA fragmentation was not detected [42]. Early changes in mitochondrial membrane potential and swelling of mitochondria indicating an active role of this structure were also associated to the apoptotic effect of YTX [43]. Effects after long time YTX incubation were also checked. After 72 h treatment, 100 nM YTX cleaved tensin and made it translocate to the cell center. This effect altered the shape of the cells and triggered apoptosis [44].

The permeability transition pore is a mitochondrial voltage-dependent calcium channel involved in cell death. This channel is present as a high conductance channel in the mitochondria with a controversial role in apoptosis [45]. As it was mentioned, no changes in mitochondrial membrane potential were observed after 12 h incubation in the presence of different YTX concentrations. Only a small change was induced in the presence of 1 μM YTX after 12 h treatment. After 48 h, a small effect was observed at lower YTX concentrations [40]. However, an important and immediately activated mitochondrial depolarization has been described in the hepatoma MH1C1 cells line after YTX addition, suggesting that mitochondria and the opening of permeability transition pores are targets for YTX [46]. The effect is blocked by cyclosporin A and requires the presence of a permissive level of calcium (10 μM). However, only 40 nM of calcium influx is activated by YTX (Figure 14.1), and in some cases the toxin inhibited calcium influx-increase, although at least 10 μM calcium is necessary to significantly activate these pores. In addition, cyclosporin A can modulate several intracellular structures. On the other hand, the permeability transition pores seem to play a more of role in necrosis than in apoptosis and they are opened as a consequence of apoptosis and not to induce it [45]. Therefore, the effect of YTX over mitochondria of normal (nontumoral) liver cells should be carefully discussed. This agrees with the total absence of liver effects reported after exposure of mice to different YTX analogs through oral and i.p. injection [2,14,47]. In this sense, only desulfated YTX showed some liver necrosis [4].

YTX induced marked intracytoplasmic edema in cardiac muscle cells after a 3 h i.p. injection. In contrast, desulfated YTX after 24 h injection causes severe fatty acid degeneration and intracellular necrosis in liver and pancreas but not in the heart. The conclusion was that the target organ of the toxin was the heart [4]. However, after oral administration only moderate changes in the heart were observed [2]. On the other, YTX induced morphofunctional changes in neurons, in particular in calcium binding proteins from the cytoskeleton, after 2 h i.p. injection [48].

Since controversial results about cytotoxicity were obtained with different YTX concentrations depending on the cellular model used, a cytotoxicity study in different cellular models was developed. YTX showed extremely high cytotoxicity to the human cervix carcinoma cells HeLa-229, to the human hepatocellular carcinoma cell line Hep-G2, and to the ovarian adenocarcinoma cell line A2780. As Figure 14.4 shows, YTX was a potent proliferation inhibitor after 24 h incubation to HeLa-229 cells and A2780 cells, with a ED_{50} of 4.5 nM, and 3.01 nM, respectively. When the toxin effect was checked in primary neuronal cultures, after 24 h treatment only a small cytotoxic effect was reported at concentrations between 10 and 100 nM (30% cells death). These results agree with other data [49] and point that higher concentrations and probably longer incubation times are necessary to induce a cytoxic effect in nontumoral cells.

From the initial E-cadherin results with different toxin analogs and taking the different observations about toxin effects in different cellular models, the existence of two separate receptorial systems involved in YTX effect was proposed [31]. One system was activated early in micromolar YTX concentrations and the other was activated after long incubation times within the nanomolar toxin

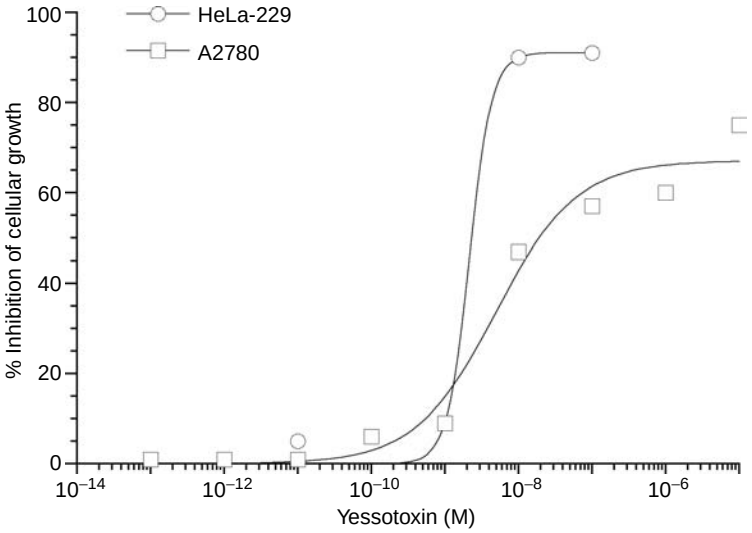

FIGURE 14.4 Effects of YTX (24 h treatment) over cellular growth in HeLa-229 and A2780 cells.

range and is related with E-cadherin. However, E-cadherin role in cellular survival is controversial and a decrease in this protein levels was observed after 3–5 days YTX-treatment, when tumoral cells start to die [30]. In addition, E-cadherin is an epithelial protein and therefore the effect of YTX only could be explained in these cells; however, the major effect observed of the toxin was in the heart. The mechanism that renders E-cadherin functional is unknown, and even its function as mediator in adhesion cells can be regulated by several signal transduction, and also the protein may regulate other process such as migration, proliferation, apoptosis, or cytotoxicity [50]. *In vitro* studies about E-cadherin expression show that the levels of this protein depend on the cellular line used, epithelioid morphology, high expression, or fibroblast-like morphology, lost expression [51]. In addition, as earlier was described no effects in E-cadherin were reported *in vivo* even after long periods of YTX treatment [32]. On the other hand, the fact that the potency is dependent not only on C9 chain observed in E-cadherin results but also in PDE-YTX binding results is probably not a coincidence and point to the same mechanism of action. Probably PDE activation, E-cadherin, and cytoskeleton effects are related in some later step to the signal transduction pathways and probably the last mechanisms are only activated in tumoral cells. The effect after PDE activation in tumoral cells can be different from the effect after PDE activation in nontumoral cells, with the same mechanism of action with different implications depending on cellular model and time. In addition, it is important to bear in mind that some cytotoxic effect after i.p. administration can be due to the combination of different lipohilic toxins. The fact that YTX coexists with DSP, pectenotoxins, and azaspiracids is important since they may show important unknown pharmacological interactions that each toxin did not show separately.

14.2 BIOLOGICAL DETECTION METHODS

Several methods have been designed to detect and/or quantify YTXs. Some of them are used to isolate and identify different YTX analogues whereas others are useful to quantify YTX concentration in shellfish or microalgae extracts. There are biological, chemical, immunological, instrumental, and functional methods. One of the most important objectives of these methods is to know if marine products are suitable for human consumption or if their YTX concentration is excessively high, in accordance with local legislation, to consume the product. In general, for these regulatory purposes the official method is the mouse bioassay (MBA), with various disadvantages such as ethical considerations, low specificity, and false results. The validation of new detection methods has been difficult

owing to different problems, such as the continuous identification of new YTX analogs with different structures, the lack of naturally isolated standards, and the presence in natural samples of multiple toxins that can interfere with the selected analytical technology [1]. For this reason, the search for new detection methods to be validated and internationally accepted is an important international research objective.

Recently, some advices of the FAO/IOC/WHO workshop on biotoxins, focusing on risk assessment, have been published [52]. YTXs are described as a group of lipophilic toxins formed by a large number of analogs, persistent in shellfish tissues and with a regulatory level in some countries of 1 mg YTX_{eq}/kg shellfish [53]. There are no data about human intoxications caused by YTXs but some studies on toxicity in animals have been done. In a short-term gavage study in mice, no toxicity of YTX at a dose of 5 mg/kg bw was observed. On the basis of this result and applying a safety factor of 100, the Expert Consultation of the FAO/IOC/WHO established a provisional acute reference dose (RfD) of 50 μg/kg bw. The maximum level of toxin in shellfish can be calculated using this RfD, a medium body weight, and the shellfish portion consumed, usually between 100 and 380 g. From these data the levels of toxins are between 30 and 8 mg/kg shellfish, far away from the regulatory level previously cited. Nevertheless, the Expert Consultation considered that there are insufficient data on the chronic effects of YTX, so no tolerable daily intake (TDI) can be established and more toxicological studies must be performed.

In general, the official YTX detection method is the MBA. Briefly, the toxins are extracted from the shellfish sample, the extract is evaporated and the residue is dissolved in 1% Tween 60 and then injected i.p. into mice. Many characteristics of the assay can vary, like the part of the shellfish extracted, the extracting solvents used, the number of injected mice, the weight of mice or the time of observation, and are usually determined by local legislation; therefore it is difficult to compare results between different countries.

In the original MBA, lipophilic toxins were extracted from HP of shellfish samples with acetone, the solvent was evaporated, the residue was suspended in 1% Tween 60 and this suspension was injected i.p. into mice [54]. This assay can detect various lipophilic toxins (okadaic acid, OA; dinophysistoxin, DTXs; pectenotoxins (PTXs), YTXs, etc) and produce false positive results owing to the presence in the extract of paralytic shellfish poisoning (PSP) toxins and high concentrations of free fatty acids.

To avoid interferences, some modifications in the extraction procedure have been proposed. The residue from the acetone extract can be dissolved with water and extracted with diethyl ether. Then the watery layer is discarded and the ether solution is collected. The solvent is removed and the residue suspended in 1% Tween 60 and injected i.p. into mice [55]. This modification eliminated interferences due to polar molecules, such as PSP toxins, Amnestic shellfish poisoning (ASP) toxins, or salts, which are dissolved in the water. However, it can produce false negatives owing to the low solubility of YTX in diethyl ether [56,57], so the substitution of this solvent with ethyl acetate or dichloromethane has been proposed. Since the first one can generate emulsions when it is partitioned with water, dichloromethane was selected, although its high toxicity and chlorinated nature can likely be a problem for the safety of laboratories with many samples to analyze.

To avoid interferences from free fatty acids, the residue from the acetone extract can be dissolved in 80% methanol and partitioned with hexane. The hexane is discarded with the free fatty acids, and the methanol is evaporated; the residue is dissolved in 1% Tween 60 and injected i.p. into mice. The problems in this case are PSP interferences, since they are not eliminated [58].

The extraction procedures previously described are done with the shellfish digestive glands but there are also extraction methods using the whole flesh. In one of them shellfish samples are extracted with acetone and methanol, extracts are mixed, solvents are evaporated, and the residue is dissolved in water. This solution is partitioned with dichloromethane, the watery layer is discarded, and the organic phase is evaporated. This residue is suspended in 1% Tween 60 and injected i.p. into mice [59]. In a modification of this method, the residue from the acetone–methanol extracts is dissolved in dichloromethane and 60% methanol. The two phases are separated and the methanol layer is selected and evaporated; the residue is suspended in 1% Tween 60 and injected i.p. into mice [59].

In this case, YTXs are separated from the other lipophilic toxins with the partition dichloromethane-60% methanol, owing to their different polarities.

Moreover, other authors propose the extraction of digestive glands or whole flesh with methanol, without the previous extraction with acetone [60].

In addition to the different extraction processes, there are also particular characteristics of the injection procedure depending on countries. The European Union (EU) regulations specified that three animals should be used in each assay and results are positive if two of the three mice die in less than 24 h after the injection of the extract obtained from 5 g of digestive glands or 25 g of whole flesh [61]. The initial use of two mice has been proposed, with two possible criteria of analysis. With the first one, result will be positive if the two mice die and will be negative if the two mice survive; if one mouse is alive and the other one is dead at the end of the test, the third mouse must be used. With the second criterion, a positive result is obtained when one of the two mice dies; this guarantees the safety of the shellfish products and reduces the number of test animals [62]. The mice weight is another interesting point; although an initial draft of EU-wide harmonized protocol suggested that only mice with 20 ± 1 g body weights could be used, some studies reveal no problems in using mice weighing 16–23 g [60].

One important characteristic of the MBA is the lack of specificity, since observed effects on mice can be produced by several lipophilic toxins: OA, DTXs, PTXs, azaspiracids, YTXs, cyclic imines (gymnodimine, spirolides), and so on [62]. Although different symptomatology has been associated with some groups of these toxins [59], it seems difficult to distinguish between all of them with the MBA. This lack of specificity becomes an advantage when the public protection is being sought, since the assay will detect new unknown lipophilic toxins that can represent a hazard for public health.

Although MBA is the assay prescribed in the legislation, it has not been validated internationally. There are many available protocols and even when using the same one different results between laboratories can appear because of factors like the strain or sex of the mice used. The ethical concerns due to the use of large numbers of mice and the lack of specificity recommend the replacement of the MBA by another kind of assay, retaining this biological test only to screen for new toxins [62].

An immunoassay (ELISA) has been designed for the quantification of YTXs [63]. This assay detects the toxins in extracts from shellfish, algal cells, and culture supernatants, using polyclonal antibodies with broad specificity for YTXs. This method has been used in Norway, analyzing different algal cultures and single cells [64]. The results obtained have been compared with chromatographic analyses, showing a high correlation and the detection of several YTX analogues that had not been previously characterized [65]. This is a rapid and sensitive analytical method that can be used in monitoring programs due to the possibility of analyzing several samples in very short times. It shows minimal matrix effects and can detect broad ranges of YTX analogues. Its disadvantages are the use of antibodies that must be synthesized and the inability to quantify each analogue of YTX specifically. In addition, the cross-reactivity studied indicates that the binding of some toxin analogues by antibodies is not accurate and it depends on YTX molecule [63].

There are also functional assays to detect YTX. These assays are based on the binding of the toxin to a molecular component or a cell that selectively recognizes it. This interaction can be quantified or it produces some events, such as cytotoxicity, protein expression, and so forth, that are measured, and from those data the toxin amount in a sample is determined [66]. In this sense, it has been described that a cytotoxicity assay uses fresh rat hepatocytes and light and scanning electron microscopy to observe morphological changes in the cells when they are exposed to toxins. In this assay, differences between OA, DTX1, PTX1, and YTX can be observed [67]. Results show that YTX does not produce changes in the shape of the cells but causes the appearance of round blebs on their surfaces, larger when YTX concentration in the medium increases. As each toxin has its own effect, this method allows YTX detection; however, it can produce confusing results when mixtures of toxins are analyzed and it is time-consuming due to the maintenance of cellular cultures.

Another cytotoxicity study explains the effect of YTX in cultured HeLa cells, by showing cellular death and activation of various caspase isoforms in the presence of the toxin [41]. YTX induced

these cytotoxic effects at lower doses and after longer duration than OA and the activated caspase isoforms were toxin specific. These effects were used to detect YTX in shellfish samples.

The treatment of MCF-7 cells with YTX produces the accumulation of a 100-kDa fragment of E-cadherin; this effect was used to quantify the YTX concentration in a sample [29,31]. This functional assay seems to be specific, sensitive, and robust, and no matrix effects were found. However, it needs the culture of MCF-7 cells, their treatment with problem samples, the preparation of cell extracts, and the final analysis to obtain the concentration of toxin; therefore the time needed to carry out the assay is 2–3 days. Owing to this detriment, it has been designed as a slot blot procedure [68] that simplifies the analytical part of the functional assay from the 2 days in the original method to only 7 h, maintaining the same limit of detection but obtaining larger standard deviations in YTX measurements. However, recently the effect over E-cadehrin was also described in the presence of the lipophilic toxin azaspiracid-1; therefore this method is not YTX-selective if that toxin is present in the sample [33].

The capacity of YTX to activate cellular PDEs [11] is used in three different functional detection methods. In the first one, PDEs activity over a fluorescent derivative of cAMP is measured, showing higher activity the samples with high YTX concentrations; this can be used to quantify the toxin with a high degree of accuracy [69]. The other two methods are based on the study of the interaction between PDEs and YTX, using two different techniques. In one of them [12], a resonant mirror biosensor is used to quantify the binding between the PDEs attached to an aminosilane surface and the YTXs added in a sample (Figure 14.3) by detecting changes in the refractive index near the sensor surface where the proteins are attached. This method can detect the binding between different PDEs [16] and various YTX analogues [13], and it can be used to quantify toxin concentrations in shellfish samples. The last method [15] uses the fluorescence polarization technique to detect the interaction, due to the change in the movement of fluorescently labeled PDEs when they bind to YTXs. This method has been applied successfully to the quantification of YTX concentration in shellfish extracts obtained through various extraction procedures [70], showing that different YTX analogs can also be quantified. This is a specific, economical, and easy detection method with a high degree of repeatability and accuracy, less expensive than LC-MS, and that avoids ethical problems.

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15 Toxicology of the Yessotoxins

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CONTENTS

15.1 INTRODUCTION

Yessotoxin, a disulfated polyether, was first reported as a contaminant of the scallop, *Patinopecten yessoensis*, in Japan in 1987 [1]. Since then, yessotoxin has been identified in shellfish from Norway [2], Chile [3], New Zealand [3], the United Kingdom [4], Canada [5], Russia [6], and Italy [7]. The levels in shellfish may be high, with concentrations up to several mg/kg edible tissue being reported [8–11]. Yessotoxin in shellfish results from ingestion of certain algae. *Protoceratium reticulatum* was the first species found to produce this substance [12], although more recent studies have shown that yessotoxin is also produced by the closely related species *Lingulodinium polyedrum* [13,14] and *Gonyaulax spinifera* [15]. Algae producing yessotoxin have been found not only in countries in which contamination of shellfish with this material has been reported, but also in organisms from Spain and the United States [14].

In addition to the parent compound, the above algae produce many yessotoxin analogues [16–26]. These same analogues have been detected in shellfish following ingestion of the algae [3,13,27–34], although there is evidence that hydroxylated and carboxylated derivatives largely result from metabolism of yessotoxin in the shellfish after ingestion [3,9,11,35,36].

Although no human intoxication is known to be caused by consumption of shellfish contaminated with yessotoxin or its analogues, because of the widespread occurrence of these compounds and their high levels in shellfish, their toxicity both *in vitro* and *in vivo* has been extensively studied.

15.2 TOXIC EFFECTS OF YESSOTOXIN AND DERIVATIVES *IN VITRO*

At nanomolar to micromolar concentrations, yessotoxin has been shown to be toxic to many mammalian cells in culture, including a BE(2)-M17 neuroblastoma cell line [37], a human neuroblastoma cell line [38], HeLa S_3 cells [39], rat L6 and mouse BC3H1 skeletal muscle myoblast cell lines [40], P388 mouse leukemia cells [41], 3T3 mouse fibroblasts [42], rat hepatocytes [43], and isolated cerebellar neurons [44]. Yessotoxin did not cause significant mortality in MCF breast cancer cells at nanomolar concentrations, although growth was inhibited [45]. Toxicity to an insect cell line (IPLB-LdFB), derived from a lepidopteran larval fat body, has also been demonstrated [42].

Little information is available on the structural changes in cells undergoing yessotoxin-induced damage. In hepatocytes, yessotoxin induced membranal alterations, as indicated by the formation of blebs on the cell surface. The severity of blebbing was dose dependent, but no gross membranal damage occurred, since Trypan Blue was not taken up by the cells, and there was no perceptible leakage of lactate dehydrogenase [43]. Bleb formation was also recorded in myoblasts exposed to low levels of yessotoxin [40]. The nuclei of the latter cells exhibited shrinkage, while condensed chromatin was observed in L6 cells, but not in BC3H1 cells [40].

In neuroblastoma cell lines [37,38], HeLa S3 cells [39], and myoblasts [40,46] yessotoxin-induced cell death occurred through apoptosis, associated with activation of caspases. In the case of myoblasts, formation of apoptotic bodies has also been observed [40], and apoptosis may occur through activation of the mitochondrial pathway [46]. In contrast, apoptosis was not the primary cause of death in IPLB-LdFB and 3T3 cells exposed to yessotoxin, and it was suggested that lysosomal damage was the trigger for cell death in these cells [42].

Yessotoxin modulates transmembranal calcium transport *in vitro*. In cultured cerebellar neurons, cytosolic calcium levels were increased in the presence of yessotoxin [44]. This change in intracellular calcium was prevented by nifedipine and verapamil, which are antagonists of voltage-gated calcium channels [44]. Yessotoxin also increased intracellular calcium levels in human lymphocytes, and both nifedipine and SKF 96365, an agonist for receptor-gated calcium channels, inhibited calcium uptake [47]. Yessotoxin also potentiated the calcium uptake induced in lymphocytes exposed to maitotoxin, although in this case, the effect was insensitive to SKF 96365 [48]. In the presence of extracellular calcium, but not in its absence, yessotoxin decreased cellular levels of adenosine 3′,5′-cyclic monophosphate, owing to activation of phosphodiesterases [38]. In isolated mitochondria, yessotoxin opened the permeability transition pore, a voltage-dependent calcium channel, at concentrations between 10^{-6} and 10^{-7} M. Again, this effect required the presence of calcium in the incubation medium [49]. In the presence of the chemotactic tripeptide *N*-formyl-Meth-Leu-Phe, yessotoxin increased the motility of mussel immunocytes, an effect that was again inhibited by verapamil [50].

The interaction of yessotoxin with purified phosphodiesterases has been studied in detail [51]. Activation of phosphodiesterases by yessotoxin involves binding of the toxin to the enzyme [52]. The affinity of 45-hydroxyyessotoxin for the enzyme was found to be lower than that of the parent compound, while that of carboxyyessotoxin was lower still [53]. Different phosphodiesterases were shown to exhibit different affinities toward yessotoxin [54].

As in isolated mitochondria, the permeability transition pore was opened in mitochondria in Morris hepatoma 1C1 cells, leading to depolarization [49]. Adverse effects on mitochondrial metabolism by yessotoxin appear unlikely, however since this substance, even at micromolar concentrations, did not inhibit formazan formation from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium in rat glioma C6 cells [55], a process that requires the presence of functional mitochondrial reductases [56]. Yessotoxin did not cause membranal depolarization in the BE(2)-M17 human neuroblastoma cell line [57].

At micromolar concentrations, yessotoxin and di-desulfoyessotoxin caused dissociation of glycopholin A, a membranal protein in human erythrocytes, and increased the degree of lysis induced by alamethacin and melittin in these cells [58]. By itself, however, similar concentrations of yessotoxin caused no lysis of mouse erythrocytes [55].

In MCF-7 breast cancer cells, yessotoxin caused the accumulation of an E-cadherin fragment [8]. This effect was seen with extremely low levels of the toxin, the EC_{50} being 0.55 nM. 1a-Homoyessotoxin was shown to be of similar activity, while 45-hydroxyyessotoxin, carboxyyessotoxin, and noroxoyessotoxin were less effective [59]. In a later study [45], E-cadherin disruption was shown to occur not only in MCF-7 cells but also in Caco-2 cells from colorectal carcinoma and Madin Darby canine kidney cells. The effect was, however, specific for E-cadherin, since no fragments derived from N- or K-cadherin were found in cells exposed to yessotoxin [45].

Yessotoxin did not disrupt the cytoskeleton in BE(2)-M17 neuroblastoma cells [60], but disruption was seen in cultured cerebellar neurons and in 3T3 cells after prolonged incubation with the

toxin [42, 44]. In another study with L6 and BC3H1 cells, yessotoxin, at a concentration of 100 nM, caused marked disruption of the F-actin cytoskeleton and cleavage of tensin after incubation for 48 h and 72 h, respectively [61]. Since tensin has a role in the maintenance of cell morphology, such changes may explain the effects of yessotoxin on the shape of cells following exposure *in vitro* [61].

Yessotoxin inhibited purified protein phosphatase 2A with an EC_{50} of 0.36 mg/mL. This activity is lower than that of the "classical" protein phosphatase inhibitors (okadaic acid, dinophysistoxins) by four orders of magnitude [55]. Unlike brevetoxins and ciguatoxins, yessotoxin and di-desulfoyessotoxin do not bind to the voltage-gated sodium channel of synaptosomal membranes from rat brain [62].

In preliminary experiments on the effect of yessotoxin on extracellular signal-regulated kinase (ERK), no increase in the levels of either total or phosphorylated ERK1 and ERK2 isoforms in cultured cells were recorded at nanomolar concentrations of the test compound [63].

Yessotoxin and di-desulfoyessotoxin inhibited the growth of *Aspergillus niger*, *Penicillium funiculosum*, and *Candida rugosa*, but neither substance caused growth inhibition in a range of gram-positive and gram-negative bacteria [55]. The di-desulfo derivative was more active against *A. niger* than the parent compound [58,64].

15.3 TOXIC EFFECTS OF YESSOTOXIN AND DERIVATIVES *IN VIVO*

15.3.1 Acute Toxicity to Mice by Intraperitoneal Injection

Results of experiments on the acute toxicity of yessotoxin and derivatives by intraperitoneal injection in mice are summarized in Table 15.1.

Estimates of the acute toxicity of the parent compound vary widely, with estimates of the LD_{50} ranging from 80–100 [55] to 500–750 μg/kg [65]. It has been suggested that such disparities could reflect the use of mice of different strains or sexes, and in order to examine these possibilities, a study of the intraperitoneal toxicity of yessotoxin was conducted as an open, randomized parallel group trial with a four-level response surface design [66]. Three mouse strains (ICR, Swiss albino, and NMRI) of both sexes were employed, with nine mice being used at each dose level. The first dosing level was 400 μg/kg. If >50% mortality was recorded, the next group of mice was dosed at 200 μg/kg. Otherwise, they received a dose of 600 μg/kg. At the third level, the mice would receive a dose either increased or decreased by 100 μg/kg. At the fourth and final level, the doses of the test compound were increased or decreased by 50 μg/kg. By regression analysis, the results indicated relatively small differences in LD_{50} among the three mouse strains, although in all strains, female mice were more susceptible to the toxic effects of yessotoxin than males (Table 15.1).

With the differences in reported LD_{50} for the parent compound, comparison with published acute toxicities of yessotoxin derivatives is difficult. Furthermore, toxicities have been quoted as "lethal dose" in many instances, rather than a properly determined LD_{50}. From the data in Table 15.1, it would appear that 1a-homoyessotoxin is as toxic as yessotoxin itself, while the 45-hydroxy, 45-hydroxy-1a-homo, 55-carboxy, 55-carboxy-1a-homo, and desulfo derivatives are less toxic than the parent compound. The 1,3-enone isomer of heptanor-41-oxoyessotoxin and the trihydroxylated amide of 9-methyl-41a-homoyessotoxin are certainly much less toxic than yessotoxin, with no deaths being recorded at a dose of 5000 μg/kg. The acute intraperitoneal toxicity of adriatoxin has been reported to be slightly lower than that of the parent compound [31], although no quantitative data were given by these authors.

Yessotoxin is a comparatively slow-acting poison. While deaths may occur within 30–50 min after injection of very high doses [55,67], at dose levels close to the LD_{50}, animals may survive 10 h or more before succumbing [68]. Restlessness, shivering, and cramps have been reported in mice given lethal doses of yessotoxin [65,67]. Labored breathing and cyanosis preceded death [68].

TABLE 15.1
Acute Toxicity of Yessotoxin and Derivatives by Intraperitoneal Injection in Mice

Compound	Mouse Strain	Mouse Sex	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
Yessotoxin	ddY	Male	LD_{50}	Between 80 and 100	55
Yessotoxin	NMRI	Female	LD_{50}	Between 500 and 750	65
Yessotoxin	NMRI	Female	LD_{50}	313	66
Yessotoxin	NMRI	Male	LD_{50}	454	66
Yessotoxin	ICR	Female	LD_{50}	405	66
Yessotoxin	ICR	Male	LD_{50}	475	66
Yessotoxin	Swiss albino	Female	LD_{50}	296	66
Yessotoxin	Swiss albino	Male	LD_{50}	334	66
Yessotoxin	CD	Female	LD_{50}	512 (312–618)*	67
Yessotoxin	ICR	Male	LD_{50}	300**	69
Yessotoxin	Swiss albino	Female	LD_{50}	112 (96–131)*	68
Yessotoxin	C57 Black	Female	LD_{50}	136 (112–166)*	68
1a-HomoYTX	CD	Female	LD_{50}	444 (315–830)*	67
1a-HomoYTX	?	?	Lethal dose	100	32
45-HydroxyYTX	?	?	Lethal dose	~500	32
45-Hydroxy-1a-homoYTX	CD	Female	Lethal dose	No deaths at 750	67
55-CarboxyYTX	?	?	Lethal dose	~500	34
55-Carboxy-1a-homoYTX	?	?	Lethal dose	~500	30
45,46,47-TrinorYTX	?	?	Lethal dose	~220	33
Di-desulfoYTX	ICR	Male	LD_{50}	301	69
1-DesulfoYTX	?	?	Lethal dose	~500	87
1,3-Enone isomer of heptanor-41-oxoYTX	Swiss albino	Female	Lethal dose	No deaths at 5000	17
Trihydroxylated amide of 9-methyl-41a-homoYTX	Swiss albino	Female	Lethal dose	No deaths at 5000	18

* Figures in brackets indicate 95% confidence limits.

** This figure is the LD_{50} at 3 h after dosing. Since deaths from yessotoxin intoxication occur at times greater than 3 h, the true LD_{50} in this experiment is likely to lower than that indicated.

No macroscopic changes were observed in mice dying after intraperitoneal injection of lethal doses of yessotoxin [67]. In contrast, the livers of mice receiving di-desulfoyessotoxin became swollen and yellow in color within hours of administration of toxic doses [69]. Consistent with the color change, an increase in hepatic fat levels was recorded in animals dosed with di-desulfoyessotoxin [69]. There was also evidence of lipid peroxidation, as reflected by a doubling of thiobarbituric acid-positive material, in the livers of these mice [69].

There was no evidence of apoptosis in the hearts of mice injected with toxic doses of yessotoxin, and no signs of myo- or hepato-toxicity, as indicated by normal plasma activities of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and creatine kinase [67].

By light microscopy, four out of five mice that died after intraperitoneal injection of yessotoxin showed vacuolation in cardiac muscle, and intracellular edema was recorded in the hearts of animals receiving high doses of the toxin. Similar changes were seen in one control mouse [65]. In another experiment, however, no changes were seen by light microscopy in the heart following lethal doses of yessotoxin [67].

In two studies, no effects were observed in the lungs, thymus, liver, pancreas, kidneys, adrenals, uterus, ovaries, skeletal muscle, brain, spinal cord, spleen, stomach, jejunum, colon, or rectum

of mice receiving lethal doses of yessotoxin [65,67]. In a later experiment, however, effects of yessotoxin on the brain, duodenum, and thymus were recorded [70,71]. While no effects on the large neurons of the cerebral or cerebellar cortex were observed [70], damage to Purkinje fibers in the cerebellum of mice was recorded after a lethal dose of yessotoxin [71]. The cells were shrunken and hyperchromic, and their distribution was disrupted. Fusion of Nissl bodies was observed, with chromatin condensation [71]. In mice given either a lethal (420 μg/kg) or a sublethal (10 μg/kg) dose of yessotoxin, there was evidence of an inflammatory response in the duodenum, while numbers of thymocytes in the thymic cortex were decreased. Apoptosis, associated with increased mitotic activity, was recorded, and necrosis of medullary epithelial cells was observed. The severity of the structural changes in the thymus was more severe at the sublethal dose. From these results, it was concluded that the thymus is the primary target organ of injected yessotoxin [70].

By electron microscopy, ultrastructural changes in the hearts of mice injected with yessotoxin have been observed. Swelling and degeneration of endothelial cells in the left ventricle have been described, with cells in the vicinity of capillaries becoming hypochromic. Myofibrils and mitochondria became separated, and the latter organelles assumed a rounded appearance [65,69]. No ultrastructural changes were seen in the liver, pancreas, lungs, adrenals, kidneys, spleen, or thymus [69]. Effects of di-desulfoyessotoxin on the heart were minimal, with only a slight deposition of fat in cardiac muscle being observed. Unlike yessotoxin, however, the desulfo derivative caused severe fatty degeneration of the liver, with swollen mitochondria, and increased phagocytic activity. Degeneration of pancreatic acinar cells was also observed, again associated with fat deposition, although no changes were seen in other pancreatic cells or in the intestines, lungs, adrenals, kidneys, spleen, or thymus [69].

15.3.2 Acute Toxicity to Mice by Oral Administration

Yessotoxin is much less toxic to mice after oral administration than after injection. No deaths were recorded in mice given yessotoxin by gavage at 1 [55], 2 [67], 10 [65], or 50 mg/kg [68]. No deaths or signs of toxicity were seen in mice dosed orally with 1a-homoyessotoxin or 45-hydroxyyessotoxin at 1 mg/kg [67]. In a pharmacokinetic experiment, known amounts of yessotoxin were administered orally to mice. Urine samples were collected at hourly intervals. The mice were killed after 6 h, and tissues were harvested. Only trace amounts of yessotoxin were found in blood, urine, and tissues. Most was recovered from the lower intestine and faeces. The difference in toxicity between injected yessotoxin and that given orally may therefore simply reflect a lack of absorption from the gastrointestinal tract [68].

Some structural changes have, however, been reported in mice dosed orally with yessotoxin. By light microscopy, slight intracellular edema was recorded in the hearts of mice given oral doses of between 2.5 and 10 mg/kg of yessotoxin [65]. Terao et al. [69] saw no ultrastructural changes in the hearts of mice given 0.5 mg/kg of yessotoxin orally. At higher doses, however, similar alterations were observed in orally dosed mice as in those injected with yessotoxin [65,67]. Cytoplasmic protrusions of cardiac muscle cells into the pericapillary space and rounded mitochondria were recorded. Damage to myofibrils and dilatation of intercalated discs were also seen, although such changes did not show a dose–response relationship [67]. Similar ultrastructural changes in the heart were observed in mice receiving 1a-homoyessotoxin or 45-hydroxyyessotoxin at 1 mg/kg [67]. Although yessotoxin by injection has been reported to cause thymic damage, oral administration of this substance did not exacerbate the toxicity of okadaic acid to the thymus [72].

Acute oral administration of yessotoxin to adult [73] or neonatal [1,55] mice did not cause diarrhea.

15.3.3 Effect of Yessotoxin on E-cadherin *In Vivo*

Effects of yessotoxin on the E-cadherin system in the lung, kidney, and colon of mice dosed orally with yessotoxin at 1 mg/kg/day for 7 days have been investigated [74]. Both intact E-cadherin and

some proteolytic fragments of this protein could be obtained from these tissues. In animals killed at the end of the dosing period, no differences between control mice and those receiving yessotoxin were seen in lung and kidney, but yessotoxin decreased the levels of fragmented E-cadherin in extracts of colon. No differences in the E-cadherin pool were observed in any of the tissues of mice analyzed 30 or 90 days after cessation of yessotoxin dosing. The stabilizing effect of yessotoxin administered by gavage on the E-cadherin pool of the mouse colon was selective and transient, indicating that some yessotoxin administered through the oral route can access certain tissues, triggering organ-selective responses, and that, at least in the case of the effects exerted on E-cadherin, the changes induced by the toxin are reversible *in vivo*.

The apparent contradiction between these results *in vivo* and previous findings *in vitro* [45] is fully reconciled by the recent demonstration that yessotoxin interferes with E-cadherin disposal in sensitive cells *in vitro*, leading to accumulation of an intermediate degradation product, rather than causing E-cadherin fragmentation [75].

15.3.4 Chronic Toxicity of Yessotoxin

No deaths or signs of toxicity were recorded in mice dosed with yessotoxin at 2 mg/kg/day or 1a-homoyessotoxin or 45-hydroxyyessotoxin at 1 mg/kg/day by gavage for 7 days. Food consumption and growth rates were normal, and no changes in plasma enzyme activities were recorded. No histological changes were observed in the liver, heart, lungs, kidneys, spleen, intestine, thymus, uterus, ovaries, skeletal muscle, brain, or spinal cord, and there was no evidence for apoptosis in the heart [73]. In a recent study [76], mice were dosed seven times by gavage with yessotoxin at 1, 2.5, or 5 mg/kg over a 21-day period, and killed 3 days after the final dose. No pathological changes were recorded by light microscopy in the heart, lungs, kidneys, small intestine, spleen, thymus, brain, pancreas testes, or adrenals, and no alterations in the heart were visible by electron microscopy.

15.3.5 Toxicity of Yessotoxin to Fish and Shellfish

Yessotoxin is of comparatively low toxicity to killifish, with no deaths being recorded at a concentration of 840 nM in the aquarium water. The di-desulfo derivative was more toxic, however, causing death at 510 nM [55]. Microgram quantities of yessotoxin killed scallops when injected into the adductor muscle [35].

15.4 EVALUATION AND RECOMMENDATIONS FOR FUTURE STUDIES

Yessotoxin is exceptionally toxic to cells *in vitro*, although the mechanism of the cytotoxic action of this substance is presently unclear. Cell death does not involve the excitotoxic activation of ionotropic glutamate receptors [44], or voltage-gated sodium channels [44,62]. While effects on calcium mobilization have been demonstrated in several cell types exposed to yessotoxin, changes in intracellular calcium concentrations, at least in the case of cerebellar neurons, are not responsible for cytotoxicity, since prevention of calcium ingress into the cells failed to prevent cell death [44]. Activation of phosphodiesterase has been suggested as the intracellular target of yessotoxin [53], although the sequence of events leading from activation of the phosphodiesterases to cell death has not been established, and the effective yessotoxin concentrations in the two responses often differ by 3–4 orders of magnitude [77].

Little information on structure–activity relationships in the cytotoxicity of yessotoxin and derivatives is available, although such information would be valuable with regard to mechanisms of *in vitro* cytotoxicity. For example, the possible role of phosphodiesterase activation in the cytotoxicity of yessotoxin could be investigated by comparing the cytotoxicity of 45-hydroxyyessotoxin and

carboxyyessotoxin with that of the parent compound, since the former compounds are much weaker activators of the phosphodiesterases [53]. Evaluation of the cytotoxicity of these analogs, together with that of 1a-homoyessotoxin and noroxoyessotoxin, would also be of interest in determining the possible role of effects on E-cadherin in promoting cell death *in vitro*, since wide variations in activity were seen with these derivatives [59].

There is evidence for death through apoptosis in several cell types treated with yessotoxin *in vitro*, although the signaling systems responsible for the initiation of apoptosis have not been fully characterized.

The relationship between effects of yessotoxin *in vitro* and toxicity *in vivo* is unclear, and none of the reported effects on isolated cells shed light on the mechanism by which yessotoxin causes the death of experimental animals. It is important, however, that information obtained *in vitro* is extended to the *in vivo* situation. In particular, the recent study of the effects of yessotoxin on E-cadherin disruption *in vivo* is of considerable importance. *In vivo*, such disruption, leading to loss of cell adhesion, could promote tumor metastasis [45], but the fact that yessotoxin actually stabilized the E-cadherin pool in mouse colon indicates that the risk of such adverse effects is minimal [74].

Yessotoxin and certain analogs are toxic to mice by intraperitoneal injection, although reported values for the LD_{50} vary widely among laboratories. Strain and sex differences are unlikely to fully account for the variations. The purity of the yessotoxin employed in the various studies may be an important factor, since it has recently been shown [78] that yessotoxin stored in the dry state undergoes significant decomposition. Because of the variations in the reported LD_{50} values for yessotoxin, comparison of the toxicity of this substance with that of its analogues is difficult. From the available data, however, it is probably fair to say that none of the yessotoxin analogues so far studied is more toxic than the parent compound when injected into mice.

Clinical signs in mice following lethal doses of yessotoxin include restlessness, shivering, and cramps, with progressive respiratory failure leading eventually to apnoea. However, histological studies on mice dying after injection of yessotoxin fail to provide an explanation for the effects on respiration. No effects on the lungs of mice dying from yessotoxin intoxication have been observed [65,67]. A central effect on respiration could be considered, and damage to the brain has been reported [71]. However, degenerative changes were restricted to the Purkinje fibers of the cerebellum, and while damage to Purkinje fibers in rodents is known to be associated with clinical effects, such as the induction of tremors and ataxia, such damage does not influence respiration and is not fatal [79,80]. Purkinje cells are especially vulnerable to ischemia [81], and the observed effects on these cells could be secondary to yessotoxin-induced anoxia. The histological changes seen in the thymus of mice following lethal doses of yessotoxin in some studies [70] but not in others [65,67] are unlikely to be responsible for death. Some authors [69] have stated that death is due to acute heart failure, based on the single-cell necrosis seen in the heart by light microscopy and the structural changes seen by electron microscopy in animals receiving lethal doses of yessotoxin. Again, it is unlikely that such comparatively minor changes are responsible for death, since rodents can survive massive chemically-induced cardiac necrosis [82,83]. It is interesting to note that while apoptosis was observed in the thymus of mice after administration of yessotoxin, no apoptosis was recorded in the heart [67], even though apoptosis is commonly induced by cardiotoxic substances [84].

Yessotoxin is much less toxic when administered orally than when injected, which appears to reflect lack of absorption from the gastrointestinal tract [68]. Intracellular edema has, however, been recorded in the hearts of mice given yessotoxin by gavage [65], and ultrastructural changes were reported in the hearts of animals given a single dose of yessotoxin, 1a-homoyessotoxin, or 45-hydroxyyessotoxin at 1–10 mg/kg [65,67]. In a later study, however, repeated administration of high doses of yessotoxin caused no ultrastructural changes in the heart [76]. Since oral toxicity is the most relevant parameter for risk assessment of food contaminants, any future studies on yessotoxin toxicology should focus upon this route of administration, and effects of longer-term oral administration of yessotoxin and analogues would be of considerable interest.

As yessotoxin is coextracted with toxins of the diarrhetic shellfish poison (DSP) group, it was formerly classified as a DSP. Yessotoxin does not, however, cause diarrhea in animals and yessotoxin is now considered separately from the DSP group [85]. The present guidance level for yessotoxin in shellfish is 1 mg/kg, combining yessotoxin, homoyessotoxin, and their 45-hydroxy derivatives [85]. With the availability of results from repeated-dose oral toxicity studies, however, this limit requires modification. In the experiment of Espenes et al. [76] the no observable adverse effect level was 5 mg/kg body weight. Application of a safety factor of 100 would give an acute reference dose of 50 μg/kg body weight, which is equivalent to a dose of 3 mg for a 60 kg human. If 250 g is taken as an estimate of the amount of shellfish consumed by an adult in a single meal, the guidance level for yessotoxin and its derivatives would be 12 mg/kg of shellfish meat, and this was the recommendation from the FAO/IOC/WHO Expert Consultation held in Oslo in 2004 [86].

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Part VIB

Pectenotoxins

16 Chemistry, Metabolism, and Chemical Detection Methods of Pectenotoxins

Toshiyuki Suzuki

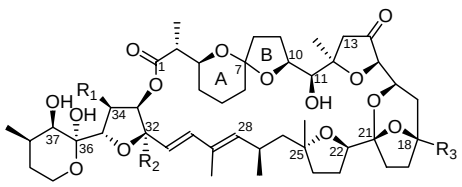
CONTENTS

16.1 INTRODUCTION

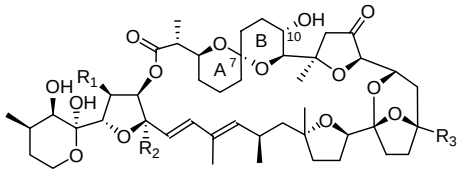
The presence of pectenotoxins in shellfish was discovered owing to their high acute toxicity in the traditional mouse bioassay after i.p. injection of lipophilic extracts [1,2]. Among the natural toxins, pectenotoxins had been grouped together with diarrhetic shellfish poisoning (DSP) toxins (i.e., okadaic acid and dinophysistoxins) [1,2] because they resemble okadaic acid and dinophysistoxins in the chemical structures and always coexist in shellfish contaminated with DSP toxins. Pectenotoxin levels have been regulated in shellfish in some countries. Animal studies indicate that pectenotoxins are much less toxic through the oral route and that they do not induce diarrhea [3]. Since pectenotoxins do not fit the clinical case definition of DSP toxins, they are recently considered as a separate group.

Pectenotoxin-2 (PTX2) has been reported to be present in the toxic dinoflagellates *Dinophysis* spp. [4–19], which is also the causative species of DSP toxins. Recently, several novel pectenotoxins, pectenotoxin-11 (PTX11) [13,20], pectenotoxin-12 (PTX12) [14], pectenotoxin-13 (PTX13) [21], pectenotoxin-14 (PTX14) [21], were found in *Dinophysis* spp. by using liquid chromatography-mass spectrometry (LC-MS). It has been shown that many of the other pectenotoxins such as pectenotoxin-6 (PTX6) and pectenotoxin-2 seco acid (PTX2SA) are formed by metabolism of PTX2 in shellfish tissues [6,22–24].

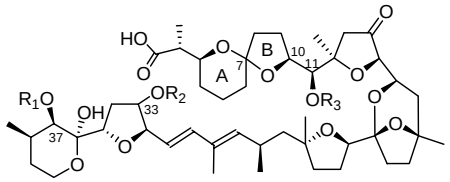
Owing to the difficulties in distinguishing between DSP toxins and pectenotoxins by the traditional mouse bioassay, several chemical analyses have been developed. These methods are based on liquid chromatography (LC) with detection by ultraviolet (UV) absorption [4–7,20,25,26], fluorescence (FL) [6,7,9–11,25–27], and mass spectrometry (MS) [5–8,10–21,23,24,28–36]. A major drawback of these LC techniques is the lack of analytical standards. In the present chapter, chemistry, metabolism, and chemical detection methods of pectenotoxins are described. Pharmacology and toxicology of pectenotoxins will be discussed in other chapters.



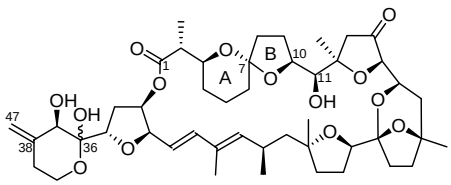
	C7	R1	R2	R3	MW
PTX2	*R*	H	H	CH_3	858.5
PTX2b	*S*	H	H	CH_3	858.5
PTX1	*R*	H	H	CH_2OH	874.5
PTX4	*S*	H	H	CH_2OH	874.5
PTX3	*R*	H	H	CHO	872.5
PTX6	*R*	H	H	COOH	888.5
PTX7	*S*	H	H	COOH	888.5
PTX11	*R*	OH	H	CH_3	874.5
PTX11b	*S*	OH	H	CH_3	874.5
PTX13	*R*	H	OH	CH_3	874.5



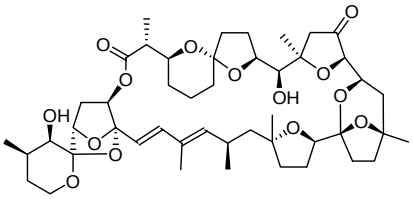
	C7	R1	R2	R3	MW
PTX2C	*S*	H	H	CH_3	858.5
PTX8	*S*	H	H	CH_2OH	874.5
PTX9	*S*	H	H	COOH	888.5
PTX11C	*S*	OH	H	CH_3	874.5



	C7	R1	R2	R3	MW
PTX2SA	*R*	H	H	H	876
7-*epi*-PTX2SA	*S*	H	H	H	876
37-*O*-acyl PTX2SA	*R*	acyl	H	H	
33-*O*-acyl PTX2SA	*R*	H	acyl	H	
11-*O*-acyl PTX2SA	*R*	H	H	acyl	



PTX12 MW 856.5



PTX14 MW 856.5

FIGURE 16.1 The structures of pectenotoxins.

16.2 CHEMISTRY

The chemical structures of pectenotoxins and some of their chemical properties are shown in Figure 16.1 and Table 16.1, respectively. Pectenotoxins resemble okadaic acid in molecular weight and in having cyclic ethers and a carboxyl group in the molecule. Unlike okadaic acid, the carboxyl moiety in many pectenotoxins is in a form of macrocyclic lactone (macrolide). All pectenotoxins absorb UV light between 235 nm and 239 nm owing to the presence of a 1,3-dienyl moiety at C-28–C-31. The infrared (IR) bands at 3400, 1760, and 1740/cm are observed due to absorption by the hydroxyl, five-membered ring ketone, and ester of lactone ring. Pectenotoxins are susceptible to isomerization due to the presence of a hemiketal (C-36) and several ketal centers (C-7 and C-21), along with a ketone α- to an ether linkage. The ketal at C-7 is especially sensitive to epimerization under acidic conditions [13,37,38]. Pectenotoxins are easily destroyed under strongly basic conditions such as those used for hydrolysis of acyl esters of the okadaic acid groups [11] while pectenotoxin-2 (PTX2) is stable for more than 24 h between pH 4.5 and 9.1 [23].

Pectenotoxin-1 (PTX1) and PTX2 were originally isolated from Japanese scallops, *Pationpecten yessoensis*, and their structures were elucidated by single crystal x-ray diffraction techniques, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS) together with UV and IR spectroscopy [1,39]. As part of the same study, three other analogs (PTX3–5) were also isolated and partially identified from UV spectra and MS [1,39]. Additional studies using spectroscopic techniques elucidated the structure of pectenotoxin-3 (PTX3) [40]. Acidic pectenotoxins, pectenotoxin-6

TABLE 16.1
Chemical and Physical Properties of Pectenotoxins Reported Previously

Name	Molecular Formula	MWt	Melting Point (°C)	UV λ_{max} (nm)	$[\alpha]^{20}D$	IR Band (cm^{-1})	References
PTX1	$C_{47}H_{70}O_{15}$	874.5	208–209	235	+17.1 (*c* 0.41, MeOH)	3400,1760,1740,970	1,39
PTX2	$C_{47}H_{70}O_{14}$	858.5	—	235	+16.2 (*c* 0.105, MeOH)	3400,1760,1740,970	1,39
PTX2b[b]	$C_{47}H_{70}O_{14}$	858.5	—	—	—	—	13
PTX2c[b]	$C_{47}H_{70}O_{14}$	858.5	—	—	—	—	13
PTX3	$C_{47}H_{68}O_{15}$	872.5	159–160	235	+2.22 (*c* 0.135, MeOH)	3400,1760,1740,970	1,39,40
PTX4	$C_{47}H_{70}O_{15}$	874.5	—	235	+2.07 (*c* 0.193, MeOH)	3500,1760,1730	1,37,39
PTX5	$C_{47}H_{72}O_{15}$	876.5	—	235	—	No absorption band at 1760/cm	1,39
PTX6	$C_{47}H_{68}O_{16}$	888.5	—	235 237[a]	+8.77[a] (*c* 0.114, MeOH) +37.1 (*c* 1.49, $CHCl_3$)	(3500,1760,1730, 1720,1700)[a]	22,37
PTX7	$C_{47}H_{68}O_{16}$	888.5	—	237[a]	+11.5[a] (*c* 0.131, MeOH)	(3500,1750,1730, 1720,1700)[a]	37
PTX8	$C_{47}H_{70}O_{15}$	874.5	—	237	+19.8 (*c* 0.126, MeOH)	3500,1760,1730	37
PTX9	$C_{47}H_{68}O_{16}$	888.5	—	239[a]	+15.2[a] (*c* 0.105, MeOH)	(3500,1760,1730, 1720,1700)[a]	37
PTX10	—	—	—	—	—	—	—
PTX11	$C_{47}H_{70}O_{15}$	874.5	—	235	—	—	20
PTX11b[b]	$C_{47}H_{70}O_{15}$	874.5	—	—	—	—	13
PTX11c[b]	$C_{47}H_{70}O_{15}$	874.5	—	—	—	—	13
PTX12	$C_{47}H_{70}O_{14}$	856.5	—	235	—	—	14
PTX13	$C_{47}H_{70}O_{15}$	874.5	—	235	—	—	21
PTX14	$C_{47}H_{70}O_{14}$	856.5	—	235	—	—	21
PTX2SA	$C_{47}H_{72}O_{15}$	876.5	—	235	—	—	42
7-*epi*-PTX2SA	$C_{47}H_{72}O_{15}$	876.5	—	235	—	—	42
Acyl-PTX2SA[c]	$C_{63}H_{102}O_{16}$	1114.7	—	—	—	—	43

[a] Measured on phenacyl ester derivative.
[b] These pectenotoxins have not yet been isolated and formally identified by NMR.
[c] 16:0-PTX2SA, the most abundant compound.

(PTX6), and pectenotoxin-7 (PTX7) were later isolated from Japanese scallops and the structure of PTX6 was elucidated by NMR spectroscopy [22]. The absolute configuration of PTX6 was later determined by NMR spectroscopy using a chiral anisotropic reagent, phenylglycine methyl ester, which was condensed with the carboxylic acid group at C-18 of PTX6, and was identified to be 18*S* [41], thereby the structures of the natural 7*R*-pectenotoxins were assigned as 2*R*, 3*S*, 7*R*, 10*S*, 11*S*, 12*R*, 15*R*, 16*R*, 18*S*, 21*S*, 22*R*, 25*R*, 27*R*, 32*R*, 33*R*, 35*S*, 36*S*, 37*R*, 38*R* as shown in Figure 16.1.

Treatment of 7*R*-pectenotoxins under acidic condition such as acetonitrile/water (7:3, v/v) with 0.1% (v/v) trifluoroacetic acid (TFA) leads to an equilibrium mixture of spiroketal stereoisomers, 7*R*-, 7*S*-, and 6-membered-B-ring-isomers [13,37,38] as illustrated in Figure 16.2. Identification and characterization of pectenotoxin-4 (PTX4) and PTX7 as the spiroketal isomers of PTX1 and PTX6, respectively, was achieved on the basis of NMR and acid-catalyzed chemical interconversion [37].

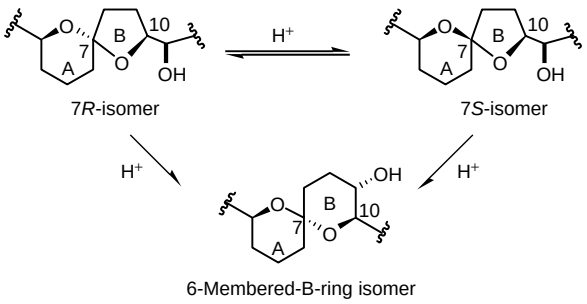


FIGURE 16.2 Acid-catalyzed interconversion of the pectenotoxins.

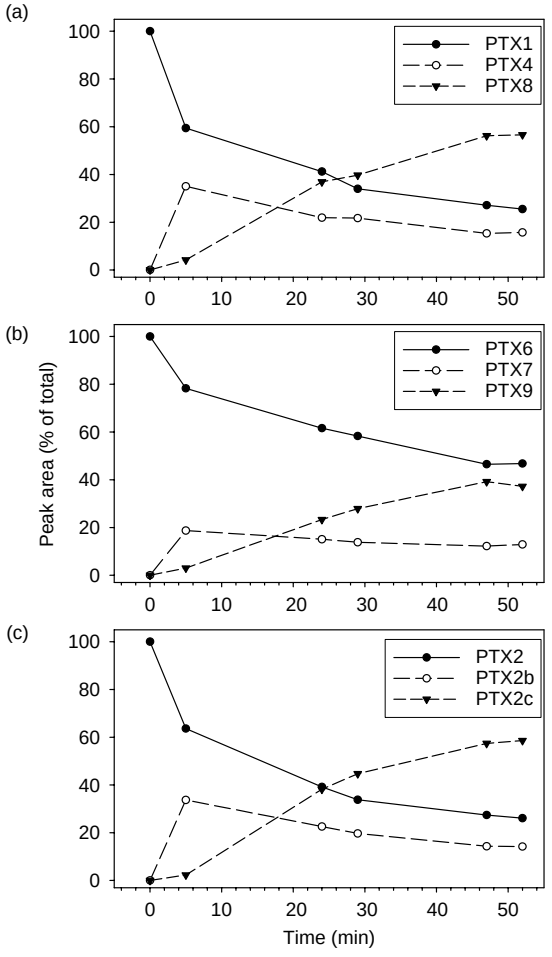


FIGURE 16.3 Kinetics of acid-catalyzed interconversion of PTX1 (a), PTX6 (b), and PTX2 (c). Peaks of pectenotoxins were obtained by selected ion monitoring (SIM) LC-MS.

Equilibration between PTX6 and PTX7 and between PTX1 and PTX4 resulted in the formation of two additional isomeric products, pectenotoxin-8 (PTX8) and pectenotoxin-9 (PTX9) [37]. It was suggested that PTX4 and PTX7 are naturally occurring toxins rather than artefacts of the extraction process, whereas PTX8 and PTX9 are artefacts obtained by acidic interconversion [37]. Analogous

spiroketal isomers of PTX2 were later tentatively identified during acidic interconversion by tandem mass spectrometry (MS/MS) coupled with chromatographic identification [13]. 7*S*- and 6-membered-B-ring-isomers of PTX2 were named as PTX2b and PTX2c, respectively. Figure 16.3 shows the kinetics for the acid-catalyzed interconversion of PTX1, PTX6, and PTX2. Spiroketal isomers of pectenotoxins reached an equilibrium ratio after 48 h in acetonitrile/water (7:3, v/v) with 0.1% TFA [13]. The kinetics for PTX6 interconversion was somewhat different from those of the other pectenotoxins [13].

PTX2SA and its epimer 7-*epi*-PTX2SA, analogues of PTX2 in which the lactone ring had been hydrolyzed, were identified in *D. acuta* from Ireland and mussels, *Perna canaliculus,* from New Zealand [42]. A compound tentatively characterized as a new pectenotoxin with the same molecular weight as PTX1 was detected by LC-MS analyses of *P. canaliculus* and *D. acuta* from New Zealand and temporarily designated as "PTX-1i" [12]. After further studies, this compound was named pectenotoxin-11 (PTX11) along with its spiroketal isomers, PTX11b and PTX11c [13]. PTX11 was isolated from *D. acuta* and identified by NMR and MS/MS [20]. The same sample of *D. acuta* also showed the presence of another compound temporarily designated PTX11x on the basis of LC-MS/MS spectra [13]. This compound was isolated from algal concentrates and identified as pectenotoxin-13 (PTX13) by NMR and MS/MS [21]. A second compound, pectenotoxin-14 (PTX14), was identified as the cyclized 32,36-dehydration product of PTX13 in the same sample, although this compound was suggested to be an artefact in origin [21]. A pair of pectenotoxin isomers with a molecular weight of two less than that of PTX2 were isolated from a bloom of *D. acuta* from Norway and identified as pectenotoxin-12 (PTX12) as a pair of equilibrating 36-epimers of 38,47-dehydroPTX2 [14]. Recently, 37-, 33-, and 11-*O*-acyl esters of PTX2SA have been reported in blue mussels, *Mytilus edulis*, from Ireland [43]. The structures of pectenotoxin-5 (PTX5) and pectenotoxin-10 (PTX10) have not been determined yet. The difficulties encountered in the synthesis of pectenotoxins are highlighted by the fact that only one total synthesis of PTX4 and PTX8 has been completed to date [38]. The absolute stereochemistry for pectenotoxins was confirmed by total synthesis of PTX4 and its isomerization to PTX8 and PTX1 [38]. The synthetic studies of pectenotoxins are reviewed by Halim et al [44].

16.3 METABOLISM

It is believed that the origin of pectenotoxins is the toxic dinoflagellate genus *Dinophysis*, which is also the causative species for DSP toxins (okadaic acid and the dinophysistoxins). PTX2 has been reported to be present in the toxic dinoflagellates *D. fortii*, *D. acuminata*, *D. norvegica*, *D. rotundata*, and *D. acuta* [4–19]. Recently, PTX11, PTX12 and PTX13 were found in *D. acuta* collected in New Zealand and Norway [13,14,20,21]. It has been shown that many of the other pectenotoxins such as PTX6 and PTX2SA are formed by metabolism of PTX2 in shellfish tissues [6,22–24].

PTX2 in bivalves is absorbed from algae and metabolized by two processes as illustrated in Figure 16.4. In the Japanese scallop, *P. yessoensis*, PTX2 undergoes step-wise oxidation of the methyl group attached to C-18 [6,22]. Thus, the 18-methyl group in PTX2 is oxidized to an alcohol (PTX1), aldehyde (PTX3), and finally a carboxylic acid (PTX6) group. This biotransformation of PTX2 in Japanese scallops was confirmed by the content of PTX6 in scallops, which was found to be significantly higher than that of PTX2 when *D. fortii* collected in the same location contained only PTX2 [6]. It is reported that PTX6 content in Japanese scallops is significantly higher than that of PTX2 and PTX1 determined by LC-MS [35,36], indicating a rapid biotransformation of PTX2 to PTX6 in Japanese scallops. The oxidation of the methyl group attached to C-18 leads to reduced toxicity in the mouse bioassay. PTX6 is half as toxic as PTX2 [25], suggesting a kind of detoxification of PTX2 in the scallops. Although very low levels of PTX7 are often found in Japanese scallops along with PTX6, it appears that PTX7 is formed by nonenzymatic isomerization of PTX6 [13,37].

In the New Zealand scallop, *Pecten novaezelandia* [24], and mussels *P. canaliculus* and *M. gallo provincialis* from New Zealand [23] and *M. edulis* from Norway [3], a different process has been

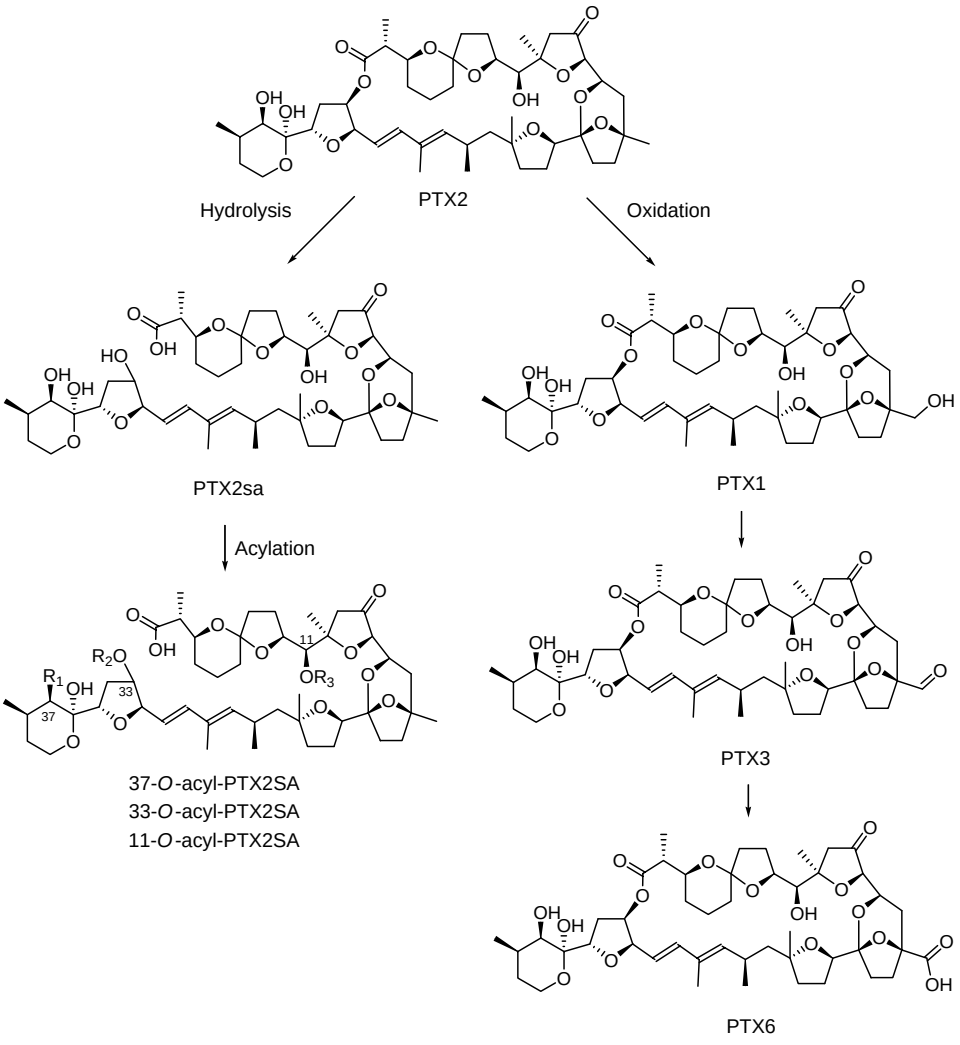


FIGURE 16.4 The metabolism of pectenotoxin-2 in shellfish.

TABLE 16.2
Pectenotoxin Profiles after the Incubation of Pectenotoxin-2 with Greenshell Mussel (*P. canaliculus*) Extracts

Hours	Profile (%) PTX2	PTX2SA	7-*epi*-PTX2SA	Other PTXs	Recovery (%)
8	9.3	90.7	—	—	121
18	4.6	93.5	1.9	—	116
24	—	95.2	4.8	—	103

Source: From Suzuki, T. et al. *Toxicon* 39, 507, 2001.

demonstrated whereby the lactone moiety of PTX2 undergoes rapid enzymatic hydrolysis to yield PTX2SA. The biotransformation of PTX2 to PTX2SA was confirmed by *in vitro* experiments by using bivalve extracts and PTX2 [3,23,24]. Table 16.2 shows the PTX profile after the incubation of PTX2 in mussel, *P. canaliculus*, extracts. The mussel extracts converted more than 90% of PTX2 to PTX2SA within 8 h. Although 7-*epi*-PTX2SA is detected in the extracts, it appears

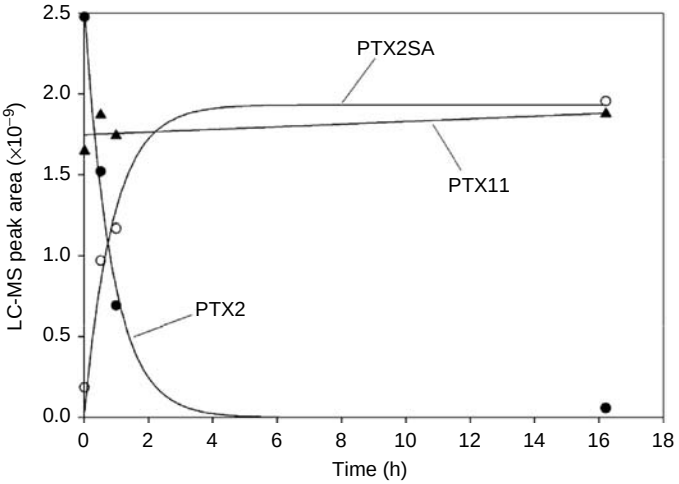


FIGURE 16.5 Conversion of PTX2 and PTX11 versus time during incubation with homogenized mussel hepatopancreas.

that this is formed by nonenzymatic isomerization of pectenotoxins [13,37]. It is interesting that PTX2 is not converted to any other pectenotoxins in Japanese scallop extracts (Suzuki, unpublished data), indicating that activities of some enzymes such as oxidases are not induced in *in vitro* extracts. The result also suggests lack of the capability to convert PTX2 to PTX2SA in Japanese scallops. Examination of the metabolite profiles reported for shellfish contaminated with pectenotoxins suggests that conversion from PTX2 to PTX2SA is the major metabolic pathway in many shellfish species [11,12,14,16,18]. Since PTX2SA was not cytotoxic to KB cells even at 1.8 μg/mL, whereas PTX-2 was cytotoxic at 0.05 μg/mL [42], the conversion from PTX2 to PTX2SA in shellfish is a kind of detoxification. Recently, metabolites of PTX2SA, 37-, 33-, 11-*O*-acyl esters of PTX2SA have been reported in the blue mussels, *M. edulis*, from Ireland [43]. The most abundant fatty acid esters in the samples were, in order, the 16:0, 22:6, 14:0, 16:1, 18:4, and 20:5 fatty acids.

PTX11 is more resistant to enzymatic hydrolysis than PTX2. PTX11 was not detectably hydrolyzed *in vitro* overnight, even though the half-life for PTX2 hydrolysis in the same preparation was ca. 15 min as shown in Figure 16.5 [20]. This result indicates that PTX11 is at least two orders of magnitude less easily hydrolyzed than PTX2 by the enzymes in the mussel hepatopancreas. This suggests that because of steric hindrance by the 34-hydroxyl group, and possibly hydrogen bonding between the 34-OH and the carbonyl oxygen of PTX11, the latter is not a substrate for the enzyme(s) responsible for hydrolysis of the lactone ring of other pectenotoxins. As a consequence, PTX11 is expected to accumulate to a much greater extent than PTX2 in mussels. This is in accord with the observation that there was a large difference between the ratios of PTX11 and the putative PTX11SA (0.63), and PTX2/PTX2SA (0.04) in naturally contaminated mussels *P. canaliculus* collected in New Zealand [12]. Analyses of contaminated mussels similarly suggest that PTX12 is more resistant to hydrolysis than PTX2 [14].

There is only limited data on depuration of pectenotoxins from shellfish. Elimination of PTX2SA from the mussel *P. canaliculus* in New Zealand occurred with half-lives of 26 days [12] and of 15 days in Akaroa Harbour [45]. Depuration of PTX2SA and 7-*epi*-PTX2SA from *M. galloprovincialis* in Portugal has also been reported [18]. When PTX6 was injected into the adductor muscle of scallops *P. yessoensis* it was rapidly transported to the hepatopancreas [46]. The residual portion of PTX6 in the hepatopancreas of the scallops was less than 20%. The residual portion of PTX6 was slightly higher than that of okadaic acid group.

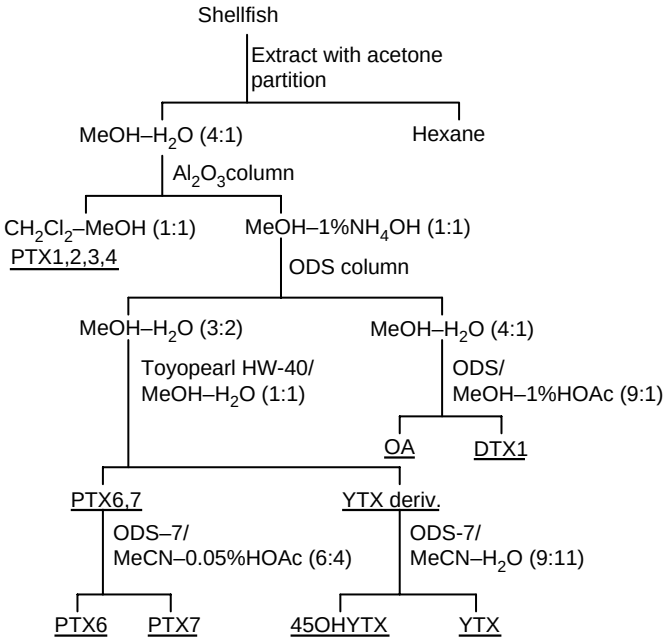


FIGURE 16.6 Purification of DSP toxins and lipophilic toxins.

16.4 CHEMICAL DETECTION METHODS

16.4.1 Extraction, Isolation, and Cleanup for Chemical Methods

DSP toxins and lipophilic toxins including pectenotoxins are usually isolated from bivalves or algal concentrates harvested from natural blooms by acetone extraction, although aqueous methanol such as 80% or 90% methanol is sometimes used. Individual toxins are then isolated through a complex fractionation and purification procedure of raw extracts on the basis of a combination of chromatographic techniques. Several procedures for the isolation of pectenotoxins have been reported [1,3,14,20,21,22,39,40,42,43,47]. Extraction and fractionation of DSP and lipophilic toxins from bivalves reported by Yasumoto [22] are shown in Figure 16.6. Acidic PTX6 and PTX7 are separated from neutral pectenotoxins on an alumina column together with okadaic acid and dinophysistoxin-1. Toxin fractions separated by the alumina column are usually chromatographed on a silica gel column (Merck) with chloroform/methanol [47], although this step is not shown in the scheme in Figure 16.6. Recent work has shown that it is relatively easy to purify pectenotoxins from algal concentrates harvested from natural blooms [3,14,20,21] or by *in vitro* enzymatic conversion of isolated PTX2 [3,48].

Preparation of extracts for chemical methods for pectenotoxins is rather simple compared to the isolation. Pectenotoxins are extracted from bivalve samples with 4–9 volumes of 80% or 90% methanol [28,29,34]. These solvent systems have been shown to be equally efficient for pectenotoxins, although 90% methanol is more efficient for DSP toxins and their esters [33]. In some LC-MS methods, methanol extracts are directly analyzed by LC-MS [34–36]. Cleanup has been accomplished using liquid/liquid partitioning between methanolic solution and chloroform. Pectenotoxins are partitioned into the chloroform layer and toxins are analyzed directly by LC-MS after solvent evaporation and dissolution in methanol [28]. The method of McNabb et al. [33] uses a hexane wash of the crude methanolic extract to remove nonpolar lipids before direct LC-MS analysis of the relatively dilute extract (0.1 g eq./mL). Solid-phase extraction (SPE) cleanup is useful in LC-MS [29] in situations where the quantification of the toxins is interfered with by coextractives from biological matrices [31]. SPE is also useful to extract pectenotoxins from plankton net samples [6,14].

FIGURE 16.7 Fluorescence HPLC chromatogram of ADAM derivative of PTX6 in hepatopancreas of scallops. *Column*: Develosil ODS-5 (250 mm × 4.6 mm I.D.); *Mobile phase*: MeCN–H_2O (8:2, v/v), *Flow rate*: 1.1 ml/min, *Excitation*: 364 nm, *Emission*: 412 nm.

16.4.2 Liquid Chromatography-Ultraviolet Detection

As pectenotoxins have a conjugated diene in the macrolide skeleton, UV detection between 235 and 239 nm is applicable to their detection after LC separation. PTXs can be separated by a C18 or C8 reversed phase column with acetonitrile/water mobile phases. Quantification of PTX2 by isocratic LC-UV detection at 235 nm has been reported [4–7,20,25,26]. The LC-UV detection is not sufficiently sensitive or specific for pectenotoxins in bivalve extracts due to biological matrices, although this approach is useful for algal extracts [4–7,20,25,26].

16.4.3 Liquid Chromatography-Fluorescence Detection

The carboxyl group in acidic pectenotoxins such as PTX6 and PTX2SA reacts rapidly with 9-anthryldiazomethane (ADAM) [6,7,10,11,25–27,42], 1-bromoacetylpyrene (BAP) [7], 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (BrDMEQ) [27], and 9-chloromethylanthracene (CA) [27]. The derivatization of toxins with ADAM has also been used for confirmation of the presence of a carboxyl group in structural studies [42]. Separation of fluorescent PTX derivatives by LC is usually carried out using C18 reversed phase chromatography with acetonitrile/water mobile phases. Figure 16.7 shows a typical LC separation of ADAM derivative of PTX6 obtained from hepatopancreas of Japanese scallops, *P. yessoensis*. Although cleanup of ADAM derivative of okadaic acid by Sep Pak silica cartridge [49] is useful, in the case of ADAM–PTX6 several peaks that may interfere with the quantification of ADAM–PTX6 were observed. A second chromatographic run on a cyanopropyl column (Capcell Pak CN SG 120 column, Shiseido, Japan) [50] was useful for

confirmatory analysis of ADAM–PTX6 [6]. The primary hydroxyl group of PTX1 and PTX4 is derivatized with 1-anthroylnitrile (AN) [25,26]. Precolumn fluorometric analysis of PTX2 by isocratic LC-FL detection has been reported [9] after derivatization of PTX2 with the fluorescent dieno phile DMEQ-TAD, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione. The fluorescent derivative of PTX2 was quantitatively detected in the range 1–200 ng. Application of this method to bivalve samples has not been reported although it has been used to confirm the occurrence of PTX2 in plankton net samples.

16.4.4 Liquid Chromatography-Mass Spectrometric Detection

The LC-MS technique has proven to be one of the most powerful tools for quantification and identification of several marine toxins including pectenotoxins [32]. Several analytical methods for the determination of pectenotoxins by LC-MS are reported [5–8,10–21,23,24,28–36,45]. Selected ion monitoring (SIM) for $[M\text{-}H]^-$, $[M+HCO_2]^-$, $[M+NH_4]^+$ or multiple reaction monitoring (MRM or MS/MS) using loss of water molecules from $[M+NH_4]^+$ are applicable to the quantification. Determination of pectenotoxins by LC-MS is usually carried out using reversed phase chromatography on a C8 or C18 column and isocratic or gradient elution with acetonitrile/water mobile phases containing acidic modifiers such as acetic acid or formic acid/ammonium formate. An LC-MS method for a wide range of toxins has been subjected to a full single-laboratory validation and a limited interlaboratory study [33,34]. The detection limit of pectenotoxins was 5–80 ng/g hepatopancreas [28,29,35] or 0.01mg/kg whole flesh [33]. There seems to be no significant difference between the results for single MS (SIM) or MS/MS (MRM) detection.

A short narrow-bore column packed with 3 μm Hypersil-BDS-C8 phase is one of the most widely used columns, which is capable of separating a wide range of toxins using rapid gradients [30, 32]. Simultaneous separation of okadaic acid analogues, yessotoxins, and pectenotoxins is possible by using Hypersil-BDS-C8 column as illustrated in Figure 16.8 [35]. Toxins were detected using SIM for $[M\text{-}H]^-$ of okadaic acid (*m/z* 803.5), dinophysistoxin-1 (*m/z* 817.5), dinophysistoxin-3

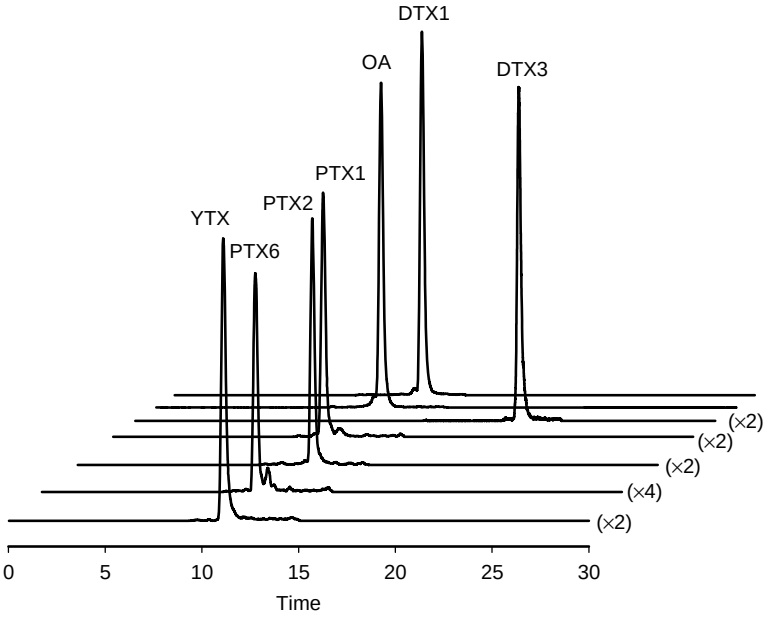


FIGURE 16.8 Selected ion monitoring (SIM) LC-MS chromatogram for a standard mixture of lipophilic toxins. Column: Hypersil-BDS-C8 (50 mm × 2.1 mm I.D.). Mobile phase: A (water) and B (acetonitrile/water [95:5 v/v]) both containing 2 mM ammonium formate and 50 mM formic acid. Gradient elution from 20% to 100% B over 10 min (step 1) and 100% B for 15 min (step 2).

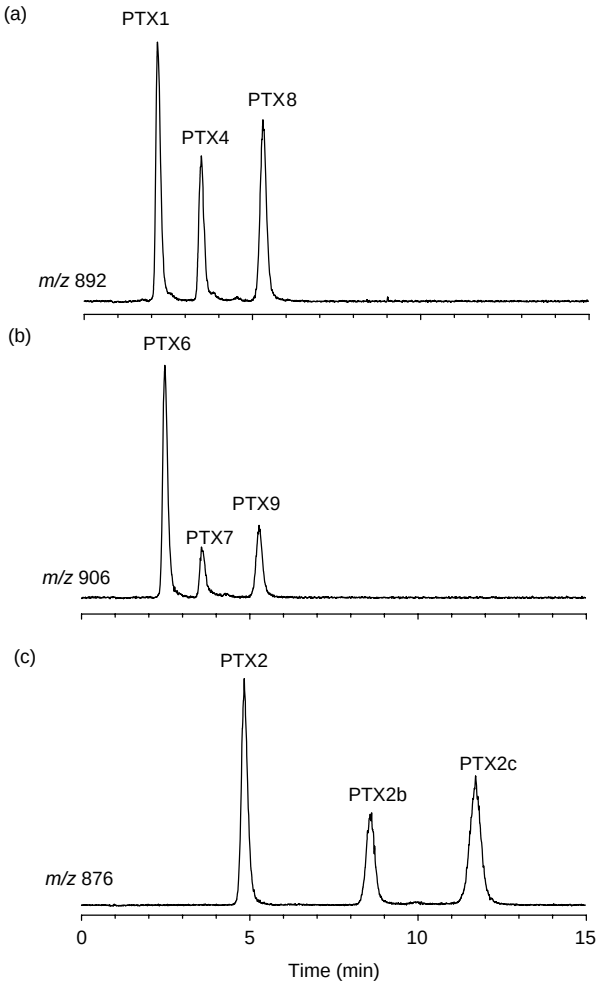


FIGURE 16.9 Selected ion monitoring (SIM) LC-MS chromatogram of spiroketal isomers of PTX1 (a), PTX6 (b) and PTX2 (c). Column: Hypersil-BDS-C8 (50 mm × 2.1 mm I.D.). Mobile phase: acetonitrile–water (48:52) with 2 mM ammonium formate and 50 mM formic acid.

(*m/z* 1055.7), for $[M+HCO_2]^-$ of PTX1 (*m/z* 919.5), PTX2 (*m/z* 903.5), PTX6 (*m/z* 933.5), and for $[M-2Na+H]^-$ of yessotoxin (*m/z* 1141.5). Pectenotoxins were also separated by the same column with an isocratic elution [13]. Figure 16.9 shows the LC-MS chromatogram of spiroketal isomers of pectenotoxins detected by SIM for $[M+MH_4]^+$ ions. Spiroketal isomers of pectenotoxins were eluted in the order of 7*R*-, 7*S*-, and 6-membered-B-ring-isomers.

LC-MS/MS spectra for $[M+NH_4]^+$ of pectenotoxins are useful for confirmation of identity and structure elucidation [7,13,14,20,21,43]. Figure 16.10 shows the product ion spectrum of the $[M+NH_4]^+$ ion (*m/z* 892) of PTX11 (a) and PTX1 (b) acquired on a triple quadrupole MS/MS. Both compounds showed many common fragment ions, but a few of the ions were shifted up or down in mass by 16 amu. Figure 16.11 presents a proposed assignment of fragment ions deduced from examination of the structure and through comparison of the spectra of PTX1 and PTX11. The higher mass regions of both spectra show a very low abundance $[M+H]^+$ ion at *m/z* 875, because of an initial elimination of ammonia from $[M+NH_4]^+$, and a series of ions, $[M+H-nH_2O]^+$ ($n = 1–5$), because of sequential water losses from $[M+H]^+$. Fragment ions in the rest of the spectrum are most easily explained by an initial opening of the macrocyclic ring at the lactone site as shown in Figure 16.11.

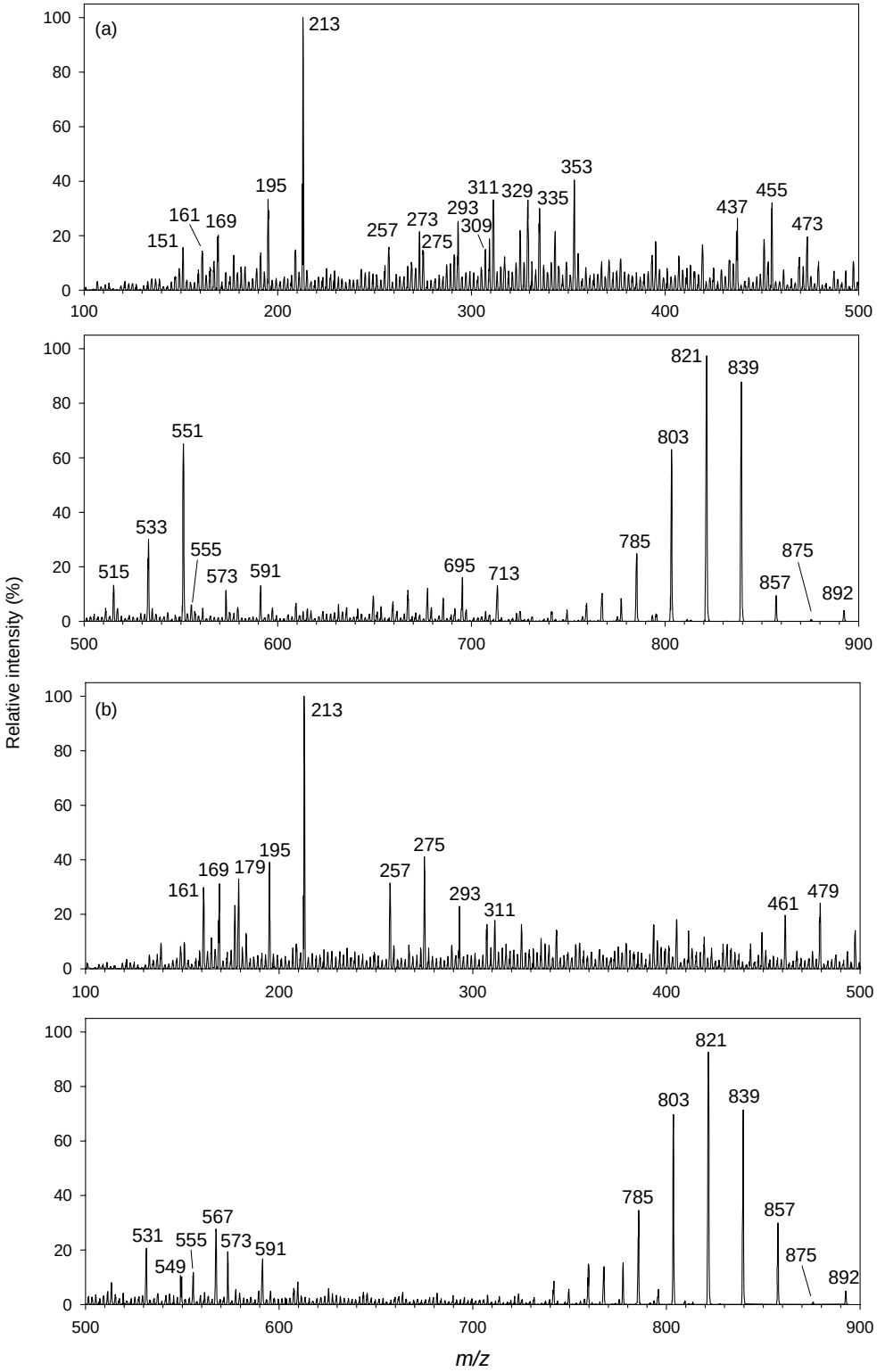


FIGURE 16.10 LC-MS/MS product ion spectra obtained for the $[M+NH_4]^+$ ions (*m/z* 892.5) of pectenotoxin-11 (a) and pectenotoxin-1 (b). All *m/z* values have been rounded off.

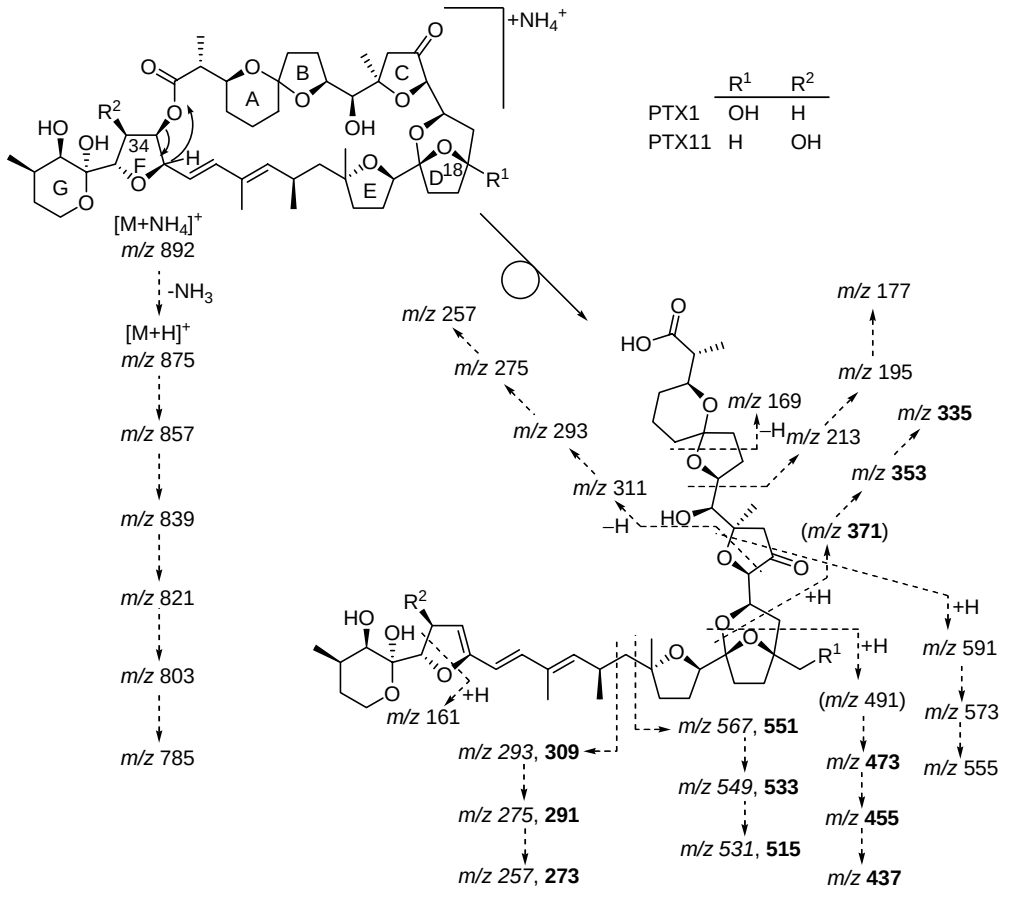


FIGURE 16.11 Proposed fragmentations observed in positive ion MS/MS spectra of pectenotoxin-1 and pectenotoxin-11. Ions in bold text arise from PTX11; ions in italic text arise from PTX1, and ion in parentheses are of very low abundance. All transitions shown involved loss of water (18 amu) unless otherwise indicated.

This was supported by the fact that the product ion spectrum of the $[M+NH_4]^+$ ion of the PTX2SA was almost identical to that of PTX2, with only an additional loss of water fragment at high mass. The base peak in both spectra was *m/z* 213, a fragment ion observed in the spectra of all PTXs. This appears to be due to cleavage of the C10–C11 bond adjacent to ring B. Losses of 1 and 2 water molecules from *m/z* 213 produced ions at *m/z* 195 and 177. Another low mass ion (*m/z* 161), observed in both spectra, was explained by fragmentation of ring F. Fragmentation of ring C followed by a series of water eliminations gives rise to one group of ions at *m/z* 311, 293, 275, and 257, and another group at *m/z* 591, 573, and 555. Fragmentation of the bridging ring connected to ring D with water eliminations gave rise to one set of ions at *m/z* 371, 353, and 335, and another set at *m/z* 473, 455, and 437 in the spectrum of PTX11. These ions do not appear in PTX1, presumably because of the influence of the hydroxymethyl function at C18. A corresponding series of ions at 16 amu less (*m/z* 457, 439, and 421) do appear in the spectrum of PTX2. A set of ions at *m/z* 551, 533, and 515 in the PTX11 spectrum were very clearly shifted 16 amu lower than the equivalent set (at *m/z* 567, 549, and 531) in the spectrum PTX1. These ions can be explained through fragmentation of the C25–C26 bond and two subsequent losses of water. Finally, the ion at *m/z* 273 in PTX11 can be explained by a scission of the C26–C27 bond to form a low abundance ion at *m/z* 309 that loses water to give ions of *m/z* 291 and 273. The corresponding ions in PTX1 would be isobaric with the previously discussed ion series at *m/z* 293, 275, and 257.

16.4.5 Screening Tests

Enzyme-linked immunosorbent assay (ELISA) methods for pectenotoxins are under development in several countries and a preliminary method has been reported [51–54]. Silica gel thin layer chromatography (TLC) is useful to identify pectenotoxins in semipurified samples [1,20,39]; however, it is difficult to use the TLC for screening tests of toxins in crude bivalve extracts owing to the poor selectivity and sensitivity.

16.4.6 Calibration Standards

Calibration standards of PTX2 and PTX2SA are available from National Research Institute, Canada (Halifax, Canada) [55]. There is a standard toxin distribution project organized by the Japanese Government for domestic use [47]. PTX1, PTX2, and PTX6 are available in Japan from Japan Food Research Laboratories (Tama, Japan).

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17 Pharmacology of Pectenotoxins

Natalia Vilariño and Begoña Espiña

CONTENTS

17.1 INTRODUCTION

Pectenotoxins (PTXs) are macrolactones, first isolated from the scallop *Patinopecten yessoensis* cultivated in Japan, that can accumulate in shellfish and intoxicate humans [1]. PTXs have been traditionally included in the diarrheic shellfish poisoning (DSP) group of toxins. Nowadays, because of their different chemical structure and inability to induce diarrhea they are classified as a phycotoxin group by themselves. Actually, in mouse toxicity studies, PTXs seem to have an extremely low oral toxicity. Since they often co-occur with other phycotoxins, no toxic episodes in humans could be unequivocally related to PTXs and therefore there is no information about their toxicity to humans. Experimental and epidemiological data suggest that the current regulatory limits for PTXs content in shellfish could be inappropriate and should be revised. In spite of their apparent lack of oral toxicity, PTXs are toxic to mice by intraperitoneal (i.p.) injection and subsequently they are a possible source of false positives in the detection of DSP toxins with mouse bioassay method. In this chapter, we compiled the available information related to PTXs pharmacology, including one of their more interesting characteristics, their cytotoxicity against certain tumor cell lines that raises them as possible new tools in the therapy of cancer. More detailed descriptions on animal and cell toxicity can be found in Chapter 18.

17.2 STRUCTURE–ACTIVITY RELATIONSHIP OF PECTENOTOXINS

To date, fifteen PTX analogues have been identified . Their chemical structures (Figure 17.1) clearly determine their pharmacologic activity. The available information about PTX structure–activity relationship has implications not only for consumer health protection and risk assessment but also for the economy of the aquaculture sector. Actually, the dependency of PTX activity on chemical structure has evidenced the existence of two shellfish metabolic routes that transform the parental compounds found in dinoflagellates to other less toxic PTX analogues [2–4].

PTX-2 shares with other two analogues the highest level of i.p. toxicity among PTXs, with a LD_{50} of 230 μg/kg of body weight in mice [5]. PTX-2 has been isolated both from algae and shellfish [6,7], being the parental compound of a series of PTXs that have been found mainly in shellfish. In the Japanese scallop *Patinopecten yessoensis* metabolic transformation of PTX-2 following an oxidation cascade of the 43-methyl group generates PTX-1, with a hydroxyl substituent in C-43, toxicity of which is slightly diminished versus PTX-2 (LD_{50} of 250 μg/kg) [2,5]. Further oxidation of the molecule yields PTX-3 and PTX-6, with C-43 aldehyde and carboxyl moieties, causing a

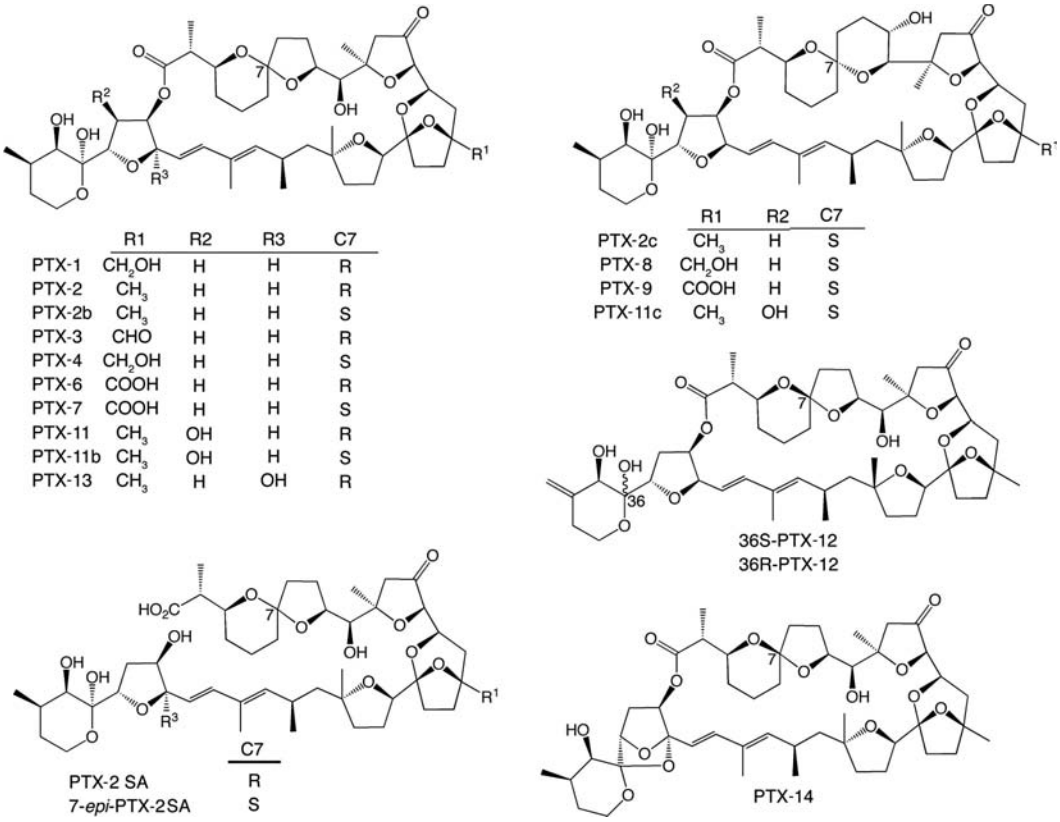

FIGURE 17.1 Chemical structures of pectenotoxins.

substantial reduction of toxic activity as the oxidation level is increased (LD_{50} of 350 μg/kg and 500 μg/kg, respectively) [2,5,8]. Not only the degree of oxidation of C-43 has an effect on PTXs activity, but the stereoisomery of some parts of the molecule is also a determinant for toxicity. Actually, PTX-4 and PTX-7, C-7 S stereoisomers of PTX-1 and PTX-6, respectively, have LD_{50} of 770 μg/kg and >5000 μg/kg, respectively, much higher than their C-7 *R* stereoisomers [5,9]. A modification of the B ring area of the molecule present in PTX-8 and PTX-9 also causes a loss of toxicity [9]. Although this structural information in the case of PTX-8 and PTX-9 is not relevant to human health, since they are generated after acid-catalyzed interconversion and their presence in nature has not been demonstrated, it can be useful for modeling of toxin–target interactions. Besides oxidation, PTX-2 is modified by hydrolysis in many shellfish species [4,10–14]. Hydrolysis of the C1-C33 lactone bond is a more effective detoxification process, since the compounds generated by this reaction, PTX-2SA (PTX-2 seco acid) and its epimer 7-*epi*-PTX-2SA, the latter appearing in part as an artifact after conversion from PTX-2SA during storage and purification, are not toxic by i.p. injection [4,11,15]. Other PTXs that are considered parental compounds for being detected in microalgae and shellfish are PTX-11, PTX-12, and PTX-13 (Figure 17.1) [12,16–18]. PTX-11 has a similar toxicity by i.p. injection to PTX-2 and PTX-1 (LD_{50} 244 μg/kg) [16]. Unfortunately, there is no toxicity information about PTX-12 and PTX-13. However, it is worth mentioning that some structural features of PTX-11 and PTX-12 are relevant to detoxification of these compounds by hydrolysis in shellfish. PTX-11 is not hydrolyzed to its seco acid form by shellfish enzymes in conditions that completely hydrolyze PTX-2 [16]. The presence of a hydroxyl moiety in C34 seems to impair this enzymatic reaction. The exocylic olefinic methylene group in C38 of PTX-12 does not impair but reduces hydrolysis since PTX-12SA has been identified also in shellfish extracts, although at a lower

ratio versus its parent toxin than PTX-2SA [17]. The lack of efficiency in detoxification of PTX-11 by shellfish has an impact on toxicity monitoring by i.p. injection in mice, mainly in algal blooms with a high presence of this PTX [12].

Intraperitoneal toxicity to mice was used to establish a structure–activity relationship since this was the piece of information available for the highest number of PTX analogues. The scarce amounts of pure PTXs has precluded the inclusion of several analogues in studies of other PTX biological activities. In every *in vitro* study that includes more than one analogue, the structure–activity relationship is similar to that obtained *in vivo* [4,19]. The *in vitro* study that includes more analogues compares the effects on actin cytoskeleton of PTX-1, -2, -11, and -2SA in human neuroblastoma and rat hepatocarcinoma cells, and the results resemble *in vivo* toxicity, with PTX-2 and -11 being the more potent compounds, followed by PTX-1 [19]. In the same experiments, PTX-2SA had no effect [19].

17.3 ORGANS TARGETED BY PECTENOTOXINS AND PHARMACOKINETICS

It is generally accepted that PTXs are hepatotoxic compounds. Histopathological studies have demonstrated that i.p. injection of PTX-1 (1 mg/kg) induces macroscopic alterations of the liver, including congestion and finely granulated appearance [20]. Microscopic analysis revealed hepatocyte necrosis in periportal regions of hepatic lobules [20]. The fact that in the same study PTX-1 caused no pathological changes in the intestine or other visceral organs has placed PTXs in the group of hepatotoxins. Regarding other analogues, PTX-2 was reported to induce a reduction of liver weight without other macroscopic or microscopic alterations, which is not surprising since in this study the dose of PTX-2 was considerably lower (200 μg/kg) [21]. However, the induction of increased levels of alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase in serum, three liver damage-related serum enzymes in mice treated with PTX-2 doses of 100 μg/kg or higher, demonstrates that PTX-2 is also hepatotoxic [21]. There is no pathological information for the other PTXs although they have been often considered hepatotoxic by analogy.

In spite of the existence of and the presence of early reports on PTX-2 diarrheic toxicity p.o., even at doses similar to those that induce i.p. toxicity [22,23], there have been more recent studies with pure PTXs that demonstrate that this group of toxins does not induce diarrhea or any other pathological signs by oral administration at doses considerably higher [4,16]. In fact, none of the PTXs tested to date seem to have intestinal toxicity by any route of administration [4,16,24]. Considering that doses as high as 5000 μg/kg of PTX-2, -11, and -2SA have been administered by oral gavage to mice without inducing any detectable symptoms, the oral toxicity of PTXs in mice can be considered extremely low, even low after knowing that this way of oral administration may lead to an overestimation of toxicity due to the lack of mixing of the administered toxin with the intestinal content [25,26]. It is possible that since some okadaic acid esters can be coextracted with PTX during the purification process [4], the contradictory initial results about PTX-2 oral toxicity were due to the inadvertent presence of okadaic acid derivatives, which would not be surprising given the fact that these two groups of toxins often coexist in the same algae blooms [6,12,27,28]. Overall, the intestine does not seem to be the organ responsible for PTXs toxicity. Another organ that might be affected after repeated administration of PTX-2 is the kidney, although the existing evidence is only an increase of urea levels in serum [29] in animals dosed with 200 μg/kg/day for several days, conditions that also caused 50% mortality and elevation of liver damage-related serum enzymes.

Although only available as a PhD discussion, it is interesting to mention some data about PTX pharmacokinetics. The body distribution of PTX-2 and -2SA was analyzed in mice after p.o. and i.p. administration [30]. Remarkably, when administered orally, both toxins were mainly detected in the gastrointestinal tract content and/or feaces, and only a small portion was found in the gastrointestinal tract tissue and an extremely low amount of PTX-2SA in the liver. After i.p. administration, the distribution of the toxins was similar, with a slightly higher presence in the liver and also detectable

levels in blood and kidney. Moreover, a considerable amount of PTX-2 was converted to PTX-2SA or 7-*epi*-PTX-2SA. These data indicate that PTXs do not seem to be readily absorbed when administered orally, which would in part explain their lack of toxic activity by this route. PTXs also seem to be rapidly excreted through feaces after i.p. administration suggesting that their hepatotoxicity is probably due to accumulation in the liver for detoxification by metabolization and excretion through the bile. Although the detection technique used in this study may not be sensitive enough for low levels of toxin, the results are certainly indicative of the compartments in which PTX is mainly distributed in the organism. The conversion of PTX-2 to -2SA supports the presence of metabolization routes that might help explain the rapid loss of total amount of PTX. However, the metabolization routes of PTXs are unknown. The unaccountability for a considerable fraction of PTX in this study could also be due to distribution to other tissues where the concentrations are under the detection limit. Actually, if we consider that it is not clear whether the hepatic failure is the cause of mouse death after i.p. administration of PTXs, since the reported lessions to the liver occurred at doses of PTX-1 much higher than its LD_{50}, it could be possible that the toxin presence in other tissues also had life threatening consequences.

Unfortunately, there are no data on PTX toxicity and distribution in humans. Although all the information available nowadays suggests that PTXs do not pose a risk for human health, it would be possible that humans were more sensitive to these toxins or had different pharmacokinetics than the species used for toxicology studies. In general, the study of the pharmacokinetics of phycotoxins has been complicated because of the relatively low sensitivity of the methods used to detect this toxins in tissues and biological fluids.

17.4 SENSITIVITY OF MAMMALIAN CELLS TO PECTENOTOXIN

Initial reports on PTXs hepatotoxicity prompted the study of *in vitro* effects of these toxins on hepatocytes. There are several reports on PTX-1 and -2 *in vitro* cytotoxicity to hepatocytes. PTX-1 has been demonstrated to induce morphological changes and apoptosis in freshly prepared rat and salmon hepatocytes [31,32]. PTX-1 toxic effects on primary cultures of liver cells have also been reported as disarrangement of actin and microtubule cytoskeleton [33]. Recently, in our laboratory, we found that actin cytoskeleton of primary rat hepatocytes was also altered by PTX-2 and -11 in the same way as PTX-1 but with a slightly higher potency [34]. Another primary cell type sensitive to PTXs is fresh enterocytes isolated from rabbit, in which PTX-6 induces a significant depolimerization of actin filaments [35].

Besides primary cells, PTXs are cytotoxic to many *in vitro* tumor models. PTX-2 elicits a potent cytotoxicity in cell lines of human colon, lung, ovarian, renal, central nervous system tumors, melanoma, and breast cancers [36], which raises this class of compounds as possible antitumor drugs. Since this first report, other groups have also described PTX-2-induced alterations in human neuroblastoma cells, human colorectal cancer cells, human hepatocarcinoma cell lines, human lung cancer cells, human oral epidermoid carcinoma KB cells, rat proximal tubule epithelial cell line, and clone 9 rat hepatocarcinoma cell line [10,19,37–39]. Not many *in vitro* studies have been done with other PTX analogues in tumor cell lines. The experiments performed with PTX-2SA to date reveal a complete absence of effects on several cell lines, the clone 9 rat hepatic carcinoma cell line, the oral epidermoid carcinoma KB cells, and the BE(2)-M17 neurobalstoma cell line [10, Ares, 2007: #27]. In Figure 17.2, confocal microscope images are presented comparing the effects of PTX-2 and -2SA on F-actin (filamentous actin) cytoskeleton of clone 9 rat hepatic carcinoma cells and BE(2)-M17 neurobalstoma cells. PTX-1 and -11 have been reported to affect actin cytoskeleton organization in a way similar to that of PTX-2 in neuroblastoma cells [19].

The toxicity exerted by PTX in several cell types indicates that the hepatocytes are not the only cellular model sensitive to this group of toxins. Therefore, unlike the fresh water toxin microcystin, hepatotoxicity of which is due to unique transport mechanisms present in the hepatocyte plasma membrane [40,41], PTXs hepatotoxicity *in vivo* seems to be determined in part by

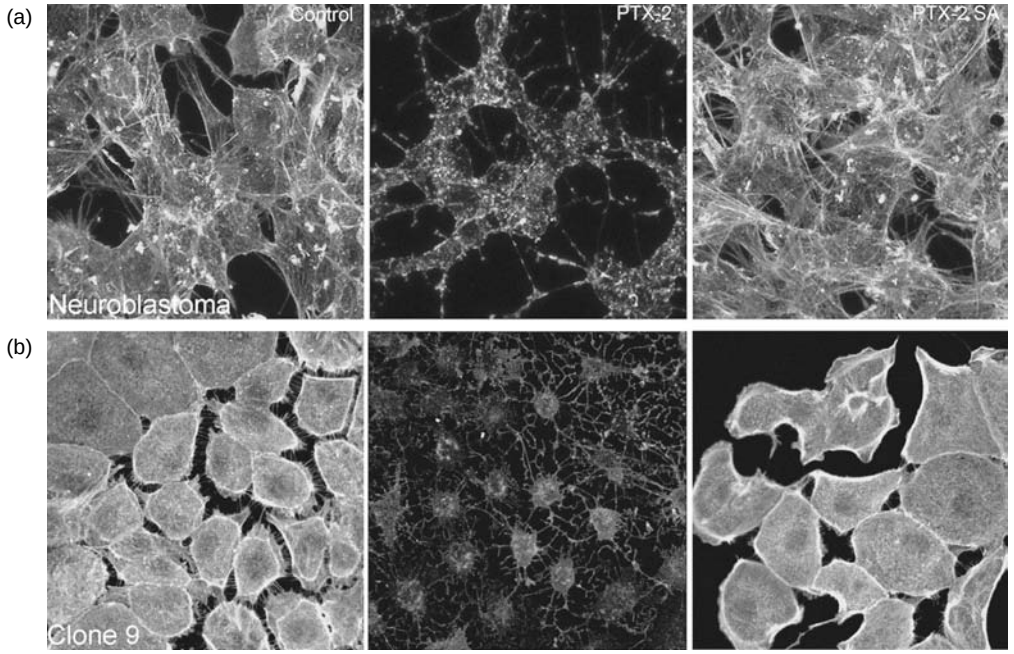

FIGURE 17.2 Effect of pectenotoxins on F-actin cytoskeleton. Confocal imaging of (a) BE(2)-M17 human neuroblastoma cells and (b) clone 9 rat hepatocarcinoma cells treated for 4 h, and 3 h, respectively with 200 nM PTX-2 or 200 nM PTX-2 SA. F-actin was stained with Oregon Green® 514 Phalloidin.

their pharmacokinetics and not so much by the cell type. In addition, not only do PTXs not need a cell-specific mechanism of membrane transport but their target also seems to be quite ubiquitous. The effective doses of PTXs for *in vitro* effects vary greatly with the cell type, although they are usually located in the nanomolar range. This different sensitivity among cell types to PTXs could be due to different levels of activation of their target.

17.5 PECTENOTOXINS MECHANISM OF ACTION

The mechanism of action of PTXs has not been completely elucidated yet, although the evidence collected since they were first described points to an involvement of the actin cytoskeleton in their effects at the cellular level. PTXs were initially classified as diarrheic shellfish poisoning toxins; however, it is generally accepted now that they do not share their mechanism of action with okadaic acid and dinophysistoxins, since PTX-1 and -6 do not inhibit protein phosphatases PP2A and PP1 [32,42]. Other aspects of cell physiology that were explored for PTX modulation but turned out not to be involved in the toxin mechanism of action were DNA synthesis, reduction–oxidation processes, intracellular calcium, or intracellular cAMP (3'-5-cyclic adenosine monophosphate) [36,38]. However, the effects of PTXs on actin cytoskeleton are well documented. The rearrangement of the actin cytoskeleton with a reduction of stress fibers and total polymerized actin has been found in various cell types, including chicken primary hepatocytes [33], rabbit primary enterocytes [35], human BE(2)-M17 neuroblastoma cells [19,38], human renal NRK52-cells [39], rat ahorta A7r5 cells [43], and clone 9 rat hepatocarcinoma cells [19]. As mentioned above, Figure 17.2 shows the effects of PTX-2 and -2SA on the organization of F-actin cytoskeleton of clone 9 rat hepatic carcinoma cells and BE(2)-M17 neuroblastoma cells. Laser scanning fluorescence measurements shown in Figure 17.3 reveal a clear decrease in F-actin in BE(2)-M17 neuroblastoma cells treated with PTX-2, but not with PTX-2SA. As a consequence of these effects on F-actin, cell functions dependent on cytoskeleton are impaired, such as contraction of isolated rat aorta or cytokinesis [37,39,44]. The reduction of the amount of

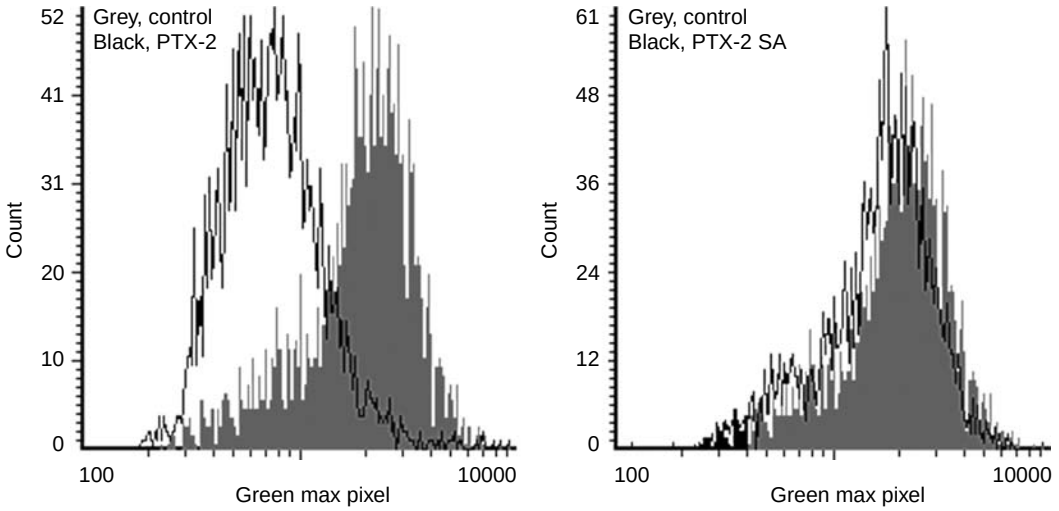

FIGURE 17.3 Effect of pectenotoxins on total amount of F-actin per cell in BE(2)-M17 human neuroblastoma cells. Histograms comparing the differences between the fluorescence intensity of cells stained with Oregon Green® 514 Phalloidin in the absence of toxin or treated for 4 h with 200 nM PTX-2 or 200 nM PTX-2 SA.

total F-actin [19,35,38,39] is accompanied by an increase in G-actin (globular actin) levels [38,39], as happens with other actin depolymerizing drugs [39]. To date, PTX-1, -2, -6, and -11 are the analogues with known actin cytoskeleton depolymerizing properties, while PTX-2SA lacks this activity [19,35,38,39]. Interestingly, the relative potency of these analogues mimics the data obtained in *in vivo* toxicology studies. The reduction of F-actin levels in live cells is probably related to the ability of PTXs, more specifically PTX-2, to inhibit actin polymerization *in vitro* [43,44]. Similarly to latrunculin, PTX-2 was suggested not to sever or cap actin filaments but to bind G-actin with a dissociation constant of 20 nM and a stoichiometry of 1:4 [39,44]. However, a direct interaction between G-actin and PTX has not been demonstrated until very recently [45]. New evidence suggests that the stoichiometry of the actin-PTX interaction is 1:1 and that PTX caps actin filaments [45]. Actually, although PTXs were postulated to have a similar mechanism of action to latrunculin, whose interaction with G-actin is well characterized, long-term effects of both drugs on the cytoskeleton are different with PTX-2 having some F-actin nucleation activity in the perinuclear region that is absent in latrunculin treated cells [39]. PTX-1 was also described to alter microtubule cytoskeleton inducing a decrease of microtubules and the loss of radial arrangement [33].

Cytotoxicity of PTXs has been reported in many cell types [31–33,36,37]. Actually, the induction of apoptotic cell death rather than necrosis by PTX-1 and PTX-2 is well established based on morphological changes, reduction of mitochondrial membrane potential, increase of cytoplasmic cytochrome C and Smac/DIABLO and caspase-3 and caspase-9 activation [32,37]. The inhibition of PTX-induced cell death by caspase inhibitors supports the apoptotic pathway [32,37]. PTX-6 was reported not to have any effect on mitochondrial membrane potential [38]; however, this is a late marker of apoptosis, and PTX-6 is not as potent as PTX-1 and -2. PTX-2-induced actin damage has been related to triggering of the above-mentioned death signals. However, the exact signaling route is unknown, although it seems to involve the sequential activation of the proapoptotic Bcl-2 family members Bim and Bax [37]. Calcium/calmodulin kinase II inhibition with KN-93 also decreases cell apoptosis induced by PTX-1, suggesting that this protein could be involved in the apoptotic route of PTXs [32]. Interestingly, PTX-2 exerts a higher cytotoxicity in p53-deficient tumor cell lines that might explain in part the difference in potency among cell types. Moreover, considering

that p53-deficient tumors are often resistant to cancer chemotherapeutic agents due to the reduction of apoptotic processes that involve triggering by p53, PTXs could be very useful as therapeutic tools in the treatment of certain cancers [37]. Actually, i.p. injection of PTX-2 reduced the growth of p53-null tumors induced by subcutaneous injection of tumor cells in nude mice [37].

The causal relationship of PTX actin depolymerizing activity with i.p. toxicity in mice has not been demonstrated as well as its causal relationship with apoptosis induction in tumor and primary cells. Further research on PTXs mechanism of action is necessary to elucidate if actin depolymerization is responsible for *in vivo* and *in vitro* effects of PTX, if PTXs have other cellular targets, and which cell types are involved in the *in vivo* toxicity of PTXs.

17.6 CONCLUSION

In vitro cytotoxicity assays have been the first step for the discovery of many drugs used in antitumor therapy. The selective cytotoxicity of PTXs against certain tumor cell lines and their ability to induce apoptosis of p53-deficient cell lines that are often resistant to other chemotherapeutic agents places them as interesting new candidates for cancer therapy. More information is needed about their toxicity to humans and their pharmacokinetics in order to make a better judgment about their usefulness in the management of the cancer patients and their possible side effects. Further investigation on the mechanism of action of PTXs will help understand toxicity and possible therapeutic benefits. The data on structure–activity relationship reveal that in many occasions metabolization of parental PTXs by shellfish diminishes the toxicity of the shellfish meat when tested by mouse bioassay. However, depending on the toxin profile of the algae bloom and the shellfish species that are contaminated, this conversion to less toxic compounds is more or less efficient and therefore in some conditions PTXs could be a source of positive results in the mouse bioassay test. Since these toxins do not seem to pose a health threat to human consumers; it is necessary to develop specific detection methods to avoid huge economic losses in the aquiculture sector.

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18 Toxicology of the Pectenotoxins

Rex Munday

CONTENTS

18.1 OCCURRENCE OF PECTENOTOXINS

Pectenotoxins (PTXs) are a family of macrocyclic polyethers that contaminate shellfish in many parts of the world, including Australia [1], Croatia [2,3], Ireland [4–7], Italy [6], Japan [8–13], New Zealand [14–17], Norway [5, 8], Portugal [19–22], Spain [23], Russia [24], and the United Kingdom [25].

Many pectenotoxin derivatives have been described. The primary source of all these substances appears to be algae of the genus *Dinophysis* and shellfish become contaminated by pectenotoxins after ingestion of such algae. The situation is complicated, however, by the fact that pectenotoxins are readily metabolized within the shellfish. Furthermore, some derivatives decompose during extraction procedures, leading to the formation of artifacts. The pectenotoxin derivatives that have been reported, and their origins, are listed in Table 18.1.

PTX-2, PTX-2b, PTX-2 seco acid, 7-*epi*-PTX-2 seco acid, PTX-11, PTX-11b, PTX-11x, PTX-11 seco acid, PTX-12, PTX-13, and PTX-14 have been isolated from various *Dinophysis* species, although PTX-2b and PTX-14 may be artifacts of the extraction process. In contrast, PTX-1, PTX-3, and PTX-6 are not present in the algae, but have been isolated from the Japanese scallop (*Patinopecten yessoensis*), in which the methyl group at position 34 of PTX-2 is progressively oxidized to an alcohol, aldehyde, and carboxylic acid. PTX-4 and PTX-7, the 7-epimers of PTX-1 and PTX-6, respectively, are also found in Japanese scallops. Although the pectenotoxin seco acids, in which the lactone ring is opened, are found in *Dinophysis* species, they are also produced from PTX-2 through enzymatic conversion in many shellfish species, including Greenshell mussels (*Perna canaliculus*) [14–17,26], blue mussels (*Mytilus edulis*) [6,22,26], Italian mussels (*Mytilus galloprovincialis*) [6], the New Zealand scallop (*Pecten novaezelandiae*) [27], oysters (*Crassostrea japonica*) [22], clams of the *Donax*, *Ruditapes*, *Venerupis*, and *Solen* genera [22] and the common cockle (*Cerastoderma edule*) [22], and in the green crab (*Carcinus maenas*) [22]. PTX-11 seco acid may be formed similarly in Greenshell mussels [17]. PTX-2c, PTX-11c, PTX-8, and PTX-9 have not been detected in nature, and are formed by acid-mediated isomerization of PTX-2, PTX-11, PTX-1 and -4, and PTX-6 and -7, respectively. Compounds described as PTX-5 and PTX-10 were isolated from Japanese scallops, but the structures of these substances have not yet been established. Similarly, PTX-11a, PTX-11b and PTX-11x, which appear to be stereoisomers of PTX-11, have not yet been fully characterized.

TABLE 18.1
Pectenotoxin Derivatives and Their Origins

Compound	Origin	References
PTX-1	Oxidation of PTX-2 in the Japanese scallop	[8,10,11,45]
PTX-2	*Dinophysis* spp.	[6–8,10,11,17,22, 23,26,65–69]
PTX-2b	*Dinophysis* spp (possible artefact) and acid-mediated isomerization of PTX-2	[67]
PTX-2c	Acid-mediated isomerization of PTX-2	[67]
PTX-3	Oxidation of PTX-2 in the Japanese scallop	[8,10,11,45]
PTX-4	Epimerization of PTX-1 in the Japanese scallop. Not an artefact	[13]
PTX-5	Isolated from Japanese scallopas. Structure unknown	[13,42]
PTX-6	Oxidation of PTX-2 in the Japanese scallop	[8,11,65]
PTX-7	Epimerization of PTX-6 in the Japanese scallop. Not an artefact	[13]
PTX-8	Artefactual isomerization product of PTX-1 and PTX-4	[13]
PTX-9	Artefactual isomerization product of PTX-6 and PTX-7	[13]
PTX-10	Isolated from Japanese scallopas. Structure unknown	[13,42]
PTX-11	*Dinophysis* spp.	[17,26,44,67,68]
PTX-11b	*Dinophysis* spp. and acid-mediated isomerization of PTX-11	[67]
PTX-11c	Acid-mediated isomerization of PTX-11	[67]
PTX-11x	*Dinophysis* spp.	[67]
PTX-12 (two isomers)	*Dinophysis* spp.	[18]
PTX-13	*Dinophysis* spp.	[70]
PTX-14	*Dinophysis* spp. Possible artefact	[70]
PTX-2 seco acid	*Dinophysis* spp. and metabolism of PTX-2 in mussels, New Zealand scallop, common cockle, oysters, clams, and crabs	[4,6,7,14,16,22, 26,27]
7-*epi*-PTX-2 seco acid	*Dinophysis* spp. and metabolism of PTX-2 in mussels, New Zealand scallop, common cockle, oysters, clams, and crabs	[4,6,7,14,16, 26,27]
PTX-11 seco acid	*Dinophysis* spp. and metabolism of PTX-11 in mussels (tentative finding)	[17]

18.2 TOXICITY OF PECTENOTOXINS TO CULTURED CELLS AND TO ISOLATED TISSUE *IN VITRO*

PTX-1 caused vacuolation in isolated rat hepatocytes *in vitro*, but unlike okadaic acid and dinophysistoxin-1, PTX-1 did not cause blebbing of the hepatocyte membrane, and it caused no membranal disruption, since no LDH leakage or Trypan Blue uptake occurred [28,29]. The cells maintained their spherical shape, but their surface was devoid of microvilli, and there was evidence for invagination of the cell membrane [29]. Incubation of primary cultures of hepatocytes from chick embryos with PTX-1 led to cell shrinkage, which was associated with loss of microtubules in the perinuclear regions, and the remaining microtubules lost their radial arrangement and became twisted [30]. Stress fibers disappeared from the cytoplasm, but cytoplasmic vacuolation was not observed. At low concentrations of PTX-1, the changes were reversible [30]. At a concentration of 2 μM, PTX-1 caused apoptosis in salmon and rat hepatocytes, associated with nuclear and cellular shrinkage [31]. The severity of these effects was similar in rat and salmon hepatocytes, and toxicity was ameliorated by the caspase inhibitor ZVAD-fmk and by a calcium/calmodulin kinase II inhibitor, KN-93 [31]. Again, no membrane disruption occurred, as indicated by the absence of Trypan Blue uptake [31].

The effects of pectenotoxins on the actin cytoskeleton in cells *in vitro* have been extensively studied. PTX-6 caused depolymerization of F-actin in neuroblastoma (BE[2]-M17) cells, although the total amount of actin in these cells, and in MCF-7 and HeLa cells exposed to PTX-6, remained unchanged [32]. This substance also disrupted F-actin in isolated rabbit enterocytes [33], rat kidney epithelial (NRK-52E) cells [34], and A-10 (rat myoblast) cells [35]. PTX-2 was also shown to cause

depolymerization of isolated F-actin [35] and to sequester monomeric actins [34–36]. Despite the cytoskeletal disruption, little effect on cell viability, biochemistry, or morphology has been observed in the aforementioned cells. PTX-6 caused no change in mitochondrial membrane potential, total DNA content, cytosolic calcium levels, or cell attachment of neuroblastoma cells. Apoptosis was not observed at any of the dose-levels investigated (1–10 μM), and cell viability was not compromised [32]. No effect on cell proliferation was observed in neuroblastoma, MCF-7, or HeLa cells incubated with PTX-6 [32], and no change in cell morphology was seen in neuroblastoma cells [32] or enterocytes [33] exposed to this substance or in A-10 cells incubated with PTX-2 [35].

PTX-2 was toxic to KB (human epidermoid carcinoma) cells *in vitro* at a concentration of 58 nM [14]. In contrast, no evidence of a toxic effect in these cells was seen with PTX-2 seco acid or 7-*epi*-PTX-2 seco acid at a concentration of 2 μM [14]. PTX-2 has been screened for selective cytotoxic activity against 60 human tumour cell lines by the US National Cancer Institute. It was selectively toxic to several cell lines of ovarian, renal, pulmonary, colonic, cerebral, and mammary tumors at nanomolar concentrations, but was ineffective against leukemia or prostate cancer cell lines [37]. PTX-2 was toxic to Vero (monkey renal epithelial) cells at a concentration of 0.58 μM [38]. At nanomolar concentrations, PTX-2 was not toxic to wild-type HCT116 (human colorectal carcinoma) cells, but decreased the viability of HCT116 lacking the transcription factor p53. Death of these cells was through apoptosis [39].

PTX-1 was inactive against the fungi *Aspergillus niger* and *Penicillium funiculosum* and against the bacteria *Staphylococcus aureus* and *Bacillus megaterium* [40].

PTX-2 inhibited the contractions elicited by potassium chloride or phenylephrine in the isolated rat aorta [35,36]. It did not cleave isolated DNA [37], and caused no disruption of electron transport across isolated rat liver plasma membranes [37], but it inhibited mitosis in mouse oocytes [39]. Neither PTX-1 nor PTX-6 inhibited purified protein phosphatases [41].

18.3 TOXICITY OF PECTENOTOXINS TO ANIMALS

18.3.1 Acute Toxicity

18.3.1.1 Acute Toxicity by Intraperitoneal Injection

The acute toxicities of pectenotoxin derivatives to mice by intraperitoneal injection are summarized in Table 18.2. PTX-1, PTX-2, PTX 3, and PTX-11 are highly toxic by injection, with lethal doses between 219 μg/kg and 411 μg/kg being reported [11,26,42–45]. PTX-4 and PTX-6 are less toxic, with minimum lethal doses of 770 μg/kg and 500 μg/kg, respectively [11]. PTX-7, PTX-8, PTX-9, PTX-2 seco acid, and 7-*epi*-PTX-2-seco acid are less toxic still, and no deaths were recorded in mice injected with these substances at a dose of 5000 μg/kg [13,26,46].

Symptoms of intoxication were observed within minutes of injection of toxic doses of PTX-2. The animals became hunched and lethargic and showed ataxia [26,43]. Subsequently, respiration became labored, with abdominal breathing, and the respiration rate progressively decreased [26]. Cyanosis and a decrease in body temperature were also recorded [43]. Deaths generally occurred between 4 h and 10 h after dosing, although one animal, receiving a dose close to the LD_{50}, survived for 22 h. At this time, it was in very poor condition, and was humanely killed [26].

PTX-1 did not cause diarrhea when injected intraperitoneally into suckling mice [47], and PTX-2 caused no diarrhea when injected into mature animals [26]. No diarrhea was observed in mice injected with high doses of PTX-2 seco acid [26,46] or 7-*epi*-PTX-2 seco acid [46], and the contents of the intestines of the treated animals were found to be entirely normal at necropsy. Furthermore, no change in intestinal weight was observed in these animals [46].

Yoon and Kim [43] reported a decrease in liver weight in mice injected with PTX-2 at a dose of 200 μg/kg. No significant changes were recorded in the weights of the liver, kidneys, spleen, heart, lungs, or intestine of mice dosed intraperitoneally with PTX-2 seco acid or 7-*epi*-PTX-2 seco acid at 5000 μg/kg [46].

TABLE 18.2
Acute Toxicity of Pectenotoxin Derivatives in Mice by Intraperitoneal Injection

Compound	Mouse Strain	Mouse Sex	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
PTX-1	?	?	MLD	250	[42]
PTX-2	?	?	MLD	260	[42]
PTX-2	?	?	MLD	230	[11]
PTX-2	ICR	Male	MLD	400	[43]
PTX-2	Swiss albino	Female	MLD	192	[26]
PTX-2	ICR	Male	LD_{50}	411	[43]
PTX-2	Swiss albino	Female	LD_{50}	219 (183–257)*	[26]
PTX-3	?	?	MLD	350	[45]
PTX-4	?	?	MLD	770	[11]
PTX-6	?	?	MLD	500	[11]
PTX-7	?	?	MLD	>5000	[13]
PTX-8	?	?	MLD	>5000	[13]
PTX-9	?	?	MLD	>5000	[13]
PTX-11	Swiss albino	Female	MLD	250	[44]
PTX-11	Swiss albino	Female	LD_{50}	244 (214–277)*	[44]
PTX-2 seco acid	Swiss albino	Female	MLD	>5000	[26,46]
7-*epi*-PTX-2 seco acid	Swiss albino	Female	MLD	>5000	[46]

* Figures in brackets indicate 95% confidence limits.

Terao et al. [47] reported that by 60 min after intraperitoneal injection of 1000 μg/kg of PTX-1, the surface of the liver of suckling mice was finely granulated. No macroscopic pathological changes were observed in mice injected with PTX-2 seco acid or 7-*epi*-PTX-2 seco acid at 5000 μg/kg [46].

Serum activities of alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase were significantly elevated in mice injected with PTX-2 at 100 μg/kg or above, but glucose-6-phosphatase activities were unchanged [43]. At a dose of 200 μg/kg, a significant decrease in total hepatic microsomal protein levels was observed, although concentrations of cytochrome P-450 and cytochrome b_5 and activities of NADPH-cytochrome c reductase and aminopyrine *N*-demethylase remained unchanged [43].

Terao et al. [47] observed multiple vacuoles in the periportal region of the livers of suckling mice 60 min after intraperitoneal injection of PTX-1 at 1000 μg/kg. The vacuoles were up to 15 μm in diameter, and did not contain fat. Similar changes were recorded in the livers of mice given 500 or 700 μg/kg of PTX-1 2 h after dosing, and eosinophilic granules were also seen in the vacuole-containing hepatocytes. Only minor changes were seen in the livers of mice receiving the test compound at 150 or 200 μg/kg. After 24 h, almost all hepatocytes containing numerous vacuoles and granules had become necrotic. Electron microscopy showed that the vacuoles resulted from invagination of the hepatocytic plasma membrane. No histological changes were observed in the small or large intestines of the mice, or in their kidneys or hearts [47]. In a later experiment, mice were dosed with pure PTX-2 intraperitoneally at 250 μg/kg. The animals died or were killed between 4.8 and 8.2 h after dosing. The major histological changes observed in these animals were splenic, renal, and hepatic congestion. Centrilobular vacuolar degeneration of the liver was also recorded, but no changes in the small or large intestine were observed [48]. No histological changes were recorded in the liver, diaphragm, duodenum, jejunum, colon, gastrocnemius, kidneys, adrenals, lungs, thyroid, trachea, heart, spleen, ovary, uterus, tongue, thymus, brain, pancreas, or urinary bladder of mice injected with PTX-2 seco acid or 7-*epi*-PTX-2 seco acid at 5000 μg/kg [46].

TABLE 18.3
Acute Toxicity of Pectenotoxin Derivatives in Mice by Oral Administration

Compound	Mouse Strain	Mouse Sex	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
PTX-2	ddY	Male	LD_{50}	~200*	[49]
PTX-2	Swiss albino	Female	MLD	>5000	[26]
PTX-2 seco acid	Swiss albino	Female	MLD	>5000	[26]
PTX-11	Swiss albino	Female	MLD	>5000	[44]

* This estimate is questionable because of the absence of a dose-response in this study (see text).

18.3.1.2 Acute Toxicity by Oral Administration

The acute toxicities of the pectenotoxins by oral administration are summarized in Table 18.3. On the basis of the study of Ogino et al. [49] it could be argued that the acute toxicity of PTX-2 administered by gavage is similar to that following intraperitoneal injection. The data of this study are difficult to interpret, however, since the incidence of death was not dose-dependent. The mortality recorded at a dose of 25 μg/kg (25%) was higher than that seen in mice given 100 μg/kg (0%) or 200 μg (20%) whereas that recorded at a dose of 400 μg/kg (25%) was lower than that seen at 300 μg/kg (40%). A later study did not confirm the apparently high oral toxicity of PTX-2. No deaths were observed at an oral dose of 5000 μg/kg, and the subsequent appearance and behavior of the dosed mice were entirely normal [26]. No signs of toxicity were recorded in mice given PTX-11 or PTX-2 seco acid by gavage at 5000 μg/kg [26,44].

PTX-1, given by gavage, did not cause diarrhea in suckling mice [38,50]. Similarly, mature mice dosed orally with PTX-2, PTX-2 seco acid, or PTX-11 showed no diarrhea [26,44]. No macroscopic changes were recorded in the intestines of mice dosed with these compounds, and the consistency of the intestinal contents of these animals was normal [48]. In contrast, Ishige et al. [51] reported viscous or watery diarrhea after oral administration of PTX-2. The diarrhea was associated with dilatation of the intestines, which were full of watery material. Furthermore, Burgess and Shaw [52] reported that a mixture of 35% PTX-2 seco acid and 65% 7-*epi*-PTX-2 seco acid caused diarrhea in mice after oral intubation. However, later work by the latter authors indicated that the previously-observed diarrhea was most likely due to contamination of the PTX-2 seco acids with okadaic acid esters [53], and no diarrhea or any other toxic effects were seen in mice dosed with a different batch of PTX-2 seco acid [54].

Histological changes in the intestine were recorded in those experiments in which diarrhea was reported [51,52]. Damage to the mucous membranes of the gastrointestinal tract, with vacuolation and necrosis of villi, was observed [51], while Burgess et al. [52] reported necrosis, edema, and hemorrhage of the glandular stomach of mice, together with necrosis of the microvilli of the duodenum, with extensive mucosal and submucosal hemorrhages. Some muscle damage also occurred in the duodenum, as reflected by edema and submuscular hemorrhage. If, as discussed above, the diarrhea sometimes observed with pectenotoxins is due to contamination with okadaic acid derivatives, this would also account for the changes recorded in the intestine. The histological changes reported in these studies resemble those described in animals receiving okadaic acid [55].

18.3.1.3 Acute Toxicity in Humans

It was suggested [56] that pectenotoxins could have been involved in outbreaks of human illness in Australia in 1997 and 2000. The symptoms of intoxication were nausea, vomiting, and diarrhoea, and shellfish responsible for the toxic effects were found to contain PTX-2 seco acid. However,

later work showed that the shellfish also contained okadaic acid esters at concentrations sufficient to cause human intoxication [54], and it is therefore likely that the symptoms observed in these outbreaks were caused not by pectenotoxins but by derivatives of okadaic acid.

18.3.2 Toxicity to Animals after Repeated Administration

Little information on the subacute toxicity of the pectenotoxins is available. Repeated intraperitoneal injections of 20 or 100 μg/kg of PTX-2 in mice over a 1- or 2-week period caused no deaths or changes in serum biochemical parameters indicative of hepatic or renal toxicity [57]. No changes in hepatic microsomal levels of cytochrome P-450 or cytochrome b_5 were observed, and activities of NADPH-cytochrome c reductase and aminopyrine *N*-demethylase were normal, although a slight decrease in the activity of *p*-nitroanisole *N*-demethylase was recorded. At a dose of 200 μg/kg/day, however, 50% of the animals died, and serum activities of alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase were significantly elevated, as were serum levels of urea [57]. Effects of modulators of P-450 enzymes on mortality were inconsistent. Phenobarbital, an inducer of these enzymes, decreased mortality, while a second inducer, 3-methylcholanthrene, had no effect. Two inhibitors of the P-450 enzymes, cobaltous chloride and SKF-525A, were also tested. The former decreased the death rate after PTX-2 administration, while the latter increased it. None of the inhibitors or inducers significantly influenced the changes in serum biochemical parameters induced by PTX-2 [57]. Intraperitoneal injection of PTX-2 at 100 μg/kg/day for 20 days in athymic nude mice was without effect on body weights, but administration of PTX-2 decreased the size of p53-deficient tumors established in these mice [39].

18.4 EVALUATION AND RECOMMENDATIONS FOR FUTURE STUDIES

Certain pectenotoxin derivatives are acutely toxic to mice after intraperitoneal injection. The degree of toxicity is, however, strongly dependent upon structure. PTX-1, -2, -3, and -6 differ only with regard to the substituent at C-43. In PTX-2, the substituent is CH_3, and this substance is one of the more toxic derivatives. Oxidation of the methyl group to CH_2OH, forming PTX-1, or to the aldehyde, forming PTX-3, has little impact upon toxicity, but further oxidation to the carboxylic acid (PTX-6) decreases toxicity. Epimerization at C-7 greatly decreases toxicity. PTX-4 (7-*epi*-PTX-1) is less toxic than PTX-1, and PTX-7 (7-*epi*-PTX-6) is less toxic than PTX-6. In contrast, the acute toxicity of PTX-11 (34-β-hydroxy PTX-2) was not significantly different from that of PTX-2, indicating that such substitution at the 34 position is without major effect on toxicity. Opening of the lactone ring of PTX-2, to yield PTX-2 seco acid, caused a pronounced decrease in toxicity. The epimer, 7-*epi*-PTX-2 seco acid, was also of very low toxicity.

The liver appears to be a major target organ of the pectenotoxins after administration by injection. Hepatic congestion and vacuolar degeneration of hepatocytes was recorded with both PTX-1 and PTX-2 after intraperitoneal injection. The observation of elevated serum activities of alanine aminotransferase, aspartate amino-transferase, and sorbitol dehydrogenase is also consistent with hepatic damage. No histological changes attributable to pectenotoxins have been identified in tissues other than the liver, although the increase in serum levels of urea recorded in mice given repetitive doses of PTX-2 indicates the possibility of a nephrotoxic effect of this compound under these conditions.

The mechanism whereby pectenotoxins cause death in experimental animals is not presently known. The rather mild hepatic changes recorded in animals given high doses of PTX-1 and -2 are unlikely to be responsible, since rodents are able to survive severe hepatic necrosis [58,59]. The symptoms of intoxication in mice suggest an adverse effect on the respiratory system, although no pulmonary changes have been recorded as sequelae of pectenotoxin intoxication. Interestingly, effects of PTX-2 in isolated hepatocytes (vacuolation, membranal invagination) closely mimicked those seen *in vivo*. Like many other natural products [60], pectenotoxins cause damage to the

cytoskeleton in several cell types. However, the relationship of such changes to toxicity *in vivo* is presently unclear, and studies on the potential of the pectenotoxins to interact with the cytoskeleton in the living animal are needed. Such studies may be highly significant, since there is evidence that effects on the actin cytoskeleton are important in cancer chemotherapy [60]. Furthermore, the selective toxicity of PTX-2 to certain cancer cells suggests that further studies on the *in vivo* anticancer activities of the pectenotoxins would be valuable.

It is unfortunate that so many of the studies on pectenotoxin toxicity in animals have focused on administration by intraperitoneal injection, since such data are of limited value in assessing the risk of these substances to human health. Pectenotoxins are eaten by humans, and oral data from animal experiments are therefore much more valuable in assessing possible harmful effects to consumers.

Recent data indicate that PTX-2 is much less toxic orally than by intraperitoneal injection. Although early studies suggested that PTX-2 was orally toxic, these data are questionable because of the absence of a dose response–relationship. In a later study, no deaths or other changes were recorded with PTX-2 at a dose of 5000 μg/kg. The low oral toxicity of PTX-2 may reflect poor absorption from the gastrointestinal tract or conversion to a less toxic material, such as PTX-2 seco acid, in the gut [26].

Little information on the metabolism of the pectenotoxins is available. A major role for the cytochrome system seems improbable in view of the observation that PTX-2 did not increase the activities or levels of components of this system and no consistent effects on toxicity were seen with modulators of the P-450 enzymes. PTX-2 is enzymatically converted to the seco acid within shellfish tissues. Mammalian tissues similarly contain lactonases [61], so a similar conversion of pectenotoxins to the corresponding seco acids is feasible. Studies of the absorption and metabolism of pectenotoxins after oral administration to animals would be valuable.

Because the pectenotoxins occur in association with okadaic acid and the dinophysistoxins, they were classified within the diarrhetic shellfish poison group [62]. However, most available information now indicates that these compounds do not cause diarrhea in experimental animals. No diarrhoea was seen in mice receiving pure PTX-2, PTX-11, or PTX-2 seco acids. It was shown, however, that the C_8-diol ester of okadaic acid is co-extracted with the pectenotoxins, so that without careful purification, contamination of the latter with diarrhetic substances is likely [26].

There is no evidence that pectenotoxin derivatives *per se* have caused toxicity problems in humans. While it was suggested that such compounds could be involved in cases of human poisoning in Australia, the evidence now indicates that okadaic acid esters, rather than pectenotoxins, were responsible.

The recent data on the oral toxicity of PTX-2, -11, and -2 seco acid allow comment on the acute reference doses of these compounds and hence tolerable levels for their presence in shellfish. No effects were recorded with any of these compounds after administration to mice by gavage at 5000 μg/kg. The lethal dose of these compounds is therefore greater than 5000 μg/kg. Application of the standard 100-fold safety factor [63] gives a dose of >50 μg/kg as a predicted nontoxic acute dose for a human. This equates to >3000 μg for a 60 kg adult, and if a shellfish intake of 380 g in a single meal is assumed, the predicted safe level in shellfish is >7.9 mg/kg.

Pectenotoxins are currently regulated against, and the European Union allows a maximum total of 160 μg of okadaic acid, dinophysistoxins, and pectenotoxin per kilogram of shellfish [64]. However, since the pectenotoxins do not belong in the diarrhetic shellfish poison group, a re-evaluation of this regulation would be appropriate, and, if it is considered necessary, regulatory limits for pectenotoxins alone should be set.

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Part VII

Domoic Acid Intoxication

19 Ecobiology of Amnesic Shellfish Toxin Producing Diatoms

Yuichi Kotaki

CONTENTS

19.1 INTRODUCTION

Domoic acid (DA) was first recognized as the causative toxin of the amnesic shellfish poisoning (ASP) accident that occurred in Canada in 1987.[1] Before this accident, DA had been recognized only as an insecticidal compound extracted from red alga, *Chondria armata*.[2] Its derivatives, isodomoic acids A, B and C[3] domoilactone A and B[4], and isodomoic acids G and H[5] have also been isolated from *C. armata*. However, the whole original ASP toxin profile has not been elucidated. After the Canadian ASP accident, the primary source of the toxin was traced to a pennate diatom *Pseudo-nitzschia multiseries*.[6] During the study, small amounts of isodomoic acids D, E, F, and 5′-*epi*-DA have been confirmed in *P. multiseries* and in shellfish that accumulated toxins from the diatom.[7] Structures of some of the toxins are shown in Figure 19.1.

After the finding of *P. multiseries*, screenings of pennate diatom that produces DA were performed mainly in temperate and cold-water areas, and resulted in reporting several kinds of *Pseudo-nitzschia* spp. as described in the next section (Table 19.1).

During the survey to screen the DA-producing diatom in tropical waters, a benthic diatom *Nitzschia navis-varingica* was isolated as a major DA producer and identified as a new species (Figure 19.3).[8,9] Detailed screening of the diatom performed later showed that *N. navis-varingica* produces significant level of DA and distributes widely in brackish water areas in eastern Asia.[10] And during the survey, this diatom was found to produce not only DA but also isodomoic acids

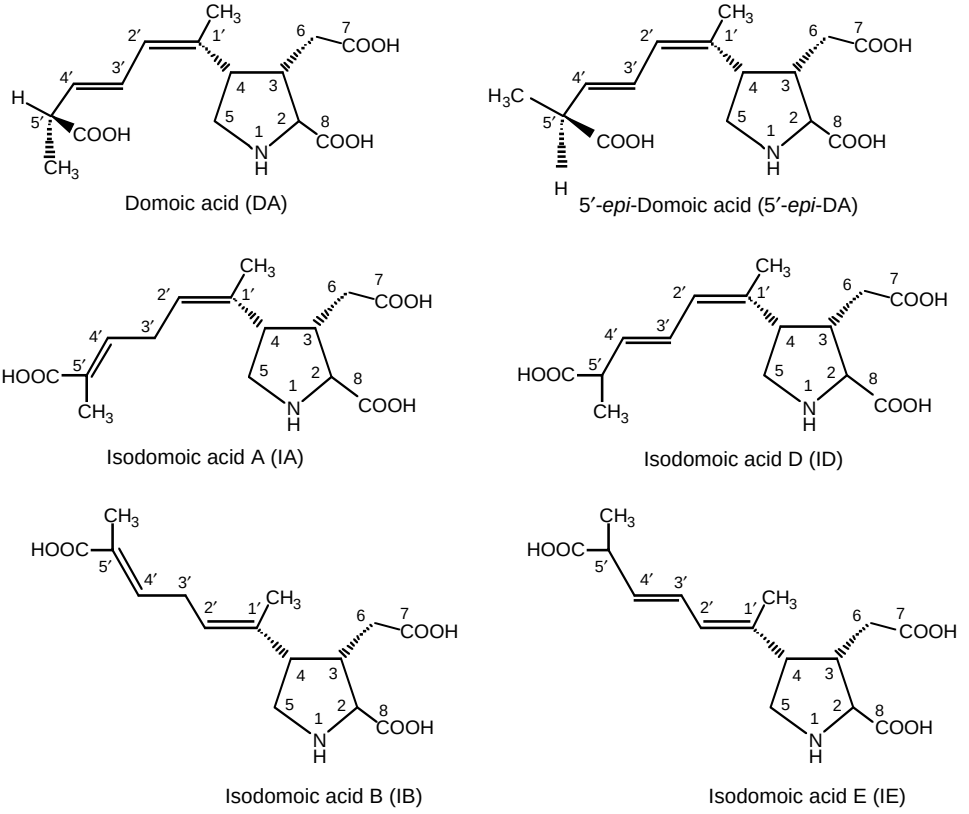

FIGURE 19.1 Structure of amnesic shellfish toxins.

A and B as major toxin components.[11] More detailed distribution and ASP toxin profiles of *N. navis-varingica* were recently investigated.[12]

In this chapter, a detailed story of this topic and the characteristics of ASP toxin production by *N. navis-varingica* are mainly described. In addition, newly investigated ASP toxin profile of some *Pseudo-nitzschian* spp. and original ASP toxin profile of *C. armata* are also described.

19.2 AMNESIC SHELLFISH TOXIN-PRODUCING ORGANISMS

19.2.1 Genus *Pseudo-nitzchia*

After the *P. multiseries* was first found as the causative organism of ASP toxin contaminated shellfish, many efforts to screen the DA-producing pennate diatoms were extensively done in recent years. More than several species of *Pseudo-nitzschia* were reported as shown in Table 19.1.[13–19] *Pseudo-nitzschia australis* and *Pseudo-nitzschia seriata* were also found as DA producers.[13,14] Especially *P. australis* is famous for its high toxicity responsible for the deaths of sea birds (pelican and cormorants) and sea lions, which ate DA-contaminated anchovies.[20–22] These three species are recognized as major DA producers (in the highest DA producers, maximum DA level is higher than approximately 10 pg/cell). The other *Pseudo-nitzschia* species including *Pseudo-nitzschia pseudo-delicatissima*, *Pseudo-nitzschia delicatissima*, and several more species showed low DA levels (in low DA producers, maximum DA level is lower than approximately 1 pg/cell). We also screened

TABLE 19.1
Domoic Acid Producing Diatoms

Diatom	DA Level[a]	Country[b]	Reference[b]
Pseudo-nitzschia			
multi series	High	Canada	6
australis	High	USA	13
seriata	High	Denmark	14
delicatissima	Low	New Zealand	15
pseudodelicatissima	Low	Canada	16
pungens	Low	New Zealand	15
turgidula	Low	New Zealand	15
fraudulenta	Low	New Zealand	17
multistriata	Low	Italy	18
cuspidata	Low	Japan	34,35
subpacifica	Low	Japan	34,35
subfraudulenta	Low	Japan	34,35
heimii	Low	Japan	34,35
Pseudo-nitzschia spp. (unidentified)	Low	Japan	34,35
Amphora coffeaeformis	Low	Canada	19
Nitzschia navis-varingica	Medium	Vietnam	8

High: >10 pg/cell, medium: 1–10 pg/cell, low: <1 pg/cell.

[a] Maximum domoic acid (DA) content of the diatom culture.
[b] First report.

DA-producing *Pseudo-nitzschia* in Ofunato Bay, Japan and reported that *P. multiseries* do exist there and its isolate produces high levels of DA in culture.[23] In addition to *P. pseudodelicatissima*, *P. delicatissima*, *Pseudo-nitzschia pungens*, *Pseudo-nitzschia turgidula*, and *Pseudo-nitzschia fraudulenta* that had been already reported as DA producer, *Pseudo-nitzschia cuspidate*, *Pseudo-nitzschia subpacifica*, *Pseudo-nitzschia subfraudulenta*, and *Pseudo-nitzschia heimii*, and two unidentified *Pseudo-nitzschia* were newly found to produce low levels of DA by culture experiment. The screening of *Pseudo-nitzschia* was performed for the production of DA[34,35] but not for DA derivatives. Recently, production of isodomoic acid C simultaneously with DA was reported in a *P. australis* culture.[24] Mechanism controlling the toxin composition seemed interesting but has not been solved so far.

19.2.2 Genus *Nitzschia*

19.2.2.1 Finding of *N. navis-varingica*, the First Major Domoic Acid-Producing Diatom Other than *Pseudo-nitzschia* Spp.

During the survey of DA-producing *Pseudo-nitzschia*, samples were collected by scoop net (mesh size of 20 μm) from the old shrimp pond, Do Son, Vietnam, followed by finding a DA-producing *Nitzschia*.[8] An aliquot (0.1 L^{-1} mL) of the net samples was inoculated to *f*/2 medium, brought back to our laboratory, and then pennate diatoms were isolated by capillary washing method under light microscope. In our study, *f*/2 medium[25] is usually made using 80% sea water (final conc. ca. 27 psu) obtained from Okkirai Bay, located next to our college. Isolates were inoculated into 30 mL *f*/2 medium in 50 mL tissue culture tube and cultured at 25°C under 60 μmol photons/m^2/sec with the L:D cycle of 16:8. Cell growth was monitored by *in vivo* chlorophyll *a* fluorescence using a

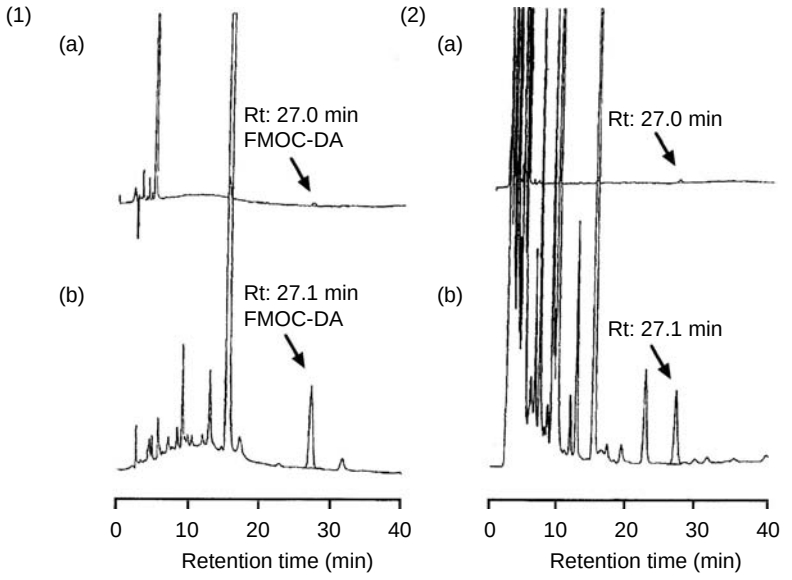

FIGURE 19.2 HPLC-fluorescence analysis of domoic acid (DA). (1) Standard DA (50 ng/mL). (2) The extract of *Nitzschia navis-varingica* (VSP 974-1) isolated from Vietnam. (a) Detected by UV monitor (242 nm). (b) Detected by fluorescence monitor (Ex. 264 nm, Em. 313 nm).

hand-made fluorometer. The DA production of the cultures were tested 10 days after they reached the stationary growth phase for the whole culture using an high performance liquid chromatography (HPLC) analysis according to Kotaki et al.[8], which is a slightly modified method of Pocklington et al.[26] Detection of DA was done by fluorescence detector after being derivatized with FMOC-Cl reagent (Figure 19.2). The toxicity was expressed as ng/mL whole culture. Two types of pennate diatoms were isolated from these samples. One type of diatom always showed the clear peak identical to DA whereas the other type did not. The DA positive diatoms were identified by N. Lundholm as a new species *N. navis-varingica*.[9] The morphological characteristics are described in this section. The other type of diatom isolates were not identified. Confirmation of the compound corresponding to DA peak was done by electrospray ionization-mass spectrometry (ESI-MS) after purifying the cell extract of 3 L mass culture. DA amount at day 15 of the cell fraction and cell-free medium fraction of the mass culture were 64 ng/mL and 11 ng/mL, respectively. The purified compound showed an ion peak at *m/z* 312 that is identical to the $[M+H]^+$ of DA.

DA production of *P. multiseries* has been intensively studied to solve the toxin production mechanism by culture experiments. These data showed that DA is mainly produced during the stationary growth phase.[6,27] However, substantial level of DA is produced during the exponential growth phase in case of *N. navis-varingica* culture. Another interesting characteristic of DA production of *P. multiseries* is that high levels of DA produced during the late stationary growth phase were observed only in nonaxenic cultures, whereas substantially less DA is always observed in axenic cultures. Douglas et al.[28] and Bates et al.[29] reported that DA amount produced by axenic *P. multiseries* cultures are 1/20 or less than that produced by nonaxenic cultures. In addition, the reintroduction of bacteria to the axenic culture of *P. multiseries* enhanced the DA production by 2- to 115-fold. These indicate that bacteria are involved in one of the triggering mechanisms of DA production by *P. multiseries*.

DA production was measured both in axenic and in nonaxenic cultures of *N. navis-varingica* (Figure 19.3). The toxicity was expressed as picogram per cell that was obtained by calculation using the data of toxin concentration in whole culture, in the filtrate (ng/mL), and of the cell concentration (cells/mL). The maximum cellular DA in nonaxenic culture (1.7 pg/cell) was within the levels reported for *P. multiseries* (0.1–21.0 pg/cell) and *P. seriata* (0.3–33.6 pg/cell) but less than that of *P. australis* (12.0–37.0 pg/cell) (Bates 1998). The maximum cellular DA in axenic culture was

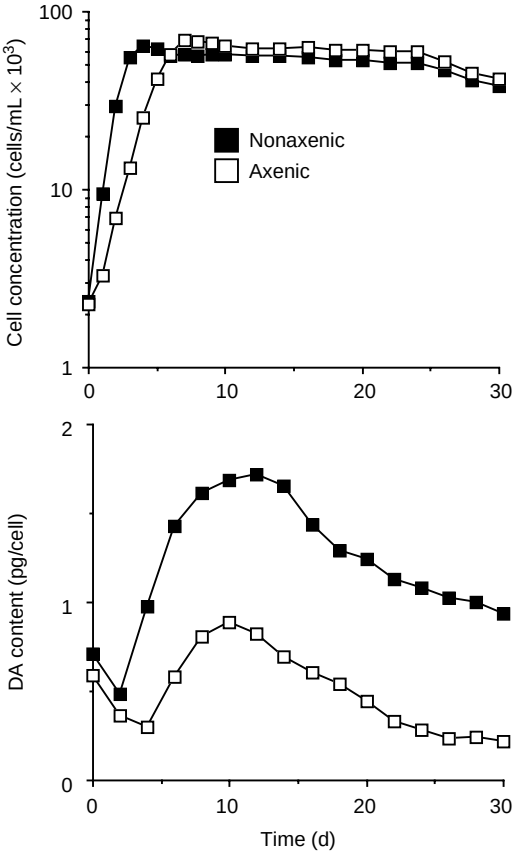

FIGURE 19.3 DA production of axenic and nonaxenic culture of *Nitzschia navis-varingica*.

approximately half (0.9 pg/cell) that of the above nonaxenic culture (Figure 19.3). This experiment was repeated and similar result was obtained. This result shows that bacterial effect on enhancing the DA production of *N. navis-varingica* is not so obvious as that of *P. multiseries*.

Fixed samples of the representative isolates positive for DA production were sent to N. Lundholm for the morphological study. Live isolates were also sent later for the entire observation of the cell.

Morphological observation for the identification of *N. navis-varingica* as a new species was done by N. Lundholm.[9] The cells of the pennate diatoms were yellow-brown and possessd two chloroplasts at each end of the cells. Cells were lanceolate in valve veiw, 38–110 µm long and 9–11 µm wide and rectangular in girdle view in the middle. The pervalver axis is wider than the transapical axis. Most cells make very short ribbon-shaped colonies while growing. Using TEM, the density of the striae, the fibulae and the stria poroids was found to be 26–30 per 10 µm, 10–12 per 10 µm and 3–4 per 1 µm, respectively. The raphe is raised on a distict keel, with the margins of the raphe slit extended as lips that flap outwards distally. The wall of the raphe canal is perforated by areolae and bearing longitudinal silica ridges. A pattern of vermiform ridges is present on the mantle. The singular bands are open and ornamented with wartlike structures. The valvocopula has 2–3 rows of areolae, the remaining copulae have 1–2 rows of areolae, with or without scattered areolae (Figure 19.4).[8,9]

19.2.2.2 Distribution of *N. navis-varingica*[10]

As *N. navis-varingica* isolates were obtained from a shrimp culture pond in brackish water area in Vietnam,[8] expanded screening of DA-producing *Nitzschia* was performed mainly in brackish water areas in Japan and the Philippines.[10]

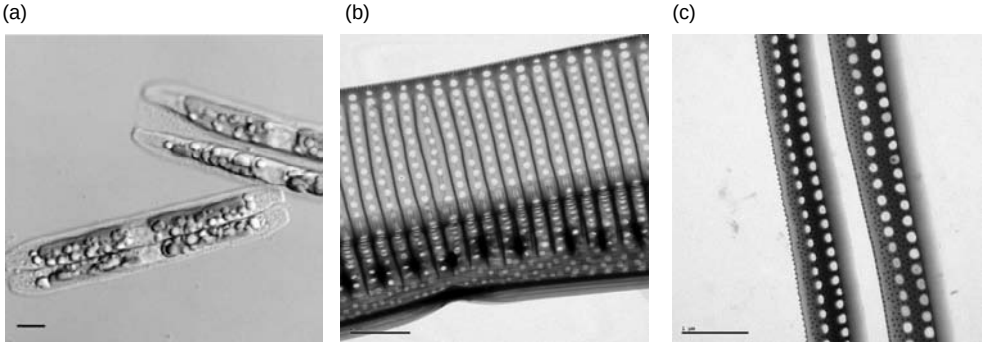

FIGURE 19.4 Photograph of *Nitzschia navis-varingica*. (a) Light microscopy. Bar; 10 mm. (b and c) Transmission electron microscopy provided by Dr. Nina Lundholm (Copenhagen University, Denmark). Bars; 1 mm. a) cells in girdle view. b) middle of valve showing indention of valve, vermiform ridges on raphe wall (below) and striae with one row of poroids. c) bands (valvocopulae) with two rows of poroids and wartlike silica structures.

Nitzschia-like diatoms were collected at two estuarine and two seawater areas of Ishigaki Island (subtropical area), Okinawa, and four estuarine areas of Tohoku district (temperate area) in Japan. Fresh water diatom samples were also collected from the area 2 km above one of the river mouths (Sakari River, Tohoku District, Japan) to examine the possibility of distribution of the *N. navis-varingica* there. Samples were also collected from one estuarine area in Manila Bay, the Philippines. Handy scoop net (mesh size of 20 μm) was used for collecting the diatoms weakly attached to the reed or mangrove root. An aliquot of (0.1–1 mL) of the samples was inoculated into medium to make crude cultures. Isolation of the *Nitzschia*-like diatom was performed from these cultures under light microscope. At least a few or several subsites were included in each sampling area. Isolation was performed directly from the net samples when *Nitzschia*-like diatoms could be observed frequently. Analyses of DA were done as described in Section 19.2.1. In this screening, DA content was expressed as cellular DA (pg per cell basis) calculated by using the data of whole culture DA concentration (pg/mL) and cell concentration (cells/mL).

From Ishigaki Island, Japan, many *Nitzschia*-like diatoms were isolated from estuarine areas, especially from Nagura estuary where 32 out of 47 isolates were positive for DA production. Their DA content range was 0.1–2.8 (average 0.7) pg/cell. All of the DA-positive diatoms were identified as *N. navis-varingica* by N. Lundholm, Denmark. *Nitzschia pellucida*, *Entomoneis* spp., and unidentified pennate diatoms were included in the 15 isolates negative for DA production. In spite of the repeated culture experiments after that, *N. pellucida* and *Entomoneis* spp. never showed DA production (unpublished data). All of the 13 isolates obtained from seawater area in Ishigaki Island were negative for DA production. These were *Nitzschia*-like diatoms but not *N. navis-varingica*.

In Tohoku district, 5 out of 25 isolates positive for DA production (2.1–3.9, average 3.0 pg/cell) were obtained from Natori estuarine area and 41 out of 41 isolates positive for DA production (1.3–4.2, average 2.1 pg/cell) were also obtained from Sakari estuary, near our college. All of the 13 *Nitzschia*-like isolates obtained from fresh water areas (2 km above from river mouth) were negative for DA production. These were not *N. navis-varingica*.

In the Philippines, ten out of twenty-seven *Nitzschia*-like isolates showed the significant level of DA production (1.4–15.3, average 5.6 pg/cell). All of the DA-positive isolates were identified as *N. navis-varingica*. Other isolates negative for DA production were not *N. navis-varingica*.

Shimizu et al. reported that *Amphora coffeformis* also produced small amounts of DA.[19] Although DA amount of *N. navis-varingica* shows the locality, the DA amount is comparable to that of *P. multiseries*, the famous species that causes ASP accidents by the consumption of shellfish contaminated with DA. These results show that *N. navis-varingica* is a major DA-producing diatom

and distributed widely in brackish water areas in eastern Asia. There is no report on DA contamination in the brackish water animals; however, it could be possible for these animals to accumulate DA from *N. navis-varingica* through food webs. As *N. navis-varingica* is a diatom weakly adhered to the mangrove root, it could be easily released by strong stream of water and flow down into seawater benthos in the bay followed by contaminating the animals there. A culture experiment on cell growth and DA production of *N. navis-varingica* was performed under conditions of several seawater ratio in *f*/2 medium. The *Nitzschia* could grow and produce DA at the seawater range of 20%–100% (7–34 psu) after getting acclimated (unpublished data). This experiment supports the earlier possibility of DA contamination of sea animals in benthos.

19.2.2.3 *N. navis-varingica* Produces Isodomoic Acids A and B and Domoic Acid[11]

To obtain more detailed information about the distribution of *N. navis-varingica* reported as a major DA producer in south eastern Asia, expanded screening of DA-producing *N. navis-varingica* was performed in the Philippines. Collection of *Nitzschia*-like diatom was done at Tanauan and San Roque estuary in San Pedro Bay near Tacloban city and at Bacoor and Bulacan estuary in Manila Bay. Collection, isolation, and culture experiment for the screening of DA-producing diatom were performed as described in the Section 19.2.2. Species identification was done morphologically by N. Lundholm, Denmark, according to Lundholm and Moestrup.[9] In addition to the identification, representative strains were analyzed for a region (D1–D3) of the nuclear large subunit rDNA gene (LSU rDNA) according to Lundholm et al.[30]

About 30 out of 31 isolates from the estuarine areas of San Pedro Bay were positive for DA production (0.04–5.3, average 3.1 pg/cell). Five out of six isolates from Bacoor estuary were also positive for DA production (3.1–5.3, average 3.8 pg/cell). All of them were morphologically identified as *N. navis-varingica*. Although 21 isolates from Bulacan estuary were identical to *N. navis-varingica* by both morphological observation and LSU rDNA analysis, none of them showed DA peak in the HPLC chromatogram. However, two peaks that seemed to be DA derivatives were observed in the chromatogram (Peaks 1 and 2 in Figure 19.5).

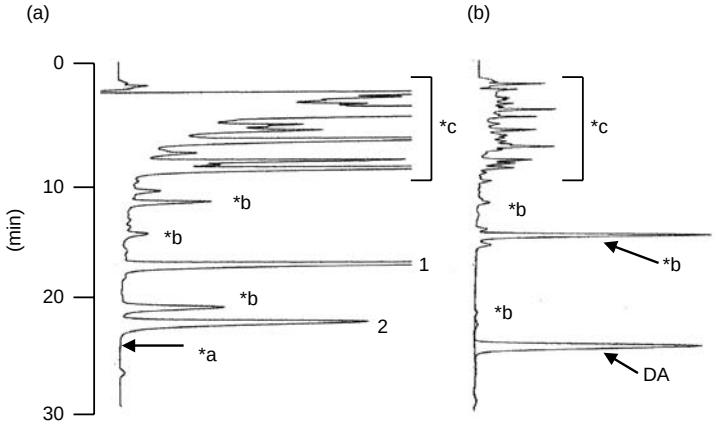

FIGURE 19.5 HPLC-fluorescence analysis of the extract of *N. navis-varingica* isolated from Bulacan estuary (a) and of the culture medium spiked with DA (59 ng/mL) (b). 1; unknown peak, 2; unknown peak,*a; Lack of DA peak, *b; Side products of the FMOC-Cl reagent Pocklington et al. (1990) *c; Reaction products derived from contaminants in the medium and other compounds in the culture (e.g., neutral amino acids) that react with FMOC-Cl reagent. Analytical conditions: Column; Develosil ODS-5 (Nomura, 4.6 mm × 250 mm), Mobile phase; 40% acetonitrile in 20 mM phosphate buffer (pH 2.5), Column temperature; 55°C, Flow rate; 1 mL/min, Fluorescent detection; Ex. 264, Em. 313 nm.

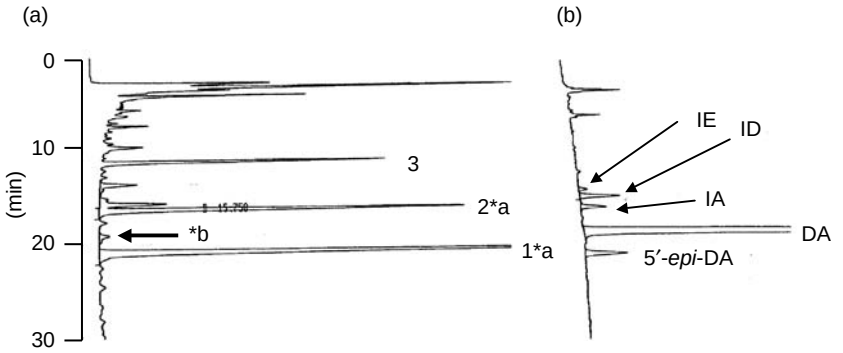

FIGURE 19.6 HPLC-UV analysis of the extract of *N. navis-varingica* isolated from Bulacan estuary (a) and the Canadian standard (DACS-1-D) (b). 1; unknown peak, 2; same retention time as IA, 3; same retention time as tryptophan. Analytical conditions: Column; Develosil ODS-5 (Nomura, 4.6 mm × 250 mm), mobile phase; 10% acetonitrile in 20 mM phosphate buffer (pH 2.5), column temperature; 35°C, Flow rate; 0.8 mL/min, UV detection; 242 nm. *a; Elution order of peaks 1 and 2 in this analysis was reverse of the HPLC-fluorescence analysis in which DA and isomers are derivatized with FMOC-Cl reagent. *b; Lack of domoic acid peak.

To confirm that the substances correspond to the peaks, one isolate from the Bulacan estuary was mass cultured in *f*/2 medium for 3 weeks. The cell fraction was extracted with 50% methanol by ultrasonication. The extract was purified successively by a Wakosil 25 C18 column chromatography (Wako Chemical Co. Ltd, 10 mm × 450 mm, mobile phase; acetonitrile:actic acid:water = 10:1:90) and Develosil ODS-5 preparative HPLC (Nomura, 8.0 mm × 250 mm, mobile phase; acetonitrile: actic acid: water = 10:1:90). During the purification, corresponding components were monitored by HPLC-ultraviolet (UV) analysis.[31,32] Detection was done with UV absorption of 242 nm that depends on the conjugated diene structure. Although some isomers do not have this conjugated diene structure, it could also be detected by the end absorption that depends on the three carboxilic moieties. No DA peak but three other peaks (peaks 1, 2, and 3) were shown in the chromatogram (Figure 19.6). Each of the retention times of peak 2 and 3 was identical to that of isodomoic acid A that is included in the commercial standard DACS-1-D (National Research Council, Canada) and that of standard tryptophan, respectively. Half-purified sample obtained from Sep-Pak C18 treatment was also analyzed by LC-MS/MS (Applied Biosystem, API-2000). Peaks 1 and 2 showed a positive ion peak of 312, identical to the $[M+H]^+$ of DA or DA derivatives with the typical fragment ion peak of 266, 248, 220, and 161. Peak 3 showed the positive ion peak of 205 identical to the $[M+H]^+$ of tryptophan. The half-purified compounds corresponding to peaks 1 and 2 were further purified, and finally 160 μg of peak 1 and 60 μg of peak 2 were obtained. The UV spectra of these two compounds showed the maximum peak at 220 nm, indicating that they do not have the conjugated diene structure as with DA. The proton nuclear magnetic resonance (NMR) spectrum of peak 1 is shown in Figures 19.7 and 19.8, supported by ^{13}C NMR to confirm the assignment (data not shown). The proton NMR spectrum of the peak 1 compound coincided well with that of isodomoic acid B reported by Maeda et al.,[3] indicating that the peak 1 compound is isodomoic acid B. The NMR data of peak 2 compound also coincided with that of isodomoic acid A reported by Maeda et al.,[3] indicating that the peak 2 compound is isodomoic acid A. These data show that peaks 1, 2, and 3 included in the extract of mass culture of one isolate from Bulacan estuary, Manila Bay, the Philippines were isodomoic acids B, A and tryptophan, respectively.

This study shows that *N. navis-varingica* isolated from Bulacan estuary, Manila Bay does not produce DA but does produce isodomoic acids A and B as major toxin components. After this confirmation, the chromatograms of the culture experiments were reobserved to clarify the toxin composition of *N. navis-varingica* isolates obtained from several areas in the Philippines. The chromatograms

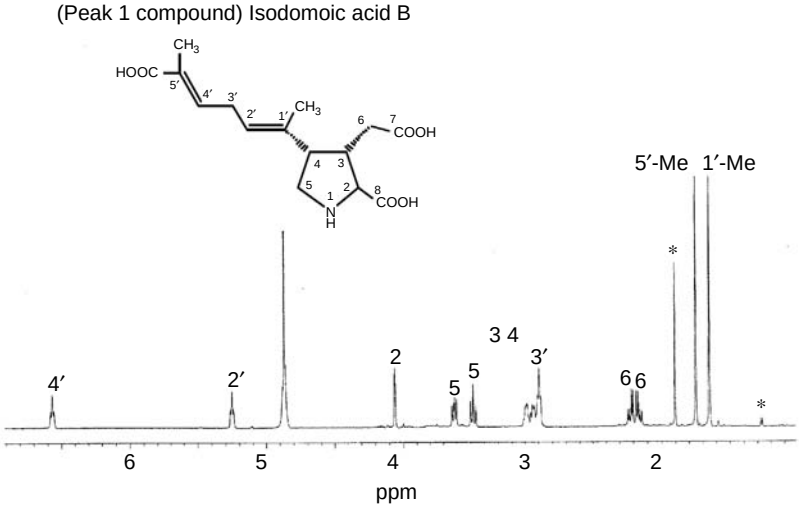

FIGURE 19.7 Proton NMR of isodomoic acid B (peak 1) in *N. navis-varingica* isolated from Bulacan estuary, the Philippines. The spectra were measured with a Varian INOVA 600 spectrometer at 20°C in D2O. *, Signals from impurity.

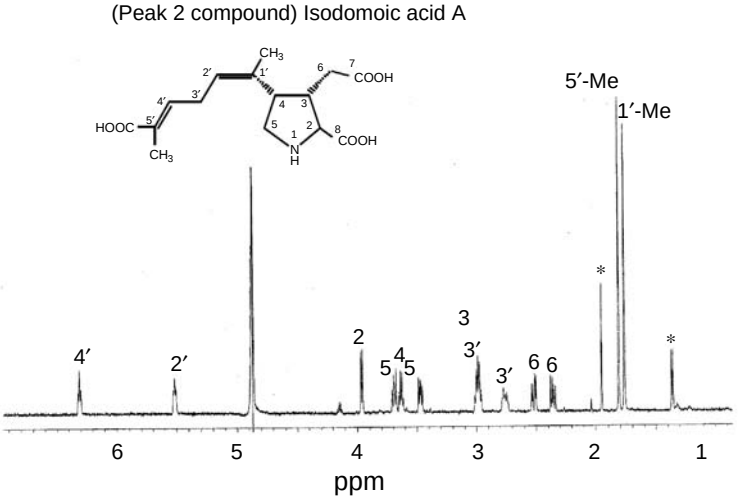

FIGURE 19.8 Proton NMR of isodomoic acid A (peak 2) in *N. navis-varingica* isolated from Bulacan estuary, the Philippines. The spectra were measured with a Varian INOVA 600 apectrometer at 20°C in D2O. *, signals from impurity.

showed that only the isolates from Bulacan estuary, Manila Bay produce both isodomoic acids A and B; however, all of the DA-positive isolates from other areas in the Philippines produce DA and isodomoic acid B.

Isodomoic acids A and B have previously been found in the red alga, *C. armata*[3], but have not been detected as major toxin components in *Pseudo-nitzschia* or in shellfish contaminated with DA from toxic diatoms. *N. navis-varingica* is the first diatom that was found to produce isodomoic acids A and B as major toxin components.

Insecticidal toxicities of isodomoic acids A and B against American cockroach are reported to be 12-fold less toxic than DA,[3] and that of IC is 20-fold less toxic than DA.[24] The affinity of isodomoic acid C to glutamate receptors was reported to be 240-fold lower than DA[24] because of the lack of

1′–2′ double bond with Z configuration.[33] This might indicate that the neurotoxicity of IB is weaker than that of IA, which has the 1′–2′ double bond with Z configuration. Measuring the affinity of isodomoic acids A and B against glutamate receptors are under way; however, preliminary result supports this speculation. It is unknown whether there is any potential of *N. navis-varingica* corresponding to ASP accident. Finding of *N. navis-varingica* that produces isodomoic acids A and B without DA may alert us to the possibility of ASP toxin contamination of marine animals even when DA is not detected in them.

19.2.2.4 Amnesic Shellfish Toxin Profile of *N. navis-varingica* and a Factor That Affects the Toxin Profile[12]

Reobservation of the HPLC chromatogram of *N. navis-varingica* isolates from several areas in the Philippines showed that only isolates from Bulacan estuary produced isodomoic acids A (IA) and B (IB) instead of DA, and all of the isolates from other areas produced DA and IB.[11] It seemed to be interesting to solve whether *N. navis-varingica* isolated from other areas outside the Philippines also produces IA or IB and DA. And it also seemed to be interesting to know whether *P. multiseries* or some other *Pseudo-nitzschia* could produce DA derivatives such as IA and IB.

To investigate the ASP toxin profile, culture experiments were performed for the isolates newly obtained from the brackish water areas in Japan and Vietnam. Philippine *N. navis-varingica* strains including previous isolates were also used for the comparison of toxin profile among different countries. Collection, isolation, and culture experiment for the analysis of ASP toxin production was done as described in the former sections, except that the standard toxins were newly prepared by using the purified DA, obtained from *P. multiseries* culture and IA and IB, obtained from *N. navis-varingica* culture from Bulacan estuary, the Philippines.

Out of 85 isolates from 8 areas in Tohoku district, northern part of Japan, 14 isolates from only 1 estuary (Ohtsuchi, Iwate prefecture) were positive for ASP toxin production with a composition of DA and IB (average total toxin content; 1.5 pg/cell). About 56 out of 148 isolates from 8 areas in Okinawa Prefecture, Japan were positive for ASP toxin production with a composition of DA and IB (average total toxin content; 2.1 pg/cell). About 86 out of 99 isolates from 16 areas near Haiphong, Vietnam were positive for ASP production with a composition of DA and IB (average total toxin content; 3.1 pg/cell). Sixty strains of *N. navis-varingica* isolated from the Philippines were positive for ASP toxin production. Among them, 29 isolates from 3 northern parts of the Philippines showed a toxin composition of IA and IB without DA (average total toxin content; 7.3 pg/cell), whereas the other 31 strains showed a toxin composition of DA and IB (average total toxin content; 5.3 pg/cell).

Morphological study on species identification showed that all of the representative strains positive for ASP toxin production were *N. navis-varingica*.

The average toxin compositions of *N. navis-varingica* strains in each country are shown in Figure 19.9. The most frequent type of ASP toxin was DA–IB. The ratio of the two toxins varied much among the sampling areas. The ratio of IB in the DA–IB type strains was 6% in Tohoku district (northern Japan), 28% in Okinawa (southern Japan), 35% in Haiphong (Vietnam), and 36% in the Philippines, indicating the tendency that the ratio of IB is higher in the isolates from southernmost areas. Among a total of 214 strains positive for ASP toxin production, the IA–IB type was restricted to only 29 strains from three areas in the northern Philippines. The average ratio of IB was ca. 60% of the whole toxin amount. In this study, no peak other than DA, IA, and IB was observed in the HPLC chromatograms.

To examine the stability of toxin composition in each strain, 20 and 16 substrains were established from two Philippine parental strains that had a DA–IB toxin composition. Ten substrains were also established from another Philippine parental strain that had an IA-IB toxin composition. Toxin composition was analyzed by culture experiment as described earlier. All of the substrains showed the same toxin composition as that of parental strains. The results show that the toxin composition is stable in each strain.

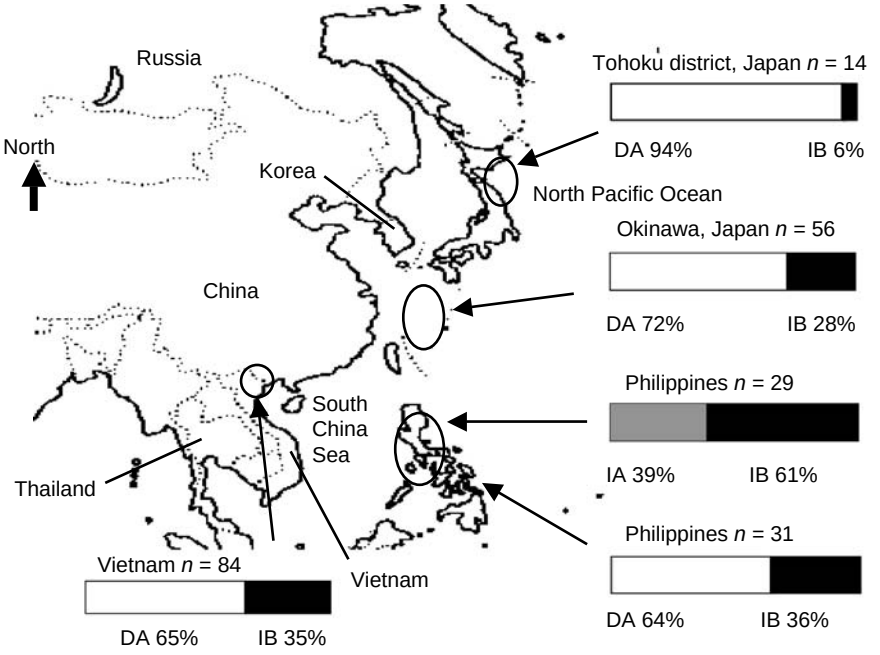

FIGURE 19.9 ASP toxin profile of *N. navis-varingica* isolated from brackish water areas in eastern Asia.

Bacteria are included in the factor that enhances the DA production in *P. multiseries* culture;[28,29] however, in case of *N. navis-varingica* culture, the effect of bacteria on enhancing the toxin production was not obvious.[8] Another interest about bacterial effect on ASP toxin composition of *N. navis-varingica* was examined. After exposure to *f*/2 medium containing mixture of antibiotics (gentamicin, penicillin G, streptomycin) followed by capillary washing, three axenic substrains were established from three Philippine parental strains showing the toxin composition of DA–IB (two strains) and IA–IB (one strain). One of the axenic substrains from DA–IB type parental strain changed its toxin composition to the IA–IB type, whereas the other two substrains did not change their toxin type. When the cell-free medium of the axenic culture that changed its composition to IA–IB was replaced with that of nonaxenic medium (filtrated by 3 μm filter) of the parental strain, the toxin composition reverted to DA–IB type. These results indicate that the toxin composition of *N. navis-varingica* might be affect by bacteria or by a combination of bacteria.

19.2.3 Reanalyses of Amnesic Shellfish Toxins in Some *Pseudo-nitzschia* Cultures[12]

Toxin composition analysis was also performed by HPLC-fluorescence analysis on some *Pseudo-nitzschia* cultures maintained in our laboratory. *P. multiseries*, *P.* cf. *delicatissima* and *P. pungens* isolated from Ofunato Bay, Japan, were cultured for 3 months and analyzed for DA, IA, and IB weekly for the first month and monthly thereafter. In a 1-month-old culture of *P. multiseries*, small amounts of IA (0.1 pg/cell) and IB (0.1 pg/cell) were detected along with DA (5.0 pg/cell). In a 3-month-old culture, IA (4.1 pg/cell) and IB (5.3 pg/cell) were also detected along with DA (82.2 pg/cell). IA and IB makes up approximately 5–6% of the total cellular toxin amount. Although some other small peaks that seemed to be isomers of DA were observed in the chromatogram, they were not included in this analysis. Analysis of these peaks was performed later by HPLC using UV detector with the Canadian standard DACS 1-D, and they were confirmed to be isodomoic acids D, E, and 5′-*epi*-DA. In a 3-week-old culture of *P.* cf. *delicatissima*, only trace amount of DA (0.04 pg/cell)

was detected, whereas DA (1.6 pg/cell), IA (0.07 pg/cell), and IB (0.19 pg/cell) were detected in a 4-week-old culture. IA and IB make up approximately 2–10% of the total cellular toxin amount in this species. These data show that *P. multiseries* and *P.* cf. *delicatissima* contains small amounts of IA and IB together with DA (Kotaki et al. in press). However, it is uncertain that IA and IB are synthesized *de novo*. *P. pungens* was not positive for the ASP toxins during the entire culture period.

19.2.4 Red Alga *Chondria armata*

Red alga *C. armata* is well known to contain high level of DA. Several DA derivatives in *C. armata* have been reported a few times separately.[2–5] However, the original toxin profile has not been elucidated yet. We tried analysis of this toxin while making an ASP toxin standard. *C. armata* was collected in Kagoshima Prefecture, Japan, and sent to our laboratory under frozen conditions. The sample was extracted with hot 50% methanol. The purification of the toxins are now under way; however, the extract was preliminary analyzed by HPLC with UV detector (242 nm), using the Canadian standard of DACS 1-D and purified IB from mass culture of *N. navis-varingica*. HPLC analysis was performed using Develosil ODS-5 column (Nomura, Japan, 4.6 mm × 250 mm) with the mobile phase of 10% acetonitrile in 20 mM phosphate buffer (pH 2.5). Six out of seven peaks were identical to those of the standards. As a preliminary result, DA (400 mg), IA (20 mg), IB (45 mg), 5′-*epi*-DA (20 mg), ID (2 mg), and IE (3 mg) were confirmed in a 1 kg sample. One peak that appeared after these six peaks has not been identified yet. ASP toxins in *C. armata* were found to consist of approximately 80% DA and 20% isomers.

19.3 SUMMARY

P. multiseries, a famous major DA producer, was isolated from Ofunato Bay, Iwate Prefecture, Japan, and was confirmed to produce high levels of DA in culture. Several other *Pseudo-nitzschia* species were also isolated and confirmed to produce low levels of DA in culture. During the survey on DA-producing diatoms in tropical area, a benthic pennate diatom *N. navis-varingica* was isolated from a shrimp-culture pond in Do Son, Vietnam, and was confirmed to produce significant level of DA in culture. Distribution of *N. navis-varingica* was surveyed outside Vietnam, and *N. navis-varingica* was isolated from the brackish water areas in Tohoku district (northern Japan), in Okinawa Island (southern Japan) and in the Philippines (tropical area), indicating the wide distribution of *N. navis-varingica* in eastern Asia. *N. navis-varingica* that produces isodomoic acids A (IA) and B (IB) instead of DA was isolated from Bulacan estuary, Manila Bay. Reobservation of the HPLC chromatogram showed that Philippine strains of *N. navis-varingica* isolated from other areas than Bulacan estuary produce DA and IB. Extensive survey for testing the ASP toxin profile in *N. navis-varingica* was performed by culture experiment using new isolates from Tohoku district, Japan, from Do Son, Vietnam, and from a few estuarine areas in the Philippines. Already isolated strains of Philippine *N. navis-varingica* were also included in the test for the comparison. As a result, *N. navis-varingica* was found to produce ASP toxins with the combination type of DA–IB and IA–IB. The major toxin type was DA–IB, whereas IA–IB type was restricted to the strains isolated from three estuarine areas in the northern Philippines. Among the isolates that showed DA–IB toxin type, the average ratio of IB showed the tendency of being higher in the isolates from southernmost areas. More than ten nonaxenic substrains were made from three Philippine strains of *N. navis-varingica* that showed two toxin types (DA–IB and IA–IB), and were tested for the toxin composition. All of the substrains showed the same toxin composition type as those of the parental strains, indicating that ASP toxin composition type of *N. navis-varingica* is stable in each strain. To investigate factors affecting the ASP toxin composition in *N. navis-varingica*, three axenic cultures were made and tested for the toxin composition comparing with those of the parental strains. One of the axenic cultures changed its parental toxin type DA–IB to IA–IB, whereas other two axenic cultures did not change their parental toxin type. In addition, when the cell-free medium of the axenic culture that

changed its toxin type was replaced with that of the nonaxenic parental strain culture (filtrated with 3 μm filter), the strain culture reverted its toxin type to DA–IB from IA–IB. These results indicate that the toxin composition of *N. navis-varingica* might be affected by bacteria or by a combination of bacteria. ASP toxin profiles were investigated using some kinds of *Pseudo-nitzschia* isolated from Ofunato bay, Japan. In the old culture of *P. multiseries*, 5–6% of IA and IB against DA were detected with high levels of DA. Small amount of other derivatives (5′-*epi*-DA, ID, IE) were also detected in the culture. In the culture *P.* cf. *delicatissima*, small amounts of DA, and trace amount of IA and IB were detected at some culture periods. However, it is uncertain that those isomers are produced *de novo*. Original ASP toxin profile in red alga *C. armata* was analyzed while making standards from it. The extract showed the toxin profile of ca. 80% DA (400 μg/g sample) and 20% other isomers (90 μg/g sample in total) including IA, IB, ID, IE, and 5'-*epi*-DA.

ACKNOWLEDGMENTS

We thank Dr. R. Terada, Kagoshima University, for collecting red alga *Chondria armata*. This work was supported in part by a grant-in-aid for research (C) from the Japan Society for the Promotion of Sciences (JSPS) and by a grant-in-aid for a multilateral cooperative research project (coastal oceanography), from the Ministry of Education, Science, Sports and Culture of Japan.

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20 Domoic Acid: Detection Methods, Pharmacology, and Toxicology

Tracy A. Doucette and R. Andrew Tasker

CONTENTS

20.1 CHEMISTRY OF DOMOIC ACID AND ITS ISOMERS

Domoic acid is a naturally occurring excitatory amino acid that was identified as the toxin responsible for an outbreak of amnesic shellfish poisoning that occurred in Canada in 1987 following consumption of contaminated blue mussels (*Mytilus edulis*) [1] as well as numerous other outbreaks worldwide. The parent compound is a tricarboxylic amino acid produced by certain marine organisms, the best characterized of which are the red alga *Chondria armata* and the planktonic diatom *Nitzschia pungens*, although other species are also known to produce domoic acid (for review, see Reference 2).

Chemically, domoic acid is (2*S*,3*S*,4*S*)-2-carboxy-4-1-methyl-5(*R*)-carboxyl-l(*Z*)-3(*E*)-hexadienyl pyrrolidine-3-acetic acid ($C_{15}H_{21}NO_6$) and has an anhydrous molecular weight of 311.14, a melting point of 215–216°C (dihydrate), and a UV (ethanol) absorption spectrum with a maximum of 242 nm, although this is pH dependent. Structurally, domoic acid is very similar to another known toxin, kainic acid, and both are analogues of the amino acids glutamate and proline. The structures of domoic and kainic acid are shown in Figure 20.1.

The stability of domoic acid in solution as well as in shellfish extracts has been a subject of considerable study, and it seems clear that improper storage of the compound has contributed to variability in the experimental literature. Extensive decomposition is observed when aqueous solutions are stored at either high temperatures (50°C) or extremes of pH (2 or 12). Decomposition is also accelerated by exposure to light or oxygen. One of the most striking features of domoic acid stability, however, is that numerous groups have reported variable rates of decomposition in aqueous solutions stored in a conventional −12°C freezer. This is believed to be due to incomplete freezing and/or freeze–thaw cycles resulting from poor temperature control or repetitive use in experimentation [3]. Aqueous solutions of domoic acid at pH 5–7 and stored in the dark at 4°C, however, are stable for periods up to 1 year [3], and Johannessen [4] reported no degradation in saline, either with or without ascorbic acid, for periods up to 15 weeks if stored in a conventional refrigerator but removed and warmed to room temperature daily. For long-term storage of either domoic acid in solution or in shellfish extracts, freezing at −80°C is recommended.

In addition to the parent compound, a number of isomers of domoic acid have been identified (Figure 20.2). Isodomoic acid A, B, and C are present in small amounts in the red alga *C. armata* [5] as are isodomoic acid G and H [6] (Figure 20.2). The isomers isodomoic acid D, E, and F, as well as the 5′ epimer (Figure 20.2) have been identified in small amounts from both plankton cells and shellfish tissue [7, 8], although it seems likely that these isomers form on exposure to UV light rather than being metabolic products produced by the plankton [3]. Although the binding of domoic acid isomers *in vitro* to kainate receptors was reported a number of years ago [9], there have been few investigations to date of the pharmacology and toxicology of these compounds. A recent report by Sawant et al. [10] described dose–response relationships between isodomoic acid A, B, and C both *in vitro* and *in vivo*.

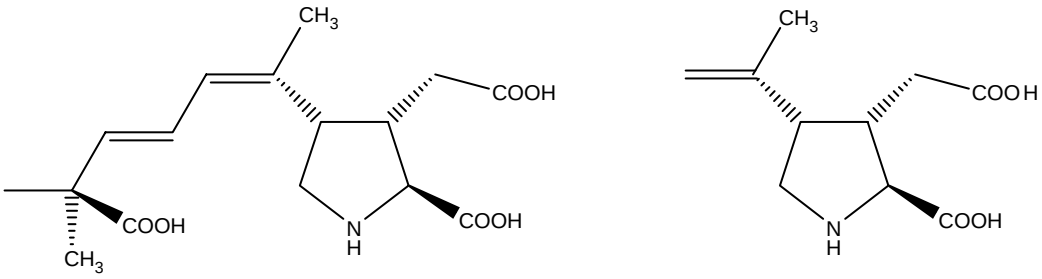

FIGURE 20.1 Structure of domoic and kainic acid.

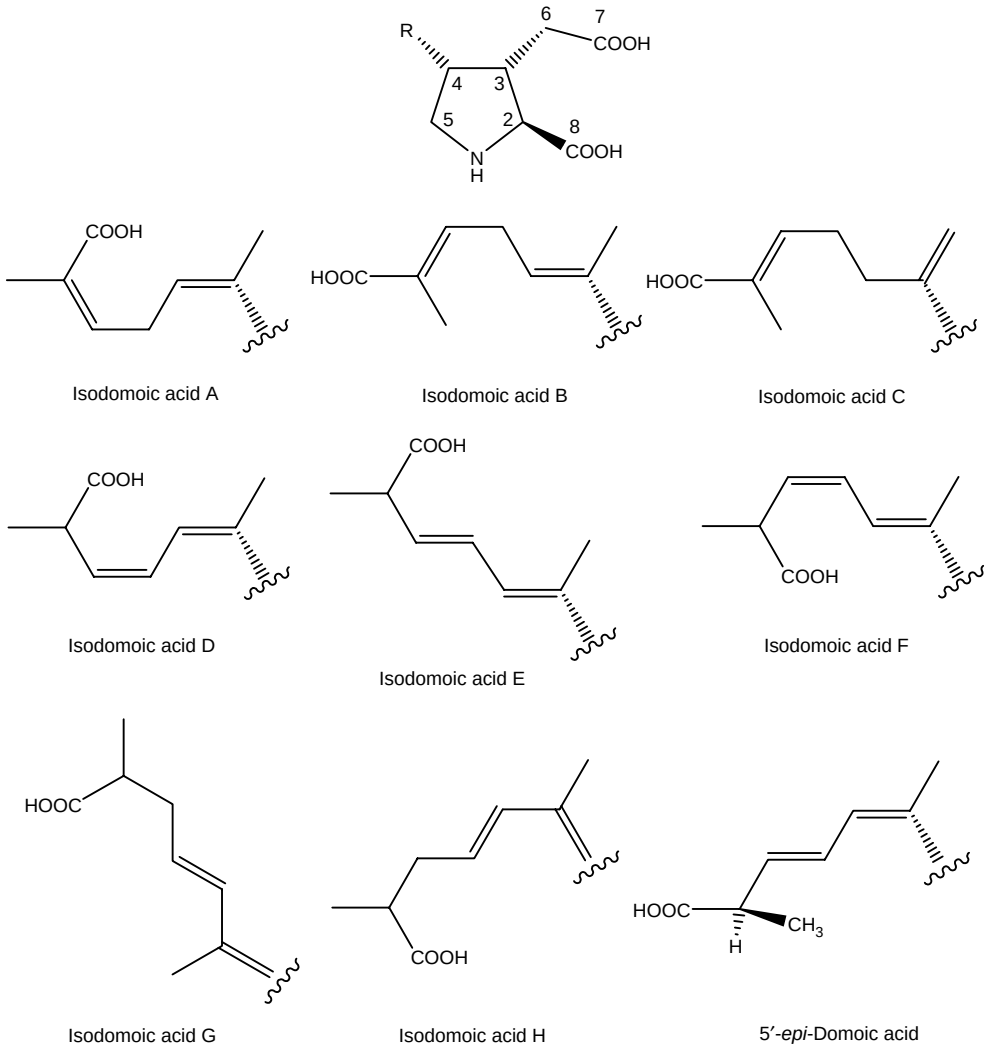

FIGURE 20.2 Structure of known isomers of domoic acid.

20.2 DETECTION METHODS

20.2.1 Sample Preparation

Domoic acid is easily extracted from crude homogenates of shellfish or other biomaterial. The most common method is to drain the tissues to remove saltwater and then to homogenize the pooled tissue in a blender. Tissue homogenates can be frozen and stored for several weeks in tightly sealed containers. Tissue homogenates are then suspended in a water:methanol (1:1) solution usually added as 4.0 mL/g homogenate, mixed, and centrifuged for several minutes at about 3000 × *g*. Extracts can be frozen in tightly sealed screw-capped containers for later analysis if necessary.

For most analytical procedures, further cleanup of the extracts is recommended. This is usually accomplished by passing the extract through a strong anion exchange (SAX) cartridge preconditioned with methanol, water, and extraction solvent (see above). Domoic acid can be eluted from the cartridge using a citrate buffer. For details, see Reference 3.

TABLE 20.1
Summary of Analytical Techniques for the Detection of Domoic Acid

Technique	Limit of Detection	Key Features	References
Thin-layer chromatography	10 μg/g	Semiquantitative Applicable to a variety of matrices Inexpensive	[4,12]
High performance liquid chromatography	20–30 ng/g (UV) 15 pg/g (Fluorescence)	Quantitative Sample cleanup usually required Can detect isomers Derivatization required for fluorescence AOAC approved method (UV)	[4,13–20]
Capillary electrophoresis	3 pg/injection 150 ng/g	Quantitative Minimal cleanup required High resolution Small volumes required	[4,21]
Mass spectrometry	1 μg/g	Quantitative and qualitative high resolution Usually requires prior separation Expensive equipment	[4,22–25]

20.2.2 Chemical Assays

A number of analytical techniques have been described for the detection of domoic acid in seawater, plankton, and shellfish/tissue. These are summarized in Table 20.1 and described briefly below.

20.2.2.1 Thin-Layer Chromatography

Domoic acid can be analyzed semiquantitatively using thin-layer chromatography (TLC) [11] (Table 20.1). The compound is detected on silica gel TLC plates as a weak UV-quenching spot that stains yellow with a 1% solution of ninhydrin. However, many other amino acids in biological samples interfere with domoic acid detection, and consequently, sample cleanup using either two-dimensional TLC or SAX cartridge (see above) methods is required.

20.2.2.2 High Performance Liquid Chromatography

Reversed-phase high performance liquid chromatography (HPLC) is the preferred method of quantifying domoic acid for both regulatory and research applications. Detection can be accomplished using either UV absorbance or fluorescence, although the latter requires derivatization of the molecule but also confers greater sensitivity (Table 20.1).

The HPLC-UV method, originally described by Lawrence et al. [12] and others [13,14] reliably detects domoic acid in shellfish homogenates and has also been adapted to mammalian serum samples [15]. This method has been adopted as an official method for domoic acid quantitation by the Association of Official Analytical Chemists (AOAC) [16]. Separation can usually be accomplished using isocratic conditions with an acidified mobile phase (typically acetonitrile:water) and absorbance measured at 242 nm, although some complex matrices require the use of gradient HPLC. If crude extracts are used, the practical limit of quantitation of domoic acid in shellfish is about 1 mg/kg, which is well below the standard regulatory limit of 20 mg/kg. However, interference by other amino acids in shellfish, notably tryptophan, can produce false positives when crude extracts are used. Consequently, the use of photodiode array detection and/or sample cleanup using SAX cartridges (see above) is preferred. The latter will typically improve sensitivity by about two orders of magnitude.

In cases where greater sensitivity is required, the use of fluorescence detection following precolumn derivatization with fluorenylmethoxycarbonyl (FMOC) reagent is indicated [17] (Table 20.1). Variations on this original HPLC-FD method using derivatization with either 6-aminoquinolyl-*N*-hydroxysuccinimidyl reagent [18] or 4-fluoro-7-nitro-2,1,3-benzoxadiazole reagent [19] have been described.

20.2.2.3 Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique with considerable potential for the analysis of marine toxins, including domoic acid and its isomers. Typically, a small volume (1–10 nL) is injected into a narrow bore (50–100 μm i.d.) fused silica capillary filled with buffer and subjected to a differential voltage of 20–30 kV. As ionic substances migrate as narrow bands, they can be detected by UV absorbance, fluorescence, or other methods. Successful application of this technique to the detection of domoic acid in shellfish has been reported [20] (Table 20.1).

20.2.2.4 Mass Spectrometry

Mass spectrometry is an accurate method for both quantitative and qualitative determination of domoic acid in a variety of matrices. The original identification of domoic acid in contaminated mussels as the cause of amnesic shellfish poisoning (ASP) was accomplished using fast atom bombardment (FAB) mass spectrometry with direct probe insertion [21,22]. Subsequently, several methods utilizing gas chromatography-mass spectrometry (GC-MS) were described [23,24], although the recent popularization and affordability of liquid chromatography-mass spectrometry (LC-MS) systems has made other methods largely obsolete. Although a number of LC-MS interfaces have been used (continuous flow FAB, thermospray, atmospheric pressure chemical ionization, electrospray), a recently published interlaboratory validation study on the use of tandem mass spectrometry (LC-MS/MS) reported sensitive and specific identification of a number of algal toxins, including domoic acid, in a variety of shellfish species [25] and must be considered the state-of-the-art method at this time.

20.2.3 Biological Assays

20.2.3.1 Immunological Assays

Assays based on specific antigen–antibody interactions have proven useful for the detection of a variety of compounds in complex systems. A polyclonal antibody to domoic acid was described a number of years ago [26] and has been used in the construction of enzyme-linked immunosorbent assays (ELISAs) [27–29]. One of these assays has been commercialized as a direct competitive enzyme-linked immunosorbent assay (cELISA) to provide high-throughput analysis of shellfish samples for determination of domoic acid content (ASP Direct cELISA kit, Biosense Laboratories, Norway). A recent report by Hesp et al. [30] validated the use of this technology in measuring domoic acid concentrations in rat serum and brain tissue also, and the same antibody has recently been used in the generation of blood collection cards for field use [31]. A different approach to detecting domoic acid (and other toxins) in seafood has recently been described by Kreuzer et al. [32]. These authors used an antibody to domoic acid conjugated to alkaline phosphatase and coated on an electrode to generate an electrochemical immunosensor that was reported to have a detection limit of 2 ppb domoic acid. It seems likely that further refinement of immunoassays for domoic acid in biological samples hold considerable potential for the development of simple, dockside tests for measuring toxin concentrations.

20.2.3.2 Radioligand Binding Assays

Much of the original classification of domoic acid pharmacology (see below) was based on the ability of domoate to displace [^{3}H]kainic acid from binding sites in brain homogenates. Kainic

acid binding in brain can be described by two dissociation constants (K_d)—one of high affinity for kainate (K_d ~ 3–5 nM) and one of low affinity for kainate (K_d ~ 25–50 nM) [33]. A notable exception is the cerebellum, where only a single binding site (K_d ~ 25 nM) has been described [33]. This differential binding is now known to be due to differences in the subunit composition of kainate receptors, whereby the subunits designated KA-1 and KA-2 are known to correspond to the high-affinity binding and the subunits GluR5/GluR6/GluR7 (often now designated GluK5/6/7) correspond to the low-affinity sites (for review, see References 34 and 35). Of particular pertinence to the current discussion is that domoic acid is known to interact preferentially with low-affinity [^{3}H]kainic acid binding sites corresponding particularly to the GluK5 and GluK6 subunits [36–38]. On the basis of this knowledge, Van Dolah et al. [39] developed a receptor-binding assay to detect domoic acid using a cloned glutamate receptor. The assay utilizes membranes from SF9 insect cells transfected to express the GluK6 (GluR6) subunit. When tested on contaminated mussels treated with glutamate decarboxylase (to remove endogenous glutamate), the assay reliably detected the presence of domoic acid with a sensitivity that should be lower than 100 ng/g shellfish on the basis of the data reported [39].

20.2.3.3 Neuronal Culture Assays

Neurons maintained in primary culture are exquisitely sensitive to any compound that alters their physiological equilibrium by changing ion fluxes [40,41] or altering their biochemical steady state [42,43]. Exposure of neuronal cultures to excitatory amino acids such as domoic acid results in a concentration-dependent neuronal degeneration that begins within minutes and is complete in 12–18 h [41,44,45]. Neurodegeneration is easily quantified using fluorescent dyes that specifically label live cells [41]. Although not currently employed in routine monitoring, neuronal cultures have been proposed as a viable and inexpensive means of monitoring amnesic (domoic acid), diarrheic (okadaic acid), and paralytic (saxitoxin) shellfish toxins [46].

20.2.3.4 Mouse Bioassay

Whole-animal bioassays provide an easy, rapid, robust, and sensitive screen for phycotoxin analysis, although the ethics of using whole animals for routine regulatory purposes has been questioned in many jurisdictions including Europe and North America. In standard mammalian bioassays, toxicity is measured as a function of the biological response of the animal using behavioral and physiological indicators that are known to be correlated with increasing toxin concentration. Routinely, crude extracts of shellfish or other foodstuffs are injected, and the response of the animal is monitored. One argument often made for the continuation of bioassay screening is that the technique inadvertently screens for previously unknown or "foreign" toxins. Indeed, this was the case for identifying domoic acid as a toxin in Prince Edward Island (PEI) shellfish in 1987. Other toxins such as saxitoxin were known to be common in PEI estuaries so that routine screening was employed. However, the appearance of unique behavioral toxicity in 1987 prompted investigators to look for a different class of toxin that was ultimately identified as domoic acid [21]. A counterargument, however, is that conventional bioassays can (a) be confounded by many variables, including inappropriate sample preparation and the presence of nontoxic irritants, (b) have poor dynamic range, especially at low concentrations, and (c) are inherently imprecise and increasingly less cost-efficient as newer diagnostic tests are developed (see above).

The accepted mouse bioassay for domoic acid is based on the AOAC bioassay for saxitoxin (PSP). In brief, shellfish tissue is boiled in 0.1N HCl, cooled and pH adjusted, and injected intraperitoneally (i.p.) in serial dilutions. Time- and dose-dependent toxicity symptoms are recorded for 4 h and used to determine concentration in the sample by interpolation from standard curves. The limit of sensitivity of the assay is considered to be 40 ppm, which is double the regulatory limit in most countries [47], although reproducible behavioral change can be detected at much lower concentrations [48] in controlled laboratory conditions.

20.3 PHARMACOLOGY

20.3.1 Neurobiology of Glutamate Receptors

20.3.1.1 Classification

Until the mid-1980s, researchers were content with two major classes of glutamate receptors, the *N*-methyl-D-aspartic acid (NMDA) receptor and the non-NMDA receptors (comprised of kainate receptors and quisqualate receptors at this time) [49,50]. However, with the development of selective agonists and antagonists and advances made through molecular biology, this receptor family, and the intricacies within each categorical subtype, have expanded dramatically [51]. The currently accepted classification scheme for glutamate receptors is shown in Figure 20.3.

Glutamate receptors are categorized into two distinct classes. Metabotropic glutamate receptors (mGluRs) are coupled to guanosine 5′-triphosphate (GTP) binding proteins, with actions mediated

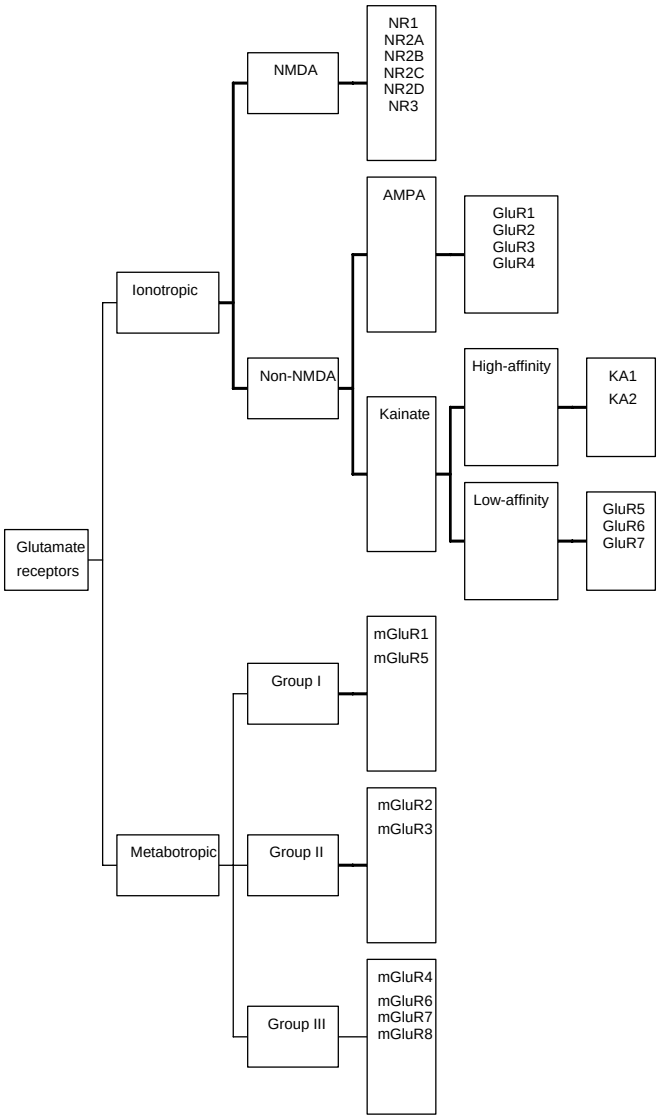

FIGURE 20.3 Classification of glutamate receptors and glutamate receptor subunits.

through an effector protein, which modulates the production of second messengers (reviewed in Reference 52). Inotropic glutamate receptors are comprised of various glutamate receptor subunits (GluRs) that form cation-specific ion channels and mediate fast synaptic transmission. However, inotropic receptors possess functional properties that extend beyond gating cation channels. The intracellular carboxy terminal has the capacity to interact with a wide variety of intracellular proteins, including those involved with spatial and functional organization of post-synaptic densities, and proteins involved in signal transduction [53].

20.3.1.2 Inotropic Glutamate Receptors

Inotropic glutamate receptors contain cation-specific channels and are further subdivided, on the basis of sequence homology and agonist specificity, into three major subtypes: NMDA; alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA); and kainite (KA) receptor channels. Traditionally, however, since neither agonists nor antagonists clearly distinguished between AMPA and KA receptors, they were collectively referred to as non-NMDA receptors (reviewed in References 49 and 50). Although cloning studies had demonstrated that AMPA and KA receptors were distinct receptor complexes, both could be activated by the same agonists; notably, AMPA receptors could be activated by KA, and KA receptors with certain constituent subunits could be activated by AMPA [49,50].

However, multiple studies subsequently confirmed that AMPA and KA receptors are indeed distinct subclasses of the inotropic glutamate receptor family. First, several antagonists that differentially block either AMPA or KA receptors were developed (reviewed in References 49 and 50). Second, electrophysiology studies demonstrated that while glutamate-induced currents show rapid and profound desensitization in all non-NMDA inotropic receptors, KA causes much more pronounced desensitization of GluR5-, GluR6-, KA1-, and KA2-containing receptors, whereas AMPA is selective for GluR1–4 containing receptors and induces strong desensitization (reviewed in References 49 and 50) Third, cyclothiazine has been shown to be a selective positive modulator of AMPA receptors, whereas concanavalin A has been shown to be much more effective on kainate-preferring receptors on for AMPA receptors (reviewed in References 49 and 50). Finally, geneticists and molecular biologists have revealed that the diversity of inotropic glutamate receptors is much larger than expected, on the basis of electrophysiological and pharmacological studies alone (reviewed in Reference 52).

Recent molecular cloning and expression studies have isolated at least 15 complementary deoxyribonucleic acids to date that code for non-NMDA glutamate receptor subunits, which include four for AMPA receptor subunits (GluR1–4); five for KA receptor subunits (GluR5–7, KA1, KA2) and six for NMDA (NR1, NR2A–D, NR3). Two additional cDNAs for δ subunits, δ1 and δ2, have been clowned, of which the functions are presently unknown (reviewed in Reference 52) (Figure 20.3). Constituent subunits of inotropic glutamate receptors are believed to form functional receptors as tetrameric subunit assemblies, with each monomer carrying its own ligand-binding site and contributing to the formation of the channel lumen with a specific sequence of amino acids in the hydrophobic M2 segment [54].

20.3.1.2.1 Non-N-methyl-D-aspartic acid receptors

Of the non-NMDA receptors, AMPA receptors have been better described in the literature to date, but kainate receptors are catching up quickly. Within the mammalian central nervous system (CNS), AMPA receptors mediate fast excitatory transmission at most synapses. Constituent subunits for AMPA receptors (Glu1–GluR4) can assemble into both homomeric and heteromeric receptor configurations with distinct functional properties. At least three separate binding sites are present on AMPA receptors, at which agonists or antagonists can act; glutamate binding, desensitization and intraion binding sites (reviewed in Reference 52).

AMPA receptors are ubiquitously distributed throughout the mammalian CNS, with rich concentrations found within the hippocampus, the dentate gyrus, and superficial layers of the cortex.

Within the hippocampus, GluR1, GluR2, and GluR3 genes express strongly in all principal cells, while GluR4 is confined to dentate granule cells. Expression of GluR1 appears to dominate within hippocampal interneurons; however, mRNA for all subtypes is detectable.

Similar to AMPA receptors, KA receptor subunits GluR5 and GluR6 form homomeric receptors and can assemble as heteromeric receptors in pairwise combinations with the KA1 and KA2 subunits (reviewed by Reference 49). For all known functional recombinant KA receptors, KA elicits a fast onset and rapidly desensitizing response. However, other pharmacological and functional properties differ depending on subunit composition [55]. Kainate receptor subunits do not form mixed channel complexes with AMPA receptor subunits; however, both types of receptors can be expressed in the same neuron [56].

Kainate receptor gene expression is widespread, including the cortex, limbic system, and cerebellum (for review, see Reference 57). More specifically, with respect to the principal cells of the hippocampus, KA1 mRNA is restricted to CA3 pyramidal cells and dentate granule cells, while KA2 mRNA is abundant in both CA1 and CA3 pyramidal cells and dentate granule cells. The GluR6 gene is moderately expressed in all CA pyramidal cells and in dentate granule cells, with expression greater in CA3 than in CA1. Finally, GluR7 mRNA is present in dentate granule cells, but not expressed in CA pyramidal cells [58]. Consequently, this pattern of expression could yield receptors with the following configurations: CA1 pyramidal cells, GluR6/KA2; CA3 pyramidal cells, GluR6/KA1, GluR6/KA2, or GluR6/KA1/KA2; dentate granule cells, receptors derived from combinations of KA1, KA2, GluR6, and GluR7. With respect to hippocampal interneurons, GluR5 expression is abundant. However, most GABAergic cells in the pyramidal cell layer also express GluR6. Thus, a specific subset of interneurons stain positive for GluR5/GluR6 [58].

20.3.1.2.1.1 Posttranscriptional modifications. As described above, non-NMDA receptors are composed of multiple protein subunits, each of which is encoded by a separate gene. In addition to the multiplicity of genes, the molecular diversity of inotropic glutamate receptors is further increased by variants because of alternative splicing and ribonucleic acid editing. The known sites for these alterations are depicted in Figure 20.4. Two alternative splice variants of GluR1–4 subunits, designated "flip" and "flop," have been shown to differ in their expression throughout the brain and during development [59] and to impart different pharmacological and kinetic properties on the resultant channel. In general, AMPA flip isoforms show somewhat slower desensitization kinetics and are more sensitive to the positive modulatory effects of cyclothiazine [60]. This splicing occurs within a 38-amino acid exon in the extracellular loop between the third and fourth transmembrane domain [60].

In situ hybridization studies on hippocampal principal cells reveal that CA1 expresses all flip versions and all flop versions, with the exception of GluR4 flip. In contrast, CA3 synthesizes only the flip version of GluR1, GluR2, and GluR3, with flip mRNA higher in CA3 than in CA1. Dentate granule cells express all flip and flop forms with the exception of GluR4 flip, but with flop mRNA levels higher than flip mRNA (reviewed in Reference 58).

The GluR5 subunit also yields two variants (GluR5-1 and -2, with the former containing an additional 15 amino acids in the extracellular N-terminal region). Further, each of the splice variants of GluR5-2 possesses one of three alternative C-terminal sequences. Two C-terminal alternative splice variants of GluR7 (a and b) have also been reported. However, no alternative splicing has been reported yet for rat GluR6, KA1, or KA2 [55].

Another form of posttranscriptional modification that generates subunit diversity is RNA editing [60–63]. In brief, the gating characteristics of non-NMDA receptor ion channels strongly depend on the amino acid sequence of the second transmembrane spanning region, which includes the Q/R site that is subject to posttranslational nuclear editing of pre-mRNA. For instance, in adult tissue, GluR1, GluR3, and GluR4 receptor subunits contain a glutamine (Q) residue in the putative pore-forming second membrane domain, and generate strongly inward rectifying calcium ion currents. Editing of

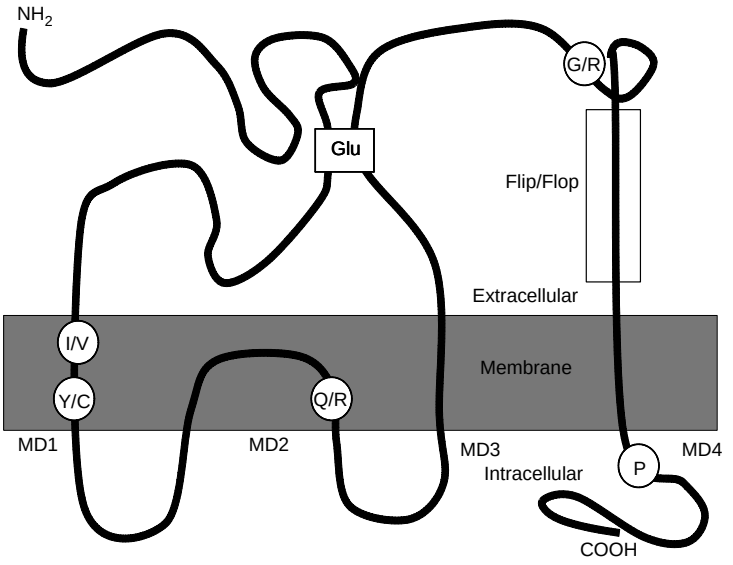

FIGURE 20.4 Glutamate receptor topology indicating sites of alternative splicing and posttranscriptional editing. MD1, MD3, and MD4 are transmembrane domains, whereas MD2 does not cross the membrane. Two domains (lobes 1 and 2) are proposed to interact to form the glutamate-binding site (Glu), as predicted from structural similarity to bacterial amino acid binding proteins. Circles indicate RNA editing; the Q/R (glutamine to arginine) (position 586) site may be edited in GluR2, GluR5, and GluR6 transcripts, the I/V (isoleucine to valine) and Y/C (tyrosine to cysteine) sites may be edited in GluR6 transcripts. Sequences affected by differential splicing are located in the extracellular N-terminal domain and at the C-terminus. GluR2, GluR3, and GluR4 may be edited at the R/G site (arginine to glycine) (position 743), while a glycosylation site that is glycosylated in GluR6 is found at approximately the same position. Phosphorylation of the NMDA receptor subunit in NR1 *in vivo* is indicated with P. The box surrounds amino acids 744–781, and indicates the region where alternative splicing variants, flip and flop, occur (nine amino acids differ between splice variants).

the RNA for GluR2, however, replaces this glutamine with an arginine (R), resulting in low calcium ion permeability and a linear *I–V* relationship [60,63,64]. Virtually no unedited GluR2 transcripts are detected in the adult brain [52]. An additional RNA editing site, located at position 743 (R/G site), has been described for GluR2, GluR3, and GluR4 [65]. Functionally, editing of arginine to glycine (R/G site) at this site confers a slower desensitization rate [65].

With respect to KA receptor subunits, GluR5 and GluR6 may also undergo posttranscriptional editing at the Q/R site [60]. Further, GluR6 monomers have two additional editing sites [66]. For instance, GluR6 can undergo subsequent editing at two additional sites in the first hydrophobic domain, where isoleucine (I) is changed to valine (V), and tyrosine (Y) to cysteine (C) [66]. Importantly, gating characteristics are controlled by the concerted action of various editing sites [66]. Thus, as with AMPA receptors, RNA editing is of crucial importance to the rectification properties of the KA channels as well as to their divalent cation permeability [62,66].

At present the physiological and clinical importance of alternative splicing and RNA editing is unclear. However, there is increasing evidence to suggest that abnormal regulation of these processes might underlie various neurodegenerative and neuropathological conditions [67–71].

20.3.1.2.1.2 Developmental changes. Many developmental shifts occur with respect to regional subunit expression and posttranscriptional modifications, many of which have potentially important implications for plasticity and neurotoxicity. For instance, before postnatal day (PND) 16, AMPA receptors in the pyramidal neurons in the neocortex are deficient in GluR2; thus, such AMPA receptors would permit the influx of calcium ions [72]. In addition, AMPA receptor subunits are expressed primarily as the flip isoform (thus exhibiting slower desensitization), with the flop module

not reaching adult levels until PND 14 [59]. Finally, posttranscriptional RNA editing at both the R/G and Q/R sites are also developmentally regulated [73–77].

20.3.1.2.1.3 Kainate receptors coupled to second messengers. Within the last 10 years, glutamate receptor classification has become even more complex. Evidence now suggests the existence of inotropic kainate receptors that possess metabotropic function [78,79]. Although still somewhat controversial, it is held that GluR5-containing KA receptor assemblies found on hippocampal GABAergic interneurons are directly or indirectly coupled to G proteins that activate PCK in the terminal region and ultimately produce a diminution in synaptic transmission [78,79]. Metabotropic kainate receptors are believed to be of functional significance in their potential role in epilepsy and long-term potentiation.

20.3.1.2.2 NMDA receptors

Of all glutamate receptors, the NMDA receptors have been best described in the literature to date, and NMDA receptors have been implicated in a vast array of physiological and pathological CNS functions. It is known that NMDA receptors mediate excitatory transmission in the mammalian CNS, and are characterized by slow gating characteristics, high calcium ion permeability, and a voltage-dependent magnesium ion block (reviewed in Reference 52). Each NMDA receptor is composed of at least one NR1 subunit in combination with one or more NR2 subunits (NR2A-D), and different recombinant receptors express distinct properties, depending on the constituent subunits within the heteromeric assembly (e.g., degree of voltage-dependent magnesium block and deactivation kinetics) (for classification, see Figure 20.3). Consequently, NR2 subunits are viewed as modulatory subunits. The NR1 subunit, however, is required to form functional channels, and consistent with this obligatory inclusion, NR1 is found ubiquitously. Adding further to the molecular diversity of this class of receptors, at least eight alternative splice variants are known to exist for NR1 (reviewed in Reference 52.)

NMDA receptors are distributed widely throughout the mammalian brain (as shown by immunocytochemistry and *in situ* hybridization), with the highest concentrations found in the forebrain (highest levels are present in the CA1 region of the hippocampus) (reviewed in Reference 52). With respect to the principal cells of the hippocampus, NR1, NR2, and NR2B are highly expressed in adult dentate granule cells and CA1, CA2, and CA3 pyramidal cells, while NR2C and NR2D are not expressed by these cell types [58]. In addition, it has been shown that NR3A is present in the CA1 pyramidal cell layer [58]. In turn, mRNA for NR1, NR2B, and NR2D are widely expressed in hippocampal interneurons.

NMDA receptors are considered to be both ligand- and voltage-gated receptor complexes. When the postsynaptic membrane is at resting potential, a magnesium ion is attached to the magnesium-binding site within the channel lumen, thus blocking the ionophore and inhibiting calcium ion influx (reviewed in Reference 80). However, if the postsynaptic membrane is partially depolarized, the magnesium ion is repelled from its binding site, and certain divalent cations (i.e., particularly calcium ions) are free to permeate. Thus, ion passage occurs only if glutamate (and glycine) is present and if the postsynaptic membrane is partially depolarized (reviewed in Reference 80).

20.3.2 Mechanism of Action of Domoic Acid

Domoic acid is generally considered a "non-NMDA" receptor agonist, and more specifically a selective kainate receptor agonist (for review, see Reference 52). Domoic acid is known to bind with high affinity to both the GluR5 and GluR6 subunits of kainate receptors [49,60,63] (see Section 20.2.3.2), although, at slightly higher concentrations, domoic acid also interacts with the KA1 and KA2 subunits as well as with AMPA receptor subunits. The relative affinity of different AMPA/kainate agonists for different subunits is summarized in Table 20.2. On the basis of this binding data, it is reasonable to assume that primary mechanism of action of domoic acid is via activation of "low-affinity" (GluR5/6-containing) kainate receptors that could be referred to colloquially as "domoate" receptors.

TABLE 20.2
Relative Affinity of Non-NMDA Agonists for Different AMPA/Kainate Receptors Subunits

Receptor Subunit	Relative Affinity
GluR1, GluR2, GluR3, GluR4	QUIS > AMPA > DOM > GLU > KA
GluR5, GluR6, GluR7*	DOM > KA > QUIS > GLU > AMPA
KA-1, KA-2	KA > DOM

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
DOM, domoic acid
GLU, glutamic acid
QUIS, quisqualic acid
KA, kainic acid

* For receptors formed by the subunit GluR7, GLU > QUIS.

Certainly, there is considerable evidence from pharmacological studies using neuronal culture [41,63], brain slices [81–83], and whole-animal toxicity [84] that domoic acid produces toxicity via these receptors and that antagonism of GluR5/6-containing receptors attenuates the actions of domoic acid. However, understanding the pharmacology of domoic acid requires a more complex interpretation of the available data. This is for two main reasons: (a) the drug is selective for GluR5/6 but will interact with other receptor types at slightly higher local concentrations, and (b) kainate receptors exist in various configurations and locations wherein they subserve different functions. A detailed discussion of the complexity of non-NMDA receptors is beyond the scope of this chapter, but there is a wealth of evidence supporting the involvement of other glutamate receptor subtypes, as well as other neurotransmitter and neuromodulator systems, in at least some aspects of domoic acid toxicity in different systems. For example, domoate toxicity can be blocked by "low-affinity" kainate receptor antagonists such as NS-102 [84,85] and NS-3763 [86], but under certain circumstances it is also attenuated by more generalized kainate receptor antagonists such as CNQX and DNQX, as well as by AMPA-selective antagonists such as NBQX [82,84,87,88]. Presumably, these effects reflect actions at postsynaptic non-NMDA receptors. Kainate receptors, however, are also located presynaptically whereby they regulate glutamate release (for review, see References 34, 54, and 89), and it is assumed that they are responsible for both domoate-induced glutamate release [90] and observations that domoic acid toxicity can be antagonized in part by NMDA receptor antagonists both *in vitro* [41] and *in vivo* [91,92]. To further complicate matters, it is now well established that GluR5- and GluR6-containing kainate receptors are found on both the soma and presynaptic terminus of GABAergic interneurons in the hippocampus [78,79,93–95] where they either promote or inhibit GABA release, respectively (see above). There is also evidence to suggest the involvement of opioid systems in domoate toxicity [96], and it is reasonable to assume that other neurotransmitter systems are similarly involved. Lastly, it is now known that kainate receptors are also present on glia as well as neurons and participate in glial excitotoxicity (for review, see Reference 97). Both reactive gliosis [98,99] and gliotoxicity [98] have been observed following administration of domoic acid. Hence, overall, understanding the mechanism(s) of action of domoic acid is complex, and varies with region and local concentration.

20.3.3 Absorption, Distribution, and Elimination

Although consumption of contaminated food products is the primary route of domoic acid intoxication, there have been very few objective studies on the absorption of this toxin following oral administration. On the basis of these limited observations, it appears that domoic acid absorption

from the gastrointestinal (GI) tract is erratic and bioavailability is approximately 10%, but there are many unanswered questions about the factors that contribute to erratic oral bioavailability.

Iverson et al. [100,101] reported on the relative toxicity of domoic acid following oral or i.p. administration in both mice and rats. These authors used both extracts of contaminated mussels and purified DOM, obtaining similar results with each. Using scratching behavior, seizures, and death as the criteria for DOM toxicity, they reported consistently reproducible toxicity following i.p. administration of doses from 2.0 to 9.5 mg/kg but variable toxicity following oral administration of doses ranging from 35 to 104 mg/kg in mice. Similar values were obtained in rats, and the authors concluded that oral doses approximately 10-fold those of parenteral routes of administration are required for toxicity but that other unidentified factors also contribute to oral bioavailability [100, 101]. Poor oral bioavailability in rats was also reported by Truelove et al. [102], who found no changes in behavior, clinical chemistry, or histopathology in rats dosed orally with 0.1 mg/kg/day (equivalent to a 50-kg human consuming 250 g of shellfish containing 20 ppm DOM each day) and only minor histopathological changes in the brains of rats dosed orally with 5.0 mg/kg/day.

In nonhuman primates observed for 17–44 days following oral doses of toxic mussel extract containing DOM in concentrations ranging from 5.21 to 6.62 mg/kg, GI disturbance and inconclusive CNS toxicity were observed [103], and toxicity was not facilitated by either monosodium glutamate (MSG) or dimethylsulfoxide (DMSO) [103]. Using purified domoic acid similar results were obtained, with nausea and vomiting being observed within 0.5–1 h of dosing with 10.0 mg/kg and 5.0 mg/kg, respectively, but "histopathological changes in orally treated monkeys were minimal, equivocal or nonexistent" [104].

Thus, one is forced to conclude that oral bioavailability of domoic acid is poor in both primate and nonprimate species, but it is clear from the outbreak of human toxicity that occurred in 1987 [1] that, under some circumstances, domoic acid can be absorbed from the gastrointestinal tract, thereby posing a risk to human health.

Once absorbed, domoic acid distributes poorly throughout the body, as would be expected for a hydrophilic molecule. In rats, the interpolated apparent steady-state volume of distribution (Vd_{ss}) ranges from about 0.2 to 0.4 L/kg [105,106]. The Vd_{ss} may be even lower in primates and has been reported as 0.16 L/kg in cynomolgus monkeys [106].

In order for domoic acid to be neurotoxic, it must gain access to the brain. The transport of both glutamate and domoic acid at the blood–brain barrier (BBB) has been studied by both *in vitro* cell uptake assays and *in vivo* perfusion methods [107]. In most regions of the brain, the uptake of these compounds from the circulation was limited by the BBB. At physiologic plasma concentrations, glutamate flux from plasma into the brain appears to be mediated by a high-affinity transport system at the BBB. Efflux from the brain back into plasma appears to be driven in large part by a sodium-dependent active transport system at the capillary membrane. The concentration of glutamate in brain interstitial fluid is only a fraction of that of plasma and is maintained fairly independently of small fluctuations in plasma concentration. This restricted brain passage was also observed for domoic acid [108]. These authors used injection of radiolabeled domoic acid in a group of ten adult rats to estimate BBB transfer constants relative to labeled sucrose, which is largely impermeable. They sampled in seven brain regions (frontal cortex, occipital cortex, striatum, hippocampus, diencephalons, cerebellum, and pons medulla) over 30 min, and the results indicated poor BBB penetration, with mean transfer constants ranging from 1.60 to 1.82 mL/g/sec $\times 10^6$ (sucrose constants ranged from 1.00 to 1.24). These data argue against the existence of a carrier protein and suggest that the highly charged state of domoic acid at physiological pH results in poor CNS penetration kinetics after "single-pass" exposure. However, if renal elimination is impaired, such as in rats subjected to nephrectomy, regional brain concentrations increase by 50–120%, largely because of elevated plasma concentrations and reduced clearance [108]. These data were accurately modeled using physiologically based pharmacokinetic calculations [109] and are consistent with the epidemiology of ASP toxicity in humans [1].

Domoic acid appears to be eliminated almost exclusively by renal excretion. Preston and Hynie [108] reported that a single i.v. dose of radiolabeled domoic acid was almost completely

eliminated from the serum of intact rats within 30 min. However, nephrectomy resulted in a significantly reduced clearance, such that detectable serum concentrations were still present at 60 min postinjection (the last time point studied). In a more robust study of renal pharmacokinetics, Suzuki and Hierlihy [105] reported that serum clearance of domoic acid was almost entirely due to renal excretion, with similar kinetics over a wide range of dosages (0.5 ng/kg–2.0 mg/kg i.v.). Analysis of serum concentration over time curves produced elimination rate constants (k) of 0.025–0.035/min (equivalent to an elimination half-life of approximately 13 min). These authors also reported total body clearance values of 7.75–10.82 mL/min/kg and renal clearance values ranging from 8.80 to 12.20 mL/min/kg. Renal excretion in this study appeared to be mainly by glomerular filtration because kinetics were not altered by the presence of probenecid, a competitive inhibitor of acid transport systems in the kidney [105]. There is no published data indicating that domoic acid undergoes biotransformation in any species studied to date.

20.3.4 Effects of Low-Dose Domoic Acid on Behavior

While most studies of domoic acid actions *in vivo* are concerned with toxicity (see below), a few studies have investigated the pharmacology of domoic acid in rodents at more "physiological" concentrations.

Doucette et al. [110] reported physiologically relevant changes in brain development in the absence of convulsions when neonatal rats were injected daily (s.c.) with very low doses of domoic acid (5 and 20 μg/kg), or pharmacologically equivalent doses of kainic acid (25 and 100 μg/kg) from PND 8–14. This study showed that while neither compound had identifiable effects on typical measures of toxicity such as weight gain, acoustic startle, ultrasonic vocalizations (UVs), or maternal retrieval, drug administration did result in significant differences in eye opening, conditioned place preference, and spontaneous activity. These authors conclude that low doses of domoic acid can produce changes in brain development if given during particular "windows" of brain development. In this case, that window is the second perinatal week in the rat, which is a particularly dynamic period for kainate receptor expression, and which corresponds roughly to the latter part of the third trimester in humans. This same research team recently reported on a follow-up study [92] in which they describe the induction of a conditioned odor preference in rats administered 0.020 mg/kg domoic acid s.c. by daily injection between PND 8 and 14 (the same time frame as the previous study). No observable toxicity was reported, but drug-treated rats spent significantly more time over a conditioning odor previously paired with drug administration. These data indicate a DOM-induced learned association. Moreover, this effect was antagonized by concomitant injection of the NMDA receptor antagonist 3-(2-carboxypiperazin-4-yl) Propyl-1-phosphonic acid (CPP), implicating a role for NMDA receptors in this phenomenon.

Surprisingly few studies have been conducted on the effects of lower (physiological) doses of domoic acid in adult mice and rats. Human patients who presented with abnormal EEG patterns following consumption of domoic acid showed a predominantly anterograde memory disorder, with particular deficits in visuospatial tasks [111]. In rats, visuospatial tasks are often measured in the Morris Water Maze task [112]. Studies in both rats and mice have demonstrated that doses of domoic acid sufficient to elicit mild seizures also produce deficits in both the acquisition and retention of this task [113,114].

20.4 TOXICOLOGY

20.4.1 Toxicity in Rodents

20.4.1.1 Whole Animal Toxicity

The acute toxicity of domoic acid has been extensively studied in both mice and rats. The results of many of these studies are summarized in Table 20.3. One important point to note, however, is that

TABLE 20.3
Summary of Whole-Animal Toxicity Studies Conducted Using Either Purified Domoic Acid or Extracts from Domoate-Contaminated Shellfish

Species and Strain	Dose/Source	Observed Toxicity	Reference
Mice Swiss Webster	5.3–60 mL/kg (i.p.) whole mussel extract	Sluggishness, scratching Hind limb stretching, clonicconvulsions, death	[161]
Mice Swiss Webster PND 15	5–30 mL/kg (i.p.) whole mussel extract 0.64–2.55 mg/kg DOM from mussel hepatopancrease extract (i.p.)	Same as above	[161]
Mice Swiss Webster PND 15	300 mg/kg (i.p.) from mussel hepatopancreas extract	Gastric and duodenal ulcers	[162] [163]
Mice Swiss Webster (male)	0.5, 0.7, 0.8, or 1 mL of whole mussel extract (i.p.)	Gastric and duodenal ulcers Peritoneal ascites	[162] [163]
Mice Swiss-Webster	2, 3, or 7 mg/kg (i.p.)	Damage confined to circumventricular organs and their environs	[164]
Mice Swiss Webster (Infant female)	0.5, 0.84, 1, 4, or 8 mg/kg (i.p.)	Gastrointestinal bleeding	[163]
Mice CF1 (male/female)	12–233 µg DOM in whole mussel extract (i.p.)	Inactivity, scratching, uncontrolled rolling/twisting, loss of righting reflex, seizures, death	[100]
Mice CF-1 (Female)	8.8–104 mg/kg in whole mussel extracts (oral; gavage)	Inactivity, scratching, uncontrolled rolling/ twisting, loss of righting reflex, seizures, and death	[100]
Mice CD-1 (male)	0.15–20 mg/kg of contaminated mussels, spiked mussels, pure DOM, or algal DOM (i.p.)	Hypo-activity, sedation, rigidity, stereotypy, loss of posture, convulsions, and death in a dose-dependent manner such that max. severity increased and latency decreased with increasing dose	[48]
Mice CD-1 (male)	Unknown	Scratching, seizures, and death, lowest toxicity at pH 3.7, highest toxicity at pH 7.4, pH effect diminished as doses increased	[165]
Mice CD-1 (male)	0.06–0.3 nmol (i.c.v.)	Wet dog shakes, hyper-motility, circling, loss of righting reflex and seizures	[166]
Mice CD-1 (female)	4.0 mg/kg in whole mussel extracts (i.p.)	Sedation, rigidity, stereotypy, balance loss and seizures, neuronal damage in hippocampus	[118]
Mice CD1 (female)	Various doses from whole mussel extracts (i.p.)	Scratching and death	[167]
Mice CD-1 Pregnant (E13)	0.6 mg/kg to dam (i.v.)	Reduced seizure threshold, neuronal damage in CA3, CA4 and DG, reduced	[130]

Continued

TABLE 20.3 (Continued)

Species and Strain	Dose/Source	Observed Toxicity	Reference
GABA, increased Glu			
Mice ICR (female)	1–4 mg/kg DOM in 100 μL PBS (i.p.)	c-fos mRNA, c-fos product, degeneration in CA1–3, lateral and medial septum, and olfactory bulb.	[115]
Mice ICR (female)	0.25–4.9 mg/kg (i.p.)	Hyperactivity, scratching, seizures, fos expression in brain regions controlling memory and gastrointestinal functions	[168]
Mice DBA (male)	1.0 and 2.0 mg/kg acutely or on alternating days for 1 week (i.p.)	Dose-related toxicity consistent with previous reports, mice receiving acute injections had impaired performance on match-to-sample task on first day posttraining.	[169]
Mice DBA PND 14 (male/female)	2.0 mg/kg (i.p.)	Swim in circles, longer latency in Morris water maze	[114]
Mice ICR and DBA (female)	0.5, 1.0, and 2.0 mg/kg (i.p) acute 0.5 and 2.0 mg/kg every 48 h for 8 days (i.p.)	Increased behavioral toxicity; DBA more sensitive	[170]
Mice	35–104 mg/kg of whole mussel extract (oral; gavage) 20–80 mg/kg DOM (oral; gavage)	Scratching and seizures	[101]
Mice (female)	12–233 μg in toxic mussels 20–200 μg DOM (i.p.)	Scratching, rolling, tremors, seizures death	[101]
Mice	0.3–2.5 g– equivalent or original wet mussel tissue (i.p.)		[171]
Rats SD (female)	0, 1, 2, 4, 7.5 mg/kg (i.p.)	Behavioral and neurological signs of toxicity, lesions in hippocampus, hypothalamus, cingulum, frontoparietal cortex, septum and olfactory nuclei	[104]
Rats SD (male)	0, 0.22, 0.65 or 1.32 mg/kg (i.p.)	Hypomotility, decrease in body weight, increased auditory startle, degenerating neurons in CA1/CA3 and gliosis	[172]
Rats Fischer (male)	2.25 mg/kg (i.p.)	Stereotyped behavior and convulsions, increased GFAP in cerebral cortex, hippocampus, septum, caudate, putamen and thalamus	[98]
Rats Long-Evans	0.5–1.0 mg/kg (i.v.)	Seizure discharge in hippocampus, tonic-clonicconvulsions and death, learning maze task was impaired after intra-ventricular injection, DOM interfered with relearning maze	[173]
Rats Long-Evans	0.04 μg (i.c.v.)	Seizure discharge in hippocampus, tonic-clonic convulsions and death, pyramidal neuronalloss in CA1, CA3 and CA4	[173]

Continued

TABLE 20.3 (Continued)

Species and Strain	Dose/Source	Observed Toxicity	Reference
Rats CD-COBS (male)	0.03, 0.1, 0.3 nmol (i.c.v.)	Complete behavioral toxicity profile comparable to i.p.	[166]
Rat pups Long-Evans PND 2 and 10	0–1.5 mg/kg (i.p.)	Seizures, c-fos expression	[117]
Rat pups SD PND 0, 5, 14, 22 (male/female)	0.05–1.0 mg/kg (i.p.)	Full behavioral toxicity profile Reduced potency with increased age	[116]
Rats SD (male/female) EM histopathology	0, 0.1, 5.0 mg/kg/day oral (gavage)	No clinical abnormalities or changes in hematology or clinical chemistry	[102
Monkeys Cynomolgous	4.0 mg/kg (i.p.)	Chewing with frothing gagging and vomiting, abnormal movements,tremors excitotoxic lesions in CNS	[103]
Monkeys Cynomolgous	0.025–0.5 mg/kg (i.v.)	Same as above	[102]
Monkeys Cynomolgous Juvenile and adult (male/female)	0.25–4.0 mg/kg (i.v.)	Extensive hippocampal neurodegeneration, juveniles appear less sensitive	[129]
Monkeys Cynomolgous	0.5–10 mg/kg for max 6 days (oral; gavage, i.p., i.v.)	Vomited, mild to moderate CNS lesions	[104]
Monkeys Cynomolgous	0.5 mg/kg/day for 15 days and then 0.75 mg/kg/day For 15 days (oral; gavage)	No change in clinical chemistry or histopathology of major organs includingbrain, 24 h urine samples indicated GI absorption of 4–7% of administered dose	[174]

many of the data in mice (and to a lesser extent in rats) derive from early studies that used injections of extracts from contaminated shellfish (usually mussels) that had been previously analyzed for demonic acid content, rather than using chemically purified domoic acid. This is an important distinction, because the method of preparing extracts varies between studies and locations, and while one must assume that domoic acid concentrations in these extracts were accurately measured, there is no way of knowing if other neuroactive compounds are also present. In fact, an early report by Novelli et al. [41] demonstrated that other amino acids in shellfish extracts can confound assessments of domoic acid toxicity *in vitro*. Similarly, there are numerous anecdotal reports, as well as one published report [4], indicating that the method of storing domoic acid, either in solution or in extracts, can alter the biological activity of the compound. For example, in our own laboratory, we have observed that repetitive freeze–thaw cycles for domoic acid solutions results in significant loss of activity (unpublished). With these provisos, however, it is clear from Table 20.3 that when domoic acid is administered to rodents either systemically (oral, intravenous, intraperitoneal) or directly into the brain (intracerebroventricular), it produces robust and highly reproducible signs of behavioral toxicity.

Acute toxicity studies to date have used either exclusively male, exclusively female, or both male and female mice. Comparisons of the data obtained indicate quite consistently that there is no

appreciable sex difference in DOM toxicity in mice. There are, however, differences of opinion on whether different mouse strains respond differently to domoic acid. Comparisons between studies suggest that there are no major differences in toxicity between studies that used Swiss-Webster, CD-1, or CF-1 strains. However, the only report to specifically compare between mouse strains concluded that the DBA strain of mouse is significantly more sensitive to DOM toxicity that are ICR mice [115]. No such comparison has been done to date in rats.

Behavioral toxicity following systemic injection of domoic acid has been studied extensively in mice, and to a lesser extent in rats. In mice, systemic injections of domoic acid elicit a graded series of behavioral changes before seizure onset [48,91,92]. The sequence of events is both highly reproducible and predictably dose related. Following injection, adult mice will present initially with hypoactivity, followed in order by sedation akinesia, rigidity, stereotypy (characteristically consisting of a repetitive scapular scratching with one hindpaw), loss of postural control, convulsions (initially forepaw tremors progressing to tonic-clonic convulsions), and death. Both the maximum behavior observed and the speed with which the animal progresses through these stages are dose-dependent [48]. Consequently, it is possible to construct a domoic acid dose–response curve based on behavioral toxicity, thereby allowing for pharmacological investigation [48].

Similar behaviors are also seen in adult rats [104] and in monkeys [103,104], indicating remarkable interspecies consistency in response. Lastly, a modified version of the "Tasker scale" was used by Doucette et al. [116] to construct dose–response curves for domoic acid and kainic acid in neonatal rats, which also display progressive dose-related toxicity but have a much more restricted behavioral repertoire [116,117].

20.4.1.2 Histopathology

There is widespread agreement between labs regarding the histopathology of the acute brain lesions associated with domoic acid toxicity in rodents. Acute administration of high-dose domoic acid results in neurodegenerative changes, consisting of neuronal shrinkage, vacuolization of the cytoplasm, cell drop out, edema, and microvacuolation of the neuropil. These changes have preferential distribution within structures of the limbic system [103,104,118]. Among other brain regions, the hippocampus appears to be a specific target site having high sensitivity to domoic acid toxicity, particularly the pyramidal neurons in the CA3 region, followed by the dentate gyrus and CA1 region [103,104,113,118]. With some exception, the CA2 region is reported as the least affected [119]. Other regions affected include the pyriform and entorhinal cortices, olfactory bulbs, nucleus accumbens, arcuate nucleus, area postrema, and the retina [100,103,104,120]. The anatomical extent of brain lesions induced by domoic acid has also been identified by magnetic resonance imaging microscopy (MRM) in both human [121] and rat [122] and is consistent with histopathology data.

Light and electron microscopy observations in animal studies show two types of neuronal degeneration associated with domoic acid toxicity. Swollen, vacuolated neurons are described as the predominant feature, intermingled with some shrunken and darkly stained electron dense neurons [103,104,118,123,124]. These findings are consistent with the view that the neuronal degeneration induced by domoic acid is mostly necrotic, at least in the short term. Studies using markers of cell injury and apoptosis also support this view [123]. At the cellular level, the dendrite appears as a preferential early target site for excitotoxicity [124,125]. Electron microscopy of the CA3 hippocampal region of rats exposed to low doses of domoic acid show dilatation of the dendrites while the body of neurons are still preserved or with minimal changes [124]. These findings are supported by immunohistochemical studies using the microtubule-associated protein (MAP2) antibody, which preferentially binds to the microtubules of the dendrite tree [124]. Furthermore, *in vitro* studies on hippocampal slices demonstrated a distinct excitotoxic sequence of events leading to acute neuronal damage, highlighting the changes in the dendrites [125].

In addition to neuronal degeneration, there is also evidence suggesting involvement and injury of glial cells, including astrocytes and microglia [124,126,127]. Acute injury of astrocytes has

been observed by light and electron microscopy in animals exposed to domoic acid [124]. The lesion includes vacuolation and cell necrosis. In experimental animals surviving the acute episode of intoxication, there are permanent structural lesions of the hippocampus represented by gliosis [103], similar to what has been observed in patients who have died as a consequence of domoic acid exposure (see below).

20.4.1.3 Electroencephalography

Abnormal EEG recordings have also been reported following parenteral administration of domoic acid to freely moving nonanesthetized rats. Fujita et al. [128] administered vehicle or one of three doses of domoic acid (1, 5, or 10 mg/kg i.p.) to male Wistar rats implanted with bipolar electrodes in the dorsal hippocampus, and recorded recurrent focal hippocampal seizures beginning within minutes (high-dose group) or within 1 h (low-dose group) postinjection and lasting for about 24 h. Rats in the two higher-dose groups displayed hippocampal seizures that progressed to full limbic seizures propagating to the sensorimotor cortex and culminating in fatal status epilepticus [128]. A more recent study by Scallet et al. [129] correlated electroencephalographic, behavioral, and c-fos responses induced in rats by acute exposure to a single intraperitoneal dose of 2.2 or 4.4 mg/kg.b.w. of domoic acid. Both doses resulted in significant electroencephalographic changes beginning at about 30 min postinjection, but behavioral changes ('rearing/praying' and scapular scratching) were only seen at the higher doses. Postmortem examination of brains using c-fos immunohistochemistry showed that c-fos was activated in the anterior olfactory nucleus by both high and low doses of domoic acid. However, only the high dose increased c-fos immunoreactivity in the hippocampus, suggesting a dose-related regional differentiation within the brain that would be consistent with predictions that circumventricular organs would be more susceptible to systemic domoate toxicity than would brain regions protected by the BBB.

Also relevant to this discussion are several reports of altered EEG and/or seizure-like behaviors that result from perinatal exposure to domoic acid. A study by Dakshinamurti et al. [130] reported that 10- to 30-day-old mice exposed to domoic acid *in utero* (0.6 mg/kg i.v. in the pregnant dams) demonstrated generalized electrocortical depression associated with diffuse spike and wave activity in basal EEG recordings and had reduced seizure thresholds to exogenous domoate. These changes were seen even in the absence of motor convulsions, and interestingly, were accompanied by severe neuronal damage in the hippocampal CA3 and dentate gyrus region. Similarly, Doucette et al. [131] described a highly reproducible "seizure-like" syndrome in adult rats that had been treated postnatally with very low doses of domoic acid, and recently a preliminary report by Gill et al. [132] has confirmed that this behavioral syndrome is accompanied by an altered EEG pattern as recorded using cortical electrodes and remote telemetry.

20.4.1.4 Effects of Age and Tolerance

During the toxic mussel poisoning incident of 1987, the short-term epileptogenic effects and the lasting neurological deficits were seen primarily in older patients, suggesting a heightened susceptibility to domoic acid toxicity in the elderly (see below). It was not clear whether the heightened vulnerability arose from an age-related alteration in neuronal response to the toxin, from alterations in the pharmacokinetics of the toxin (e.g., impaired clearance due to age or preexisting disease) or from a combination of these factors. In addition, although not part of the clinical epidemiology of ASP, it seems reasonable to assume that there would be greater risk to either the fetus or newborn exposed to domoic acid. This is because the kinetics studies described above (Section 20.3.3) clearly demonstrate that the BBB is a major impediment to the movement of domoic acid into the CNS and that neurotoxicity is directly related to brain concentration. In mammalian species, the BBB lacks integrity until some time after birth [133] and renal function is reduced; consequently, both the fetus and the neonate should have greater susceptibility than do normal adults. To address these two questions of greater risk in the elderly and the very young,

a number of studies in recent years have focused on the relationship between age and domoic acid toxicity.

In vitro studies support the view that there is an age-dependent susceptibility to excitatory amino acid (EAA) excitoxicity and provide evidence for induced tolerance in the young, but not in the old, rat [83,134]. Hippocampal slices from young (3 months) and aged (26–29 months) rats assessed by CA1 field potential analysis before and after preconditioning with domoic acid were used to investigate this phenomenon [134]. The authors show that in naive slices from young animals, domoic acid produced initial hyper-excitability, followed by significant dose-dependent reductions in population spike amplitude during prolonged application. Following toxin washout, only small changes in neuronal activity were evident during a second application of domoic acid, suggesting that a resistance to the effects of domoic acid occurs in hippocampal slices that have undergone earlier exposure to domoic acid. However, aged CA1 failed to exhibit any tolerance to domoic acid following preconditioning, suggesting that a loss of inducible neuroprotective mechanisms may account for increased sensitivity to excitotoxins during aging [134]. A follow-up study from the same laboratory demonstrated that tolerance is triggered by a selective reduction in constitutive KA-sensitive G-protein activity, and that this potential neuroprotective mechanism is lost with age [83]. This age-related loss of tolerance to excitotoxins may serve to explain some aspects of the supersensitivity of aged animals and humans to brain damage associated with domoic and kainic acid toxicity.

Alterations in the pharmacokinetics of domoic acid as a function of age have not been systematically investigated to date. However, it is known that domoate is very hydrophilic and, consequently, exhibits relatively poor BBB permeability [108]. It has also been established by Suzuki and Hierlihy [105] that domoic acid is cleared systemically almost exclusively by renal clearance. Both BBB integrity and renal function are known to be impaired in very young and very old animals relative to normal adults.

There is also considerable evidence for a higher susceptibility to domoic acid toxicity during early postnatal development in rats [92,110,116,117,131]. Domoic acid induces a time-dependent behavioral toxicity in neonatal rats that is characterized by hyperactivity, stereotypic scratching, convulsions, and death at exposures 40–50 times lower by body weight in neonates than that reported in adults [116,117]. However, acute toxicity with domoic acid does not appear to cause structural alteration in the brain of neonates as assessed by Nissl staining and cupric silver histochemistry [117]. This is consistent with numerous other studies of neonatal seizures that demonstrate a generalized resistance to excitotoxic damage [135,136].

Comparative toxicity–response data for domoic acid and kainic acid in neonatal rats using a five-point behavioral rating scale adapted to neonates have been described for rats ranging in age from PND 0 to PND 22 [116]. While both compounds are markedly more potent in neonates than adults, the dose–response curve shifted dramatically to the right with increasing age, particularly after PND 14 [116]. Interestingly, however, the relative potency of domoic and kainic acid does not change throughout neonatal development (i.e., the dose response curves (DRCs) shift in parallel) [116], indicating that changes in potency are due to the physicochemical properties of the compounds (e.g., reduced BBB permeability and/or reduced clearance), rather than being due to developmental changes in glutamate receptor composition. However, interestingly, studies by Xi et al. [117] and Tasker et al. [92] have provided evidence for an involvement of NMDA receptors in neonatal domoate toxicity, which could also contribute to some of the observed effects.

One interesting "twist" to this theme is that there is an increasing body of evidence demonstrating that neonatal rats may not display signs of acute toxicity at the time of injection but do experience altered brain development. Studies by Doucette et al. [131] and Levin et al. [137] have demonstrated persistent behavioral changes in adult rats exposed to low-dose domoic acid during perinatal development. The paper by Doucette et al. [131] also reported on changes in hippocampal morphology in these rats, and subsequent publications by Bernard et al. [99] and Gill et al. [132] have confirmed alterations in hippocampal morphology and excitability arising from domoate-induced changes in brain development. While fascinating in terms of advancing our understanding

of neurological disease, these studies are also disturbing when considered within the context of risk assessment for shellfish toxin exposure.

20.4.2 Toxicity in Nonhuman Primates

The toxicity of domoic acid in nonhuman primates has been described in a limited number of publications, but is remarkably consistent with both investigations in rats and mice (see above) and the clinical manifestations of ASP reported in humans (see below). Cynomolgus monkeys (*M. fascicularis*) dosed either intraperitoneally (4 mg/kg) or intravenously (0.025–0.5 mg/kg) with domoic acid in extracts from contaminated mussels display clinical signs of neurotoxicity preceded by a short presymptomatic period (2–3 min) and an even shorter prodromal period (0.5–1 min). The symptomatic period proper is characterized by persistent chewing with frothing, varying degrees of gagging, and vomit. Monkeys receiving higher doses exhibit additional signs, including abnormal head and body positions, rigidity of movements and loss of balance, and tremors [103,104,119,120]. The duration of the symptomatic period is dose dependent. Thus, the nature and timing of dose-dependent toxicity in primates is comparable to that described previously in mice [48] and rats [104]. This is especially true when one considers that the gagging and vomiting seen in monkeys has no equivalent behavior in rodents (mice and rats do not vomit), but is presumably mediated by the lower brainstem regions such as the area postrema, known to be affected early in the DRC in mice (see above).

Histopathologically, the response of nonhuman primates is also comparable to both rodent and human patients. Excitotoxic lesions consisting of vacuolation of the neutrophil, astrocytic swelling, and neuronal shrinkage and hyperchromasia were detected in the area postrema, the hypothalamus, the hippocampus, and the inner layers of the retina in monkeys given domoic acid [103,104,119,120].

20.4.3 Genotoxicity

There is only very limited information on the potential for domoic acid to produce genotoxicity. In a hepatocyte-mediated assay with V79 Chinese hamster lung cells, domoic acid added to lung cell growth medium at doses of 27.2 or 54.4 μg/mL medium acid did not cause an increase in the frequency of mutations to thioguanine resistance or to oubain resistance, either alone or in the presence of rat hepatocytes, nor did it increase the frequency of sister-chromatid exchange or micronucleus frequency. This was in contrast to the effects produced by both a direct-acting (ethylmethanesulfonate) and indirect-acting (7,12-dimethylbenz[a]anthracene) genotoxin in the same assay [138]. These data would argue that the risk of genotoxicity and/or carcinogenicity following domoic acid is low or nonexistent, although a recent study by Carvalho et al. [139] has challenged this view. More work is clearly needed before firm conclusions can be drawn, but if domoic acid is a potential carcinogen it would seem logical that cells in the gastrointestinal epithelium would be most at risk.

20.4.4 Effects in Wildlife

It is now recognized that at least five species of *Pseudo-nitzschia* are able to produce domoic acid under certain environmental conditions and, as described previously, monitoring of both alga and marker species for the appearance of domoic acid is increasingly common. Correspondingly, it is now apparent that the toxin enjoys a worldwide distribution and has been identified as either a food contaminant or a causative agent in marine wildlife toxicity events on a global scale. Some of the more well-documented outbreaks of domoic acid toxicity in wildlife are summarized in Table 20.4 and described briefly below.

20.4.4.1 Marine Invertebrates

Although the most common vector for domoic acid, including the source in the 1987 human intoxication incident in Canada, is the blue mussel (*M. edulis*) [140–142], several authors have also reported

TABLE 20.4
Chronology of Documented Outbreaks of Domoic Acid Toxicity in Wildlife

		Affected Species	
Location	**Year**	**Common Name**	**Scientific Name**
Invertebrate species			
Prince Edward Island, Canada	1987	Blue mussel	*Mytilus edulis*
Bay of Fundy, Canada	1988	Soft-shell clam	*Mya arenaria*
		Blue mussel	*Mytilus edulis*
		Horse mussel	*Volsella modiolus*
		Sea scallop	*Placopecten magellanicus*
Coastal Washington and Oregon, USA	1991	Razor clam	*Siliqua patula*
		Dungeness crab	*Cancer magister*
Monterey Bay, California, USA	1991	Northern anchovy	*Engraulis mordax*
Pacific Coast of the USA	1991–1993	Blue crab	*Cancer spidus*
		Rock crab	*Cancer pagurus*
		Stone crab	*Menippe adina*
		Spiny lobster	*Palinurus elephas*
Coastal New Zealand	1993–1997	Maori scallop	*Pecten novaezealandiae*
		Greenshell mussel	*Perna canaliculus*
		Pacific oyster	*Crassostrea gigans*
		New Zealand cockle	*Austrovenus stutchburyi*
		Chilean oyster	*Tiostrea chilensis*
		Tuata surf clam	*Paphies subtriangulata*
Galicia, NW Spain	1994	Mediterranean mussel	*Mytilus galloprovincialis*
Georges, German and Browns Banks, Gulf of Maine	1995	Sea scallop	*Placopecten magellanicus*
Baja California Peninsula, Mexico	1995	Pacific mackerel	*Scomber japonicus*
Offshore Portugal	1996	Blue mussel	*Mytilus edulis*
		Common cockle	*Cerastoderma edule*
		Peppery furrow shell clam	*Scrobicularia plana*
		Pullet carpet shell	*Venerupis pullastra*
		European oyster	*Ostrea edulis*
		Razor clam	*Ensis* spp.
		Clam	*Ruditapes decussate*
Chinhae Bay, South Korea	1998	Various shellfish	Not specified
Washington and Oregon coasts, USA	1991–2005	Razor clam	*Siliqua patula*
Central Coast, California, USA	1998	Northern anchovy	*Engraulis mordax*
Offshore Scotland	1999–2000	King scallop	*Pecten maximus*
Offshore Ireland	1999	King scallop	*Pecten maximus*
Western Brittany, France	1999	Wedge shell clam	*Donax trunculus*
Vertebrate species			
Monterey Bay, California	1993	Brant's cormorants	*Phalacrocorax penicillatus*
		Brown pelicans	*Pelecanus occidentalis*
		Double-breasted cormorants	*Phalacrocorax auritus*
		Pelagic cormorants	*Phalacrocorax pelagicus*
		Western gulls	*Larus occidentalis*
Cabo San Lucas, Mexico	1997	Various seabirds	Not specified
Central Coast	1998	California sea lions	*Zalophus californianus*
California, USA	2000		
	2002	Gray whales	*Eschrichtius robustus*
		Blue whales	*Balaenoptera musculus*
		Sea otters	*Enhydra lutris*
		Sea lions	*Zalophus californianus*
		Humpback whales	*Megaptera novaeanglia*
		Various fish species	Not specified

on the presence of domoic acid in other shellfish [143–145] and crustaceans [146]. Among these, the most comprehensive study is that of Vale and Sampayo [147] in which they sampled a variety of Portuguese shellfish and fish for the presence of domoic acid. These authors reported considerable accumulation of domoic acid in a variety of shellfish including the common cockle (*Cerastoderma edule*), the peppery furrow shell (*Scrobicularia plana*), the carpet shell (*Venerupis pullastra*), oysters (*Ostrea edulis*), razor clams (*Ensis* spp.), and common clam (*Ruditapes decussate*). Although it is well established that shellfish and marine crustaceans can bioaccumulate domoic acid, there is no evidence to date of toxic consequences to the animals themselves. The reason for this lack of toxicity in invertebrates is currently unknown, but there are two dominant theories: (a) that domoic acid is not absorbed from the gastrointestinal organs of invertebrates, and (b) that invertebrates have configurations of AMPA/kainate receptors that differ from those of mammals. Regardless of which, if either, is correct, marine invertebrates appear to be insensitive to domoate toxicity.

20.4.4.2 Birds

There have been numerous anecdotal reports of domoic acid intoxication in piscivorous seabirds; however, the best-documented cases are those described by Work and colleagues in 1993 and by Sierra Beltran et al. in 1997. Work et al. [148,149] described the epidemiology of a domoic acid epidemic in Monterey Bay, California, in which 95 Brant's cormorants (*Phalacrorax penicillatus*) and 43 brown pelicans (*Pelecanus occidentalis*) died. They also noted the deaths of several other species, including double-breasted cormorants, pelagic cormorants, and western gulls in the same incident. The source of domoic acid in these cases appeared to be anchovies, which were recovered from the stomachs of many of the dead birds and were shown by HPLC analysis to have high concentrations of domoic acid. Analysis of blood samples from 12 intoxicated pelicans and 5 cormorants yielded detectable concentrations of domoic acid (0.5–48 μg/g) in 11 of 12 pelicans and in 2 of the cormorants (1.4 and 6.3 μg/g). In terms of behavioral toxicity, intoxicated cormorants showed few overt signs of CNS toxicity but were generally lethargic or docile. In contrast, affected pelicans displayed many signs of toxicity that were reminiscent of ASP in other species. These included side-to-side head motions, fine motor tremors, scratching, vomiting, loss of awareness, loss of postural control, convulsions, and death. In a similar incident involving 150 dead seabirds in Cabo San Lucas, Mexico, Sierra-Beltran et al. [150] reported the presence of domoic acid in viscera from five dead pelicans, which they analyzed by both mouse bioassay and HPLC. The other birds were not tested. In this case, the source of domoic acid appeared to be consumption of contaminated mackerel. Lastly, on a more esoteric note, there is a widespread belief that the events dramatized in Alfred Hitchcock's famous movie "The Birds" (©Universal Pictures, 1963) were caused by California seabirds consuming shellfish contaminated with domoic acid.

20.4.4.3 Marine Mammals

Domoic acid has been presumed to be the cause of an incident in which many California sea lions (*Zalophus californianus*) died and many others displayed signs of neurological dysfunction along the central California coast during May and June 1998. A bloom of *Pseudo-nitzschia australis* (diatom) was observed in the Monterey Bay region during the same period, and this bloom was associated with the production of domoic acid [151]. This toxin was also detected in planktivorous fish, including the northern anchovy (*Engraulis mordax*) [152,153], and in sea lion body fluids. Interestingly, blue mussels (*M. edulus*) collected during the outbreak contained no domoic acid, or contained only trace amounts. Such findings reveal that monitoring of mussel toxicity alone does not necessarily provide adequate warning of domoic acid entering the food web at levels sufficient to harm marine wildlife and perhaps humans [154].

Scholin et al. [154] provide a detailed account of the clinical and histopathology observations of animals affected during the 1998 outbreak. Briefly, clinical signs in affected sea lions were ataxia, head weaving, muscle tremor, titanic convulsions, rubbing, and lethargy, with 48 (69%) of the

initially affected sea lions either dying owing to toxicity or having to be euthanized. Seizures, often progressing to *status epilepticus* for over an hour, were common in the most severely affected animals and ranged in duration from a few minutes to 30 min, with frequency recorded between 1 and 30 seizures over a 24-h period. In animals that survived, the frequency of seizures gradually decreased over a 1-month. Histopathology showed that the principal lesions that were considered unique to this stranding event were in the brain and in the heart, with the most severe necrosis being recorded in the dentate gyrus and pyramidal layers of the hippocampus in the anterior ventral region, with other areas for the brain appearing less severely or consistently affected [154]. However, it is possible that the extent of the lesions in other brain areas was underestimated.

In 2000, a further 184 sea lions stranded with similar clinical signs [155], both during detectable algal blooms and after the blooms had subsided. The clinical signs in these sea lions included seizures, ataxia, and head weaving, decreased responsiveness to stimuli, and scratching behavior. Affected animals had high hematocrits and eosinophil counts, and high activities of serum creatine kinase. They were treated supportively by using fluid therapy, diazepam, lorazepam, and phenobarbitone. Fifty-five of the 81 sea lions (68%) affected in 1998, and 81 of the 184 (44%) affected in 2000 died despite treatment. Three of the 23 sea lions that survived in 1998 were tracked with satellite and radio transmitters; they traveled as far south as San Miguel Island, California, and survived for at least 3 months. Eleven of the 129 animals that were subsequently released were found stranded within 4 months [155].

In addition to sea lions, domoic acid has been implicated as a causative toxic agent in several other marine mammal species notably whales and sea otters. A 2002 report by the Working Group on Marine Mammal Unusual Mortality Events [156] describes investigations into the deaths of 350 gray whales, 7 sea otters, 90 sea lions, and 2 humpback whales. Domoic acid was detected in body fluids from 2 of 11 gray whales (*Eschrichtius robustus*) sampled and in the urine of affected sea otters. While such detection is not evidence of a causal relationship, it does demonstrate bioaccumulation of domoic acid in the marine food chain. Similarly, a report by Lefebvre et al. [157] describes domoic concentrations from 75 μg/mL to 444 μg/mL in the viscera of various fish species that had been consumed by three humpback whales (*Megaptera novaeanglia*) and concentrations from 10 to 207 μg/g in whale feces collected from one humpback and two blue (*Balaenoptera musculus*) whales; the blue whales were presumed to have been feeding on contaminated krill, although this was not confirmed. By extrapolating these concentrations, Lefebvre et al. [157] arrived at estimated toxin loads of 1.1 mg/kg domoic acid for humpback whales feeding on fish and 0.62 mg/kg domoic acid for blue whales feeding on krill.

20.4.5 Effects in Humans

Late in 1987, an outbreak of a newly recognized acute illness caused by eating blue mussels and characterized by gastrointestinal and unusual neurological symptoms occurred in Canada. More than 107 people (47 men and 60 women) were affected, mostly from Quebec. A case was defined as due to the presence of gastrointestinal symptoms (vomiting, abdominal cramps, and diarrhea) within 24 h and neurological symptoms within 48 h (severe headache and memory loss) [1,111]. The etiologic agent was found to be domoic acid, produced by *N. pungens f. multiseries.*, and present in the digestive glands of the causative cultivated mussels [21], harvested from the eastern coast of Prince Edward Island, and shipped to other parts of Canada [1].

Of the 107 persons that met the case definition, 19 were hospitalized, of which 12 were admitted to intensive care, and 3 died in hospital. Another patient died after 3 months. Data on symptoms and the quantity of mussels consumed was collected on 99 patients, 87 of whom were 40 years of age or older. Symptoms of illness included nausea (77%), vomiting (76%), abdominal cramps (51%), diarrhea (42%), headache (43%), and memory loss (25%). None of the younger patients (20–39 years) suffered memory loss, and their only symptoms were of a gastrointestinal nature. Hospital charts were available for 16 of the 19 hospitalized patients, indicating that all severely ill patients less than 65 years of age had preexisting illnesses, including diabetes (3), chronic renal disease (2),

and hypertension with a history of transient ischemic attacks (1), considered to be the predisposing factors. All patients admitted to intensive care had serious neurological dysfunctions, including coma (9), mutism (11), and seizures (8).

On the basis of the patients' recall of portion sizes and analysis of unconsumed portions of mussels, it was possible to estimate the exposure to domoic acid in nine patients and one person who did not become ill. Increasing exposure correlated with the clinical course of events. All patients reported gastrointestinal illness, but only one of six patients who consumed between 60 and 110 mg domoic acid suffered memory loss, and none required hospitalization. All three patients who had consumed 270–290 mg domoic acid suffered neurological symptoms and were hospitalized. One person who consumed only 20 mg domoic acid did not become ill. The cognitive impairment observed in this new disease, attributed to domoic acid, appeared to be persistent and led to the term "amnesic shellfish poisoning" [1,158].

The neurologic manifestations of domoic acid intoxication from the 1987 Canadian outbreak were studied over a period of several years in 14 of the patients, all from the Montreal area [159]. This study included neuropsychological testing, an assessment of motor function, and positron emission tomography (PET) to assess glucose metabolism in specific regions of the brain. The detailed findings of these investigations are too extensive to be fully described herein, but the acute phase of poisoning was characterized by gastrointestinal illness, and seven patients became comatose and suffered severe complications, including hemodynamic instability with hypotension, and arrhythmias unrelated to cardiac disease. All 14 patients were confused and disoriented 1.5–48 h postexposure, and their behavior ranged from agitation or somnolence to coma, with maximal deficits between 4 (least affected) and 72 h (for comatose patients) postexposure. Most improved within 24 h–12 weeks. Patients characteristically had anterograde memory disorder with relative preservation of other cognitive functions. Those individuals with a moderate memory disturbance were generally able to encode information, but had difficulty with delayed recall. More severely affected individuals had some difficulty learning verbal and visuospatial material, and their delayed recall was also very poor. In addition, some of these latter patients also had retrograde amnesia extending several years back. Electroencephalograms (EEGs) in seven patients obtained within 1 week postexposure revealed moderate to severe generalized slowing of background activity. At about 4 months postexposure, the electroencephalograms of 11 patients showed mild to moderate generalized disturbance of background activity, and the other 3 had normal readings. In addition to central changes, there was also considerable evidence of peripheral nerve damage and changes in sensory thresholds. The authors suggested that domoic acid induced acute nonprogressive neuronopathy involving anterior horn cells or diffuse axonopathy predominantly affecting motor axons.

Neuropathological examination of the four patients who died indicated neuronal necrosis and astrocytosis, particularly in the hippocampus and the amygdaloid nucleus. All four victims also had lesions in the claustrum, secondary olfactory areas, the septal area, and the nucleus accumbens septi. Two had prominent thalamic damage, especially in the dorsal medial nucleus. The subfrontal cortex was also damaged in three of the patients. The authors noted that the pattern of damage in the hippocampus appeared to parallel that seen in animals that suffered neurotoxic reactions after administration of kainic acid (and domoic acid; see above).

One final interesting report of the 1987 incident involves the initial findings and follow-up of an 84-year-old man who suffered domoic acid intoxication during the 1987 outbreak in Canada and who subsequently died, 3.25 years after exposure [121]. The patient's symptoms during the phase of acute toxicity were comparable to those of others (see above). However, approximately 1 year postintoxication, he experienced complex partial seizures consisting of staring and twitching of the left lower part of the face, and then clonic movements of the left arm and leg developed. Gross examination of the patient's brain postmortem (death due to pneumonia) revealed atrophy of the hippocampi and slight dilatation of the ventricular system and of the Sylvian fissure. Histologically, the hippocampi showed complete neuronal loss in the H1 and H3 (equivalent to CA1 and CA3 in rodents) regions, almost total loss in H4 (equivalent to CA4) and moderate loss in H2 (equivalent to CA2). The amygdala

showed patchy neuronal loss in medial and basal portions with neuronal loss and gliosis in the overlying cortex. Mild-to-moderate neuronal loss and gliosis were seen in the dorsal and ventral septal nuclei, the secondary olfactory areas, and the nucleus accumbens. It was suggested that temporal lobe epilepsy following domoic acid exposure might develop after a "silent period" of 1 year [121].

Apart from the 1987 incident in Canada, the only other presumed case of domoic acid poisoning in humans was an outbreak that may have occurred in October/November 1991 in Washington State (although unreported incidents of toxicity presumably also occur worldwide). Approximately two dozen people became ill after ingesting razor clams harvested along the Washington and Oregon coasts. Although gastrointestinal or neurological symptoms were observed within 36 h of ingestion, no other symptoms consistent with domoic acid intoxication were reported. A total of 21 possible domoic acid victims and 43 nonvictims were interviewed. On the basis of the concentrations of domoic acid measured in razor clams, the mean total domoic acid consumption by the 21 victims would have been 17 mg (estimated to be 0.28 mg/kg b.w.) and 8 mg by the unaffected individuals (0.13 mg/kg b.w.). A total of 13 patients developed neurological symptoms, but all recovered. Unfortunately, the incident was not well reported and was unsubstantiated [160].

20.6 CONCLUSIONS

Domoic acid is a naturally occurring compound produced by several species of algae and phytoplankton. Algal-derived domoic acid has been used therapeutically for centuries in Japan, but phytoplankton-derived domoic acid can accumulate in shellfish and herbivorous fish species to extremely high concentrations. Consumption of contaminated shellfish or fish then leads to gastrointestinal, and at higher doses, neurological toxicity in higher species, including humans. A number of analytical methods have been developed for monitoring and quantifying domoic acid, and new methods for both high-resolution or high-sensitivity detection as well as convenient "dockside" monitoring are emerging regularly. Pharmacologically, domoic acid exhibits poor oral bioavailability and limited BBB transit, but on gaining access to the mammalian central nervous system the compound acts preferentially as an agonist at "low-affinity" kainate receptors. With increasing concentration, other subtypes of glutamate receptors contribute to toxicity, complicating therapeutic options. The toxicology of domoic acid in rodents and nonhuman primates has been extensively described, and in general there are few interspecies differences in the symptoms of toxicity. One area of great potential concern, however, is that the very young and the very old are much more sensitive to domoate toxicity, and there exists a growing body of literature suggesting that exposure to low concentrations around the time of birth can lead to persistent neurological deficits and possibly neurological disease. Regulatory agencies may want to give this issue greater consideration, but as our knowledge of glutamate receptor pharmacology in the CNS increases, domoic acid should continue to emerge as a valuable experimental tool for understanding the role of kainate receptors in health and disease.

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Part VIII

Non-PSP Neurotoxic Episodes

21 Ecobiology of the Brevetoxin, Ciguatoxin, and Cyclic Imine Producers

Lincoln MacKenzie

CONTENTS

21.1 ECOBIOLOGY OF BREVETOXIN PRODUCERS

21.1.1 Introduction

Davis (1948) was the first to formally describe the relationship between the occurrence of blooms of a planktonic dinoflagellate, that he named *Gymnodium brevis*, and mass mortalities of marine animals off the west coast of Florida. In the 1970s the major brevetoxin (BTX) components (then referred to as GB-1 and GB-2) produced by the dinoflagellate were isolated (Shimizu et al., 1974) from cell cultures. Since then this dinoflagellate has become notorious, especially in the western coastal regions of Florida, for causing major fish kills, human respiratory distress, neurological illness to consumers of shellfish, and significant economic disruption to local economies. In recent years problems with blooms appear to have become more common in the western Gulf of Mexico (1996–2000 was one of the most active period for blooms in recorded history) although accounts of red tides and associated fish kills in this region date back to the seventeenth century (Magaña et al., 2003). During the austral summer of 1992–1993 there was an extensive dinoflagellate bloom in northern New Zealand that resulted in contamination of shellfish with BTXs and an epidemic of neurotoxic shellfish poisoning. This remains the only known documented occurrence of this phenomenon outside the United States. It is probable that various species within the plankton produce BTX or BTX-related compounds and it is expected that with the adoption of more sophisticated chemical analyses these compounds will be detected more widely.

21.1.2 Taxonomy and Global Distribution of Brevetoxin Producers

The major BTX-producing dinoflagellate, now known as *Karenia brevis* (Figure 21.1), has had several name changes that has left a legacy of various names in the literature for the same organism and, conversely, several species under one name. The genus names have also influenced the names applied to the various toxic compounds produced by this organism. The resulting confusion is because the classification of dinoflagellates was previously based mainly on morphological features visible under the light microscope. Unique distinguishing features in naked dinoflagellates are difficult to see and were poorly described until recently. Consequently many species were lumped together in genera that did not reflect true phylogenetic relationships.

Gymnodinium brevis was renamed *Gymnodinium breve* in the mid-1960s and then Steidinger (1979) presented a new description of the genus, on the basis of ultrastructural features, and proposed the name *Ptychodiscus brevis*. It was at this time that the chemical structure of the various brevetoxin analogues were being described, and they came to be referred to as *Ptychodiscus* toxins (PbTxs). The *P. brevis* designation was adopted until further study of the morphology of the cells (Moestrup and Larsen, 1990; Steidinger, 1990) resulted in restoration of the name *G. breve*. Finally, Daugbjerg et al. (2000) revised the phyologeny of the naked gymnodinoid dinoflagellates on the basis of ultrastructural and partial large subunit (LSU) ribosomal RNA (rRNA) gene sequence data, and erected three new genera (*Karenia*, *Karlodinium*, and *Akashiwo*). *G. breve* was renamed

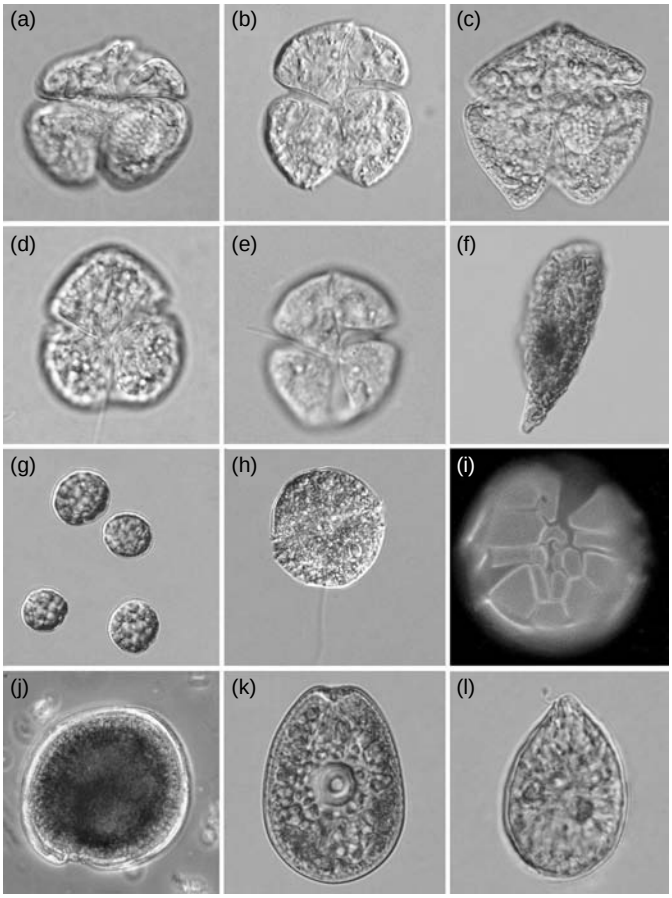

FIGURE 21.1 Photographs of live specimens of species referred to in this review. (a) *Karenia brevis* (b) *Karenia selliformis,* (c) *Karenia bidigitatum,* (d) *Karenia mikimotoi,* (e) *Karenia brevisulcata,* (f) *Chattonella antiqua,* (g) *Heterosigma akashiwo,* (h) *Alexandrium ostenfeldii,* (i) *Al.ostenfeldii,* (calcofluor stained), (j) *Gambierdiscus toxicus* (courtesy of Y. Fukuyo), (k) *Prorocentrum lima,* and (l) *Ostreopsis lenticularis*.

K. brevis. Yang et al. (2000, 2001) then published descriptions of *K. digitata* and *K. longicanalis* from Hong Kong waters, Haywood et al. (2004) and Chang and Ryan (2004) published the description of four new species of *Karenia* from New Zealand waters (*K. selliformis*, *K. papilionacea*, *K. bidigitata*, and *K. concordia*), and a previously described species (*G. brevisulcatum*; Chang, 1999) was renamed *K. brevisulcata*. Botes et al. (2003) published the description of two new species of *Karenia* (*K. bicuneiformis* and *K. crisata*) from South Africa (*K. bicuneiformis* is probably synonymous with *K. bidigitata*) and De Salas et al. (2004a,b) published the description of two new species (*K. umbella* and *K. asterichroma*) from Tasmania. *K. mikimotoi* generates blooms that have caused mass mortalities of marine fauna in many regions and has carried a variety of names in the past (*G. mikimotoi*, *G. nagasakiense*, *Gyrodinium aureolum*; Hansen et al., 2000). *K. mikimotoi* produces a ladder form polyether toxin (Gymnocin-A) similar to brevetoxin (Satake et al., 2002). Not all *Karenia* species have genetic and biochemical (e.g., pigment) data to support discrimination based on morphological descriptions, but currently there are 12 described species within the genus *Karenia*. *Karenia* belongs within a group of naked dinoflagellates that do not contain peridinin, the typical dinoflagellate accessory carotenoid, but instead produce fucoxanthin and fucoxanthin derivatives characteristic of diatoms and brown algae. Not all *Karenia* species have been shown to have toxic or noxious properties but many do. Most recently Chang et al. (2006) have reported that

K. concordia produces various brevetoxin analogues. Interestingly recent reports of blooms in the Gulf of Mexico (Heil et al., 2006) mention that other species in addition to *K. brevis* (*K. mikimotoi*, *K. papilionaceae*, *K. selliformis*, and unidentified *Karenia* species) occur within these blooms and may have been previously overlooked owing to the difficulty of identification.

There is still no confirmed record of *K. brevis* outside the Gulf of Mexico and Southeast USA. There is a record of a very similar species from Japan (Takayama, 1981), but the illustrations of this species in Fukuyo et al. (1990) are similar to *K. papilionaceae* (Haywood et al., 2004) and even in the accompanying text it is acknowledged that it may be a new species. There is one record of the occurrence of *G. breve* from the Aegean Sea (Pagou and Ignatiades, 1990); however, the identification appears tentative, and to our knowledge its identify has never been confirmed. There is a claim, on the basis of immunohistochemical evidence, that brevetoxicosis was responsible for the deaths of Common Murres (a relative of the Puffin) in Monterey Bay, California. This may have been the result of toxins produced by raphidophytes (*Heterosigma*, *Fibrocapsa*, and *Chattonella*) or *K. mikimotoi* (O'Halloran et al., 2004); however, no peer reviewed evidence to substantiate this is available.

For simplicity, in the following discussion the designation *K. brevis* has been used even when referring to published work where other names (i.e., *G. brevis*, *G. breve*, and *P. brevis*) have been used by the authors. It is assumed they are the same species.

21.1.2.1 *K. brevis* Life Cycle

Steidinger et al. (1998) reviewed what is known about the life cycle of *K. brevis* and presented a diagrammatic representation of various life cycle stages. It is surprising, given the length of time that isolates of *K. brevis* have been in culture, and the intensity with which the field ecology of this species has been studied, that the complete life cycle has yet to be elucidated. Although the fusion of isogamous gametes and the formation of a planozygote has been observed (Walker, 1982), no hypnozygote or meiocytes have been positively identified. Thick walled rounded cells and clumps of rounded cells within mucilagenous sheaths have been observed in seawater samples and old cultures that may represent resting cyst stages, but this has yet to be confirmed. To have a complete understanding of how blooms of *K. brevis* originate, it is important that a resting cyst stage, if any, is identified and quantified. Recent advances in the use of genetic probes (Gray et al., 2003) for identification of cryptic species and life cycle stages may assist in further research in this area. Interestingly, it has been suggested that some species of *Karenia*, such as *K. papilionaceae* and *K. bicuneiformis*, are closely related to and may even be life cycle stages of oceanic naked dinoflagellates within the genus *Asterodinium-Brachidinium* (Gomez et al., 2005).

21.1.3 Culture Studies on the Environmental Determinants of Growth and Toxin Production in *K. brevis*

Until relatively recently most culture studies on *K. brevis* were carried out on a single clone (Bigelow culture collection isolate CCMP718) isolated by W. B. Wilson from a water sample collected from Johns Pass, St. Petersburg on the Gulf coast of Florida in 1953. It is only in the last 5–6 years that new clones have been isolated and comparative physiological/biochemical studies been carried out. In fact, the Wilson clone has proved broadly representative of other clonal isolates, though some important characteristics (e.g., toxin profiles) vary significantly (Table 21.1).

21.1.3.1 Effects of Temperature and Salinity on Growth of *K. brevis* Cultures

The temperature tolerance of *G. breve* was defined in early work by Eng-Wilmot et al. (1977). *G. breve* proliferated over a temperature range of 17–30°C, with optimum growth at 22°C. Below 17°C cultures declined and at 4°C they died within 5 h. Above 31°C there was rapid decline

TABLE 21.1
Summary Table of *Karenia brevis* Characteristics

	(pg/cell)			Salinity Tolerance		
***Karenia brevis* origin**	**PbTx-2**	**PbTx-3**	**μ max (div/d)**	**(lower)**	**(upper)**	**Reference**
Texas strains	13.2–41.3	3.9–24.5	0.58–0.94	24	45	Loret et al., 2002
Florida-"Wilson strain"	11.2	3.5	0.2–1.0	22.5	46	Loret et al., 2002
Florida and Texas strains	4.9–12.6	0–2.3				Baden and Tomas, 1988
Florida and Texas strains	nd	nd	0.17–0.36	22.5	46	Magaña and Villareal, 2006
Florida (Wilson and Texas clones	4.4–22.0 (total PbTxs)		0.04–0.26	17.5–20.0	45	Maire Brown et al., 2006
Florida "Rogers strain	23					McNabb et al., 2006
Florida strain (Wilson?)	8.7	0.42	nd	nd	nd	Roszell et al., 1990*
Gulf of Mexico wild cells	12–126 (total PbTxs) PbTx2 = 67–75%		nd	nd	nd	Greene et al., 2000** Loret et al., 2002.

* PbTx-1 = 1.7 pg/cell, PbTx-7 = 0.026 pg/cell, PbTx-9 = 0062 pg/cell.
** Includes soluble plus particulate toxins recovered by DCM partition.

in viability and at 33.5°C cells died within 24 h. Although most studies have shown that *K. brevis* does not thrive in water below 19°C, Lamberto et al. (2004) found that while cultures grown at 15°C decreased in cell numbers they showed an increase in toxin (PbTx-2, PbTx-3, brevenal) concentration/cell [measured by high-pressure liquid chromatography with UV detector (HPLC-UV)]. Cultures grown at 22°C, 24°C, and 26°C showed no differences in cell growth or toxin production.

Before the observation in 1996 of blooms of *K. brevis* in low salinity waters in the northern Gulf of Mexico, near the Mississippi outflow, it was believed that a salinity barrier of 24 psu prevented *K. brevis* survival and proliferation (Geesey and Tester, 1993). Lekan (2006) examined the effect of salinity on the growth of *K. brevis* in culture and found no growth occurred below 20 psu but good growth occurred at 25–40 psu. The highest growth rates were attained at a salinity of 35 psu and lowest at 25 psu (0.64–0.36 div/day). Salinity was the primary factor affecting growth, with nutrient concentrations of secondary importance. Nutrient stress (N-limitation) may stimulate toxin production, but salinity was the primary factor regulating toxin production at salinities between 25 psu and 40 psu. Maier Brown et al. (2006) found that the minimum salinity at which *K. brevis* growth occurred was between 17.5 and 20 psu. Depending on the clone, optimum salinities for growth occurred between low values of 20–25 psu and high values of 37.5–45 psu. At all salinities brevetoxin levels, measured using a receptor binding assay (Van Dolah et al., 1994), were highest during stationary phase. Magaña and Villareal (2006) showed that while individual clones of *K. brevis* varied considerably in maximum growth rates there were only minor differences between Texas and Florida strains in their temperature and salinity tolerances.

21.1.3.2 Effects of Light on *K. brevis*

K. brevis is positively phototactic during the day and positively geotactic at night (Kamykowski et al., 1998) and cell concentrations often develop at the sea surface during the day. Evens et al. (2001) demonstrated that *K. brevis* has an inherent UV resistance. Exposure to UV under intense sunlight in the field did not result in differences in oxygen production or other biochemical parameters. They demonstrated that *K. brevis* has a photoprotection system involving the induction and

relaxation of the xanthophyll cycle that protects them from the harmful effects of the build up of oxidative radicals resulting from excessive photon fluxes. There were only minor diurnal changes in chlorophyll a and fucoxanthin content of the cells.

Higham et al. (2004) examined the photopigment content of three clones of *K. brevis* analyzed after acclimation to high and low irradiances. Photosynthetic pigment and carotenoid concentrations decreased with increased irradiance. Carotenoid/chlorophyll ratios differed within and between clones and irradiance treatments but the gyroxanthin-diester/chla ratios were not significantly different between clones exposed to high and low light. Evens and Leblond (2004) carried out preliminary investigation of the relationship between fatty acid synthesis and cellular processes such as photosynthesis and reproduction. They examined the effect of light intensity on thylakoid membrane permeability and fluidity and found that the differences between low and medium light grown *K. brevis* are quantifiable and significant. Shanley and Vargo (1993) showed that photosynthesis in *G. breve* (Wilson clone) was saturated at rather low light levels, although cells are frequently found in high concentrations exposed to very high light at the sea surface. Schaeffer et al. (2002) compared the photosynthetic capabilities of eight *K. brevis* clones using pulsed amplitude modulated chlorophyll fluorometry (PAM-FL). Clones were isolated from different geographic regions of the Florida coast and their photosynthetic capabilities compared with the Wilson strain. Variations among the photosynthetic capabilities of the eight clones suggested that different clones may represent different functional groups though these different groups did not represent a geographic segregation. Some clones, in contrast to the group containing the Wilson clone, were capable of maintaining their photosynthetic capabilities at natural surface light intensities. McKay et al. (2006) demonstrated that different strains of *K. brevis* displayed opposite photosynthetic responses to high and low acclimated temperatures, but their responses to high and low light intensity were similar.

21.1.3.3 Nutrient Assimilation by *K. brevis*

K. brevis reaches bloom proportions in oligotrophic waters of the Gulf of Mexico and populations persist for months, maintaining themselves under low nutrient conditions. Vargo and Howard-Shamblott (1990) showed that uptake rates of phosphorus (P)-deficient cells (Wilson clone) were almost twice that of P-sufficient cells (0.73 pg and 0.4 pg tam P/cell/h respectively), relating to cellular P turnover rates of 4.4–19 h. It seems that the P uptake rate, the half saturation constant for growth, and the long generation time of *K. braves* allows it to maintain growth in waters where the concentration of dissolved inorganic P is low. *K. brevis* does not appear to have any unusual P storage capability.

21.1.3.4 Brevetoxin Production in *K. brevis* Cultures

Baden and Tomas (1988) analyzed the breve toxin content (PbTx1–3) of cultures of six *G. breve* clones in late logarithmic phase and found significant variation in toxin profiles, although PbTx-2 (4.9–12.6 pg/cell) was the predominant form in all isolates (74–93%). The mean ratio of PbTx-1 and PbTx-3 to PbTx-2 was 0.11 and 0.09, respectively, although the absolute toxin content of these analogues varied substantially between the clones. Total toxin content ranged from 6.6 pg/cell to 16.6 pg/cell. There did not appear to be a relationship between the geographical origin of the cultures and toxin profiles. It was suggested that the clonal variation in toxin profiles may reflect variations in the metabolic capacity of the clones. The toxin content and profile of the Wilson clone (W53DB) was close to the average shown by the various other clones but the variability between these clones was much greater than what existed between extracts of replicate cultures of the Wilson strain.

Roszell et al. (1990) described changes in toxin profile (by HPLC-UV) within batch cultures of *P. brevis* (clone unspecified but probably Wilson's). PbTx-2 was the predominant toxin with lesser amounts of PbTx-1 (~30%) and PbTx-3 (~10%) in logarithmic phase cultures but in the stationary and decline phases there was an increase in PbTx-3 with a concurrent decline in PbTx-2. As cells entered stationary phase other analogues (PbTx-7) began to appear. It was concluded that

the aldehydes (PbTx-2 and PbTx-1) are the parent form of the toxins that become reduced to their corresponding alcohols (e.g., PbTx-3 and PbTx-7) as the cultures age. DNA sequence analysis of the 18S rRNA gene and internal transcribed spacer (ITS) regions of five strains of *K. brevis*, that exhibited different growth rates and toxin content under identical conditions, was carried out by Loret et al. (2002). PbTx-2 (11.2–41.3 pg/cell) was the predominant toxin analogue over PbTx-3 (3.5–24.5 pg/cell) in all strains. Despite variation in toxin content between strains, a genetic basis for this variability could not be identified using the 18S and ITS markers. McNabb et al. (2006) screened 25 dinoflagellate isolates, most from New Zealand waters, in the genera *Karenia*, *Karlodinium* and *Takayama* for BTXs using liquid chromatography-mass spectrometry (LC-MS)/MS. Only one *K. brevis* strain (Rogers) from the Gulf of Mexico was shown to produce any BTX analogues (predominately PbTx-2). Interestingly, three separately sourced cultures of the Wilson strain of *K. brevis* that had been in culture in GP medium (containing soil extract) at 17–18°C for at least a decade tested negative. When first imported to New Zealand they were shown to produce BTXs. It appears that these strains may have lost their toxin producing capability by being cultured under suboptimal conditions for an extended period.

The ichthyotoxic potency of *K. brevis* blooms does not always correlate with the abundance of *K. brevis* cells, implying that there may be natural variations in toxicity in nature. To examine this question Campbell et al. (2002) studied the production of BTX and BTX-like compounds during various growth phases of *K. brevis* (Wilson clone) in culture. Their data shows a great deal of scatter with large, though apparently nonsignificant, variation in PbTx-3 production over the growth cycle and slightly higher production of PbTx-2 in stationary phase. Their most interesting observation was that the nonichthyotoxic compound brevenal (Bourdelais et al., 2004a,b) that acts as a competitive inhibitor against BTXs in sodium channel receptor binding assays was generally higher in stationary phase cultures. Brevenal has a polyether ladder form structure that resembles hemibrevetoxin, it can be obtained from cultures and natural water samples during blooms. It is suggested it may influence the potency of *K. brevis* blooms and the marine fauna mortalities associated with these, and may provide a therapy for brevetoxin poisoning.

21.1.3.5 Biochemistry and Molecular Biology of Brevetoxin Synthesis

Early work by Shimizu et al. (1990) led to a speculation that the brevetoxins were a new type of mixed polyketide, synthesized by the condensation of dicarboxylic acids, acetate, and proprionate, and that acetate units are incorporated only after they have been converted to other metabolites such as succinate and α-ketogluterate. Subsequently, Shimizu and Wrensford (1993) discovered that growth and toxin production by *G. breve* (Wilson strain) were greatly influenced by the addition of certain amino acids (aspartate, leucine). They showed that the cells deaminate the exogenous amino acids and utilize the nitrogen and carbon moieties after degradation to smaller organic molecules, such as acetate. Following further experimentation, Shimizu et al. (1995) hypothesized that *G. breve* is capable of switching from an autotrophic to heterotrophic mode of nutrition. The addition of urea and glycine to mid growth phase cultures had a major influence on cell growth (three to four times cell yield) and toxin production (~2.5–6 times toxin production of controls) although these compounds were toxic to the cells when added at an early growth stage. They speculated that after a certain growth period the cells evolve a mechanism to utilize organic nitrogen. The switch in metabolic mode was accompanied by a loss in nitrate reductase activity and an increase in urease activity within the cultures.

Reviews of the biosynthesis of the dinoflagellate polyethers are provided by Wright and Cembella (1998), Shimizu et al. (2001), Shimizu (2003), and Rein and Snyder (2006). Stable isotope feeding experiments by Chou and Shimizu (1987) provided evidence that established the biochemical origin of the dinoflagellate polyether toxins through a polyketide synthase (PKS) mechanism, similar to that involved in fatty acid synthesis, but with various unique features (Wright and Cembella, 1998). It is believed that many dinoflagellates contain PKS genes that have the potential to produce a variety of polyether compounds. Recently, Snyder et al. (2005) have provided the first definitive evidence of endogenous PKS genes in *K. brevis*. The first data has also been collected now on the

expressed genome of *K. brevis* (Lidie et al., 2005), by the partial sequencing of a cDNA library, that has revealed 5280 unique gene groups. These sequences will be used to construct an oligonucleotide microarray to study the molecular mechanisms that regulate the expression of the PKS enzyme complex. Preliminary experiments with nutrient limited cultures of *K. brevis* have shown up regulation of PKS genes in stationary phase in parallel with up to threefold increases in BTX production (Monroe et al., 2005).

21.1.3.6 Dissolved Brevetoxins

Where BTX concentrations in the soluble and particulate phases of cultures has been measured, it is apparent that significant amounts of toxin are found in solution. Pierce et al. (2001) found that in a culture of *G. breve* about 60% of the brevetoxins were dissolved in the medium (the growth stage of the culture was not stated) and that large amounts of toxin were present in solution during a natural bloom, the proportion increasing as the bloom aged. The well-known respiratory irritation syndrome, caused by seawater aerosols generated during *K. brevis* blooms, occurs as a result of the concentration of BTXs in aerosol particles. Pierce et al. (2005) found that PbTx-2 was the most abundant analogue in the seawater (3 µg/L PbTx-2; 1 µg/L PbTx-3) while PbTx-3 was the most abundant analogue in the aerosol (5 ng/m^3 PbTx-2; 20 ng/m^3 PbTx-3). Brevenal was also detected in seawater and aerosols. Blum et al. (2000) partitioned intra and extracellular brevetoxins during a *K. brevis* bloom. They also found that in the early stages of the bloom, most toxins were intracellular, but extracellular toxins increased as the bloom progressed.

21.1.4 The Ecology of *K. brevis* Blooms in the Gulf of Mexico

Steidinger et al. (1998) comprehensively reviewed what is known of the bloom dynamics and physiology of *K. brevis* in the Gulf of Mexico and the west Florida continental shelf. Major blooms are an annual event in some areas of the west Florida coast and can be transported to the western North Atlantic coast. Most blooms occur in late summer/fall (August–February). Originally, it was thought that blooms developed close inshore, because that is where their effects were most noticeable, but it is now known that *K. brevis* blooms originate 18–74 km off the west Florida coast in depths of 12–37 m. Robbins et al. (2006) present a sequence of satellite images that clearly illustrate offshore initiation and inshore *K. brevis* bloom progression in this region in January–February 2005. Bloom initiation may be linked to eddies spawned by the Gulf Loop current that introduces deep nutrient enriched waters into the euphotic zone. During the initial offshore growth phase populations may reach >10^6 cells/L. Optimum growth occurs between 22°C and 28°C with limits of 9–33°C.

21.1.4.1 Nutrients and *K. brevis* Blooms

Evidence of links between freshwater runoff and generation and maintenance of *K. brevis* blooms has proved tenuous, although some analyses (Dixon and Steidinger, 2004) have revealed significant correlations with selected rainfall data and river flows in the central Florida coastal region. However, because there was no significant correlation between the largest discharges (Apalachicola River) it was hypothesized that the relative nutrient loads carried by different rivers may be more important than the size of the discharge alone.

K. brevis grows well at the low concentration of macronutrients typical of oligotrophic oceanic waters, and various studies have shown that it is very efficient at utilizing inorganic and organic N and P nutrients, especially the latter (Vargo and Howard-Shamblott, 1990). Bronk et al. (2004) tested the hypothesis that dissolved organic and inorganic nitrogen compounds regenerated from the nitrogen-fixing cyanobacterium *Trichodesmium* play an important role as a source of nitrogen that fuels high biomass accumulations of *K. brevis* on the Florida shelf. Their data showed that *K. brevis* can assimilate NH_4^+, NO_3^-, urea, glutamate, and ^{15}N labeled dissolved organic nitrogen (DON) produced as a result of N_2 fixation by *Trichodesmium*. Stable isotope signatures of *K. brevis* bloom

biomass has also shown that N_2 fixation by *Trichodesmium* could be an important source of regenerated nitrogen (Havens et al., 2004). Mulholland et al. (2004) demonstrated that a direct nutritional link between *Trichodesmium* and *K. brevis* may exist by showing that ^{15}N labeled NH_4^+ and DON regenerated from $^{15}N_2$ fixed by *Trichodesmium* was assimilated by other phytoplankton including *K. brevis*. Observations (Heil et al., 2001) of N:P stoichiometry in the phytoplankton of the west Florida shelf (including *K. brevis* bloom samples) support these data. Cells had N:P ratios close to the Redfield ratio or tended toward P limitation. Given the low inorganic N concentrations and low N:P ratios in shelf waters the authors concluded that there must be an unidentified nitrogen source available to the phytoplankton in this area and suggest one such source may be leachates from decaying sea-grasses. Liu et al. (2001) applied a numerical model to simulate *K. brevis* populations as these grew and moved inshore across the west Florida shelf. Their simulations suggested that as long as nitrogen was continuously available, a population sufficient to cause fish kills ($1–2.5 \times 10^5$ cells/L) could develop from a background concentration of <1000 cells/L in 1 month, at a maximum growth rate of 0.16 doublings/day. There are ongoing efforts to develop numerical models of coupled physical and biological dynamics (Ly et al., 2004) to assist with the forecasting and monitoring of blooms on the west Florida coast.

Lester et al. (2001) found relatively high levels of DON and DOP associated with waters supporting a *K. brevis* bloom. They suggested that some of this DOM may originate from near bottom diatom populations at the 30 m isobath, as there was no indication that it originated from estuarine sources. Their data suggested that DOM may be a source of nutrition for *G. breve* although they did not directly demonstrate DOM utilization. Vertical migration probably plays a critical role in efficient nutrient utilization and *K. brevis* population distribution. Vargo et al. (2004) analyzed the nutrient and hydrographic regimes during four *K. brevis* blooms between 1998 and 2000. All blooms appeared after the breakdown of vertical stratification. Cellular C:N ratios were close to the Redfield value but C:P and N:P ratios suggested P deficiency. Calculations suggested that estuarine nutrient flux may play some role in near-shore bloom maintenance but are barely sufficient to do this. Vertical migration enables cells to disperse through the entire water column at night to scavenge nutrients and may be an important means by which the blooms maintain nutrient sufficiency.

Bacteria may also be important to the growth of *K. brevis*. Nonaxenic cultures will maintain themselves over a year, but *K. brevis* cannot be sustained in axenic culture for long periods (Wilson, 1966). *K. brevis* can use a variety of organic nitrogen sources (e.g., vitamins, urea, and amino acids). Shimizu and Wrensford (1993) and Shimizu et al. (1995) showed substantial increase in growth and brevetoxin production with the addition of organic N as urea, glycine, leucine, and aspartate in mid log phase. The few kinetic studies of inorganic N assimilation by *K. brevis* (Vargo quoted pers comm. by Steidinger et al., 1998) indicate that it has a high affinity for inorganic N, demonstrating its adaptation for low nutrient conditions.

21.1.4.2 Hydrography and Bloom Progression

K. brevis is so dominant and seasonally abundant in the waters of the West Florida shelf, that it makes a substantial contribution to total annual phytoplankton primary productivity (Vargo et al., 1987; Bendis et al., 2004). When blooms that have developed offshore intrude into inshore nutrient enriched regions such as bays and lagoons with limited water exchange, they may increase in intensity and duration (Vargo et al., 2001). In western Florida, the cross-shelf movement of blooms from the mid-shelf region, is generally influenced by local circulation patterns and winds. The onshore southerly movement of blooms in late summer–autumn has been repeatedly observed, and is associated with southward tending winds at this time of year that cause local up- and down-welling events leading to concentration or dispersion of blooms. Many offshore blooms of *K. brevis* decline before they become established in near shore waters. Reasons for this may include; poor growth conditions that are insufficient for maintenance, failure of inshore transport processes, short-term shifts in wind patterns that may induce alternating up-/down-welling phenomena that nourish the blooms

but maintain them mid-shelf (Steidinger and Haddad, 1981). Dense *K. brevis* populations may be entrained in offshore fronts and transported over considerable distances. Atypical configurations of the Gulf Loop current are believed to be the mechanism that occasionally transports water from the mid-Florida shelf to the southwest Florida coast. From there, cells are transported into the Atlantic Ocean and northwards up the east coast through the Florida current and Gulf Stream (Tester et al., 1991; Tester and Steidinger, 1997; Steidinger et al., 1998). The maintenance of integrity of the water masses containing *K. brevis* blooms determines their duration and extent (Tester et al., 1991).

A recent modeling study (Janowitz and Kamykowski, 2006) has indicated that chemotaxis may play an important role in accumulation of *K. brevis* cells at offshore fronts. In the model, cell aggregation was driven by a combination of advection in currents, and the swimming behavior of cells. This involved chemotaxis toward waters with higher nutrient concentrations in outwelling waters from embayments, and from a near bottom source associated with upwelling or sediment flux. Another numerical model, incorporating wind driven surface flows in the Gulf of Mexico (Campbell and Hetland, 2006), has suggested that the primary mechanism responsible for near-shore bloom formation is not growth but physical processes alone. Simulations show that coastal down-welling can result in up to 1000 times concentration of cells.

The cause of bloom decline and dispersion are probably a combination of physical and biological factors, involving declining temperatures as the season progresses, and mixing and disruption of the water mass containing the bloom. Algicidal bacteria may play an important role in *K. brevis* bloom termination (Doucette et al., 1999), however, bacterial community interactions are crucial factors that influence their effectiveness (Mayali and Doucette, 2002). A filterable lytic agent, possibly a virus, has also been obtained from seawater affected by a *K. brevis* bloom that caused cell lysis of *K. brevis* cultures (Paul et al., 2002).

Molecular probes targeting the LSU rRNA gene (Mikulski et al., 2005) and pigment absorption signatures (Mille et al., 1995, 1997) are being explored as means of monitoring the dynamics of *K. brevis* blooms. Satellite ocean color imagery, together with the analysis of field and meteorological data, has been used to monitor and successfully provide forecasts of *K. brevis* bloom events (Stumpf et al., 2003; Tomlinson et al., 2004).

21.1.5 Interactions of *K. brevis* with Other Flora and Fauna

21.1.5.1 Allelopathy

In the oligotrophic waters of the Gulf of Mexico *K. brevis* can out-compete or exclude other phytoplankton and form nearly monospecific blooms, covering thousands of square kilometers that last for months (Steidinger et al., 1998). How it manages to achieve such persistent dominance is still not clear.

Freeberg et al. (1979) showed that media preconditioned by the earlier growth of *G. breve,* significantly inhibited the growth of 18 of 26 phytoplankton species (diatoms, dinoflagellates, other flagellates) assayed. The sensitivity to the growth inhibitor in the medium was variable within the group of species affected. A partially purified chloroform extract (crude toxin preparation) resulted in the complete growth inhibition of four diatom and four dinoflagellate species, algal growth inhibition and ichthyotoxicity were inseparable after partial purification. Kubanek et al. (Kubanek and Hicks, 2005; Kubanek et al., 2006) assayed allelopathic interactions between *K. brevis* and 12 other co-occurring phytoplankters. Nine of the 12 phytoplankton species were suppressed when grown with live *K. brevis.* Extracellular filtrates or lipophilic extracts inhibited six species, though inhibition was weaker than with live cells. Additions of purified BTXs cause autoinhibition of *K. brevis*. Growth suppression by live cells and culture filtrates was variable between species and BTXs at realistic water-borne concentrations accounted for the modest inhibition of only one competitor (*Skeletonema costatum*). *K. brevis* was susceptible to inhibition by several species (e.g., *P. minimum*) that co-occur during *K. brevis* blooms. Prince et al. (2006) showed that allelopathy

is common among Gulf of Mexico phytoplankton, but that compounds other than the BTXs are involved, and suggested that allelopathy may be induced by the presence of specific competitors.

21.1.5.2 Other Effects on Flora and Fauna

Apart from the well-documented mass mortalities of fish, and the aerosol effects of *K. brevis* blooms on humans, a variety of other effects on other species have been observed. Some of these are clearly side effects that do not contribute to the ecological competitiveness of the species, but others possibly do. Landsberg and Steidinger (1998) reviewed the incidence of mass mortalities of manatees associated with *G. breve* red tides in Florida. In the spring of 1996, there were an unusually large number of manatees affected, probably due to the early seasonal movement of bloom concentrations of *K. brevis* into inshore low salinity regions of manatee habitat. There was pathological evidence that it was the effect of BTXs that was the cause of the Manatee mortalities (Kirkpatrick et al., 2004).

K. brevis blooms may affect recruitment in bivalve populations through their harmful effects on larvae. Leverone et al. (2006) exposed the larvae of several bivalve species to cultures of whole and lysed cells of *K. brevis*, with mean total BTX concentrations of 53.8 μg/L and 68.9 μg/L, respectively. At high *K. brevis* cell numbers (5000 cells/L) 7-day-old larval survival was significantly affected but not at 1000 cells/L, though larval development was protracted. Early-stage larvae were more affected, by lysed cells. Tester et al. (2000) traced the transmission of BTXs through experimental food chains from dinoflagellates through copepods to fish. *K. brevis* was not directly toxic to the copepod *Acartia tonsa*, but it was not utilized as a food item and no difference in survivorship, behavior, or egg production was observed between starved controls and *K. brevis* treatments (Collumb and Buskey, 2004). Speekmann et al. (2006) also showed that the harmful effects of *K. brevis* on *A. tonsa* were probably due to grazing suppression and the poor nutritional quality of the cells, and not a direct toxic effect of BTXs as such. Giner et al. (2003) described novel sterols from *K. brevis* and suggested that the structural peculiarities of these molecules may render them of poor nutritional quality for predatory invertebrates, and so selectively enhance their survival. Smith (1975) described the devastating effect on reef communities in the eastern Gulf of Mexico caused by *K. brevis* blooms and the subsequent recovery and recolonization. He suggested that certain blooms, especially those generated beneath strong thermoclines, may be important regulators of the composition, abundance, and distribution of shallow water reef biota in this region, to the extent that they may prevent the establishment of climax or equilibrium reef communities.

21.1.6 Ecobiology of Brevetoxin Producers in New Zealand

21.1.6.1 The Hauraki Gulf/Northland *Karenia* spp. Bloom Event, Summer 1992–1993

Beginning between November 1992 and January 1993, an algal bloom, within which a *Karenia* species (referred to at the time as *Gymnodinium* cf. *breve*) was prominent, caused widespread biotoxin contamination of shellfish on the northeastern coast of the North Island, New Zealand (Figure 21.2) including the Coromandel marine farming area (Chang et al., 1995; MacKenzie et al., 1995). The bloom was associated with mass mortalities of marine fauna on subtidal reefs, and aerosol-borne respiratory irritation syndrome on some surf beaches. The bloom contaminated shellfish with toxins that caused symptoms consistent with neurotoxic shellfish poisoning (NSP) in a large number of people (>100) who had consumed shellfish in the northern part of the region (Jasperse, 1993). The rates of toxin elimination from cultivated mussels were slow, and a residue of the toxicity existed 2 months after the disappearance of the dinoflagellate from the plankton. The role of BTXs in this incident was confirmed in subsequent chemical analyses of shellfish collected at that time (Satake et al., 1996). This is the only known incidence of NSP caused by BTXs outside the

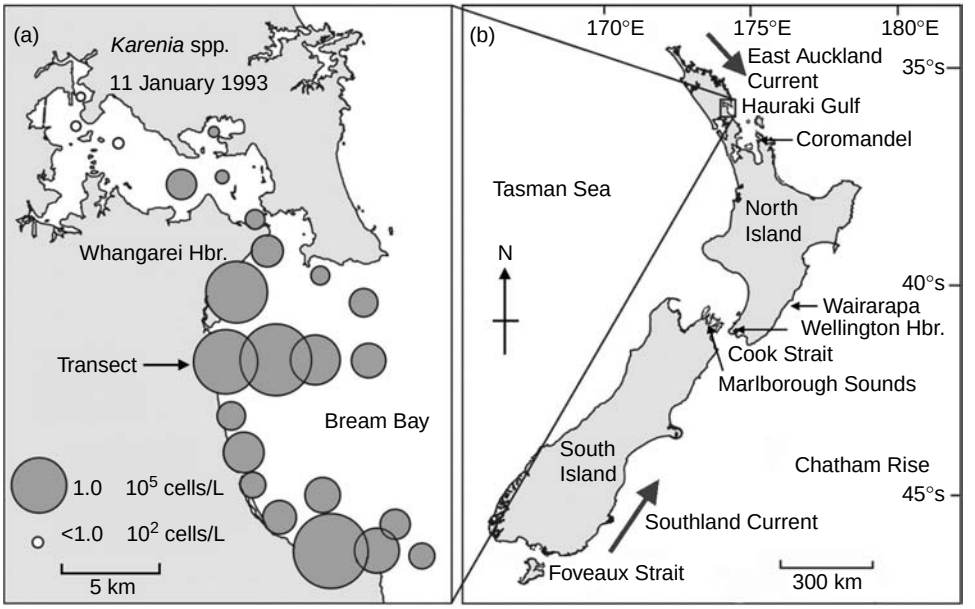

FIGURE 21.2 Maps showing geographic locations referred to in the text (b), the spatial distribution of maximum *Karenia* spp. cell numbers in the water column of Bream Bay, January 11, 1993 (a) and geographical locations referred to in the text (b).

United States of America. Unfortunately, the dinoflagellate was not definitively identified, or successfully established in culture at the time, although it had a close resemblance to *K. mikimotoi* and *K. brevis* (MacKenzie et al., 1995).

In mid-1993, as a result of this event, weekly nationwide toxic phytoplankton, and shellfish biotoxin monitoring (by mouse bioassay) programmes were established (Trusewich et al., 1996). The monitoring programme has confirmed that there has been no repetition of high-level BTX toxin contamination associated with *Karenia* spp. blooms since 1993. However, in 2002, phytoplankton surveillance combined with LC-MS/MS shellfish-flesh analysis was adopted as the preferred method of routine monitoring for BTX contamination in New Zealand (McNabb and Holland, 2003; McNabb et al., 2005). This has revealed the true incidence of this form of contamination. Traces of BTXs do appear seasonally within shellfish from the Hauraki Gulf region, but there has not been a clear association with causative phytoplankton, and concentrations in shellfish have never exceeded regulatory action levels (McNabb, pers. comm.).

21.1.6.2 Hydrographic Conditions Associated with the 1992–1993 *Karenia* spp. Bloom in the Hauraki Gulf, New Zealand

It is not known for certain what the circumstances were that led to the unusual development of the NSP-toxic 1993–1994 Hauraki Gulf/Northland *Karenia* spp./ bloom but it is generally believed that it was influenced by large scale, offshore, oceanographic processes. The seed source for the bloom may have originated in coastal or offshore waters, however there were signs indicating that coastal seawaters were especially fertile during the spring preceding the dinoflagellate bloom. The first was the report of an usually luxuriant diatom spring bloom in Bream Bay (MacKenzie, unpublished data) followed by an extensive raphidophyte bloom (*Fibrocapsa japonica* and *Heterosigma akashiwo*) throughout the Hauraki Gulf, associated with anomalously low sea surface temperatures and a negative phase in the southern oscillation index, typical of El Niño conditions in the tropical Pacific (Rhodes et al., 1993).

Sharples (1997) made observations on the intrusion of subtropical water into the coastal zone of northeast New Zealand from late winter to midsummer 1994–1995, 1 year after the *Karenia* spp. bloom. He showed that the intrusion of subtropical water into the gulf was associated with high salinity nutrient depleted surface layers and near bottom waters with high nitrate N levels (3–5 mmol/m^3). Southeasterly winds that occurred in late-November and mid-December 1992, prior to the bloom, are consistent with the hypothesis that Ekman transport effects on the East Auckland current induced the movement of a subtropical water mass, possibly carrying an established dinoflagellate community, into the Hauraki Gulf in late-December 1992.

Observations were made on the composition and distribution of the phytoplankton community within Bream Bay (Figures 21.2 and 21.3) at the peak of the bloom on January 11, 1993, when shellfish on adjacent surf beaches and inlets (Whangarei Harbor) contained high levels of brevetoxins (e.g., Ishida et al., 1995). At this time *Karenia* spp. formed a subdominant population within an abundant, diverse, and highly stratified dinoflagellate community. This was dominated, in terms

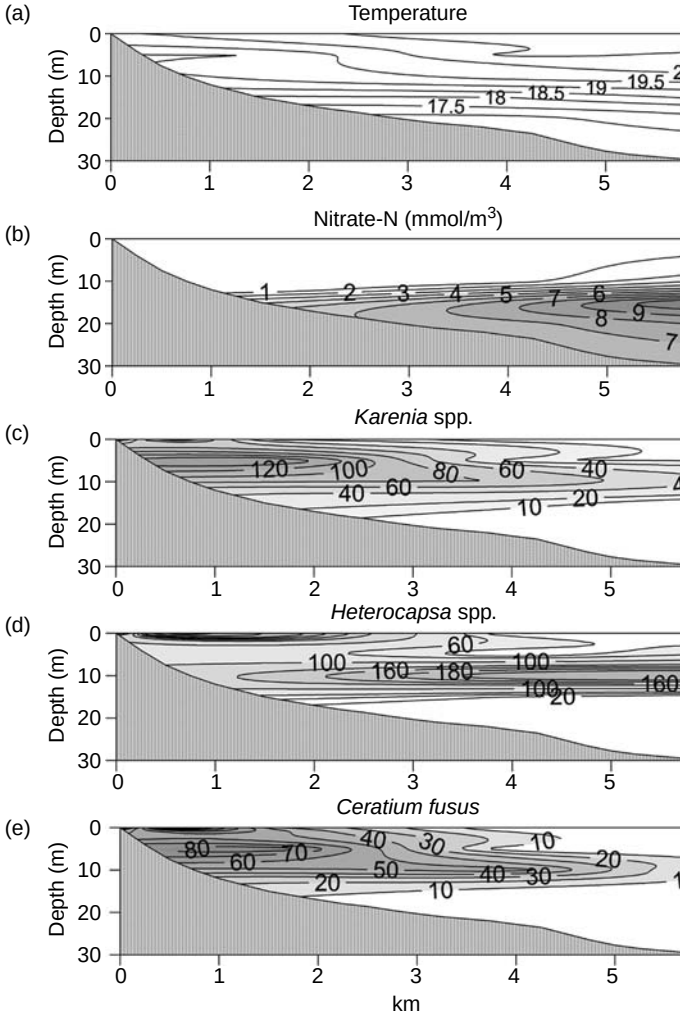

FIGURE 21.3 The vertical distribution of temperature (a), nitrate-N (b), and species within the dinoflagellate community (c–e) on a transect across Bream Bay at the peak of brevetoxin contamination of shellfish on adjacent surf beaches and within Whangarei Harbor, January 11, 1993. The location of the transect is indicated in Figure 21.2.

of cell numbers, by a small thecate species belonging to the genus *Heterocapsa* (formerly referred to a *Cachonina halli*) and in biomass by *Ceratium fusus* (Figure 21.3). The highest *Karenia* spp. cell numbers (1.8×10^5 cells/L) were observed within 1 km of the shore, and water column profiles along a mid-bay transect (Figure 21.3) revealed a continuous subsurface layer between 5 m and 15 m extending at least 5 km offshore. The salinity throughout the bay was high (35.1–35.2 psu) and uniform with depth. Salinities throughout much of Whangarei Harbor were likewise high, and *Karenia* spp. was only found in low numbers in water with salinity below 34.8 psu. Temperature stratification resulted in a pycnocline between 10 m and 20 m. The highest concentrations of phytoplankton occurred within and just above this zone and cells may have been actively transported shore-ward along the shallow pycnocline (Figure 21.3 c through e). Unusually high concentrations of nitrate-N (6.4–11.4 mmol/m^3) nitrite-N (0.4–0.7 mmol/m^3), and dissolved reactive phosphorus (0.4–0.8 mmol/m^3) existed in waters below the pycnocline (Figure 21.3b). Near surface water was depleted of nutrients with concentrations below the limits of detection. The shallow nutricline and high levels of inorganic nutrients in midsummer within Bream Bay suggest that the bloom was being sustained by intrusion of deep, nutrient-enriched, offshore waters.

21.1.6.3 Bioconversion of Brevetoxins by Shellfish during the 1992–1993 Neurotoxic Shellfish Poisoning Event in New Zealand

The chemistry of the toxin residues in shellfish samples collected during the bloom were intensively studied, and provide a well documented case study (Figure 21.4) of BTX metabolism in different shellfish species (Ishida et al., 2006).

Ishida et al. (1995) first described a new water-soluble polyether BTX analogue (B1) isolated from New Zealand cockles (*Austrovenus stuchburyi*). BTX-B1 is the taurine conjugate of PbTx-2 and was the predominant BTX metabolite produced within the cockle. BTX-B1 was not ichthyotoxic. It was only found in low levels in Greenshell™ mussels (*Perna canaliculus*) and not at all in Pacific oysters (*Crassostrea gigas*). PbTx-3 was found in all three species (Nozawa et al., 2003) and parent PbTx-2 in *Cr. gigas* (Ishida et al., 1996).

Murata et al. (1998) isolated a further BTX analogue (BTX-B2), from *Pe. canaliculus* collected from the Coromandel during the 1993 bloom. BTX-B2 is a cysteine conjugate of PbTx-2

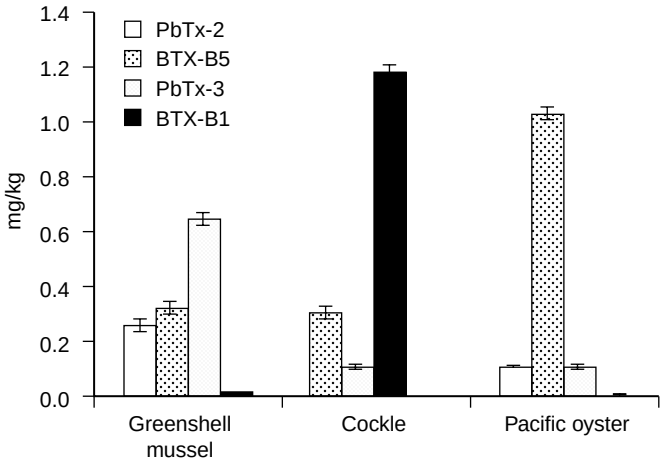

FIGURE 21.4 Profiles of BTX derivatives in Greenshell™ mussels (*Perna canaliculus*), Cockles (*Austrovenus stutchburyi*) and Pacific Oysters (*Crassostrea gigas*) collected during the *Karenia* sp. bloom event from Whangarei Harbor (*A. stutchburyi*) and the Cormandel (*P. canaliculus* and *C. gigas*), January–February 1993 (After Ishida et al., 2004b).

that appears to be unique to *Pe. canaliculus*. BTX-B2 retains its Na channel-activating activity but looses its ichthyotoxicity. Neither PbTx-2, PbTx-3 (parent compounds in *K. brevis*), nor BTX-B1 were found in *Pe. canaliculus* in this study.

Morohashi et al. (1995, 1999) described two other novel BTX analogues (BTX-B3 and BTX-B4) isolated from *Pe. canaliculus*. Both analogues are believed to be a product of enzymatic biotransformation by the shellfish. BTX-B3 results from cleavage of the D ring of the Type I backbone, with esterification of the resulting alcohol and oxidation of the aldehyde terminus. It is the only biotransformation product known so far where there is oxidation, ether ring opening, and esterification within the one molecule. BTX-B4 is a fatty acid conjugate (*N*-myristoyl or *N*-palmitoyl) of the S-cysteine conjugate of PbTx-2 (BTX-B2). BTX-B4 was the most toxic brevetoxin analogue in *Pe. canaliculus*.

Ishida et al. (2004a) identified a new brevetoxin analogue, brevetoxin B5 (BTX-B5), from the New Zealand cockle *Au. stutchburyi* harvested from Whangarei Harbor during the 1993 bloom. BTX-B5 is an oxidized form of PbTx-2 that has a carboxyl group at C42 in terminal side chain. Ishida et al. (2004b,c) provide plausible metabolic pathways for the biotransformation of PbTx-2 in New Zealand shellfish.

21.1.6.4 Other *Karenia* spp. Blooms in New Zealand

Since the 1992–1993 *Karenia* spp. bloom and BTX contamination event in northeastern New Zealand, there have been several similar extensive *Karenia* spp. blooms in the same and different coastal regions. These have affected long stretches of coastline, and in each case appear to have originated offshore and been initiated and driven by large-scale oceanographic processes.

In January 1994 an extensive bloom of *K. selliformis* (Figure 21.1), originating off the south west coast of the South Island, extended over 500 km of coastline (MacKenzie et al., 1996a). The circumstances surrounding this bloom are dealt with in more detail in the following section on the ecobiology of the cyclic imines.

Chang (1999) described a new species of gymnodinoid dinoflagellate (*G. brevisulcatum* spp. nov.) isolated from a toxic bloom that occurred in Wellington Harbor (Figure 21.2) during the summer of 1998. Subsequent to the revision of the genus by Daugbjerg et al. (2000) the dinoflagellate assumed the name *K. brevisulcata* (Chang) G. Hansen and Moestrup. The dinoflagellate resembles *K. mikimotoi* but it has several significant features that make it morphologically distinguishable (Figure 21.1). *K. brevisulcata* caused die-off of substantial portions of the subtidal and intertidal benthic biota within the harbor (Wear and Gardner, 2001). It was also responsible for cases of respiratory irritation in people exposed to *K. brevisulcata*-contaminated aerosols; and massive amounts of foam were generated after the bloom collapsed. Cell numbers as high as 3.3×10^7 cells/L were observed during the bloom. Subsequent analysis of the benthic communities have shown a remarkable recovery, probably because there was no toxic residue that prevented postbloom recruitment to the numerous vacant ecological niches (Gardner pers.comm.). The toxin or toxins produced by *K. brevisulcata* have proved difficult to identify and there is still no definite structural solution. Chang et al. (2001) documented the distribution of *K. brevisulcata* along the north, central, and southeast coast of the North Island over the period that the dinoflagellate was having such a devastating effect in Wellington Harbor. *K. brevisulcata* was observed 200 km offshore and over 1000 sea-km from Wellington Harbor off the northern tip of the North Island. The dinoflagellate appeared to be widely distributed down the entire east coast of the North Island up to at least 320 km offshore of the southeast coast and as far south as the Chatham rise. Although offshore cell numbers were generally low (>300 cells/L) a sample in early-February collected ~250 km off the Wairarapa coast had cell concentration of 1.2×10^5 cells/L. The occurrence of *K. brevisulcata* occurred during a transition from anomalously low to anomalously high sea surface temperatures in the region resulting from a phase shift from El Nino conditions. It is thought that the dinoflagellate originated in offshore northern New Zealand waters and was transported south in warmer and stronger than usual current flows.

Interaction of this warm water with more southern cooler nutrient-enriched waters may have led to favorable growth conditions for the dinoflagellate. From offshore regions, the dinoflagellate became entrained in near-shore currents and was widely distributed along the coast. Reports of the progression of toxic aerosol syndrome along the southeast coast closely matched the rate of travel of the currents.

Chang et al. (2002) reported on a *Karenia* spp. bloom on the northeast coast of New Zealand that cause extensive fish kills between October and November 2002. Very high *Karenia* spp. cell numbers were recorded (4.0×10^6 cells/L) at some locations. The bloom was associated with several intrusions of warm subtropical water into the Hauraki Gulf from the offshore East Auckland current (Figure 21.2) between August and early October 2002. The main species in the bloom was a cell closely resembling *K. mikimotoi*, though other species including *K. brevisulcata* were also observed. Subsequently, Chang and Ryan (2004) described the *K. mikimotoi* like dinoflagellate from this bloom as a new species that they named *K. concordia* on the basis of unique features of its morphology, such as the drooping epicone and very long apical groove displayed by these cells. Recently, it has been confirmed that this species produces five brevetoxin derivatives (Chang et al., 2006) The major analogue is PbTx-2, but the toxin content of the cells is low at ~1% of that contained by *K. brevis* (Chang, pers. comm.).

21.1.7 Brevetoxin Production by Raphidophytes

Karenia spp. may not be the only group of planktonic microalgae that produce BTXs. The icthyotoxic effects of the raphidophytes (*Chattonella* spp., *Heterosigma* spp., *Fibrocapsa* spp.) have been known for many years, and these species are notorious for causing mass mortalities of wild and farmed fish around the world. BTXs have been implicated in this toxicity, however their role remains controversial. Onoue et al. (1990) were the first to report the production of fat-soluble BTX-like neurotoxins from cultures of *Ch. marina*. Ahmed et al. (1995) showed a relationship between the toxicity of cultures of *Ch. marina* to fish and the production of BTX analogues (oxidized PbTx-2 and PbTx-3) from mid logarithmic to early stationary phases. Toxins were quantified by thin layer chromatography (TLC)/HPLC-UV and their identity reportedly confirmed using fast atom bombardment (FAB) ionisation mass spectrometry by comparison with authentic PbTx standards. The greatest fish toxicity was shown to occur just after cultures reached stationary phase, after which toxicity declined and disappeared by late stationary phase. The oxidized form of PbTx-2 was the predominant toxin (~10× that of reduced PbTx-3) during the logarithmic phase and increased by two to three times during stationary phase while PbTx-3 declined. Khan et al. (1996a) carried out a toxicological study of *Ch. antiqua* during various stages of growth of a unialgal culture. Three neurotoxic compounds ("CaTxs"), reportedly corresponding to PbTx-2, PbTx-3, and oxidized PbTx-2, were analyzed by TLC/HPLC. Oxidized PbTx-2 was the predominant form (2.5–6.9 pg/cell in log and stationary phases, respectively). This study was followed by that of Khan et al. (1996b) who described neurotoxin production by *F. japonica*. They identified five neurotoxic components ("FjTxs"). After TLC purification and comparison of rf values with authentic PbTx standards after C-18 reverse phase HPLC these compounds were reported to correspond to brevetoxins PbTx-1, PbTx-2, PbTx-9, PbTx-3, and oxidized PbTx-2. PbTx-2 was the predominant component (~1–2 pg/cell) followed by its oxidized form, with lesser amounts of other analogues. Khan et al. (1997) also described the finding of four neurotoxic compounds ("HaTxs") that they reported corresponded to PbTx-2, PbTx-9, PbTx-3, and oxidized PbTx-2 in cultures of *H. akashiwo* isolated from Kagoshima Bay, Japan. Haque and Onoue (2002) compared BTX composition of *Ch. antique* and *Ch. marina* grown at different salinities. Both species showed highest growth rate and icthyotoxicity at 25 ppt. Toxin profiles varied substantially with salinity. In *Ch. antiqua* putative PbTx-3 was predominant (2.9 pg/cell), and total toxin content was maximum at 25 ppt. There was a similar pattern of toxin abundance and variation with salinity in *Ch. marina*. Haque (2006) recently reported that an isolate

of *Ch. marina* from Kagoshima Bay, Japan, contained toxic components ("CmTxs") reportedly corresponding PbTx-2, PbTx-9, PbTx-3, and oxidized PbTx-2.

Bourdelais et al. (2002) identified BTXs; PbTx-2, PbTx-3, and PbTx-9 in seawater samples from the Delaware coast, USA associated with fish kills and a bloom of *Chattonella* cf. *verruculosa*. The identity of the toxins was confirmed by chromatographic, immunochemical, nuclear magnetic resonance spectroscopy and mass spectrographic analyses, and the toxin content of cells was inferred from cell counts and toxin concentrations, to be on the order of 6 pg /cell. This is comparable to the levels found in Florida *K. brevis* (Table 21.1). However, despite this strong circumstantial association, it has not yet been possible to demonstrate BTX production by cultures of *Ch*. cf. *verruculosa* isolated from Delaware coast waters (Bourdelais, pers. comm.)

In addition to these reports, there are also a number of other studies that have failed to confirm the production of BTXs by raphidophytes. Glasgow et al. (2001) found no detectable BTXs by HPLC-MS in clonal cultures (analyzed for PbTx-1, 2, and 3) of *H. carterae* (*akashiwo*) and *Ch.* cf. *marina* isolated from blooms during fish kill/disease events in the US Atlantic coast estuaries. Shellfish, fish-flesh, and seawater samples collected during the mass mortality of sea-cage salmon in Norway in 2001, caused by a bloom of *Chattonella* spp., were tested for BTXs by ELISA, mouse assay, and LC-MS without revealing any trace of these toxins (Briggs, 2001). McNabb et al. (2006) found no evidence of any brevetoxins (>0.003 pg/cell) within isolates of *Ch. antiqua* (2), *F. japonica* (3) and *H. akashiwo* (4), from New Zealand waters, screened using an LC-MS/MS triple quadrupole mass spectrometer calibrated against authentic PbTx-2, PbTx-3, PbTx-1, and PbTx-9 reference standards. There are various plausible alternative hypotheses (anoxia, mucus production, interference with respiratory and cardiovascular physiology) for the harmful effects of raphidophytes on fish, including a synergistic role of reactive oxygen species and free fatty acids (Marshall et al., 2003).

21.2 ECOBIOLOGY OF THE CIGUATOXIN PRODUCERS

21.2.1 Introduction

There is a large body of literature on ciguatera fish poisoning, including past reviews on the ecology of dinoflagellates associated with the phenomenon (Bomber and Aikman, 1989; Faust, 1995a; Tindall and Morton, 1998), the origin of the toxins (Lewis and Holmes, 1993; Lewis, 2001; Lewis, 2006), and their chemistry, toxicology, analysis, and pharmacology (Lewis, 1996; Lehane 1999; Lehane and Lewis, 2000; Lewis et al., 2000).

Ciguatera is a disease, caused by the consumption of the toxin-contaminated flesh of tropical and subtropical fish which cause a range of gastrointestinal and neurological symptoms in humans. It has a circumglobal distribution in warm seas between 35°N and 35°S latitude (Nakajima et al., 1981; Bagnis et al., 1985; McMillan et al., 1986) with significant socioeconomic consequences, especially for some small island communities (Lewis, 1992a). There are regional differences in the symptomology of ciguatera (neurological symptoms predominate in the Pacific, gastrointestinal symptoms in the Caribbean) that reflect differences in the structural chemistry of the ciguatoxins themselves (reviewed by Lewis, 2006).

Randell (1958) first published the hypothesis that ciguatera was the result of transmission and amplification of toxins through the food chain. In the late-1970s Yasumoto et al. (1977) showed that a dinoflagellate, epiphytic on the calcareous red alga (*Jania* spp.), isolated from the Gambier Islands in French Polynesia, produced lipid-soluble compounds that resembled ciguatoxins, and the dinoflagellate was subsequently described and named by Adachi and Fukuyo (1979) as *Gambierdiscus toxicus*. Yasumoto et al. (1993) provide a brief historical account of the sequence of events that led to the discovery of the cause of the ciguatera syndrome. Murata et al. (1989) were the first to describe the molecular structures of ciguatoxins.

Ciguatoxins (CTXs) are lipid-soluble polyether compounds that in their structural chemistry and physiological effects (activation of sodium channels in cell membranes), are closely related to the BTXs. *Ga. toxicus* produces lipid-soluble gambiertoxin-4A (GTX-4A) that is the precursor to the more oxidized CTXs that accumulate in the tissues (liver, skin, muscles, bone) of fish. Invertebrates may play some role in food chain transmission of ciguatoxins (Kelly et al., 1992; Lewis et al., 1994a) but it is generally accepted that the primary vectors are herbivorous and carnivorous fish (Lewis and Holmes, 1993). *Ga. toxicus* also produces other types of polyethers such as gambierol (Satake et al., 1993) and gambieric acids (Nagai et al., 1992a,b) that have potent antifungal properties. Interestingly, gambierol and gamberic acids interact competitively with BTXs on sodium channels in the same way as brevenal (Inoue et al., 2003). It is untested, but probably a fair assumption, that they inhibit ciguatoxin binding in the same way. It has been suggested that gambieric acid-A (GA-A) is an autocrine type growth stimulator. It has been found to enhance the growth of *Ga. toxicus* in culture (Sakamoto et al., 1996), and this effect is inhibited by anti-GA-A antibodies (Sakamoto et al., 1998).

The potency of CTXs are amplified as they pass through the food chain due to oxidation through cytochrome enzymes in the fish's liver (Lewis and Holmes, 1993). CTX-1, which is the dominant ciguatoxin found in Pacific carnivorous fish, is ten times more toxic (0.25 versus 2 μg/g) than its precursor (GTX-4A) produced by *Ga. toxicus* (Satake et al., 1997; Lewis, 2001). CTX-1 is the main toxin in terms of potency and quantity in carnivorous fish, but in herbivorous fish less polar CTXs and GTXs accumulate, GTXs are not found in carnivores. Ironically, given their food chain transmission and accumulation in fish tissues, CTXs are potent ichthyotoxins (Lewis, 1992b). It has been suggested that the lethal effects on fish may impose an upper limit on the concentrations that can develop in fish tissues.

Maitotoxins (MTXs) are also produced by *Ga. toxicus*. These are more water-soluble compounds than GTXs, and though they accumulate in the gut of herbivorous fish, they are probably not directly involved in ciguatera fish poisoning because of their low oral potency and poor ability to accumulate in fish flesh (Lewis, 2006). This is fortunate since MTXs are large (MW 1060–3422), structurally complex polyether compounds, that are very potent Ca^{2+} channel activators and are among the most toxic (LD_{50} 50 ng/kg) nonprotein compounds known (Takahashi et al., 1983). Although they are analogous in structure to GTXs, MTXs have no partial structure corresponding to CTXs. Curiously, only a minority of isolated *Ga. toxicu*s strains produce GTXs (Holmes et al., 1991), but most if not all have been shown to produce MTXs. Different strains of *Ga. toxicus* produce different types of MTXs (Holmes and Lewis, 1994).

The ciguatera phenomenon is a manifestation of the complex chemical ecology of the coral reef food web, containing diverse assemblages (Carlson and Tindall, 1985) of benthic and epiphytic dinoflagellates, producing a variety of potent bioactive polyether secondary metabolites (Bagnis et al., 1985; Ballantine et al., 1988). In addition to GTXs and MTXs, these include okadaic acid and its analogues (Murakami et al., 1982), prorocentrolides produced by *Prorocentrum* spp. (Hu et al., 1996a), coolia toxins produced by *Coolia monitis* (Holmes et al., 1995), amphidinols produced by *Amphidinium* spp. (Paul et al., 1996), palytoxins produced by *Ostreopsis* spp. (Tindall et al., 1990; Usami et al., 1995), and undoubtedly numerous yet to be described bioactive compounds (Tindall et al., 1998). All these compounds have the potential to contaminate seafood, and in the case of the palytoxins there are well-documented cases where human consumption of fish and invertebrates containing these toxins (Yasumoto et al., 1986) has resulted in sickness and death (Noguchi et al., 1987; Kodama et al., 1989). Palytoxin poisoning is apparently less common than ciguatera and the symptoms are different and more severe (Lewis et al., 2000). Some authors (e.g., Tosteson et al., 1998) have maintained that *Ostreopsis* spp. are the main cause of fish poisoning in some regions. For the sake of brevity, the discussion that follows focuses mainly on *Gambierdiscus* spp., and reviews what is known of the taxonomy and genetics of the genus, their biogeography and ecology, and culture studies on the environmental determinants of toxin production.

21.2.2 Taxonomy and Genetics of *Gambierdiscus toxicus*

It is only since the late 1990s that the diversity of species within the genus *Gambierdiscus* has become apparent, and distinguishing between them remains difficult because of the subtlety of the morphological details that characterize each species. Many older accounts of the ecology and toxicity of *Ga. toxicus* may in reality be accounts of a species complex.

Chinain et al. (1999a) described three new species of *Gambierdiscu*s; *Ga. australes*, *Ga. Pacificus*, and *Ga. polynesiensis*. The shape and size of the cells and the architecture of the apical pore plate distinguished them from each other and from *Ga. toxicus*. These species were characterized by isozyme electrophoreseis and DNA sequencing of the D8–D10 region of their LSU rRNA genes. Groupings based on the isozyme analysis did not relate to the morphotypes. This was consistent with observations by Chinain et al. (1997), where isozyme analysis of 19 isolates of *Ga. toxicus* from the Pacific and Caribbean showed that this species were comprised of numerous biochemically distinct strains. The DNA sequence data clearly distinguished between *Ga. toxicus*, *Ga. australes*, and *Ga. polynesiensis*, with *Ga. toxicus* more closely related to *Ga. pacificus*. The toxicity of fractionated extracts of cultured cells, tested by mouse bioassay, showed that all three new species produced toxic responses indicative of CTXs and MTXs. *Ga. polynesiensis* had particularly high levels of CTX-like toxicity and this was later confirmed by HPLC analysis. All three strains of *Ga. toxicus* used as controls tested negative for CTX-like activity. A morphologically distinct species named *Ga. yasumotoi* was isolated by Holmes (1998) from the fringing coral reef of Palau Hantu, Singapore. The species was distinctive in that the cells were smooth, the thecal pores lacked areolae, and they were not anterior-posteriorly compressed. Methanol extracts of *Ga. yasumotoi* were lethal to mice and induced symptoms similar to those produced by MTXs. Faust (1995b) described a new species that she named *Ga. belizeanus,* from a mixed assemblage of sand-dwelling dinoflagellates in a shallow tropical lagoon in Belize. These cells were ellipsoid in anterior view, anterior-posteriorly compressed, smaller than *Ga. toxicus* and with a deeply areolated cell surface. The toxicity of this species is not known.

Babinchak et al. (1996) partially characterized the LSU rRNA gene from 20 Pacific and Atlantic isolates of *Ga. toxicus*. Their results indicated, that of the 20 isolates examined, only four did not share a common haplotype with at least one of the other isolates. Different haplotypes were observed between isolates from the same site, suggesting a possible genetic basis for phenotypic differences (e.g., toxin production) between these strains. Sako et al. (1996) found a partial 18S rDNA sequence discriminated well between *Ga. toxicus* and other dinoflagellate species but could not resolve differences between different populations, although Pacific and Caribbean isolates were separated. They found that 5.8S rDNA and internal transcribed regions (ITS) provided more useful genetic markers for discrimination at the population level. Chinain et al. (2001) obtained D8–D10 LSU and 5.8+ITS rRNA gene sequences for the characterization and classification of 11 Polynesian isolates of five of the six described species of *Gambierdiscus*. Sequencing of both regions permitted discrimination between different geographic clades of the same species. The LSU sequences defined four molecular types that were consistent with the different morphotypes, with the exception of marginal discrimination between *Ga. toxicus* and *Ga. pacificus*. A recent effort to resolve the taxonomy of the *Gambierdiscus* species complex (Tester et al., 2006) using morphological and rRNA gene sequencing data supports the existence of at least five new species, plus five of the six currently described species. However, the researchers found that the main problem was in resolving *Ga. toxicus* itself. This was because type material was not available and there is a possibility that the original *Ga. toxicus* description (Adachi and Fukuyo, 1979) might actually have included several species, since it describes a large range in cell sizes of material collected from a number of sites.

21.2.2.1 Life Cycle of *G. toxicus*

The cycle of cell division in *Ga. toxicus* is phased to the diurnal cycle with division occurring during a 3 h window, beginning 6 h after the onset of the dark phase (Van Dolah et al., 1995). Cell division is

accompanied by activation of the cell cycle regulatory protein CDC2 kinase, indicating that cell division in *Ga. toxicus* is regulated by the same molecular controls as in higher eukaryotes. *Ga. toxicus* reproduces asexually and no diploid resting cyst stage (hypnozygote) has been observed, though possible sexual conjugation of isogamous gametes has been reported (Taylor, 1979). The complete life cycle of *Ga. toxicus* in nature has yet to be elucidated and a resting cyst stage may exist. Faust (1993a) has described sexuality and the formation of a zygotic resting cyst in natural mangrove populations of *Prorocentrum lima*; however, the zygotes only had a short resting period of 5–7 days.

21.2.3 Growth and Toxin Production by *G. toxicus* in Culture

A major impediment to earlier research on CTXs was the rarity of pure toxin for toxicology and structural determination, and large quantities of contaminated fish had to be extracted to obtain sufficient quantities (Yasumoto et al., 1993). Early studies (e.g., Durand et al., 1985) on cultures of *Ga. toxicus* showed activities that suggested that CTX-like and MTX-like compounds were being produced. But it was not until Satake et al. (1993) isolated and determined the structure of a ciguatoxin analogue from a *Ga. toxicus* culture, that it was conclusively demonstrated that it was possible to produce these compounds in the laboratory. Bomber and Aikman (1989) reviewed the media useful for growing epiphytic dinoflagellates.

Explanation of the environmental and genetic factors that regulate growth and toxin production by *Ga. toxicus* remains one of the most important outstanding questions regarding the ecobiology of ciguatoxins (Lewis, 2006). Perhaps one of the most puzzling aspects is that only a small proportion of cultured strains of *Ga. toxicus* have been shown to produce ciguatoxin precursors, and the specific toxicity of cells collected from the wild usually greatly exceeds the toxicity of cultured cells. Although there seems to be good evidence that variability of toxicity between clones is genetically determined, induction may also be involved. Holmes et al. (1994) suggested that "super producing" strains may explain the patchy sporadic distribution of ciguatera in nature.

Early work on the role of temperature, light, and salinity on the growth of *Ga. toxicus* (Bomber et al., 1988a) in the Florida Keys showed that cell numbers were maximal when water temperatures were about 30°C. However, culture experiments showed that at temperatures >29°C and <26°C cell division was limited. Optimal growth occurred at a salinity of 32 psu. Under optimal light, temperature, and salinity conditions >0.5 divisions/day could be sustained. Growth was optimal under blue light; cultures grown at 27°C were more toxic (by mouse assay) than those at 21°C, and toxin production could take place at high inhibitory irradiance levels.

Cultures of *Ga. toxicus* (Durand et al., 1985) were shown to produce both CTX-like and MTX-like compounds (fractionation based on aqueous/ether, methanol/hexane, and gel filtration partition). The CTX-like fractions inhibited growth of some microalgae species, and stimulated or had no effect on others. MTX-like compounds had lesser positive and negative growth effects on the same species. Both extracts had toxic effects on sea urchin eggs. Durand-Clement (1987) demonstrated high levels of toxicity (by mouse bioassay) in water soluble (MTX-like) and lipid soluble (CTX-like) fractions and found no variation in the specific toxicity of their cultured *Ga. toxicus* strain over 3 years. Growth rate was optimal at 26°C and was largely influenced by the total amount of illumination received per day. An intensity of 10 W/m^2 and light dark cycle of 10/14 h was optimal. The cells would not grow in continuous light and increases in growth were observed with antibiotic treatment. Bomber et al. (1989a) attempted to separate environmental differences from genetic differences in toxicity (mouse assay of methanol extracts) by geographically separate clones of *Ga. toxicus*. They found a high level of toxicity variation between clones from the same collection sites (four to ten times), but this was not as large as that between sites (up to 30 times). Comparison of growth rates at four light intensities indicated that there were differences between clones adapted to specific light regimes. Data on the relationship between growth rate, toxic potency, pigment synthesis, and photosynthesis is complex and contradictory. Bomber et al. (1989a) showed a negative correlation

between toxicity (mouse assay) and chlorophylls a and c2, and a suggestion of a positive correlation between potency and growth rate, while the study of Morton et al. (1993) suggested a positive relationship between toxicity and pigment content and photosynthesis.

Maitotoxins with different chromatographic behaviors and bioassay signs in mice were observed in Australian and French Polynesian strains of *Ga. toxicus* (Holmes et al., 1990), though no CTX precursors were produced by these isolates. Large and small molecular weight MTXs with different potencies were purified and characterized from cultures of *Ga. toxicus* by Holmes and Lewis (1994). Similar to other dinoflagellate cultures, high nutrient levels of standard growth media (e.g., F_2) induced aberrant cell morphologies but different nutrient concentrations (i.e., F_2 versus F_{10}) did not affect the spectrum of MTXs produced by the different strains. Subsequently, Holmes et al. (1991) tested the production of GTXs (mouse assay and ^{3}H PbTx-3 binding inhibition assay) by 13 strains of cultured *Ga. toxicus* from the Pacific and Caribbean. No CTXs as characterized in moray eels were found, and only two Queensland strains and a wild *Ga. toxicus* cell concentrate produced GTXs that competitively inhibited the binding of ^{3}H PbTx-3 to rat brain membranes. Wild cells produced 100 times greater GTX/cell. They concluded that the production of GTXs is limited to certain genetic strains of *Ga. toxicus*. Sperr and Doucette (1996) measured the variation in growth rates and GTX and MTX production in Pacific and Atlantic isolates of *Ga. toxicus* at various N:P ratios. All isolates showed an increase in total toxicity by mouse bioassay from mid- to late-log phase. Ca^{2+} channel activity (MTX-like) was observed in all isolates at all N:P ratios but Na^+ channel activity (GTX-like) was detected in only two isolates (Pacific and Atlantic) at all N:P ratios. In the other strains, GTX-like activity was detected only at the highest N:P ratios (50:1). The clearest trend they observed was an increase in total toxicity from mid- to late-log phase with a peak in toxicity at an N:P ratio of 30:1. It appeared there were intrinsic genetic differences in toxin production by different clones.

It has been hypothesized that bacteria associated with toxic dinoflagellates act synergistically to synthesize toxins. Tosteson et al. (1986, 1989) examined the associated bacterial flora, growth and toxicity of cultured *Ostreopsis lenticularis*, and *Ga. toxicus*. *Ga. toxicus* clones were characterized by the presence of *Pseudomonas* spp. but extracts of dinoflagellate associated bacteria in pure culture were not toxic. Peak toxicity of *O. lenticularis* occurred during stationary phase and correlated with increases in bacterial biomass.

21.2.4 Ecology of Ciguatera Dinoflagellates

Ciguatera fish poisoning outbreaks are believed to be due to environmental perturbations that lead to the proliferation of *Ga. toxicus* on coral reefs. However, the complex food chain interactions involved in the transfer of toxins from dinoflagellate to human consumer make the phenomenon spatially and temporally unpredictable. *Ga. toxicus* lives on a range of inorganic and organic substrates including dead coral, sandy bottoms, sea grasses, and macroalgae (Bagnis et al., 1980; Lobel et al., 1988), including cyanobacteria and turf algae (Gillespie et al., 1985). It grows within temperature and salinity ranges of 20–34°C and 25–40 psu respectively, at depths of 0.1–30 m (Bomber, 1988a), but it is most abundant in shallow waters with full oceanic salinity. *Ga. toxicus* populations are patchy and variable, and may or may not show seasonality in abundance.

Tindall and Morton (1998) classified habitats supporting tropical epiphytic dinoflagellate communities into four catagories. These comprised of high-energy reefs exposed to ocean swells (Type 1), permanently submerged reef flats, algal reefs, and tide pools (Type 2), low energy sand-silt habitats with high macrophyte biomass (Type 3) and mangrove island lagoons with high organic loading and diverse benthic microalgal mat communities (Type 4). The implication for ciguatera risk evaluation from this classification was that biomass estimates do not necessarily reflect the real production of dinoflagellate epiphytes and toxins. For example, in Type I systems with high water velocities, epiphytes are constantly being produced and sloughed off, and they may have low dinoflagellate standing crops but high toxin production rates.

Drift algae have been suggested as a dispersal mechanism for ciguatera associated epiphytic dinoflagellates (Bomber et al., 1988b). *Ga. toxicus* has been reported as an epiphyte on 56 genera of macroalgae, two cyanobacteria, one diatom, and one seagrass (Carlson et al., 1984; Inoue and Raj, 1985; Cruz-Rivera and Villareal, 2006). Cruz-Rivera and Villareal (2006) provided a comprehensive list of all macroalgae genera known to be a host of *Ga. toxicus* and their relative palatability and production of bioactive secondary metabolites. They pointed out that risk assessment of ciguatera on the basis of quantifying dinoflagellates on macroalgae may be prone to error, because many algal hosts of *Ga. toxicus* have poor nutritional quality, chemical defenses, or morphological and structural defenses that make them unpalatable to fish grazers. Heil et al. (1998) found different benthic dinoflagellates, including *Ga. toxicus*, had distinct macroalgal substrate preferences on a coral cay on the Great Barrier Reef, Australia. Holmes et al. (1998) described a diverse assemblage of benthic dinoflagellates, including *Ga. toxicus* on macroalgae and sandy bottoms on Singapore reefs. Normal sampling techniques involve shaking macroalgae in seawater to dislodge epiphytes, then counting and relating counts to the biomass of the macroalgae sampled or surface area of sediments, coral, and so forth. Vacuuming of turf algae using an airlift has also been used (Lewis et al., 1994b).

Macroalgal hosts may provide exudates (e.g., nitrogenous compounds, vitamins, chelating agents) that are beneficial to the growth of dinoflagellate epiphytes, and the close association of epiphytes with their macroalgae host may help moderate environmental extremes (Nakahara et al., 1996). Tosteson et al. (1989) and Bomber et al. (1989b) demonstrated enhanced growth of epiphytes with the addition of macroalgal extracts; however, similar growth rates of the same species were also observed in media without algal extracts but with the addition of EDTA (Morton, 1992). Villareal and Morton (2002) showed that macroalgal shading can prevent photoinhibiton in *Ga. toxicu*s, permitting them to flourish in shallow, high light tropical environments. Grzebyk et al. (1994) observed that several species of red algae supported a higher abundance of epiphytic dinoflagellates than others but the highest dinoflagellate abundance, particularly of *Ga. toxicus*, occurred on dead corals especially on the inner slope of the barrier reef and on lagoonal patch reefs. Experimental bioassays showed seawater preconditioned by incubation with macroalgae could be both inhibitory and stimulatory depending on the species of macroalgae and dinoflagellate. A variety of antifungal compounds with different selectivities are produced by different strains of *Ga. toxicus*. Lewis et al. (1998) suggested, that these may play a role in ciguatera outbreaks by allowing different strains to dominate, depending upon fungal pathogens present in the environment at any given time. Wright and Cembella (1998) make the observation that production of allelopathic compounds may be particularly advantageous to epibenthic species, enclosed within mucilaginous sheaths that are subject to colonization by pathogenic bacteria and fungi.

The abundance and distribution of ciguatera dinoflagellates may be influenced by abrupt changes in environmental conditions or by more gradual seawater temperature, nutrient, and salinity changes. After 4 years intense monitoring of benthic dinoflagellate assemblages within turf-algae communities on Reunion Island in the Indian Ocean, Turquet et al. (1998) concluded that the period during which water temperatures increased in spring was the most favorable period for the development of *Gambierdiscus* populations, but that the more specific environmental conditions necessary for bloom development were difficult to identify. Dead coral formations provide an ideal environment for colonization by macroalgae and their associated dinoflagellate epithytes (De Sylva, 1994), and natural events (storms, coal bleaching) and anthropogenic disturbance (e.g., increased sedimentation) of coral reef environments (Lewis, 1986; Bagnis et al., 1988) are commonly associated with ciguatera outbreaks. Coral bleaching occurs when the temperature tolerance of corals and their photosynthetic symbionts (zooxanthellae) are exceeded and these events are predicted to increase with increasing global temperatures (Hoegh-Gulberg, 1999). Coral bleaching episodes in the Indian Ocean (Turquet et al., 2001) and Carribean (Kohler and Kohler, 1992) have been documented to result in proliferation of filamentous algae that harbored high numbers of epiphytic dinoflagellates. In the former case an unusually large mono specific bloom of *Ga. toxicus* (>60,000 cells/g

macroalgae) developed, possibly because nutrients normally taken up by the coral zooxanthellae became more available, but this did not result in any noticeable ciguatera outbreak.

One of the most comprehensive long-term studies of the seasonal abundance and toxicity of *Ga. toxicus* was carried out at a site in Tahiti, French Polynesia over a period of 5 years between 1993 and 1997 (Chinain et al., 1999b). Their data confirmed that *Ga. toxicus* was always present at the study site and was the predominant toxic species within the benthic dinoflagellate community. They revealed a seasonal trend over some years, of maximum cell abundance at the beginning and end of the hottest period of the year (28.2–30.9°C), though the seasonal temperature range was small with minimum temperatures between 25.2°C and 26.4°C. Peak cell densities and the highest incidence of blooms was preceded by an unusually prolonged period of high water temperatures (>29°C) that was followed by a severe coral bleaching episode. They found no direct correlation between salinity and *Ga. toxicus* abundance though at their study site the range of salinity was relatively small (34.3–36.1 psu). No correlation was found between the toxicity of blooms (i.p. mouse assay of dichloromethane-soluble fractions) and biomass, or between toxicity and the seasonal temperature regime. They suggested that the severity of ciguatera incidents relates more to the genetic make up of the cells within the *Gambierdiscus* spp. communities rather than to external environmental influences. Similar relationships between *Ga. toxicus* abundance and water temperature has been described elsewhere (Bomber et al., 1988a; Bagnis et al., 1990; Morton et al., 1992) although these contrast with other studies (e.g., Ballantine et al., 1985, Hokama et al., 1996) where no seasonal pattern of *Ga. toxicus* densities were observed. The relationship between highest toxicities, highest cell densities, and cases of ciguatera poisoning from fish consumption during the Tahiti study (Chinain et al., 1999b) support the hypothesis that damage to coral reefs, resulting in the provision of new surfaces for colonization by macroalgae that are hosts for *Ga. toxicus*, is a primary factor in the ciguatera phenomenon. Large-scale environmental perturbations may also influence the incidence of ciguatera. Hales et al., 1999 found a strong positive correlation between the annual incidence of ciguatera poisoning and local warming on Pacific islands that experience warming during El Niño conditions. In islands that experience cooler sea temperatures during El Niño a weaker negative correlation was found. The most recent analysis of the relationship between seawater temperature, variations in *Gambierdiscus* spp. population fluctuations, and the incidence of ciguatera poisoning in French Polynesia (Chateau-Degat et al., 2005) has shown that temperatures were positively associated with dinoflagellate growth with a lag of 13–17 months. In turn, *Gambierdiscus* spp. growth was followed by a peak in the number of ciguatera cases 3 months after maximum cell densities. These data have enabled the construction of a predictive model of the temporal link between *Gambierdiscus* spp. and human cases of ciguatera. The model can predict the emergence of ciguatera cases and identify peak risk periods, but it cannot successfully describe the magnitude of outbreaks.

21.3 ECOBIOLOGY OF THE CYCLIC IMINE PRODUCERS

21.3.1 Introduction

The cyclic imines are a group of macrocyclic compounds, produced by planktonic and benthic dinoflagellates, that have a seven-member spiroimine moiety (a cyclic functional group containing a carbon–nitrogen double bond) that gives these compounds their biological activity (Hu et al., 1996b). Toxicity is generally manifested by a fast-acting toxicity in laboratory, mice when samples are administered by intraperitoneal injection (Munday et al., 2004), and they have a strong affinity for the muscarinic acetyl choline receptor (Richard et al., 2001). Cyclic imines include the gymnodimines (Seki et al., 1995), spirolides (Hu et al., 2001), pinnatoxins (Uemura et al., 1995; Takada et al., 2001a), pteriatoxins (Takada et al., 2001b), prorocentrolides (Torigoe et al., 1988),and spiro-prorocentrimines (Lu et al., 2001). Prorocentrolides and spiro-prorocentrimes are macrocyclic lactones. Various analogues of most of the cyclic imines have been described and there are undoubtedly many more yet to be discovered.

21.3.2 *Prorocentrum* Cyclic Imines

The ecobiology of the dinoflagellates responsible for the production of most of the cyclic imines is little known. Prorocentrolides have been isolated from tropical isolates of the benthic/epiphitic dinoflagellates *Pr. lima* (Torigoe et al., 1988) and *Pr. maculosum* (Hu et al., 1996a). *Pr. lima* has a cosmopolitan distribution, and is better known for its production of okadaic acid. The distribution of *Pr. maculosum* is unknown but it was first isolated from surface sediments and floating detritus on an intertidal mangrove island in Belize, Central America (Faust, 1993b). Spiro-prorocentrimine, structurally closely related to prorocentrolide, was isolated from an unidentified *Prorocentrum* species from Taiwan (Lu et al., 2001). The various closely related analogues of the pinnatoxins (Chou et al., 1996a,b) and pteriatoxins (Takada et al., 2001a) have only been isolated from shellfish extracts (from the bivalves *Pinna [Atrina] pectinata* and *Pteria penguin* respectively) and their attribution to dinoflagellates is circumstantial. Interestingly, pteriatoxins have a cysteine moiety like the brevetoxin analogue BTX-B2 (Murata et al., 1998).

21.3.3 Gymnodimine

In January 1994, a fast acting lipid soluble toxicity was detected by mouse assay in dredge oysters (*Tiostrea chilensis*) from Foveaux Strait, New Zealand (Figure 21.1). The toxicity occurred in association with a bloom of a dorsoventrally flattened dinoflagellate, (referred to as *Gymnodinium* spp.; MacKenzie et al., 1996a), and reports of marine fauna mass mortalities (abalone, fish, and surf clams). The novel cyclic imine, gymnodimine, was subsequently identified from contaminated oysters and cultures of the dinoflagellate (Seki et al., 1995). Haywood et al. (2004) described the gymnodimine-producing dinoflagellate as *K. selliformis* (Figure 21.1). *K. selliformis* contains the characteristic gyroxanthin diester, chlorophyll C3, and the acyl fucoxanthin derivatives 19′ butanoyloxy- and 19′ hexanoyloxy-fucoxanthin of other *Karenia* spp. (Haywood, 2001). Molecular phylogenetic analysis, based on sequencing of the D1–D2 regions of the LSU rRNA gene, showed *K. selliformis* to be distinct from but closely related to these species. Miles et al. (2000, 2003) has identified gymnodimine B and C as isomeric oxidized analogues of gymnodimine A from cultures of *K. selliformis*.

The spatial extent and progression of the 1994 *K. selliformis* bloom was well documented by water sampling and shellfish toxin analysis (MacKenzie et al., 1996a). It was an extensive bloom that progressed within the Southland Current, from Foveaux Strait up the entire east coast of the South Island to the Marlborough Sounds, covering a distance of over 500 km within 2 months (Figure 21.2). The bloom probably orginated in the Tasman Sea offshore of the southwest coast of the South Island. Stirling (2001) surveyed the occurrence of gymnodimine in a variety of shellfish, and found it occurred, at low levels, in >70% of samples from around New Zealand. Gymnodimine retention by different shellfish species varies considerably. Data from nationwide weekly shellfish toxin monitoring by LC-MS (P. McNabb pers. comm.) in New Zealand has shown that in Greenshell™ mussels (*Pe. canaliculus*) low levels of gymnodimine are almost ubiquitous, while it is rarely observed in blue mussels (*Mytius galloprovinicialis*). Dredge oysters (*T. chilensis*) may retain substantial residues for years. The origin of gymnodimine residues in Greenshell™ mussels is unexplained as the occurrence of *K. selliformis* is rare. It is speculated that there may be another, unidentified, producer of the toxin in the plankton. Gymnodimine has now been identified in shellfish in the Mediterranean Sea (Biré et al., 2002; Kharrat, 2006) and has accumulated in solid phase toxin tracking (SPATT) bags (MacKenzie et al., 2004a) in Nova Scotia, Canada (N. Lewis pers. comm.). In neither case has the causative organism been identified.

Batch culture studies of *K. selliformis* (MacKenzie et al., 2004b) have shown that gymnodinime is produced at a relatively constant rate throughout most of the growth cycle. During exponential and stationary phases, ~30% of the total gymnodimine is dissolved in the medium. Gymnodimine appears to be a rather stable constituent of the cells; however, because significant amounts of extracellular toxin exist, a possible ectocrine function for it is suggested. Experiments to examine the effect

of organic acid additions to *K. selliformis* batch cultures (Mountfort et al., 2006) have shown that the combinations most effective in increasing growth rate and toxin production were in decreasing order: glycolate/alanine>acetate>glycolate. Enhancement of gymnodimine cell content (16 pg/cell cf. 9.8 pg/cell for the control) suggests that acetate has a role in gymnodimine synthesis. Recently, Beuzenberg et al. (2006) developed an automated photo-bioreactor system for gymnodimine production, which through control of media pH, resulted in significant improvements in growth rate, cell biomass, and toxin yield.

21.3.4 Ecobiology of the Spirolides

There has been more research on the ecobiology of the spirolides producers than the producers of the other groups of cyclic imines. Spirolides were first identified as a new group of macrocyclic imines from shellfish and plankton in Nova Scotia, Canada (Hu et al., 1995), and several years later the planktonic dinoflagellate *Alexandrium ostenfeldii* was identified as the origin of these compounds (Cembella et al., 2000). Six major spirolide analogues (A, B, C, D, E, F) are known (Hu et al., 1995, 1996b, 2001) in addition to des methyl derivatives of spirolides C (13-deMeC) and D (13-desMeD), and 13 deMeC derivatives SP-1 and SP-2 (MacKinnon et al., 2004).

The early taxonomic history of *Al. ostenfeldii*, its geographic distribution, and questions regarding its toxicity were summarized by Hansen et al. (1992). This species was first described by Paulsen (1904) from Iceland and subsequently from numerous locations on the west coast of Europe, the Faroe Islands, Denmark and Norway (Balech and Tangen, 1985; Moestrup and Hansen, 1988), Spain, (Fraga and Sanchez, 1985), Egypt, west coast of the USA (Balech, 1995), eastern Russia (Konovaloa, 1991) and New Zealand (MacKenzie et al., 1996b). Since the discovery of its involvement in the production of spirolides it has been identified in numerous other locations, such as Nova Scotia (Cembella, 2000), Scotland (John et al., 2003), France (Amzil et al., 2006), the Gulf of Maine, (Gribble et al., 2005), Finland (Lindholm et al., 2006), the Adriatic (Pigozzi et al., 2006), and western Mediterranean (Franco et al., 2006) seas. *Al. ostenfeldii* is clearly a cosmopolitan inhabitant of cool high latitude and warm/temperate mid-latitude environments. The gross morphology of *Al. ostenfeldi* is similar to that of other species such as *Al. tamerense* and may go unnoticed in mixed dinoflagellate assemblages. Oligonucleotide probes offer a means of distinguishing *Al. ostenfeldii* from other species (Rhodes et al., 2001; John et al., 2003).

21.3.4.1 Spirolide Production by *A. ostenfeldii*

Despite the high variability in toxin production by different strains (Table 21.2.), evidence from culture studies indicates that the biosynthesis of spirolides is constitutive. High concentrations of inorganic nitrogen, high salinity (~30 psu), and moderate light levels (100 μmol/m^2/s) increased spirolide yields in culture and growth was strongly limited under conditions of high light (>150 μmol/m^2/s), low salinity (<15 psu), and low (<20 μM initial nitrate) nutrients (Maclean et al., 2003). Photoperiod may also influence toxin production. John et al. (2001) found there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase and corresponding decrease during the light phase, and concluded that synthesis was governed by light-dependant events during the cell cycle. Growth of Danish and New Zealand strains of *Al. ostenfeldii* occurred between 11.3°C–23.7°C and from 10 to 40 psu with maximum division rates (0.3 divisions/d) at 20°C and 15 psu–20 psu (Jensen and Moestrup, 1997). Sexuality was induced in nutrient-deficient cultures but did not lead to the formation of resting cysts. Analysis of spirolides by LC-MS is now so sensitive that their production by single cells can be estimated (Lewis et al., 2006). Gribble et al. (2005) found that spirolide profiles differed not only between isolates from different area of the Gulf of Maine but that different clones from the same site had very different profiles. The cell content of spirolides in field samples were up to two times higher than the maximum levels obtained in cultures. In surveys of the coast of Maine they found cells mainly in surface waters with a range of salinities <29 and >32 psu. Cell numbers were generally low (maximum of 400 cells/L). Spirolides were

TABLE 21.2
Examples of Spirolide Composition and Cell Quota in Wild Cells and Cultures of *Alexandrium ostenfeldii* from Various Geographic Locations

Reference	Location	Spirolide Content (pg/cell)	Spirolide Composition	
Alexandrium ostenfeldii cultures (MacKenzie et al., unpub.)	New Zealand	5.2	desMe-C: desMe-D: Other analogues:	37% 60% 3%
Alexandrium ostenfeldii culture (GB-42) (Cembella et al., 1999, 2000)	Nova Scotia Canada	65.1–74.6 54.0	desMe-C: desMe-D: Other analogues (C, C3):	90% 2% 5%
Alexandrium ostenfeldii culture Ship Hbr isolate AOSHI (Maclean et al., 2003)	Nova Scotia Canada	High N 120.9 Low N 220 l	desMe-C C3 C desMe-D	82% 12.9% 2.8% 1.8%
*Alexandrium ostenfeldii c*ultures (Ciminiello et al., 2006)	Adriatic Sea	3.7	desMeC + unidentified isomers	
Alexandrium peruvianum cultures (Franco et al., 2006)	Western Mediterranean Sea	(Not stated)	desMeC B D desMe-D C	90% 6% 2.7% 2.0% Trace
Alexandrium ostenfeldii in field samples (Gribble et al., 2005)	Gulf of Maine (GOM) USA	0–195 pg/cell	Spiro-A Spiro B Spiro C2 Spiro D2	~50–100% 0–18% <10% Trace
Alexandrium ostenfeldii GOM cultures (Gribble et al., 2005)		19.4–78.3 pg/cell	Group 1. C, C2, D2 Group 2 A, C2, B desMeC, D2 Group 3 A C2, B, D2 Group 4 A, B Group 5 >80% A, B, D	
Plankton field samples (Cembella et al., 1999, 2001)	Nova Scotia Canada			
Ship Harbour plankton		20.4–66.2	A, B, desMe-C, main C and D minor components	
Grave Shoals plankton		14.6–26.5	B, D and D2 isomer main components	

observed in 60% of seawater samples and 83% of these samples contained detectable *Al. ostenfeldii* cells. The calculated total spirolide content of wild cells ranged from 0 to 282 fmol/cell (0–195 pg/cell) and spirolide A was the predominant analogue. Cultured cells contained spirolides C and D and desMeC (field samples did not contain these congeners) and were adapted to lower temperature and light conditions than other *Alexandrium* spp. Mixotrophy may play a role in the ecology and toxin production of *Al. ostenfeldii,* since most of the wild cells that Gribble et al. (2005) observed contained food vacuoles. Ciminiello et al. (2006) recorded *Al. ostenfeldii* cell numbers up to 1.5×10^4 cells/L in the Adriatic Sea and cultured cells isolated from this region contained 13-desMeC and several other unidentified spirolide isomers (3.7 pg/cell). Hummert et al. (2002) found detectable levels of spirolides in seawater samples up to distances >100 km off the east coast of Scotland.

21.3.4.2 Field Ecology of Spirolides

Cembella et al. (1999, 2000, 2001) have carried out the most comprehensive studies so far of the field ecology of *Al. ostenfeldii* and associated spirolide production. Spirolide toxicity in shellfish in Nova Scotia was a highly seasonal and repeatable phenomenon though maximum spirolide concentrations did not coincide with high plankton biomass (i.e., high Chla) blooms. Spirolides appeared in the water column in late spring (early May to late June) following the decline of the diatom spring bloom and establishment of water column stratification. The development of *Al. ostenfeldii* blooms was more related to the formation of a pycnocline, than the direct effect of either salinity or temperature on cell growth or excystment of benthic cysts. They observed that at some sites, when diatoms (*Chaetoceros* spp.) regained dominance of the plankton later in the season, spirolides disappeared. However, in other years diatoms continued to dominate the plankton throughout the spring and co-occurred with large thecate dinos (including *Alexandrium* spp.) and spirolide production. Spirolide profiles were similar over time and depth within a site over a particular season, but the composition of spirolide analogues was substantially different between sites (e.g., desMeC was dominant at one site when spiro-B and spiro-D were dominant at another). In Nova Scotia, spirolides are often found with paralytic shellfish poison (PSP) toxins, probably as a result of *Al. ostenfeldii* co-occurring with PSP-toxic *Al. tamarense*. Tests of Canadian *Al. ostenfeldii* isolates have found no evidence of PSP-toxin production. In two *Al. ostenfeldii* culture studies, where the proportion of dissolved spirolides was measured (Maclean et al., 2003; MacKenzie et al., unpublished), 15%–17% of total spirolides were found free in the medium. The proportion of dissolved spirolides was consistently higher in the cultures supplied with low nitrogen concentrations (Maclean et al., 2003) and the profiles in dissolved and particulate fractions (88% desMeC) remained the same regardless of environmental conditions.

Earlier research on *Al. ostenfeldii* in New Zealand coastal waters (MacKenzie et al., 1996b) first led to the identification of the resting cyst of this species and described wide variations in PSP-toxin content and composition in isolates from different sites. These ranged from highly toxic isolates producing only saxitoxin to completely nontoxic isolates. It was subsequently shown that none of the isolates described in this study produced spirolides (D. Stirling pers. comm.). More recent southern New Zealand isolates of *Al. ostenfeldii* (MacKenzie et al., unpublished data) have been shown to produce spirolides (predominately desMeD and C), although the specific cell yields are low in comparison to what is typically found elsewhere (Table 21.1). These isolates also, simultaneously, produce saxitoxin derivatives (predominately GTX3). Shellfish contaminated with trace levels of spirolides occur occasionally in New Zealand shellfish (A. Selwood, Cawthron Institute, pers.comm.) with consistent seasonal trends in some areas. Over the last decade, during the course of phytoplankton monitoring, vegetative cells of *Al. ostenfeldii* have been observed from numerous locations around the coast of New Zealand, although cell numbers are always low (<600 cells/L) and little is known in any detail about its ecology. Surveys of dinoflagellate cyst types from numerous sites around the New Zealand coast (MacKenzie et al., 1996b) showed that the hypnozygote is widely distributed. It can reach high numbers (up to 7.0×10^4 cells/m^2) and be the dominant dinoflagellate cyst species in some area. The indications are that in New Zealand *Al. ostenfeldii* occurs only briefly as a haploid vegetative cell in the plankton and exists predominately as a diploid hyponozygote within the sediments.

21.4 SUMMARY

Over the last two decades there have been several hundred studies, many of which have been cited here, dealing with the ecobiology of the BTX, CTX, and spiroimine producers and there is now a good understanding of many taxonomic, ecological, and physiological features of these organisms. Many advances have been led by the application of new sampling, observation and molecular biology technologies, elucidation of toxin chemistry, and improved chemical analytical techniques.

However, there are still basic questions to be answered about the life cycles and physiology of these organisms, and the environmental circumstances that lead to the development and sustenance of blooms.

The Gulf of Mexico and the northeastern coast of New Zealand remain the only regions in the world where brevetoxin contamination associated with *Karenia* spp. blooms have been documented. Large-scale offshore oceanographic processes appear to be important in providing the conditions necessary for *Karenia* spp. bloom generation and propagation. It is possible that similar events have gone undetected in other places given the historical difficulties with definitive brevetoxin analysis. There have been various experimental studies on optimizing the growth of *K. brevis* but surprisingly few have examined the dynamics of BTX production in any detail and attempted to manipulate this in culture. Until relatively recently almost all experimentation was undertaken on a single clone. Identification of the PKS genes responsible for toxin synthesis, development of techniques for measuring gene expression, the application of mass spectrographic analysis of toxins, their precursors and other associated polyethers (e.g., brevenal) will undoubtedly lead to greater insights into the mechanism and control of synthesis. The puzzling and conflicting data regarding the putative production of BTXs by raphidophytes needs to be resolved.

The taxonomy of the ciguatera causing dinoflagellate (*Ga. toxicus*) is currently under review and new species within this complex will be described in the near future. This may, or may not, help reveal the reasons behind the variability in production of CTX precursors by *Gambierdiscus* isolates and differences in the toxicology of the phenomenon in different regions, such as between the Caribbean and Pacific. To fully understand the ecology of *Gambieriscus* spp. it is important that the complete life cycle is elucidated, and there is still a great deal to be learnt about the genetic and environmental factors that regulate toxin production. There have been only a small number of studies of toxin production by *Ga. toxicus* and most studies have used activity assays that did not definitely identify CTX precursors. With the availability of very sensitive and definitive mass spectrographic analysis, it should be possible in the future to describe more rigorously how the synthesis of CTX precursors and related compounds (e.g., gambierols, gambieric acids) are regulated. To our knowledge, there has not yet been an attempt to identify and determine expression patterns of PKS genes in *Ga. toxicus*. The most recent field studies on the incidence of ciguatera and proliferation of *Ga. toxicus* have in general supported the hypothesis that damage to coral reefs by natural (e.g., coral bleaching) or human disturbance allow the proliferation of macroalgae, which support the dinofla gellate epiphytes that produce the toxins. However, variations in specific toxicity, and the many complexities involved in food chain transmission make the phenomenon sporadic and unpredictable.

The capability to produce bioactive compounds containing a cyclic imine moiety is present in many distantly related planktonic and benthic dinoflagellates and there does not appear to be any unusual common feature that links the ecobiology of these species. The producers of these compounds range from species living on the sediments in shallow mangrove habitats to oceanic species that propagate blooms over thousands of square kilometers. Apart from the gymnodimines and the spirolides, little is known about the ecobiology of cyclic imine producers, although their diversity suggests that new compounds and new producers will be discovered in the future. Several PKS genes putatively related to spirolide synthesis in *Al. ostenfeldii* have recently been identified (Jaeckisch et al., 2006) and it is likely that the factors regulating spirolide synthesis will become clearer in the near future.

Little attention has been paid to the fact that significant amounts of most, if not all, of these toxins are found in solution during blooms. This realization has led to the development of the passive SPATT technique (MacKenzie et al., 2004a). This approach provides a means of monitoring and forecasting the development of blooms, provides unique information on the spatial and temporal distribution of toxin producers (it could for instance be used to locate ciguatoxin "hot spots" on coral reefs) and enables collection of dissolved compounds from natural seawater for investigation of allelopathic interactions (MacKenzie et al., 2006).

21.4.1 Does Quorum Sensing Provide a Unified Hypothesis for the Ecological Role of Dinoflagellate Toxins?

From a fundamental point of view, the most important outstanding questions relate to the ecological role of the BTXs, CTX precursors, and cyclic imines, and how environmental factors interact with the genetics and biochemistry of the dinoflagellates to influence their production. Why do which have independently evolved over an extended period of geological time to occupy different ecological niches (planktonic, benthic, epiphytic), find it advantageous to produce these complex and unusual bioactive molecules? The most common explanations center on the role of these compounds as allelopathic agents that enhance the ability of the dinoflagellate to compete for space and nutrient resources with other microalgae, or as chemical defenses against predation. Other hypotheses include roles as antibacterial and antifungal agents, involvement in sequestration of other metabolites, aides to dinoflagellate predation, modulation of photosynthesis, and ion channel regulation. It is possible that the toxins have multiple roles, but tests of these hypotheses have not yet provided convincing evidence that any of these deliver a complete explanation.

Another possibility, which has not been experimentally evaluated, is that the primary role of these compounds may be in cell-to-cell communication, that enables single cells to sense the density of the population and coordinate their behavior. The quorum-sensing hypothesis (Juhas et al., 2005) has in recent years altered perceptions of bacterial ecology. It is now clear that many microbial activities, including growth in biofilms, expression of virulence factors (toxins), genetic exchange, antibiotic production, motility, and swarming behavior, induction and repression of enzyme coding genes, are coordinated processes controlled by cell–cell communication, mediated by signaling molecules. The mechanism involves secretion of an autoinducer that accumulates in the medium until a threshold concentration, correlating with cell density, is reached that initiates population wide gene expression. Quorum sensing has been identified as the mechanism regulating the biosynthesis of polyketides in *Streptomyces* and *Pseudomonas* (El-Sayed et al., 2001; Recio et al., 2004). Where it has been measured in culture or in the field (MacKenzie et al., 2004a,b), it is now apparent that during dinoflagellate blooms a significant proportion of dinoflagellate polyketide secondary metabolites are free in solution. Quorum sensing may explain various characteristics of toxic dinoflagellate populations including

- Biofilm development by benthic and epiphytic species (e.g., Gambierdiscus spp.).
- Swarming behavior of planktonic species and formation of microlayers.
- Mediation of sexual contact and induction of encystment.
- Typically highest toxin content under stressed conditions and in late log and stationary growth phases.
- High variability in clonal isolates from a single population. Toxin production (e.g., GTXs) may be induced in "nontoxic" clones by the toxin produced by a small proportion of permanently expressing cells.
- Observations of higher specific toxicity in cells from natural populations than from clonal cultures. Interclonal interactions may be necessary for optimum expression of toxin genes.
- The autocrine growth enhancing and toxin neutralizing effect of toxin related polyethers such as gamberic acid-A and brevenal suggests a possible role in quorum sensing modulation.

The quorum-sensing hypothesis may provide a new model for investigating the ecological role of polyether microalgal toxins that can be tested by experimentation. This may lead to new insights into the mechanism and regulation of their production and possibly the development of new techniques for the mitigation of their effects.

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Part VIIIA

Ciguatera Toxins

22 Ciguatera Toxins: Chemistry, Toxicology, and Detection

Robert W. Dickey

CONTENTS

22.1 INTRODUCTION

Coral reef ecosystems are known for highly concentrated species diversity and fascinating survival strategies. The lower orders of marine flora and fauna in particular are replete with examples of allelopathic, feeding deterrent, and otherwise behavior-modifying secondary metabolites, presumed or demonstrated, to confer some competitive advantage for survival (Williams et al., 1989). Secondary metabolites are so named because they are not essential to the life of the organisms producing them. Nevertheless, the abundance and diversity of chemical structures and physiological activities of secondary metabolites are immense (Firn and Jones, 2003). This diversity is a reflection of equally diverse challenges to species survival encountered over time and the metabolic responses to these forces. The pathways of secondary metabolite production are presumably retained through enhancement of species survival over like species that do not produce survival enhancing secondary metabolites. The analogy of "arms race" is often invoked to describe such biochemical competition for survival within and among species. The production of bioactive secondary metabolites, however, can have ramifications beyond the immediate challenge to species survival. In the case of certain dinoflagellate species from coral reef ecosystems, toxic secondary metabolites traverse the food web with devastating impact on trophic levels well removed from the presumed initial forcing. One dinoflagellate genus in particular, *Gambierdiscus*, for which the type species *Gambierdiscus toxicus* was discovered by Yasumoto et al. (1977) and described by Adachi and Fukuyo (1979), is the source of secondary metabolites responsible for ciguatera fish poisoning in humans. These particular secondary metabolites and their piscine metabolic products are called ciguatoxins.

Ciguatera fish poisoning is a human disease that is endemic to many tropical and subtropical coral reef regions of the world. It is contracted by consumption of finfish that have accumulated lipid-soluble ciguatoxins, which are transmitted through the food web from the primary production level (i.e., *Gambierdiscus* spp.) to apex piscine predators. With its characteristic gastrointestinal,

neurological, and cardiologic symptoms, ciguatera has been described in literature from the West Indies and South Pacific since the eighteenth century, with fragmentary reports dating back to the 1500s. In the United States, it is a significant public health problem in Hawaii, Puerto Rico, the U.S. Virgin Islands, the Florida Keys, Guam, American Samoa, and the Marshall Islands. The number of cases in these areas is sufficient to result in ciguatera being consistently reported by the U.S. Centers for Disease Control and Prevention as a leading cause of finfish-associated food-borne disease in the United States.

At a global level, Fleming et al. (1998) recognized the paucity of epidemiological data and ventured a broad estimate of 50,000–500,000 poisonings per year worldwide. Tosteson (1995) argued that there are 20,000–40,000 illnesses per year in Puerto Rico and the American Virgin Islands alone, while other investigators estimate that the annual incidence approaches 10% of local island populations (Lewis 1992; Pearn 2001). There are suggestions that the incidence and worldwide distribution of ciguatera are increasing (Levine, 1995; Lehane and Lewis, 2000; De Haro et al., 2003; Poon-King et al., 2004), paralleling a worldwide increase in toxic or harmful algae bloom events (Anderson 1989; Hallegraeff 1993; Lechuga-Deveze and Sierra-Beltran, 1995). Some argue that such increases are linked to anthropogenic (Ruff, 1989) and naturally occurring environmental changes, including global warming and increased nutrient loading (Smayda, 1989). Both natural and anthropogenic environmental factors have the potential to affect dinoflagellate abundance and toxicity and thus human morbidity from ciguatera. Increases in sea surface temperature (SST) have been linked with increased dinoflagellate abundance and fish toxicity in Puerto Rico (Tosteson et al., 1988). Chateau-Degat et al (2005) demonstrated a positive correlation between SST and *G. toxicus* abundance in Tahiti and used those data and human case incidence to develop a predictive model for disease. Hales et al. (1999) observed strong positive correlations between the annual incidence of ciguatera and local warming of the sea surface in a group of Pacific Islands that experienced heating during El Niño Southern Oscillation events. At another group of islands that experienced cooling during El Niño events, the opposite was observed. Several mechanisms were offered as explanations for this observed relationship between elevated temperatures and increased ciguatera incidence, including the enhancement of denuded coralline substrate for *G. toxicus* through coral bleaching and disease. Physical disturbances of coral reefs (e.g., dredging and harbor construction) have also been associated with increased *G. toxicus* abundance (Lewis 1986a,b; Bruslé 1997) and outbreaks of ciguatera (Ruff 1989; De Sylva 1999). More recently, petroleum production platforms and state-sponsored artificial reefs located within or near subtropical–tropical latitudes provide substrate that support coral and other components of the tropical benthos, including *Gambierdiscus* spp. (Villareal et al., 2007). These structures create a new habitat in the upper euphotic zone and serve as fish aggregation points. The authors suggest that the structures could have unintended consequences for human health, particularly if rising SST over the next century alter benthic biota distributions and fish migration patterns. These concerns also extend to proposals for offshore aquaculture operations or offshore wind farms, which would also add substrate for benthic flora. A number of other natural and anthropogenic influences can lead to degradation of the reef environment as well, including tourism, eutrophication, sedimentation due to erosion or dredging, and ship groundings (Lehane and Lewis, 2000). Some have argued that ciguatera is a sensitive indicator of environmental disturbance in tropical marine ecosystems (Hales et al., 1999). Unfortunately, data to assess these issues in sufficient detail are lacking for most harmful algal bloom associated illnesses. If such data are to be collected, ciguatera, given its worldwide distribution and sufficiently high incidence to permit statistical studies, may be a good place to start.

Ciguatera is a consistently underappreciated and underreported problem. Many affected communities are at marginal socioeconomic or subsistence levels. Tourist hotels in tropical regions can afford to import their fish (and, in areas of high endemicity, such as the Virgin Islands, almost all do so), while local populations remain at risk. The public health impact of ciguatera is particularly high in remote atoll countries of the Pacific where daily intake of reef fish may exceed 100 g/person per day (Lewis, 1992) and where fish represent a major protein source that cannot be fully exploited,

either locally or internationally. In a broad altruistic sense, the impact of ciguatoxins on human activities, that is, fishing in fragile coral reef ecosystems, has the ecological and societal benefit of retarding commercial exploitation of these wonderfully complex, diverse, and attractive ecosystems. Be that as it may, the extended consequence of ciguatoxins production by species of *Gambierdiscus* is ciguatera, the disease. This malady is not unlike many other diseases that have resisted our understanding, eradication, or control.

In many underdeveloped island communities, nutrition has always been a challenge. Protein sources are limited, represented in many remote islands, for example, by goats, a few chickens, the occasional iguana, and fish. Fish are by far the most abundant protein source, but the risk of ciguatera is broadly recognized by resident populations. Residents in most cases prefer smaller reef fish caught in "fish pots" because the smaller fish are less likely to be poisonous and because this is an energy- and time- efficient way to obtain protein. Some also fish by hand line or pole, and smaller apex predators (e.g., barracuda) caught in pots or by line are consumed, again under the presumption that the smaller specimens are not poisonous or at least less poisonous. Residents are aware and selective of where they fish. Areas known for poisonous fish are avoided and this knowledge is passed on from generation to generation. Island residents waste very little of the fish they catch when preparing meals (e.g., fish stew with head and viscera), a practice that in many cases negates the margin of safety sought by selection of the smaller species because of higher toxin concentrations in organ tissues.

The odds are indeed better for smaller fish to be less toxic or even nontoxic, but smaller species and smaller specimens of apex predators can be as toxic as larger species of known repute. Residents of the ciguatera-endemic Caribbean and Pacific consume subthreshold levels of toxin on a consistent basis. The toxins accumulate in their systems until that point where toxicity threshold is reached and symptoms appear. The afflicted then stop eating fish for a time, take a local remedy (e.g., tea made from bark of the buttonwood bush in the Virgin Islands), and when symptoms subside they resume fish consumption. Many island residents have chronic low-level ciguatera symptoms. They generally recognize what is happening but accept a certain level of discomfort and risk in order to survive.

The toxicity of tropical fish depends on where the fish are caught, and how long the fish were feeding in the area before they are caught. Some areas around tropical islands are very toxic and others are not, and most tropical fish species are territorial, moving to new locations infrequently. The length of time fish reside in a toxic area correlates with toxicity for most species. Conversely, length of time in a nontoxic area will correlate with safety. The same surmise can be made for the semipelagic species of fish known for ciguatera (e.g., *Scomberomorus cavalla* and *Caranx latus*). For the fisherman or consumer, however, there are no methods to discern how long a fish has resided in a toxic or nontoxic area. Experience and historical knowledge of the region appear to be the only meaningful mitigating factors.

The path of the ciguatoxins from dinoflagellate to human seafood consumer begins in the assemblage of benthic dinoflagellate species described from tropical latitudes (e.g., Yasumoto et al., 1980, 1987; Tindall et al., 1984; 1989; Carlson and Tindall, 1985; Holmes and Lewis 1994; Tosteson 1995; Tindall and Morton, 1998). Species of *Prorocentrum*, *Ostreopsis*, *Coolia*, *Amphidinium*, and most significantly, species of the genus *Gambierdiscus* occupy benthic and epiphytic niches in low-energy habitats, contributing primary production to coral reef and inshore mangrove ecosystems. These toxic dinoflagellates differ from the better known, open-water "red-tide" dinoflagellates common to temperate latitudes. The tropical benthic and epiphytic dinoflagellates are "poor swimmers" and they flourish only in low-energy, somewhat protected locations. They are much less abundant in high-energy (e.g., ocean current and wave exposed) locations. The distribution of ciguatoxic finfish, long characterized as "spotty" or "patchy" around tropical islands and a few continental landmasses (e.g., Australia), is consistent with the localization of benthic dinoflagellate communities in low-energy mangrove systems, on leeward sides of coral reefs and atolls where physical structure provides protection from energetic water movement. By virtue of the low physical energy

required to maintain position and maneuver in relatively protected environments, these areas are also ideal nursery grounds for fishes and invertebrates, which ultimately populate higher energy coral reef areas. Further, these low energy areas are also productive grounds for predation by intermediate piscine predators. As a result of this dynamic trophic interaction, toxin accumulation in tropical fish is both initiated and sustained by the movement and predation patterns of piscine ciguatoxin vectors. Furthermore, the effects of these toxins on smaller fish, herbivores, and omnivores, accelerate predation and the transfer of these toxins to apex predators and ultimately to humans. Ciguatoxins have been shown to disturb fish equilibrium and by extension, predation escape abilities (Davin et al., 1986). The toxins have also been shown to impair embryonic and larval stages of development in fish, decreasing larval survivability, and expediting toxin transfer from *Gambierdiscus* spp. to higher trophic levels (Colman et al., 2004).

22.1.1 Clinical Features of Ciguatera

"Ciguatera" is a clinical syndrome. Despite considerable efforts to develop facile diagnostic assays (e.g., immunoassays: Hokama 1993; Hokama et al., 1996), specificity and sensitivity remain significant obstacles. Only recently have laboratory-based *in vitro* methods succeeded in detecting sodium- channel-specific activity, consistent with ciguatoxins, in clinical samples. Using a murine model, progress has also been made in the use of blood collection cards for sample collection and transport to analytical laboratories where highly sensitive *in vitro* assays for detection of ciguatera toxin activity has been refined for a blood matrix (Bottein-Dechraoui et al., 2005). The application of these methods, however, is very recent and clinical diagnosis is still based on a history of reef fish consumption and a clinical presentation defined by both gastrointestinal and neurologic symptoms (Perkins and Morgan, 2004). Within this definition, however, variation has been observed in the clinical syndrome and severity of disease. This appears to reflect equally variable individual risk factors among patients in different parts of the world and the geographic differences in ciguatoxin profiles within and among species of ciguatoxic fish.

Onset of ciguatera commonly begins with gastrointestinal problems such as nausea, vomiting, diarrhea, and abdominal pain within 12 h of eating a toxic fish, and often abates within 24 h (Hokama, 1988). Cardiovascular problems may be present during this acute period (generally a combination of bradycardia with hypotension). In the Pacific, there are reports of rapid progression to respiratory distress, coma, and occasionally death (Lange 1987; DeFusco et al., 1993; Habermehl et al., 1994). From a few hours to 2 weeks after exposure, subjective neurological complaints may emerge. Paresthesias (numbness and tingling of perioral region and extremities) and paradoxical disturbance of temperature sensation are considered pathognomonic symptoms of ciguatera fish poisoning (Pearn, 2001). Early epidemiological studies reported paradoxical disturbance of temperature sensation, that is, temperature sensation reversals with cold objects feeling hot and hot feeling cold. Studies have suggested that temperature sensation is actually intact and that the unusual sensations represent tingling or "electric shock" pain rather than a true reversal of hot and cold perception (Cameron and Capra, 1993). These authors concluded that the ciguatera toxins induced abnormal discharges in the peripheral fibers involved in temperature sensation and may also be involved in the intense itching that patients report. Nerve conduction studies have been performed in a limited number of patients with acute illness, demonstrating a generalized disturbance in both sensory and motor conduction with significant prolongation of the refractory periods and the supernormal period of excitability (Cameron et al., 1991).

Other subjective neurological symptoms reported include metallic taste, pruritus, arthralgia, and myalgias (muscle aches, especially in the legs) and sensations of loose teeth (Poon-King et al., 2004). Cerebellar signs and tremors have also been reported, which may present up to 10 days after initial exposure (Chungue et al., 1977). Headache, which appears nonlocalized, intense, and prolonged may be a presenting sign (Pearn, 2001). General weakness, hyporeflexia, and dysphagia may also be found. While cranial and peripheral nerves appear to dominate the clinical picture,

Karalis et al. (2000) reported that 11% of the neurological symptoms observed were indicative of central nervous system involvement. Central nervous system involvement (paralysis, ataxia, stupor, and confusion) is usually indicative of the most severe cases (Cameron and Capra, 1993). A wide range of neurobehavioral symptoms have been reported following ciguatera poisoning including fatigue, anxiety, depression, hysteria, "neurosis," memory disturbance, and mental inefficiencies (Gillespie et al., 1986; Lipkin 1989; DeFusco et al., 1993; Karalis et al., 2000; Arena et al., 2004). Depression in many patients accompanies slow regression of the paresthesias, weakness, fatigue, and complaints of general malaise, suggesting a secondary reaction to chronic illness. In contrast, neurocognitive complaints (memory, mental inefficiencies) may be the direct result of neurotoxic exposure, that is, a general cognitive inefficiency as a result of diminished cerebral functioning or secondary to fatigue, depression, or general malaise.

Acute gastrointestinal problems typically resolve within a day or two and cardiovascular disorders reverse within 48–72 h (Hokama 1988; Butera et al., 2000). Recovery from neurologic symptoms is longer and less predictable, ranging from 1 week to 6 months (Morris et al., 1982a,b; Lange et al., 1992; Butera et al., 2000; Poon-King et al., 2004). In a subset of patients, chronic illness may occur, characterized by a vague and poorly defined combination of recurring neurologic and neuropsychological symptoms. Pruritus, arthralgia, and fatigue can persist for months or years (Gillespie et al., 1986). The fatigue can be so debilitating that it resembles a chronic fatigue syndrome (Pearn, 1997). Chronicity may reflect lengthy persistence of ciguatoxins in the body (Chan and Kwok, 2001) or lowered neural thresholds responding to dietary or behavioral stimuli unrelated to ciguatoxins. The persistence of symptoms in some patients for several years has been documented and is not unusual. In one series (Lange et al., 1992), 65% of the patients had symptoms 6 months or longer with reported recurrence up to 2 years.

On the basis of observations suggesting that repeated exposures to ciguatera toxins are associated with a more severe clinical illness, it has been hypothesized that the fat-soluble ciguatoxins accumulate in humans and lowers threshold tolerances (Bagnis et al., 1979; Hokama, 1988; Glaziou and Martin, 1993). Increasing age and weight, associated with greater lifetime exposures and greater capacity for toxin storage, have also been linked to the duration and severity of symptoms (Katz et al., 1993). An alternate explanation is that the ciguatoxins cause a slowly or irreversible subclinical level of damage. Physical or dietary behaviors (e.g., exercise, alcohol consumption, or excessive caffeine) or repeat exposure to subthreshold levels of ciguatera toxins may induce symptom recurrence.

22.1.2 Ciguatoxins from *Gambierdiscus* spp.

A benthic microorganism origin of ciguatoxins was first hypothesized by John Randall (1958). Randall's detailed observations of feeding behavior by fish of ciguatoxic repute in the Pacific led him to the premise that ciguatera toxins must be produced by benthic microorganism(s), consumed by grazing herbivores, and accumulated by higher piscivorous fishes through predation. Field observations and laboratory evidence consistent with his surmise were compiled and elaborated by several investigators, for example, Helfrich and Banner (1963), Helfrich et al. (1968), Banner (1976), Banner and Helfrich (1964), Yasumoto et al. (1971, 1976). An important proof of Randall's hypothesis was achieved by Banner (1974) demonstrating transmission of ciguatera toxicity from fish to fish in controlled feeding studies using red snapper, *Lutjanus bohar*. Shortly thereafter, Yasumoto et al. (1971, 1976) selected the small herbivorous Surgeonfish, *Ctenochaetus striatus*, for detailed study and correlated disc-shaped dinoflagellates in stomach contents with toxicity of viscera extracts. Extracts of this dinoflagellate collected from dense populations epiphytizing calcareous algae and encrusting dead coral in the Gambier Islands yielded two toxic fractions chemically consistent with ciguatoxin and maitotoxin. Further examinations of field and laboratory cultured samples of this dinoflagellate confirmed the benthic link to ciguatera toxins (Yasumoto et al., 1977, 1979; Bagnis et al., 1980). Initially identified as a species of *Diplopsalis*, the dinoflagellate was later designated

a new species and monospecific genus, *G. toxicus* (Adachi and Fukuyo, 1979). Since the initial discovery of *G. toxicus* several new species have been added to the genus, all appearing to be geographic or morphological variants of the type species.

Extracts from wild and cultured cells of *G. toxicus* from French Polynesia yielded ciguatera-like toxins, designated gambiertoxins, which are less polar than the ciguatoxins isolated from fish (Bagnis et al., 1980). A major toxic constituent of such nonpolar fractions was designated GTX-4B (CTX-4B: $M+H^+$, *m/z* 1061.6; Figure 22.1). On structure elucidation, it appeared to be a precursor to P-CTX-1 from moray eel (Murata et al., 1989, 1990). The C-52 epimer of CTX-4B, designated CTX4A, and a deacylated-deoxy congener of P-CTX-1, designated CTX3C, were soon after identified from cultured *G. toxicus* (Satake et al., 1993, 1997, 1998). All appear to be precursors to oxidized P-CTX-1 congeners identified from toxic fish (Murata et al., 1990; Lewis and Holmes, 1993). These less polar P-CTX-1 precursors identified from *G. toxicus* exhibit median lethal potencies in the mouse model approximately 10-fold less potent than P-CTX-1 from fish. In addition to the lipid-soluble gambiertoxins, *G. toxicus* produces the water-soluble toxins, maitotoxin, gambierol, and gambieric acids (see review by Wright and Cembella, 1998). Due to the water-soluble nature of the latter toxins, however, these compounds are not thought to be involved in ciguatera fish poisoning.

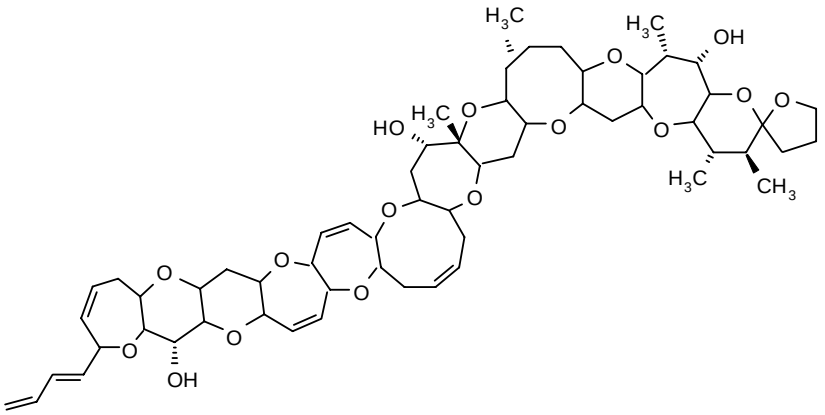
G. toxicus has been the primary focus of toxicity studies of ciguatera dinoflagellates largely due to the fact that *G. toxicus* was the only described species of *Gambierdiscus* for almost 20 years. In the last decade, however, taxonomic studies of ciguatera endemic areas described a number of new *Gambierdiscus* species including *Gambierdiscus belizeanus* (Faust, 1995), *Gambierdiscus yasumotoi* (Holmes, 1998), *Gambierdiscus pacificus, Gambierdiscus australes,* and *Gambierdiscus polynesiensis* (Chinain et al., 1999a). The toxicity of *G. belizeanus* is not known, and the description of *G. yasumotoi* includes a brief mention of maitotoxin-like activity. Chinain et al. (1999b) reported ciguatoxin-like activity from *G. pacificus, G. australes,* and *G. polynesiensis*, with low levels of activity in *G. pacificus* and *G. australes* and "exceptionally high" activity in the two *G. polynesiensis* strains examined. All of the known species except *yasumotoi* have been reported to occur in Belize (Faust, 1995; Chinain et al, 1999). Because the different *Gambierdiscus* species co-occur and can vary with respect to their toxicity, changes in the proportion of *Gambierdiscus* species in a population appear to contribute to ciguatoxin variability in natural populations.

In addition to species level differences in toxicity, the toxicity of individual *Gambierdiscus* strains can be highly variable and is dependent upon a number of factors. Holmes et al. (1991) showed that the production of gambiertoxins was limited to certain genetic strains of *G. toxicus* and that wild cells produced 100-fold greater quantities of gambiertoxins than that produced by cultured cells. This finding was believed to reflect the culture conditions used. Variations in toxin yield have also been observed in field populations in the Caribbean (McMillan et al., 1986) and Pacific (Chinain et al., 1999b) where *G. toxicus* biomass is not correlated with toxicity. This has led to speculation that ciguatera outbreaks occur when environmental conditions favor the growth of highly toxic clones within a population (Holmes et al., 1994; Chinain et al., 1999b). Indeed, laboratory studies suggest that individual *G. toxicus* strains are adapted to particular environmental regimes. Bomber et al. (1988) observed different relationships between light intensity and growth rate for a variety of strains, with reduced growth rates at high irradiance. Morton et al. (1993) showed a 200-fold difference in *G. toxicus* potency depending on the light, salinity, and temperature conditions used during culture. Higher temperatures (>28°C) increase toxicity in both *G. toxicus* (Bomber et al., 1988) and *Ostreopsis* (Tosteson et al., 1989). These results are consistent with the observed distributions of *G. toxicus* in the field, as this species is rarely found close to the surface where light levels are high (Bomber et al., 1988; Villareal and Morton, 2002).

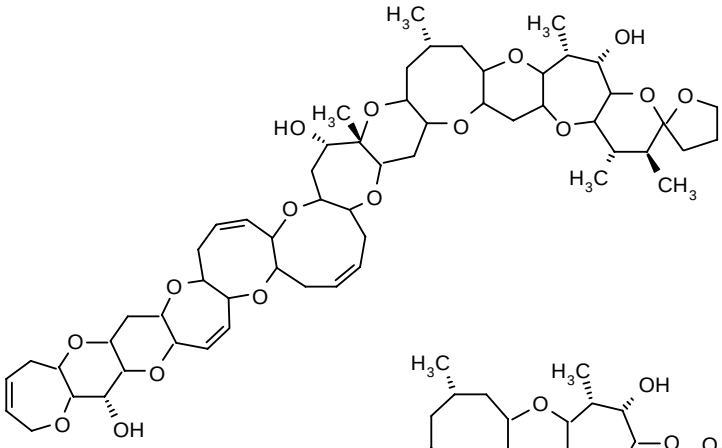
22.1.3 Ciguatoxins in Finfish

Ciguatoxins in finfish comprise an assemblage of principal ciguatoxins and numerous closely related structural isomers and congeners. The Pacific ciguatoxins were structurally characterized before the Caribbean ciguatoxins, and the latter differ slightly in structure and toxicity. Both Pacific

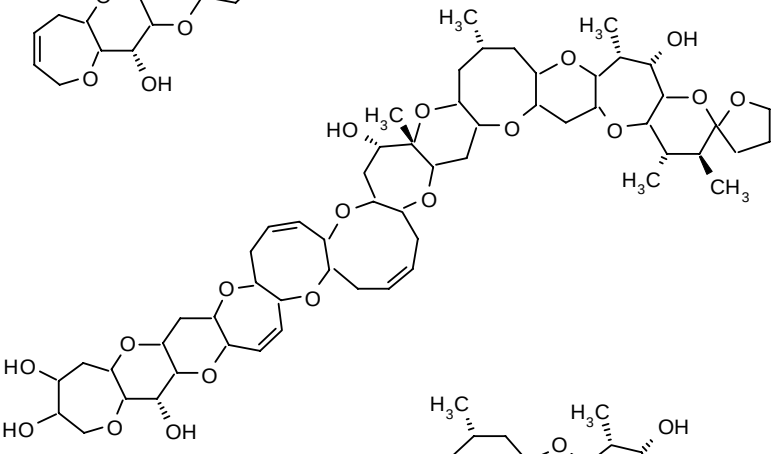
P-CTX-4B (-4A)



P-CTX-3C



2,3-Dihydroxy P-CTX-3C



51-Hydroxy P-CTX-3C

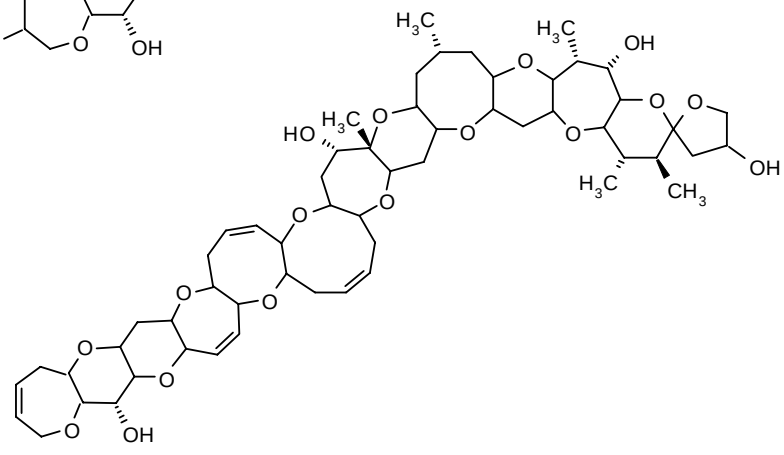


FIGURE 22.1 Ciguatoxin structures from *G. toxicus*.

and Caribbean ciguatoxins are piscine metabolites of the gambiertoxins as they are assimilated and metabolized through multiple trophic levels of the marine food web. A principal Pacific ciguatoxin was first isolated from moray eels in 1967 (Scheuer et al., 1967; Tachibana et al. 1987). One decade later, Yasumoto et al. (1977) discovered the origin of ciguatoxins in the Gambier Islands, and named the producing organism *G. toxicus* (Adachi and Fukuyo, 1979), a benthic dinoflagellate that lives on denuded coral reef surfaces and epiphytizes seaweeds. Structural elucidation of the principal Pacific ciguatoxin from moray eel and its precursor-toxin (gambiertoxin 4B or CTX-4B) from *G. toxicus* was accomplished in 1989 (Murata et al., 1989, 1990). The ciguatoxins are lipid-soluble polyether compounds with skeletal structures comprised of 13–14 transfused ether rings (Figure 22.2). They are odorless, colorless, devoid of heteroatoms other than oxygen, and bear few conjugated bonds. The first CTX structure elucidated and the principal ciguatoxin in the Pacific (P-CTX-1, Figure 22.2), has a mass of 1110.6 Da and a molecular formula of $C_{60}H_{86}O_{19}$ (Murata, 1989, 1990). It is a potent sodium channel agonist and exhibits extreme lethality in mouse models with a median lethal dose of 0.25 μg/kg.

Further investigations of ciguatoxic fish from the central and western Pacific, led by Yasumoto (Japan) and Lewis (Australia), determined that multiple structurally related toxins (i.e., CTX isomers and congeners) were present in toxic fish. However, the body of work also suggested that a select few were truly abundant, and in most cases, a single ciguatoxin structure dominated toxin profiles. P-CTX-1 is the most abundant and most toxic of the ciguatoxins present in piscivorous Pacific fish (Legrand et al., 1990, 1992). The contribution of P-CTX-1 to composite toxicity in mice, and

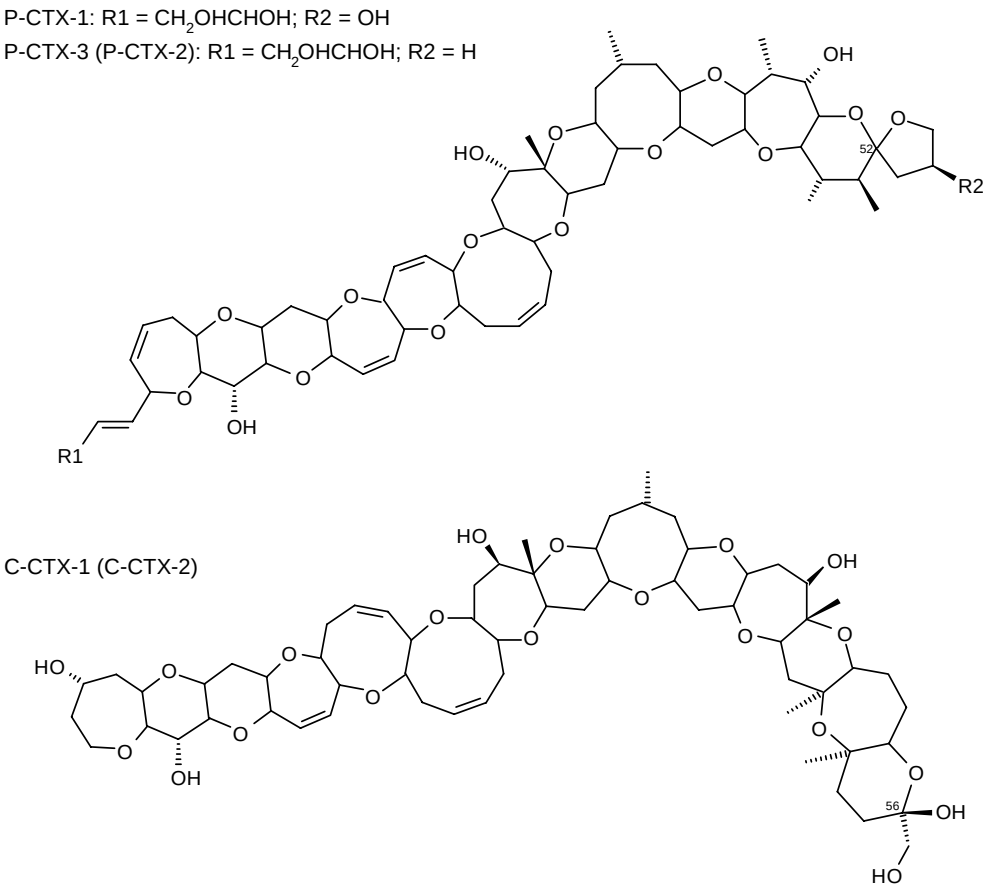


FIGURE 22.2 Principal Pacific (P-CTX-1) and Caribbean (C-CTX-1) fish ciguatoxin structures.

by extension to humans, was estimated at 90% (Lewis et al., 1991). In subsequent studies of toxin profiles from Pacific fish, numerous precursors, congeners, and isomers were identified. In all cases, insufficient amounts of these minor toxins were recovered for structural elucidation by nuclear magnetic resonance (NMR), but using fragmentation patterns of the known structures P-CTX-1 and CTX-4B as templates, many congeners were identified by FAB/MS/MS of sodiated products or via synthetic conversions to known structures (reviewed in Yasumoto, 2001). In all, approximately 29 congeners have been identified that comprise products of epimerization, hydroxylation, and oxidation of P-CTX-1 or CTX-4B. A new system for annotation (i.e., P-CTX-# for Pacific and C-CTX-# for Caribbean) was proposed to distinguish CTX source and structural variants.

Similar studies were performed from the Caribbean (Crouch et al., 1995; Vernoux and Lewis, 1997) (2002), the initial isolation and characterization of Indian Ocean CTX was reported by Hamilton et al. (2002). Caribbean ciguatoxin (C-CTX-1) was first described by Lewis et al. (1998) with a mass of 1140.6 Da and a molecular formula of $C_{62}H_{92}O_{19}$. Like Pacific CTX, it is a potent sodium channel agonist and exhibits a median lethal dose in the mouse model of 3.7 µg/kg, approximately 10-fold less potent than P-CTX-1. The case for dominance of a single Caribbean ciguatoxin appears less convincing than the case in the Pacific. A single, and apparently dominant, Caribbean ciguatoxin (presumptive MH^+, *m/z* 1123.6) was isolated and partially characterized from *Sphyraena barracuda* and *C. latus* (Crouch et al., 1995). This dominant ciguatoxin and at least two minor toxins were also observed in meal remnants of *S. cavalla* from a ciguatera outbreak (Poli et al., 1997). In both of the studies, the toxins were not obtained in sufficient quantities for structural elucidation, and there was some question as to the mass assignment of the principal toxin (i.e., *m/z* 1123.6). In 1997, five ciguatoxins were isolated from *C. latus* (Vernoux et al., 1997) and the structures of two of these, C-CTX-1 (MH^+, *m/z* 1141.6) and its C-56 epimer C-CTX-2, were elucidated by NMR and MS (Lewis et al., 1998). It was determined in the later studies that C-CTX-1 and C-CTX-2 contribute only partially (40%–60%) to total fish toxicity and that a minimum of three additional toxins may contribute to ciguatera in the Caribbean. The principal toxin isolated by Crouch et al. (1995) (*m/z* 1123.6) was later identified by M. Quilliam (NRC-Canada, personal communication) as a dehydration product of the C-CTX-1 defined by Lewis et al. (1998). More recent studies identified seven additional C-CTX congeners and three additional isomers of C-CTX-1 or C-CTX-2 (Pottier et al., 2002).

22.1.4 Sample Preparation and Detection of Ciguatoxins in Fish

The scarcity of purified ciguatoxin standards and the challenging nature of analyses for ciguatoxins in fish tissues precluded development or adoption of ciguatoxin methods in most laboratories. While many are certainly capable, only a few laboratories have produced the necessary standards and sustained support required for development and routine application of screening assays and confirmatory analyses for ciguatera toxins. Protocols for *in vitro* assay and LC-MS/MS analysis of fish tissues have been developed in U.S. Food and Drug Administration (FDA) and National Oceanic and Atmospheric Administration (NOAA) laboratories. Laboratories in Japan (T. Yasumoto) and Australia (R. Lewis) use similar protocols that predate those in the United States.

22.1.4.1 Sample Preparation

Fish tissue samples are prepared by homogenizing in high performance liquid chromatography (HPLC) grade acetone (2 mL/g tissue). After homogenizing, acetone is separated from tissue by centrifugation or by filtration through filter paper for larger samples. Tissue residues are homogenized a second time with acetone at 2 mL/g tissue and the combined acetone extracts are chilled at –20°C for at least 12 h. Nontoxic precipitate is then removed by centrifugation or filtration for larger samples using filter paper in a chilled buchner funnel. The nontoxic precipitate is discarded and the acetone and water supernatant or filtrate is dried under a stream of nitrogen or by rotary evaporation yielding a dry residue. The dry residue from acetone extraction is dissolved in 80% methanol in water (1.0 mL/g extracted tissue) and defatted by washing with 95% *n*-hexanes (HPLC grade,

2 × 0.5mL/g tissue). The hexane wash is discarded. The aqueous methanol phase is dried by nitrogen stream (1–3 g samples) or by rotary evaporation (larger samples). The resulting residue is suspended in water (1.0 mL/g tissue) and extracted with chloroform (3 × 0.5 mL/g tissue). The water phase is discarded and the chloroform phase is dried under a stream of nitrogen or by rotary evaporation. Residue weight is determined for successive steps in the protocol.

A silica gel solid phase extraction (SPE) cartridge is selected to give a sorbent/residue weight ratio of at least 20:1. The cartridge is conditioned by passing successively one column volume each of 5% water in methanol, methanol, and chloroform. If necessary, additional chloroform is passed through the cartridge until the sorbent is uniformly translucent. The tissue sample residue is dissolved in chloroform at ca. 2 μL/mg residue weight (minimum of 100 μL chloroform), applied to the cartridge and allowed to pass into the sorbent bed by gravity flow. The sample vessel is rinsed with the same volume of chloroform and applied to the cartridge, again allowing the solvent to pass into the sorbent bed. The cartridge is then washed by passing five void volumes of chloroform (void volume = 1.0–1.2 μL solvent per mg sorbent). The chloroform wash is discarded. Ciguatoxins are eluted from the cartridge by passing ten void volumes of 10% methanol in chloroform. This eluate is collected, solvent is removed by nitrogen gas stream or rotary evaporation, and the residue is dissolved in 300 μL chloroform.

The silica gel product is further purified by SPE on a 500 mg aminopropyl (NH_2) column. The 300-μL chloroform solution is loaded on a NH_2 column previously conditioned with hexane. The analytes are eluted with 2-propanol/chloroform (1:2). The eluant is evaporated under nitrogen gas stream and residues dissolved in methanol (150 g tissue weight equivalents (TE) per mL) for analysis.

22.1.4.2 *In Vitro* Cell Assay

Mouse neuroblastoma cells (N2a CCL-131, American Type Culture Collection, Rockville, MD) are cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 50 μg/mL streptomycin, and 50 units/mL penicillin as described by Manger et al. (1995). Stock cultures of N2a cells are maintained in 75 cm^2 flasks at 37°C in 5% CO_2 enriched atmosphere. Cultures are prepared for assay by lifting and suspending cells in trypsin-phosphate buffered saline (PBS) and diluting to a concentration of 5×10^5 cells/mL in RPMI supplemented with 5% FBS. Polystyrene 96-well plate cultures are initiated with the diluted cell suspension (200 μL per well) and incubated for 24 h before assays. After the 24 h incubation period the 96-well plate cultures are examined microscopically to ensure confluent monolayers of cells before use. One 96-well plate is used for the serial dilution and assay of each test sample. A standard CTX-1 dose-response (i.e., calibration) plate is prepared for each set of test samples assayed. Perimeter wells (×36) of the 96-well plates are not used because of response variation caused by CO_2-related edge effects. One-half of the interior wells (×30) receive 10 μL each of 5 mM ouabain and 0.5 mM veratridine (+O/V wells). Six of the 30 +O/V wells receive an additional 10 μL PBS and are used as +O/V controls with no further treatment. The remaining 30 interior wells receive 20 μL PBS (–O/V wells). Six of the –O/V wells receive an additional 30 μL PBS and are used as –O/V controls. See 96-well plate template in manual (appendix) for organization of dosing wells.

Fish test sample working solutions are adjusted to 2 g TE/mL in RPMI medium and then serially diluted (8×) in RPMI medium in separate vials. 10 μL from each dilution is added in triplicate to the 24 +O/V and 24 –O/V wells. Similarly, CTX-1 standard (0.5 ng/mL) is serially diluted (8×) and 10 μL from each dilution added in triplicate to 24 +O/V and 24 –O/V wells of the toxin standard plate. The 96-well plate cultures are then incubated 22–24 h. The treatment medium is removed at 22–24 h by hand-flick, replaced with (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) (MTT) solution (0.83 mg/mL RPMI medium; 60 μL/well), and incubated for 30 min. The MTT solution is removed from the plates after 30 min by hand-flick and replaced with DMSO (100 μL/well). The plates are agitated gently side-to-side to disperse the formazan reduction product of MTT before measuring absorbance at 570 nm (reference wavelength of 630 nm) using a microplate reader.

Assay responses to CTX-1 standards and test sample treatments are determined by measuring the intensity of formazan color development in viable +O/V cells following exposure to standards or test sample. Absorbance in treated wells is expressed as a percentage of formazan color development measured in the untreated +O/V control wells (no sample or standard treatment). The linearity of assay response is tested by using eight dilutions of CTX-1 standard. Assay responses are linear, reproducible, and repeatable between ca. 25% and 75% of control. The responses of −O/V cells to standards and test samples are evaluated to ascertain the sodium channel specificity of cell response. Standard CTX-1, CTX-1 spiked matrix extracts, and ciguatoxic test samples have no effect on the viability of cells in the absence of ouabain and veratridine. This is consistent with the sodium channel specificity of the assay. Quantitative estimation of CTX-1 equivalent response in toxic test samples is made by substitution of CTX-1 standard dose causing 50% reduction in cell viability (standard ID_{50}) for the mean test sample dose causing 50% reduction in cell viability (test sample ID_{50}). CTX-1 equivalent values in test samples are then normalized to give CTX-1 equivalent activity in ng/g TE (ppm).

22.1.4.3 LC/MS/MS Analysis of Caribbean and Pacific Ciguatoxin-1 (C-CTX-1 and P-CTX-1)

Gradient reverse phase HPLC with mass spectrometric detection is used to confirm Caribbean or Pacific CTX-1 in the fish tissue extracts. Identification is based on mass and retention time equivalent to C-CTX-1 or P-CTX-1 reference materials. The concentration of reference standard used is 10 ng/mL. The LC/MS/MS system consists of an LC system (Agilent Technologies Model 1100, Palo Alto, CA) coupled to a 4000 Q Trap mass spectrometer (Applied Biosystems, Foster City, CA). LC separations are performed on a Luna C8 (2) column (2.0 × 150 mm, 5 µm, Phenomenex, Torrance, CA) at a column temperature 40°C. Mobile phase is water (A) and acetonitrile (B) in a binary system, with 0.1% formic acid as additive. The elution gradient is 35% B for 2 min, linear gradient to 80% B at 30 min, 95% B at 35 min, hold at 95% for 10 min, return gradient to 35% B at 50 min, and hold for 10 min before the next injection.

A highly specific MS/MS detection, multiple reaction monitoring (MRM), is used to detect C-CTX-1 by mass spectrometry. Three precursor/product transition pairs of $(M+H-H_2O)^+$ *m/z* 1123.6 (*m/z* 1105.6, 1087.6, and 1069.6) are monitored and the response summed. The gas parameter settings are nebulizer gas (50 psi), turbo gas (50 psi at 400°C), curtain gas (20 psi), and collision gas (medium). The ion spray (IS) voltage is 5 kV and the declustering potential is 120 V. The collision energy for each precursor/product transition pairs are 35 eV for 1123.6/1105.6, 37 eV for 1123.6/1087.6, and 1123.6/1069.6. The dwell time for each monitored transition is 200 ms.

Detection of P-CTX-1 by mass spectrometry requires different parameters from C-CTX-1. For P-CTX-1 three precursor/product transition pairs of $(M+H)^+$ *m/z* 1111.6 (*m/z* 1093.6, 1075.6, and 1057.6) and $(M+NH_4)$+ 1128.6 (*m/z* 1093.6, 1075.6, and 1057.6) are monitored and the response summed. The gas parameter settings are nebulizer gas (50 psi), turbo gas (50 psi at 400°C), curtain gas (20 psi), and collision gas (medium). The IS voltage is 5 kV and the declustering potential is 100 V. The collision energy used for each precursor/product transition pairs are 15 eV for 1111.6/1093.6, 20 eV for 1111.6/1075.6, 25 eV for 1111.6/1057.6, 30 eV for 1128.6/1093.6, 35 eV for 1128.6/1075.6, and 1128.6/1057.6. The dwell time for each monitored transition is 150 ms.

22.1.5 Analysis of Ciguatera Case/Outbreak Samples

The study of ciguatera fish poisoning outbreaks and individual case histories is essential to the clinical recognition and epidemiology of ciguatera. An integral component of case study is the analysis of implicated fish samples to support clinical diagnostic capabilities. From 1990 through 2005, the FDA Gulf Coast Seafood Laboratory has examined ca. 300 case samples from approximately 100 suspect ciguatera outbreaks in the United States. Initially, mouse bioassay was used for assessment of suspect fish tissues. In 1994, *in vitro* cell assay (Manger et al., 1995) was adopted as

a replacement for mouse bioassay. The cell assay has proven to be a rugged, reproducible, and high-throughput method for ciguatera case study. Results over the years have been consistent with published contamination rates. In 1999, LC/MS was incorporated as a confirmatory method for samples testing positive by cell assay. Approximately 44% of the FDA investigated outbreaks were positive for ciguatera toxins in either meal remnants or implicated fish tissues. Outbreaks that were not confirmed ciguatera were most often scombrotoxic (decomposition, histamine-biogenic amine poisoning), lacked authentic case-related fish tissues for analysis, or predated the specificity and sensitivity of methods used today. Ciguatoxin concentrations in fish tissues (some cooked) have ranged from 0.1 to 52.9 ppb Caribbean or Pacific CTX-1 equivalents. These semiquantitative contamination rates derived from *in vitro* cell assay were confirmed in most, but not all, fish tissues by using LC/MS. This two-part protocol, cell assay followed by LC/MS, is highly informative and versatile, and is currently being assessed for use on serum and urine samples obtained from clinical cases.

A representative outbreak of Caribbean ciguatoxicosis (FDA outbreak sample 061201) may serve to illustrate the two-part protocol. Three residents of south Florida, USA, consumed portions of a 45 lb black grouper (*Mycteroperca bonaci*) on March 17 and 18, 2006. The grouper was caught near Key Largo, FL, by a fisherman friend and given to these residents as a gift. Four other groups of friends were reportedly gifted by the fisherman with portions of the same fish. Among this group, eight seafood consumers reported onset of ciguatera symptoms between 4 and 30 h following the meals. Symptoms reported by all interviewed included diarrhea, abdominal pain, circumoral paresthesias, paresthesias of the extremities, reversal of temperature sensation, dizziness, itching, and rash. A 335.3 g sample of the black grouper was made available for analysis.

Outbreak samples are initially subjected to *in vitro* sodium channel specific activity screening to ascertain toxicity consistent with ciguatoxin(s) mode of action. Outbreak sample 061201 was positive for sodium channel specific toxicity, and the estimated contamination level, 0.58 ppb C-CTX-1 equivalent activity, was determined by extrapolation of the standard C-CTX-1 ID_{50} (Figure 22.3). Outbreak/case samples that assay positive for sodium channel specific toxicity are subjected to LC-MS to determine ciguatoxin(s) content. MRM was used for outbreak sample 061201. By selectively detecting the product ions from the ciguatoxin precursor ion, the signal-to-noise ratio is increased and limits of detection are lowered. This mode of detection is useful for the rapid screening of complex samples where analytes are known and reference standards are available. LC-MS/MS (MRM) analysis of the grouper tissue extract confirmed the presence of C-CTX-1 in outbreak

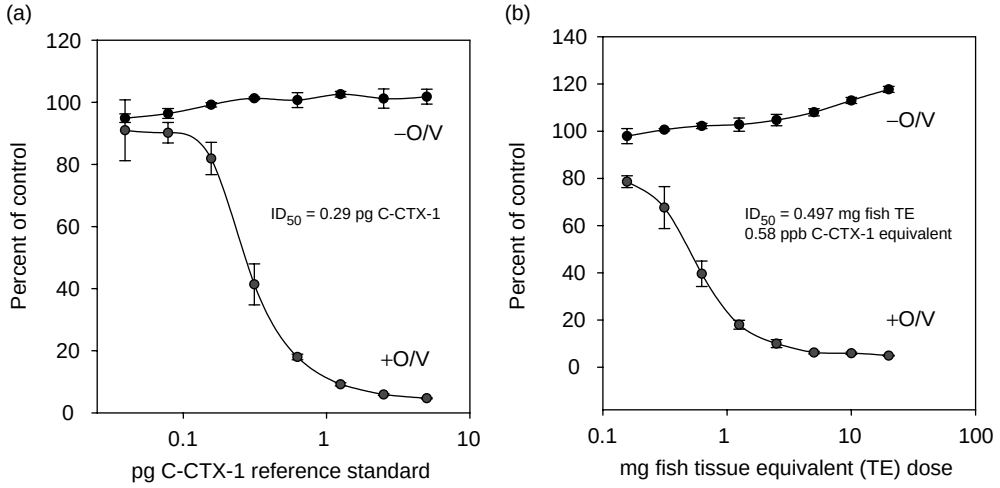


FIGURE 22.3 *In vitro* cell assay of (a) Caribbean C-CTX-1 reference standard, and (b) Caribbean ciguatoxicosis case sample 061201 (*Mycteroperca bonaci*).

sample 061201 (Figure 22.4). HPLC fractionation of the extract followed by *in vitro* cell assay of 0.5 min fractions provided further evidence of activity consistent with the retention time of C-CTX-1 reference standard, and two additional peaks of activity (Figure 22.5). The later peak is consistent with the retention time of C-CTX-2, the C-56 epimer of C-CTX-1. The lead peak of activity was not identified in this case.

A second example of Caribbean ciguatoxicosis (FDA outbreak sample 042401) can be used to illustrate apparent variability of Caribbean ciguatera and ciguatoxin congeners in fish. Three residents of Miami, Florida purchased a "Coronado" (Kingfish, *S. cavalla*) from a South Florida marina and consumed it on October 3, 2004 at 1300 h. At 1500–1800 h; all three experienced nausea, vomiting, diarrhea, abdominal pain, increased perspiration, and hypersalivation. Between 1800 h and 2200 h all developed dry mouth, light-headedness, dizziness, stiff neck, upper and lower extremity numbness or tingling, lower extremity pain "in bones," headache, and palpitations. At 2400 h, all exhibited bradycardia. The consumers sought treatment at a hospital and were diagnosed with ciguatera fish poisoning. A 393.7 g portion of the implicated fish was provided for analysis.

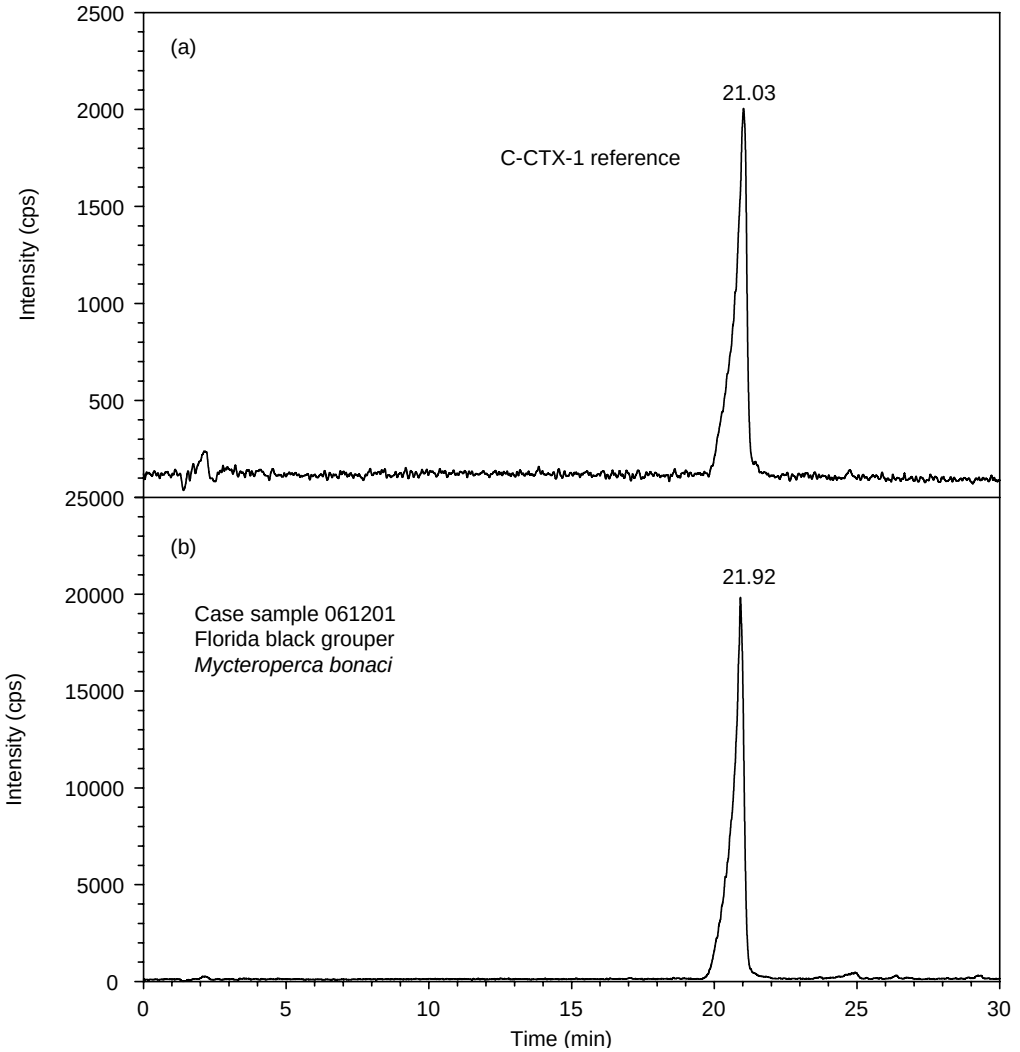


FIGURE 22.4 LC-MS/MS (MRM) analysis of (a) C-CTX-1 reference standard, and (b) FDA case sample 061201, Florida black grouper (*Mycteroperca bonaci*).

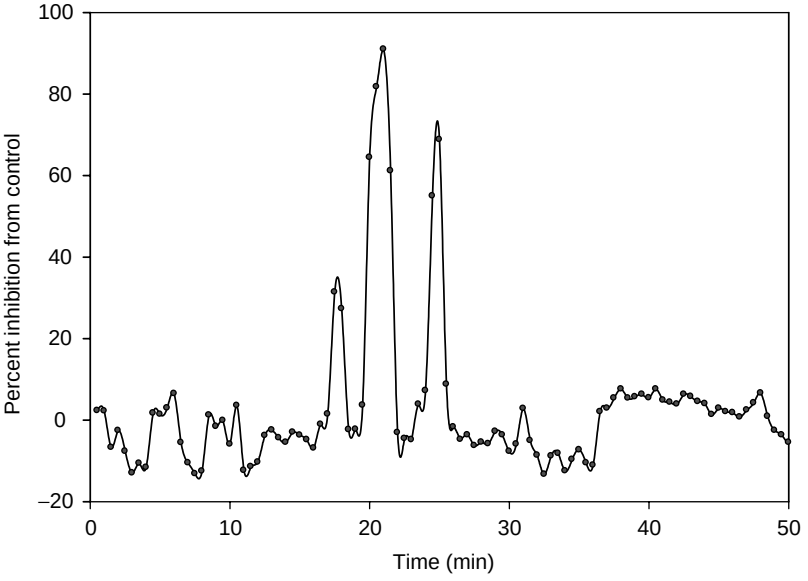


FIGURE 22.5 *In vitro* cell assay of HPLC fractionation products (0.5 min/fraction) from Caribbean ciguatoxicosis case sample 061201, Florida black grouper (*M. bonaci*).

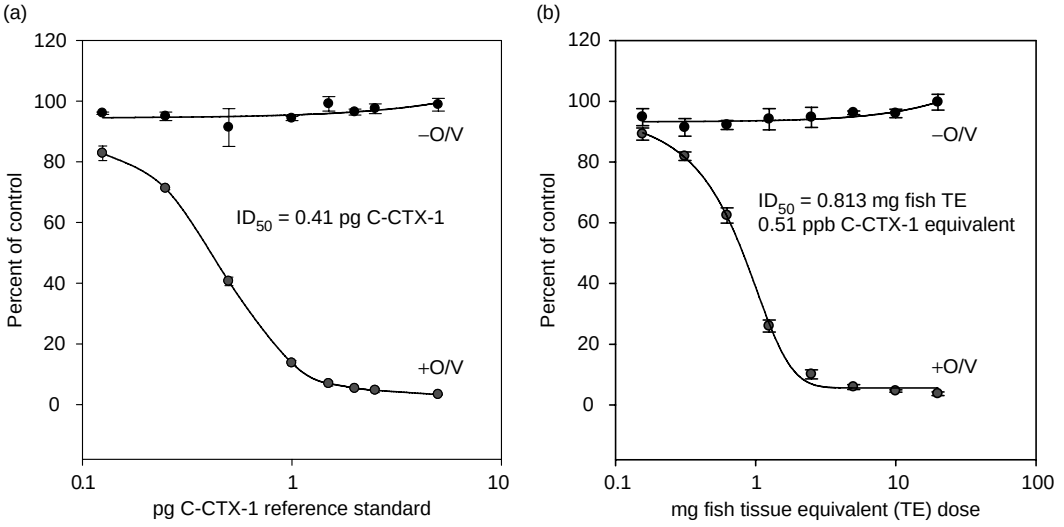


FIGURE 22.6 *In vitro* cell assay of (a) Caribbean C-CTX-1 reference standard, and (b) Caribbean ciguatoxicosis case sample 042401 (*S. cavalla).*

Outbreak sample 042401 was positive for sodium channel specific toxicity (Figure 22.6a) and the estimated contamination level, 0.51 ppb C-CTX-1 equivalent activity, was determined by extrapolation of the standard C-CTX-1 ID_{50} (Figure 22.6). The C-CTX-1 reference standard contains approximately 5% C-CTX-2 which is the C-56 epimer of C-CTX-1, and C-CTX-1 and C-CTX-2 were confirmed in sample 042401 in approximately equal proportion (Figure 22.7).

Analysis of a Pacific ciguatoxicosis case sample (FDA outbreak sample 061001) may be useful for comparison to Caribbean cases. Military personnel in Hawaii purchased frozen "Coral Cod"

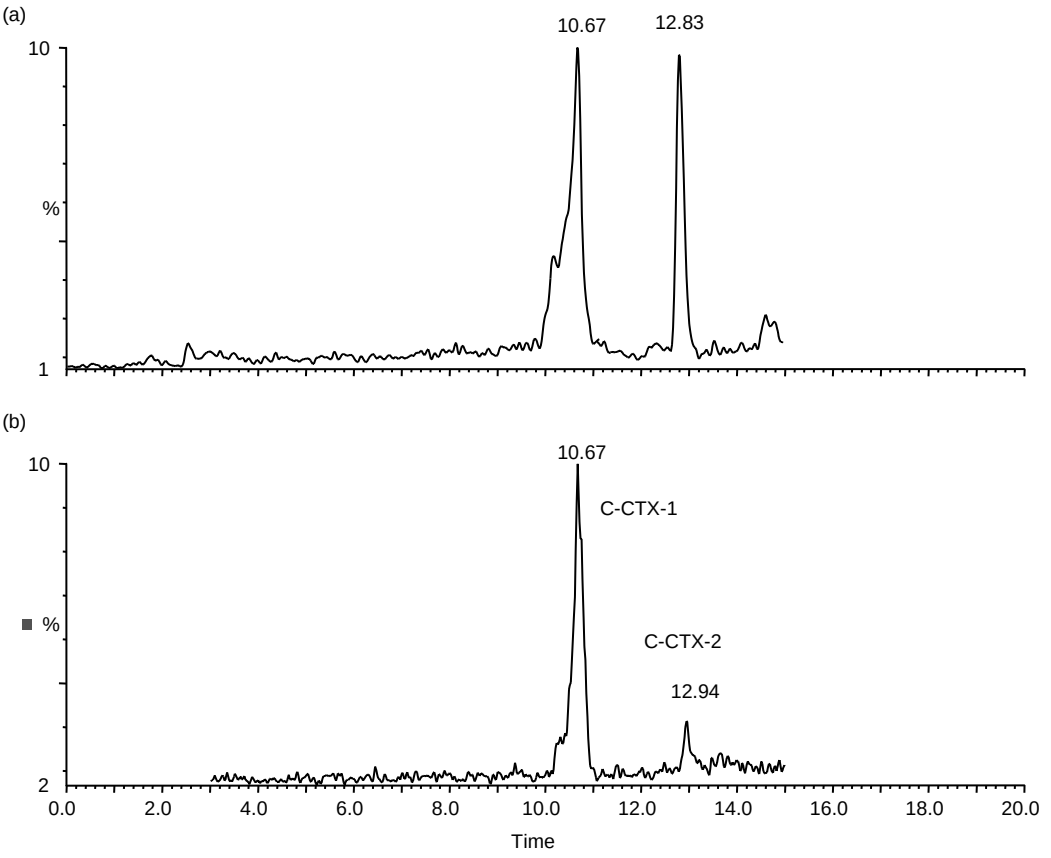


FIGURE 22.7 LC-MS/MS (MRM) analysis of (a) Caribbean ciguatoxicosis case sample 042401 (*S. comberomorus cavalla*), and (b) Caribbean C-CTX-1 reference standard 0.2 ppm.

(*Cephalopholis miniata*) on March 5, 2006 from a local grocery store in Oahu. The fish was consumed the following day at 1800 h. Onset of ciguatera symptoms were reported at 1600 h on March 7. Symptoms reported to medical personnel included diarrhea, circumoral paresthesia, paresthesia of the extremities, taste disturbance, muscular weakness and myalgia, itching, burning sensation in throat, and temperature sensation reversal. The implicated fish was traced by FDA field investigators and it was determined to have originated in Fiji, entered the United States in California through commercial channels, and was distributed to Hawaii for public sale. The fish was sold with head-on, tail-off, entrails removed, and un-scaled in a plastic bag. Only the head of the Coral Cod meal remnant was recovered for analysis, and a second whole specimen from the same shipment was also recovered. Outbreak sample 061001 (head of the cooked meal remnant) was screened by *in vitro* assay and 1.6 ppb P-CTX-1 equivalent activity was determined by extrapolation of the standard P-CTX-1 ID_{50} (Figure 22.8a and b). The whole specimen from the same shipment was also positive by *in vitro* assay and 0.3 ppb P-CTX-1 equivalent activity was detected in the fillet tissue. LC/MS-MS analysis of the head tissue extract from the meal remnant confirmed the presence of P-CTX-1 and several presumptive isomers of P-CTX-1 (Figure 22.9). Sodium channel specific activity of the presumptive P-CTX-1 isomers was verified by fractionation of the extract by HPLC and *in vitro* assay of 0.5 min fractions (Figure 22.10). The activity of fractions corresponded well with retention times of the presumptive P-CTX-1 isomers.

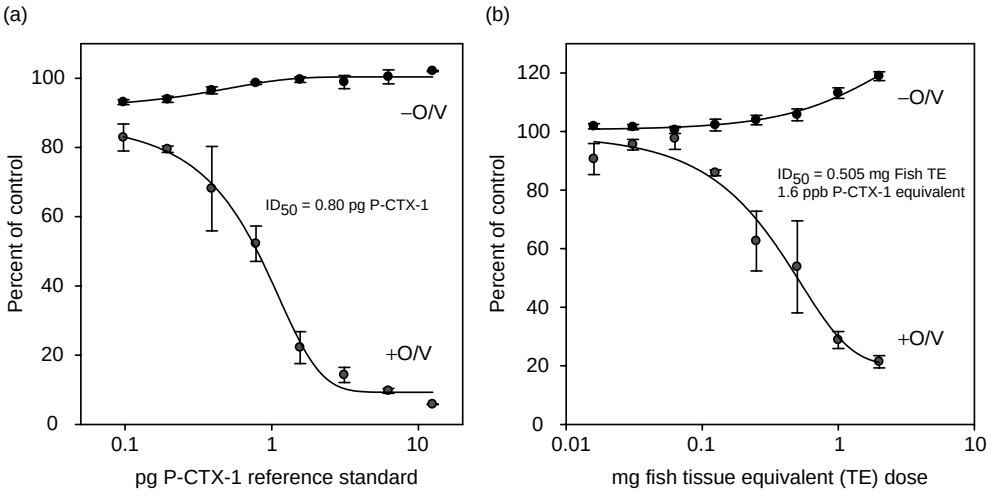


FIGURE 22.8 *In vitro* cell assay of (a) Pacific ciguatoxin P-CTX-1 reference standard, and (b) Pacific ciguatoxicosis case sample 061001 (*C. miniata*).

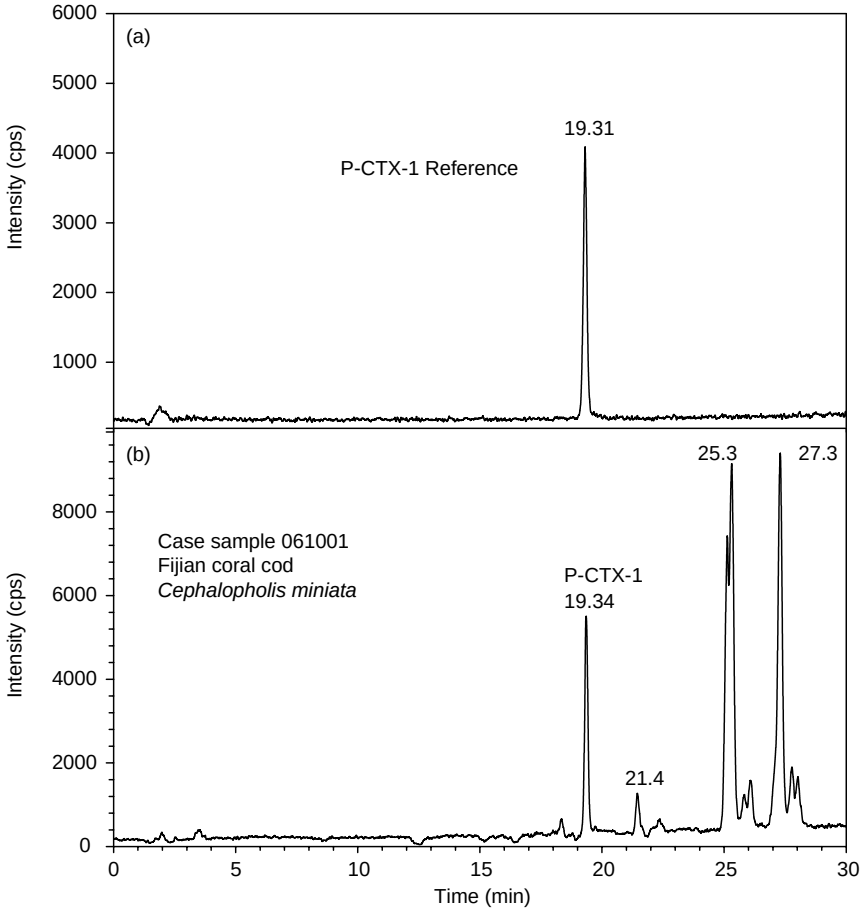


FIGURE 22.9 LC-MS/MS (MRM) analysis of (a) P-CTX-1 reference standard, and (b) FDA case sample 061001, Fijian coral cod (*C. miniata*).

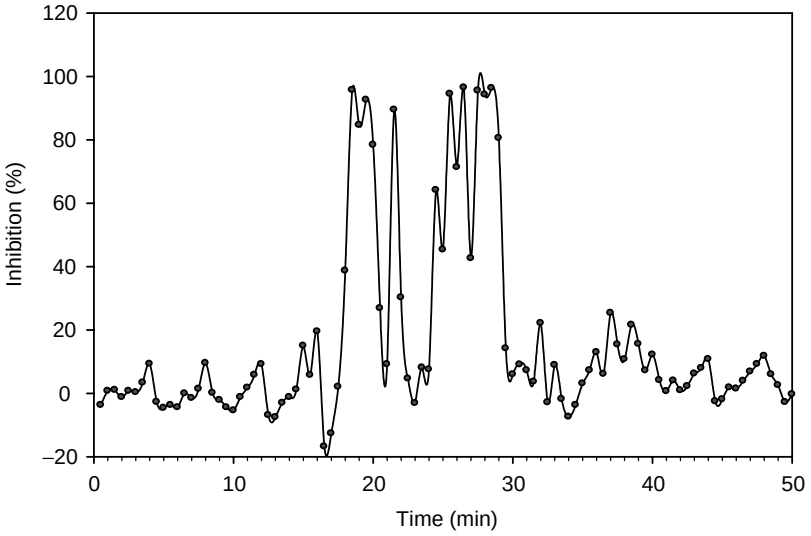


FIGURE 22.10 *In vitro* cell assay of HPLC fractionation products (0.5 min/fraction) from Pacific ciguatoxicosis case sample 061001, Fijian coral cod (*C. miniata*).

22.2 SUMMARY

The preceding discussion and presentation of data may serve to illustrate the long-standing public health challenge that ciguatera represents. This challenge to human welfare is not unlike many other diseases, some of much greater significance, in terms of resistance to progress. Indeed, and in contrast to highly significant human health challenges (e.g., cancers, malaria, HIV, etc.), perhaps as "globalization" incorporates expanded distribution of safe foods, exploitation of highly diverse and attractive coral reef ecosystems for protein, and consequently the incidence of ciguatera, will become less frequent.

Nevertheless, information derived from the study of ciguatera case histories and the analysis of implicated fish tissues will continue to stimulate improvement in the clinical confirmation and timely treatment of ciguatera. Such study is equally important for the distinction of ciguatera toxin profiles from one region to the next, the determination of toxin thresholds in humans, and the development of policy and guidance for adoption by public health and regulatory organizations. The study of ciguatera has historically been resistant to research progress, largely because of the difficulty in working with trace levels of highly potent natural toxins imbedded in complex fish tissue matrices. Consumers, physicians, public health departments, and marine resource organizations have lacked the necessary tools to address ciguatera effectively. However, technological advances in the chemical, biological, and material sciences, and perseverance by the research community, are reducing the obstacles at an increasing rate. It is hoped that soon consumers, and public health and resource management can adopt a proactive posture to mitigate the hazard of ciguatera.

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Part VIIIB

Maitotoxin

23 Maitotoxin: A Unique Pharmacological Tool for Elucidating Ca^{2+}-Dependent Mechanisms

C. L. Trevino, L. Escobar, L. Vaca, V. Morales-Tlalpan, A. Y. Ocampo, and A. Darszon

CONTENTS

23.1 INTRODUCTION

In the beginnings of the fourteenth century, some sailors traveling across the Atlantic Ocean became sick after eating fish, even if they were fresh. Upon other neurological disorders, some of these sailors felt an inversion of thermal sense, a characteristic symptom named "dry ice sensation." Upon touching the cold seawater, the sailor would feel as if he was receiving an electric shock. This mode of poisoning was later called "ciguatera" after *cigua*, a snail commonly occurring in the Caribbean Sea [1,2]. However, most of the neurological symptoms of ciguatera are due to ciguatoxin (CTX), a very potent brevetoxin-type polyether compound [3,4].

In 1965, Bagnis reported that the human symptoms caused by ingestion of herbivorous fish were distinct from those due to carnivorous fish and mainly involved gastrointestinal illnesses and not neurological disorders. A new toxin was discovered upon examination of the toxic constituents in the surgeonfish *Ctenochaetus striatus.* This toxin was named maitotoxin (MTX) since the Tahitian name of this surgeonfish is "Maito" [5]. MTX contributes, although mildly, to the toxic effects developed in ciguatera caused by herbivorous fishes.

Ciguatera occurs worldwide in tropical regions causing more than 20,000 victims per year. During the late 1960s, it was proposed that the agents causing this disease accumulated in fish as a

result of the food chain. Disc-shaped dinoflagellates were found to build up in the guts of poisonous *C. striatus* fish. These dinoflagellates grew densely in the calcareous algae found around the Gambier Islands where these fishes originated [6].

MTX is a very interesting compound since it displays an extremely potent biological activity. Mice are killed by intraperitoneal injection of 50 ng/kg: the lethal dose depends on mice strain, sample source, and preparation procedure since MTX binds to glass and plastic. This toxin shows diverse pharmacological actions, which seem to be derived by its ability to activate Ca^{2+} uptake into a variety of cell types [6]. Furthermore, this is the largest and most toxic nonbiopolymer toxin possessing a molecular weight of 3422 Da ($C_{164}H_{256}O_{68}S_2Na_2$), which exceeds palytoxin (PTX) by 748 Da. MTX is a polyketide-derived polycyclic ether constituted by four extended fused-ring systems termed polyether ladders [7]. This toxin is the most potent inducer of the acrosome reaction in mammalian sperm [8].

23.2 TOXICOLOGY

A number of human illnesses are caused by ingesting seafood contaminated with toxins produced by marine phytoplankton [9–11]. The phytoplankton is the base of the marine food web, and the toxins it produces can accumulate and concentrate in higher organisms that can become lethal if ingested. Although mainly neurotoxins, these toxins can cause a wide range of acute and chronic health effects in humans and other species. They are tasteless, odorless, and heat and acid stable. Therefore, conventional food methods are unable to detect and destroy them in contaminated seafood.

Ciguatera ranges from mild to severe intoxication with symptoms lasting from a few days and up to 2 years. A large number of fish species can be ciguateric (at least 400) and contain distinct combination and quantities of toxins giving rise to a huge diversity in symptoms and duration of the illness. Classical symptoms include gastrointestinal and neurological disorders, abdominal cramps, diarrhea, nausea, vomiting, temperature reversal, itching, and so forth. In addition, chemical modifications of the toxin as it passes the food chain may account for the differences observed. Therefore, it is difficult to establish a direct relationship between symptoms and a particular toxin. Usually, diagnosis is based only on the presence of the general symptoms associated to patients with a recent history of fish ingestion. Nowadays, there is a commercial kit called cigua-check® that is being used by fishermen, restaurants, and to facilitate clinical diagnosis; unfortunately this kit only detects ciguatoxins. In principle, diagnosis can be confirmed by detecting CTXs and MTX in contaminated fish samples by high-performance liquid chromatography and mass spectrometry.

To date, there is no antidote to treat ciguatera; only palliative treatments are usually applied such as intravenous injection of mannitol. Patients are also advised to avoid alcohol, nuts, and nut oil for at least 6 months after the intoxication to avoid reappearance of symptoms.

In contrast to ciguatoxins, which are lipid soluble, maitotoxin is water-soluble and does not apparently accumulate in the flesh of fishes but rather in organs such as the liver [12]. Therefore, MTX appears to contribute little to ciguatera. In addition, it has a very low oral potency as compared to its high lethality when injected intraperitoneally. Mice injected with MTX display reduced body temperature, piloerection, dyspnea, progressive paralysis, slight tremors or convulsions, and long death times. High doses of MTX produce ciguatoxin-like symptoms such as gasping with convulsions and shorter death-times.

Interestingly, three different MTX molecules have been isolated from different strains of *Gambierdiscus toxicus* [13,14]. MTX 1 and 2 produce similar symptoms when injected in mice except that MTX-2 exhibits shorter death times. MTX-3 additionally induces intense gasping that ameliorate near death; however, HPLC-purified MTX-3 does not produce the gasping phase, suggesting additional bioactive components in the crude preparation. The death time produced by MTX-3 is very similar to MTX-1. Solvolisis (desulfonation) of MTX molecules reduces the toxicity of all three forms about 200-fold. It is interesting that MTX-3 is about one third of the size of MTX-1 and 2.

23.3 SOURCE

The marine dinoflagellate *G. toxicus,* a single-celled plantlike organism that grows on algae in tropical waters worldwide, produces CTX and MTX. These toxins accumulate through the food chain as carnivorous fish consume contaminated herbivorous reef fish. MTX toxin accumulates in liver and viscera of fishes but not in the flesh [15]. Toxin concentrations are highest in large, predatory fish such as barracuda, grouper, amberjack, snapper, and shark. Because fish caught in ciguatera-endemic areas are shipped to several countries worldwide, ciguatera fish poisoning can occur almost anywhere.

The temperatures of the northern Caribbean and extreme southeastern Gulf of Mexico have been predicted to increase 2.5–3.5°C during the next years [16]. Higher temperatures favor *G. toxicus* growth [17] and are likely to alter fish migration patterns. Ciguatera outbreaks have been correlated with sea-surface temperature increases in the south Pacific Ocean [18] and Tahiti [17].

After Yasumoto discovered in 1977 that the dinoflagellate *G. toxicus* was the organism responsible for producing MTX, he cultivated this organism for 10 years to isolate enough of this toxin and determine its structure [19,20].

Although MTX was commercially available for some time, it is worrisome that currently it is not available.

23.4 CHEMISTRY AND STRUCTURE

Maitotoxin (MTX), whose structure was first reported in 1993 by Murata et al. [21], has a molecular weight of 3422 Da. It is the largest nonbiopolymeric and most lethal nonpeptide natural product: 0.05–0.17 μg/kg i.p. injection in mice [19,22]. MTX is soluble in water, methanol, and dimethylsulfoxide and relatively stable in alkaline but not in acidic conditions [20]. Quantitation of MTX in biological experiments can be difficult, since it adheres to the glass or plastic surfaces [22]. However, addition of albumin to the assay helps to avoid this problem [23].

Though the basic molecular skeleton as well as the complete stereochemistry (relative disposition of atoms at chiral centers) of MTX have been worked out (Figure 23.1; [24]), its full three dimensional structure has not yet been solved. MTX contains 32 saturated rings, which, with the exception of two, are transfused and expected to be conformationally rigid on the basis of nuclear magnetic resonance (NMR) studies [25]. The polycyclic structure of MTX is labeled by the rings A, B, C, and beyond Z to A′, B′ and so on up to F′. The presence of numerous oxygen atoms is remarkable: there are 32 ether linkages and 28 hydroxyl groups, which are expected to be involved in the interactions of the molecule with cations. MTX is an amphiphilic compound: a hydrophilic poly hydroxyl portion from ring C to ring Q and a hydrophibic fused-polycyclic portion from ring R to ring F′. The presence of sulfate esters in the molecule is also a distinctive feature of MTX [20].

MTX has a complex structure containing four rigid polyether ladders (A, B, C, D, in the nomenclature of) [7] connected by mobile hydrocarbon chains; also, it has flexible groups at both ends. The main difficulty to obtain a three dimensional structure of MTX is precisely related to such mobile groups that give rise to several free to rotate bonds (rotors) in the structure. In addition to these rotors, it is necessary to add those associated to –OH and $–CH_3$ functional groups located along the rigid ladders. A systematic conformational analysis of MTX was performed recently [26] with a molecular dynamics method using the extensible systematic force field (ESFF) [27], as implemented in the Discover program (Discover version 4.0.0 msi, San Diego, CA, USA). The cardinal conclusion of the analysis of the minima obtained by the simulated annealing procedure is that MTX is a compact folded structure stabilized by hydrogen bonds involving the –OH groups. Figure 23.2 shows the three most stable structures. Their energies vary by less than 3 kcal/mol and they clearly have different folding patterns. However, they share an interesting feature: all of them have a cavity 5 Å wide. The cavity for the lowest energy minimum is clearly displayed in Figure 23.3 using as accessibility criterion the van der Waals radii. It is important to point out that, during the simulation, several stable structures with energies well above the minimum of Figure 23.2 (~40 kcal/mol) conserve the cavity, suggesting

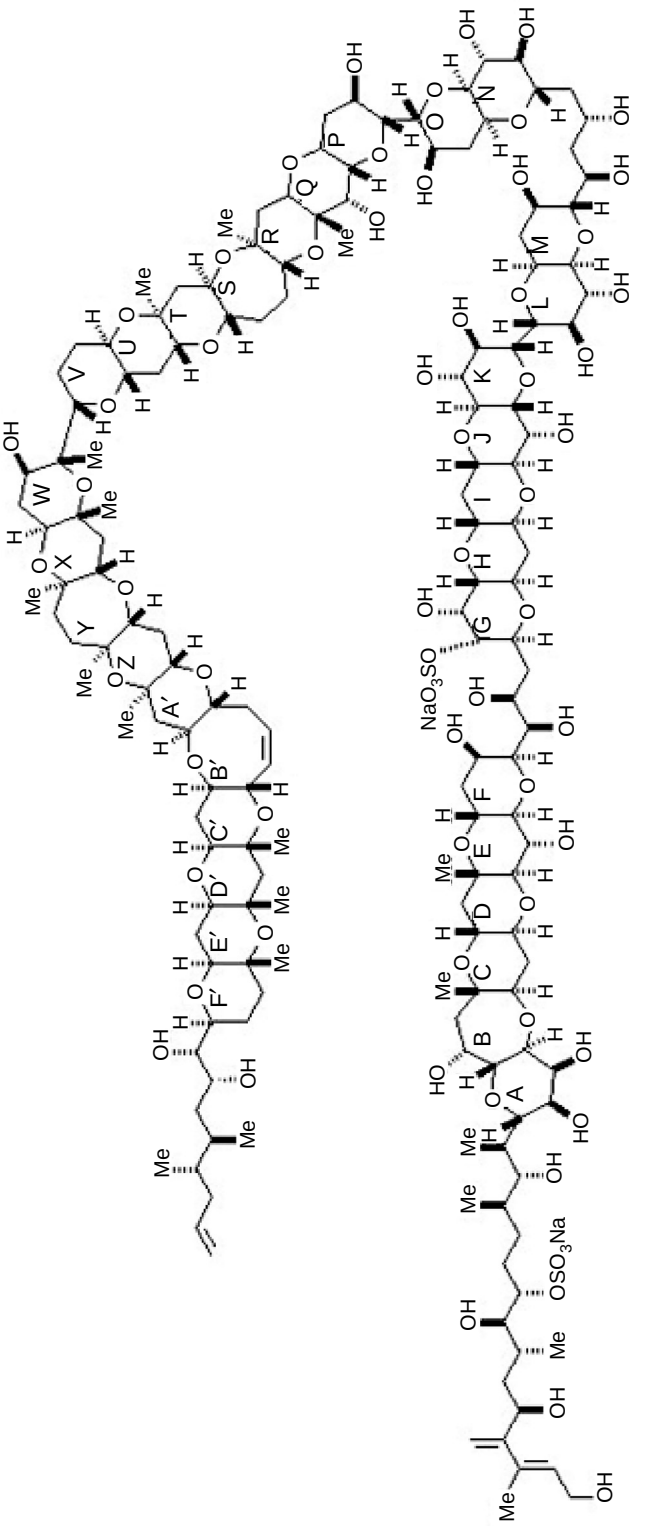

FIGURE 23.1 Stereochemistry of maitotoxin.

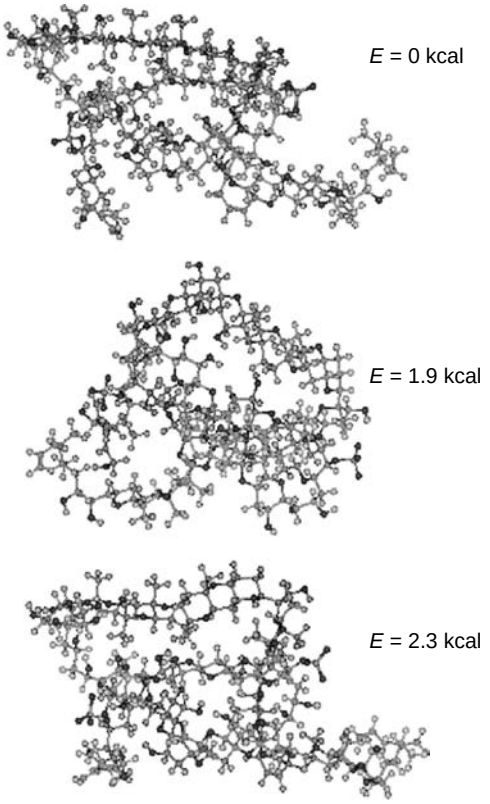

FIGURE 23.2 Structures of the three minima with lower energy, as obtained by simulated annealing procedure with the ESFF force field, [26]. The energies in the right correspond to the values relative to the lowest energy minimum indicated by $E = 0$ kcal/mol. The color indicates atom type: gray corresponds to carbon and sulfur atoms, black to oxygen, and white to hydrogen.

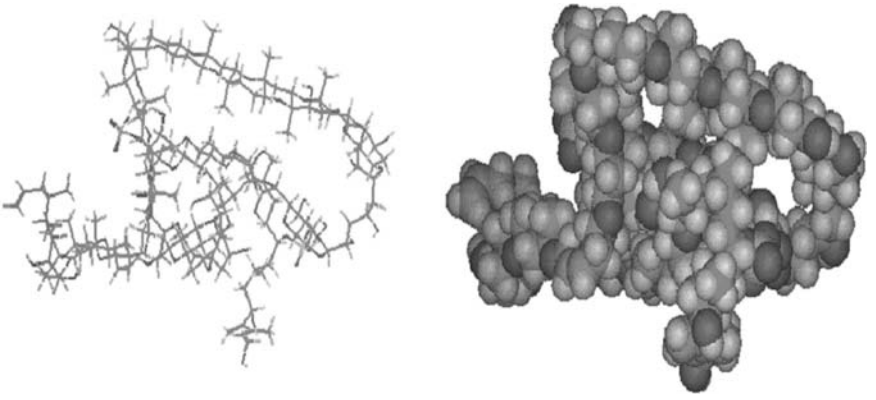

FIGURE 23.3 Minimum energy conformation of MTX. The left panel is in wire frame representation and the right one in overlapping spheres representation, each sphere having the van der Waals radius of the corresponding atom. The orientation is different from that in Figure 23.2 to enhance the view of the cavity formed in the folded state. The cavity has a size of the order of 5 Å. The atom type is given by gray scale: dark gray corresponds to oxygen atom, middle gray to carbon, and pale gray to hydrogen [26].

that this is a characteristic of the folded MTX even in high energy structures. Further theoretical and experimental work is required to determine whether or not this cavity could play a role in the biological function of MTX. There is an additional structural feature of MTX that is worth mentioning: in its ladder shaped structures, the ether oxygen atoms are separated by a distance that matches with the helix length per residue measured along the axis of alpha-helix structures. This pattern can be seen in the top ladder of Figure 23.3 (see the horizontal line of dark spots in the structure). The exposed ether-like oxygen atoms could be acceptors in the hydrogen bond interaction of MTX with an alpha-helix; this could be related with the recent findings about the interaction of ladder-shaped polyether compounds with alpha-helix peptides [28]. This remarkable conclusion suggests that the recognition process between MTX and its receptor comprises helical structures.

In relation to the intrinsic chemical reactivity of MTX, one may analyze it by looking at the features of the rigid polyether ladders. Fused tetrahydropyrane units mainly form these blocks that contain methyl and –OH groups as substituents. Recently, the electronic structure of such kind of blocks has been obtained [29]. The intrinsic reactivity of a chemical species is related to the behavior of the frontier molecular orbitals: the highest occupied molecular orbitals (HOMOs) and the lowest unoccupied molecular orbitals (LUMOs). The LUMO are crucial when the system accepts electrons as in reduction processes and nucleophilic attacks. In contrast, the HOMO are involved in electron detachment (oxidation) and electrophilic attacks. For systems such as MTX, the HOMO band is localized at the oxygen atoms and corresponds to the lone pairs framework. In contrast, the LUMO band is associated to the double bonds and is of antibonding nature [29]. According to these findings, one may expect that nucleophilic attacks on MTX rigid blocks will cleave the double bonds; meanwhile the electrophilic ones occur at the oxygen atoms.

23.5 PHARMACOLOGY

Maitotoxin activates Ca^{2+} entry in virtually every cell tested. The identity of the channels involved in MTX action remains unknown. Experimental evidence suggests that MTX has no ionophoretic activity *per se* [8,30,31]. Originally, it was thought that MTX was a specific activator of voltage-gated Ca^{2+} channels, since the rise in $[Ca^{2+}]i$ depended on the presence of extracellular Ca^{2+}, and it could be attenuated by organic and inorganic Ca^{2+} channel antagonists [30,32,33]. Later on, diverse experimental evidence suggested that MTX activates a nonselective voltage independent cation channel (reviewed in [31]). Several subsequent reports indicated that MTX activates a voltage-independent Ca^{2+} channel, probably a store-operated channel from the transient receptor potential (TRP) family [8, 34–36].

It has been reported that Maitotoxin-induced Ca^{2+} influx is inhibited by the imidazole SKF96365 [8,37,38]. Daly et al. [39] tested 25 different Ca^{2+} blockers, which included the following categories: imidazoles, phenylalkylamines, dihydropyridines, benzothiazepines, diphenylbutylpiperidines, diphenylpropylpiperidines, piperazines, and others. The most potent inhibitors of the MTX-induced Ca^{2+} influx, were clotrimazole (IC_{50} 0.56 μM), loperamide (IC_{50} 1.6 μM), RMI12330A (IC_{50} 1.8 μM), and SKF96365 (IC_{50} 1.8 μM). But also, fluspirilene (IC_{50} 3.9 μM), penfluridol (IC_{50} 3.2 μM), and proadifen (IC_{50} 3.9 μM) were quite effective. Some of the inhibitors displayed a biphasic behavior suggesting the participation of more than one channel in the MTX response. Interestingly, low concentrations of Ni^{2+} (15–200 μM) have been reported to stimulate or potentiate the Ca^{2+} entry induced by MTX [8,40]. However, higher Ni^{2+} concentrations (500 μM–5 mM) strongly inhibit the response [8,41].

Establishing a specific pharmacological profile for the MTX-induced Ca^{2+} entry is difficult, since this toxin seems to activate more than one cation channel depending on the cell type. As already indicated, MTX can apparently activate voltage dependent and voltage independent Ca^{2+} channels depending on the cell type. For example, Freedman et al. [32] showed that in neuroblastoma-glioma hybrid cells the MTX-induced Ca^{2+} increase was at least partially blocked by dihydropyridines selective for L-type Ca^{2+} channels at low concentrations (10^{-9}–10^{-6} M). In contrast, the

MTX response was not abolished in smooth muscle BC13 cells treated with 1 μM verapamil, 1 μM nifedipine, or 1 mM La^{3+} [42]. Similar results were found in human lymphocytes treated with 2 μM nifedipine [40].

Estacion and Schilling [43] investigated the participation of PLC in the MTX-induced cell death in endothelial cells using the Phospo Lipase (PLC) blocker U732122 and its structural inactive analogue U73343. Unexpectedly, they discovered that the two compounds were potent blockers of the MTX-induced Ca^{2+} increase. The IC_{50}'s for U73122 and U73343 were 1.9 μM and 0.66 μM, respectively. The mechanism underlying this inhibition is still unknown although it seems that these antagonists can directly block the pore of the channel.

23.6 MECHANISMS OF ACTION

23.6.1 Does Maitotoxin Have a Receptor?

Finding the receptor for a given toxin is of utter importance, not only because the toxin can be used as a pharmacological tool to explore the receptor function or properties, but also because finding the receptor provides essential clues about the mechanism of action of such toxin. One of the greatest conundrums in MTX research is, precisely, the identity of its putative receptor. After 15 years of research, we know little about such putative receptor.

What is the experimental evidence suggesting that MTX has a receptor? (1) The MTX effects saturate [44], (2) Treating cells with trypsin abolishes the MTX effects [44], (3) In all cell types explored (from insect to human cells), the common denominator of MTX action is an increase in $[Ca^{2+}]i$ [30], (4) MTX has no effect on liposomes [45] or planar lipid bilayers [8]. These findings altogether suggest that the MTX effects are mediated by a receptor, most likely a protein.

Definitive evidence supporting the presence of a receptor would include classic binding studies, which due to scarcity of MTX are difficult to conduct. Nevertheless, if MTX acts like most known nonpeptide marine toxins, one would expect a receptor. For instance, toxins with structural similarities to MTX, like brevetoxins and palytoxin (PTX) have recognized receptors [46–49]. The case of PTX is of particular interest, since it shares some of its cellular effects with MTX, such as membrane depolarization, $[Ca^{2+}]i$ increases and oncotic cell death [50].

Like MTX, PTX exerts its effects in almost every single cell line explored. This is due to the fact that the PTX receptor is the plasmalemmal Na^+,K^+-ATPase (NKA) pump, a highly conserved protein in eukaryotes [51]. Yeast lack the NKA pump and are insensitive to PTX but become sensitive to it when made to express this ATPase [49, 51]. As expected, the cardiac glycoside ouabain, a classic inhibitor of this pump, abolishes the PTX response. If there is a receptor for MTX, it should also be highly conserved, but it is not the NKA pump, since the MTX response is insensitive to ouabain [52]. The molecular identity of the MTX receptor remains to be discovered.

23.6.2 MTX Modulates Cell Membrane Ionic Permeability

Maitotoxin causes two highly conserved changes in a wide variety of cells. It induces a rapid and sustained elevation of $[Ca^{2+}]i$ and Na^+ ($[Na^+]i$), and as a result, a strong depolarization. The influx of Ca^{2+} and Na^+ induced by MTX appears to involve the activation of Ca^{2+}-permeable, nonselective cation channels (CaNSC). Simultaneous electrophysiological and fluorescent measurements of $[Ca^{2+}]i$ and $[Na^+]i$ upon MTX-CaNSC activation reveal similar kinetics indicating that this channel is responsible for the flux of both ions.

Notably, CaNSC activation by MTX requires a minimum concentration of intracellular Ca^{2+} and Na^+. Though we and others have carefully explored the monovalent and divalent cations needed for the MTX response, a clear explanation for these requirements is lacking. The $[Na^+]i$ increase induced by MTX occurs only when millimolar free extracellular Ca^{2+} is present. On the other hand, reducing extracellular Na^+ dramatically increases the influx of Ca^{2+}. Complete removal of extracellular Na^+ results in faster and stronger Ca^{2+} influx after MTX application [53].

Increasing $[Ca^{2+}]i$ by depleting intracellular Ca^{2+} stores with thapsigargin (a selective blocker of the microsomal Ca^{2+} ATPase), potentiates MTX-induced Ca^{2+} influx, shifting the cell sensitivity to MTX toward lower concentrations. This effect is saturable. Surprisingly, the extracellular Ca^{2+} requirement for MTX to induce Na^+ influx remains even when $[Ca^{2+}]i$ is increased with high concentrations of thapsigargin [53].

The massive influx of Na^+ and Ca^{2+} result in strong cell membrane depolarizations. In many cases, cell depolarization produces the activation of voltage-gated Ca^{2+} and Na^+ channels (when MTX is tested in excitable cells), providing extra pathways for the further influx of these cations. Eventually, the cell deteriorates and dyes when its $[Ca^{2+}]i$ buffering mechanisms are overwhelmed by the dramatic Ca^{2+} uptake. In all reported studies, prolonged exposure to MTX has deleterious effects on cell viability.

The activation of Ca^{2+} influx by MTX appears to involve an intracellular second messenger cascade. As we illustrate here for the first time (Figure 23.4), tyrosine kinase inhibitors such as genistein and tyrphostin diminish MTX-induced Ca^{2+} influx and activation of the cationic channels in different cell lines (CHO, HELA, HEK293, and human skin fibroblasts). Furthermore, over-expressing a dominant negative mutant of the Sarcoma virus Kinase (src) tyrosine kinase, which has a mutated kinase domain, prevents MTX-induced Ca^{2+} influx (unpublished observations). These results indicate that src is involved in the activation of the cationic channel responsible for Ca^{2+} influx after MTX application. Finally, genistein and tyrphostin prevent MTX induced cell dead (unpublished observations), suggesting that src may be involved in many of the cellular effects of MTX.

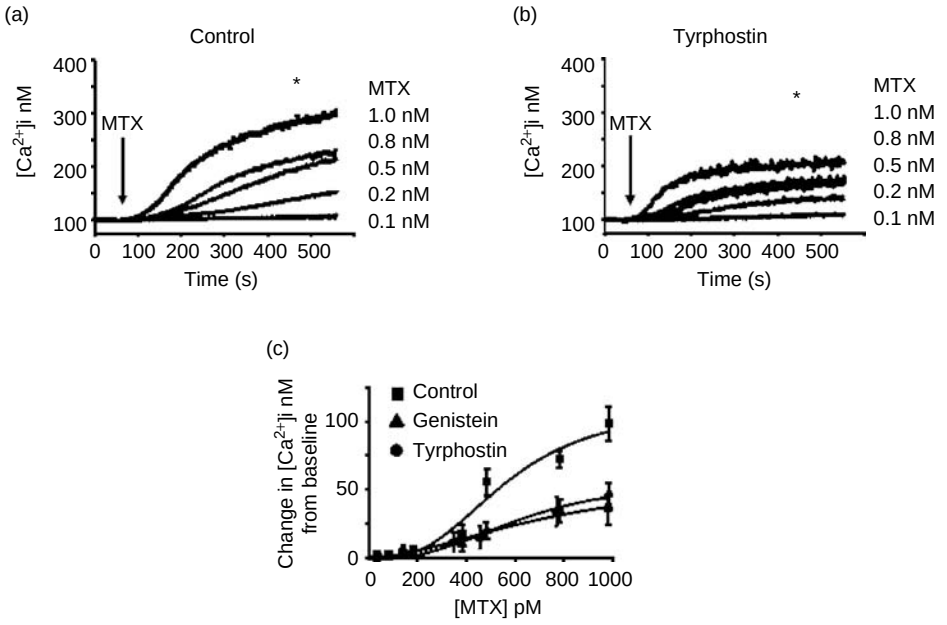

FIGURE 23.4 Effect of tyrosine kinase inhibitors on MTX-induced Ca^{2+} influx. (a) Dose–response curve to MTX in cultured human skin fibroblasts. Cell populations (3 million/mL) were loaded with the fluorescent Ca^{2+} indicator FURA-2 and Ca^{2+} increments in response to different toxin concentrations (shown on the right) were measured as previously reported [44]. (b) Similar experiments in the presence of the tyrosine kinase inhibitor tyrphostin. Asterisks indicate the time at which the effect of the inhibitors was measured. (c) MTX dose–response curves obtained under control conditions (filled square) and in the presence of 100 mM genistein (triangle) and tyrphostin 5 mM (circle). Data illustrates the mean ± standard deviation of at least five independent observations. Lines show a fit to a Hill equation. Both inhibitors reduced the cell sensitivity to MTX by fourfold. Dadzein (100 mM), a congener of the genistein, which is not an inhibitor of tyrosine kinases, had no effect on MTX-induced Ca^{2+} influx (data not shown).

23.6.3 Maitotoxin Activates Cationic Channels with Different Properties

There is general consensus that MTX activates CaNSCs. Though some studies report that MTX activates voltage-gated Ca^{2+} channels, it seems more now a secondary effect of cell membrane depolarization induced by this toxin.

The single channel conductances of the MTX-induced CaNSC (MTX-CaNSC) reported range from 12 to 40 ps, depending on the experimental conditions (for review see [31]). Although Na^+ is the main charge carrier, there is substantial Ca^{2+} permeability in the MTX-CaNSC. We recently calculated the *P*Ca/*P*Na and *P*Ca/*P*K relative permeabilities for the MTX-CaNSC in human skin fibroblasts and found them to be 0.87, indicating that the channel is also substantially permeable to K^+.

The extracellular Ca^{2+} requirement for MTX-CaNSC in human skin fibroblasts appears to be only for its activation and cannot be substituted by other divalent cations. External Ca^{2+} removal after activation does not affect the amplitude of the MTX-CaNSC ionic current [54]. Thus, extracellular Ca^{2+} may be needed only for the binding of MTX to its putative receptor or to MTX-CaNSC, if this channel is the receptor itself [54]. In contrast, other studies in β-cells [55], GH4C1 pituitary cells [56] and guinea-pig myocytes [57], have not found that external Ca^{2+} is required to activate MTX-CaNSC. The reasons for this discrepancy remain to be determined and may simply indicate that MTX can activate different channels. Indeed, it remains controversial if MTX activates the same, highly conserved CaNSC in different cells.

To complicate things further, a recent report shows that the $[Ca^{2+}]$i increases induced by MTX result in the insertion of different channels into the plasma membrane of *Xenopus leavis* oocytes [58]. We have observed that MTX induced Ca^{2+} uptake also stimulates a Ca^{2+}-dependent Cl^- current in these cells in a reversible manner [23]. Thus, MTX can activate several Ca^{2+}-dependent processes involving ion transport in many cells, which complicates the identification of the initial MTX target. The kinetic resolution of the MTX activated conductances should be very helpful to determine the receptor that initiates the signaling cascade.

Regardless of the identity of the conductances activated by MTX, it is clear that this toxin is a powerful tool to explore Ca^{2+}-dependent mechanisms. For example, MTX is the most potent inducer of the mouse sperm acrosome reaction. This exocytotic process involves the fusion of the acrosome, a vesicular structure in the head of many sperm species, to the head plasma membrane, releasing components necessary for sperm to penetrate the outer egg envelope. This reaction transforms the sperm surface to allow sperm to recognize and fuse with the egg [59].

We have recently taken advantage of MTX to study the channels involved in the acrosome reaction. The pharmacology of the MTX-activated channels characterized in mouse sperm is consistent with the involvement of TRP channels. Namely, the MTX-induced Ca^{2+}-increase was inhibited by Ni^{2+} and SKF96365 with the same potency with which these compounds inhibit the mouse sperm acrosome reaction induced by the zona pellucida, the outer envelope of mammalian eggs containing the physiological trigger of this reaction. There is substantial evidence indicating that TRP channels are responsible for the sustained $[Ca^{2+}]$i increase necessary for the acrosome reaction [59]. In addition, MTX induces this exocytotic process and a Ca^{2+} increase in capacitated and noncapacitated human sperm (see Figure 23.5). Activation of TRP channels in particular TRPC1 by MTX has been also postulated by other groups [35,36].

However, other studies show that the current–voltage relationships and the electrophysiological properties of the whole-cell current induced by MTX in human skin fibroblasts differ from those described for TRPC channels [54]. Selectivity, experiments point out some differences also, the most remarkable is a substantial permeability to the large cation *N*-methyl-D-glucamine (NMDG), which is not observed with TRPC channels expressed in heterologous systems.

One cannot discard the possibility that the activation of a yet unidentified cation channel by MTX may induce activation of TRPC channels via elevation of $[Ca^{2+}]$i or some other mechanism. Also, as mentioned earlier, it is still not clear that MTX acts in the same manner in different cell types, in some cells a member of the TRP family could be the target.

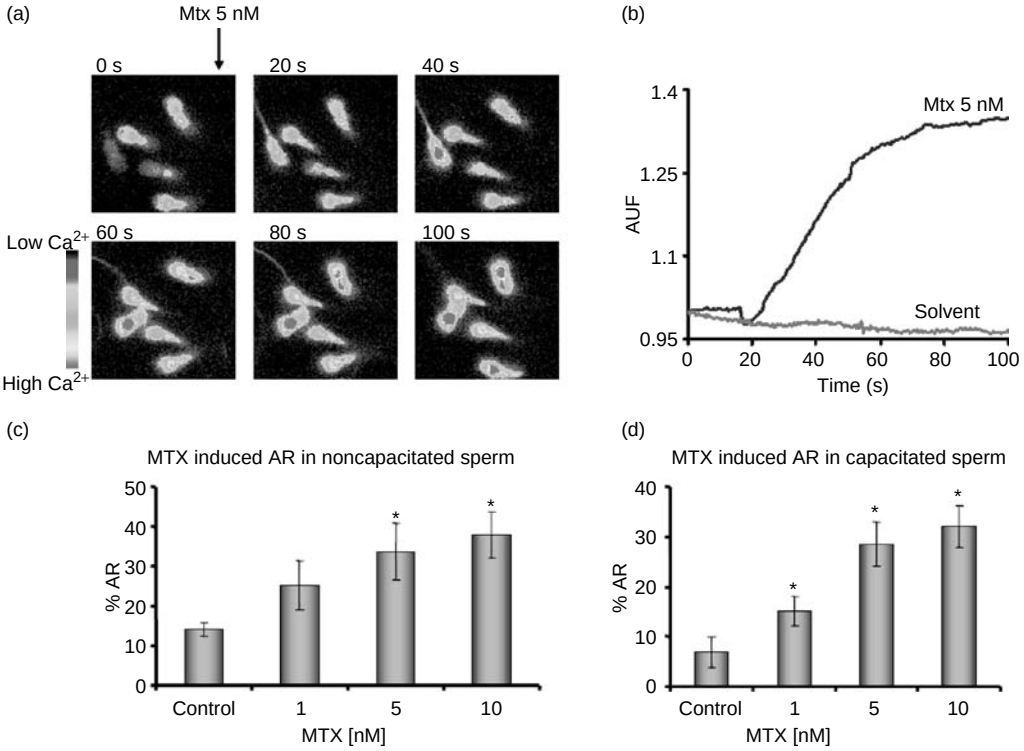

FIGURE 23.5 MTX induces an intracellular Ca^{2+} increase and the AR in human sperm. Capacitated sperm were loaded with Fluo4-AM and $[Ca^{2+}]i$ changes induced by the addition of 5 nM of MTX were monitored. (a) Representative images of the Ca^{2+} increase in individual sperm cells, time (s) after MTX addition is indicated in each image. The qualitative color scale indicates $[Ca^{2+}]i$. (b) Traces of the Ca^{2+} increase measured in a group of cells (UAF = arbitrary units of fluorescence), solvent addition is shown as a control. Capacitated (d) and non-capacitated (c) sperm were incubated in the presence of increasing concentrations of MTX and the percentage of acrosome reacted sperm was measured (c) and (d) Induction of the acrosome reaction (AR) was observed in both populations with similar potency. Data represent mean ±SEM of at least three independent experiments. $*p < .1$.

23.6.4 Maitotoxin Is a Potent Activator of Phosphoinositide Breakdown

Ca^{2+}-Dependent accumulation of inositol phosphates is another well preserved phenomenon induced by MTX stimulation in a wide variety of cells [60]. The mechanism of inositol production may involve activation of a Ca^{2+}-dependent phospholipase C [60]. Increments in inositol phosphates follow the external Ca^{2+} dependent elevation of $[Ca^{2+}]i$ [60]. Increases in inositol phosphates, in particular IP3, further augment $[Ca^{2+}]i$ releasing Ca^{2+} from internal storage compartments via IP3 receptors.

23.6.5 Maitotoxin Induces Oncotic Cell Death

The recent elegant studies of Schilling and co-workers have unmasked a rather unusual effect of MTX on mammalian cells [43,61] Schilling, [62]. These researchers have shown that following the activation of CaNSC, MTX triggers the formation of cytolytic/oncotic pores, now known as COPs [61]. These COPs allow ethidium and propidium-based vital dyes to enter the cell, preceding the release of lactate dehydrogenase, an indication of oncotic cell death [63]. The final MTX effect is cell membrane blebbing and cell death [63]. Interestingly, COP formation is modulated by $[Ca^{2+}]i$ [63]. If intracellular Ca^{2+} is chelated, COP formation is significantly delayed, although not prevented.

As indicated earlier, CaNSC activation requires intracellular Ca^{2+} and internal Ca^{2+} removal prevents activation of this channel [54]. In this particular case, both studies can be compared, since they were conducted with human skin fibroblasts [54, 61]. Therefore, COPs are not formed from CaNSC channels. Another distinct difference between CaNSC activation and COP formation is their temperature sensitivity. Reducing temperature has little effect on CaNSC activation but greatly attenuates COP formation [61].

It is worth noting that MTX-induced cell lysis is prevented by the amino acids, glycine, and L-alanine [64] by unknown mechanisms that may involve COP blockade by these amino acids while trying to pass through the pore. The cytoprotective effect of these amino acids was concentration dependent, rapid, long lasting, selective, reversible, and stereospecific [64].

Even though, recent work indicates that the MTX-induced cell death cascade is indistinguishable from that produced by activation of purinergic receptors of the P2Z/P2X7 subtype, it is still controversial if COPs are formed by these receptors [65].

These findings point out that MTX is a unique tool amongst marine toxins to explore oncotic/necrotic cell death.

23.6.6 A General Model of Maitotoxin Action

A cellular model for action of MTX could be summarized. During the first seconds of exposure, MTX activates CaNSC responsible for the massive influx of Ca^{2+} and Na^{+}. The increment in $[Ca^{2+}]i$ potentiates further the influx of Ca^{2+} from the extracellular space. In some cells, MTX induces phosphoinositide breakdown independently of $[Ca^{2+}]i$ increases by an unknown mechanism. After prolonged exposure, MTX triggers the formation or activation of large endogenous pores that allow passage of low-molecular-weight molecules (≤800 Da) through the plasma membrane. These large pores (COPs) appear to be a prelude of oncotic cell death [62]. It is not clear yet, if the large pores are the result of the multimerization of CaNSC, or if they represent independent molecular entities. Although some data reviewed here argues in favor of COPs as separated entities, other less explored events include MTX effects on mitochondria [66] and possible effects of MTX on apoptosis.

23.7 CONCLUSION

Even though MTX was identified over 15 years ago and a significant body of research has provided a rich and complex picture of its mode of action, many mysteries remain unsolved about this powerful toxin, perhaps only as complex as its structure. Probably the most fundamental problem is identifying its putative receptor(s). This would lead to a better understanding of the molecular mechanisms involved in MTX action and explain apparent discrepancies in its functional modalities.

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Part VIIIC

Brevetoxins

24 The Molecular and Integrative Basis to Brevetoxin Toxicity

John S. Ramsdell

CONTENTS

24.1 INTRODUCTION

Episodes of massive marine animal kills and noxious ocean vapors have been associated with discolored water in the Gulf of Mexico since the earliest records. In the late 1940s, two seminal reports commenced research on brevetoxins; the first identified the dinoflagellate *Gymnodinium breve* as the micro-organism responsible for discolored water and fish kills[1], and the second demonstrated that when vapors of water containing the organism were introduced to volunteers, it caused the characteristic respiratory irritation.[2] Nearly two decades later, a toxic activity was isolated from *G. breve* and *G. breve*-contaminated shellfish and found to cause a neurological poisoning similar

to ciguatera.[3] It was not until 1979, with the introduction of high pressure liquid chromatography, that the primary congeners from each of the two classes of brevetoxin were isolated[4], and within the next 5 years, their long cyclic polyether structures were elucidated by x-ray crystallography and nuclear magnetic resonance (NMR).[5,6] Concurrently, the molecular action of brevetoxin was described as binding to the voltage-gated sodium channel and interfering with the sensitive movements that transition the channel's closed, open, and inactivated states.[7] Structure–function activity analysis of synthetic derivatives identified the molecular moieties of the toxin that affect specific channel transitions that yield up to four discrete effects on ion conductance.[8] The integration of multiple molecular interactions between brevetoxin and the sodium channel induces membrane depolarization and initiates action potentials in nerve and muscle, yet shows a preference for nerve sodium channel, where it first activates and then inhibits activity.[9] Respiratory exposures lead to depolarization of cholinergic nerve endings where the release of acetylcholine causes constriction of smooth muscle lining the passages of the airways.[10] Oral exposure to the toxin leads to distribution to liver[11] where it is metabolized to new, bioactive molecular forms as a result of oxidation, reduction, and conjugation before transport to the peripheral and central nervous tissues via the blood.[12] The most severe systemic action of brevetoxin is also respiratory, where it disrupts breathing by an action on autonomic afferents of the nodose ganglion that regulate midbrain breathing centers, followed by flaccid paralysis of the diaphragm, which may involve additional direct action on the midbrain and/or a conduction block of the phrenic nerve.[13] The molecular and integrative basis to mammalian brevetoxin toxicity is the focus of this chapter, which follows the conceptual design of an earlier analysis of the domoic acid literature to analyze the primary biochemical, physiological, and toxicological literature relevant to these marine algal toxins.[14]

24.2 DINOFLAGELLATE BREVETOXINS

Brevetoxins are neurotoxic polyether toxins produced by *Karenia brevis* (formerly known as *G. breve* and *Ptychodiscus brevis*), a dinoflagellate originating in the Gulf of Mexico.[1] Bioactive principals were isolated from cultures and contaminated shellfish by McFarren and coworkers, and determined to cause neurological poisoning in mice, similar to ciguatera fish poisoning.[3] For the next 20 years, substantial effort was dedicated to purifying brevetoxins; however, no consistent results were reported. Difficulties encountered with the purification of brevetoxins from cultures were due in large part to the mixture of different toxic activities (e.g., ichthyotoxic, hemolytic) as well as toxin congeners of similar activity produced by *K. brevis*. High-pressure liquid chromatography was proved to be a requisite to resolving the brevetoxin congeners and in 1979, Risk and coworkers provided the first definitive purification of the two classes of brevetoxins: fraction T46 for brevetoxin A aldehyde and fraction T47 for brevetoxin B aldehyde.[4] The structure for T47 was solved using x-ray crystallography and proton NMR and shown to be a cyclic polyether containing 11 fused cyclic ether rings, each ring containing the following number of carbon and oxygen atoms (6, 6, 6, 7, 7, 6, 6, 8, 6, 6, 6).[5] In the following year, the structure was solved for the reduced K-ring aldehyde of brevetoxin B.[15] Brevetoxin C, a chloromethyl ketone derivative of brevetoxin was described the same year, but subsequently determined to be an artifact of chloroform extraction.[16] The structures for two oxidative products of brevetoxin B, including a K-ring acetate and H-ring epoxide, were next solved.[17] The structure of the brevetoxin A was solved a year later and determined to have a backbone of ten fused cyclic ether rings (5, 8, 6, 7, 9, 8, 8, 6, 6, 6), including a unique 9-membered ring in the E-position that linked the A–D and F–J rings with greater flexibility than found in the brevetoxin B backbone.[6,18] Yet, the brevetoxin A backbone still contained modifications of the same functional groups on the each end of the molecule and the same reductions of the K-ring substitutions as brevetoxin B. In that same year, Poli et al. proposed the designation of PbTx-1 to -9 for the brevetoxins that are currently in use for the full-ring algal toxins.[19] (Figure 24.1).

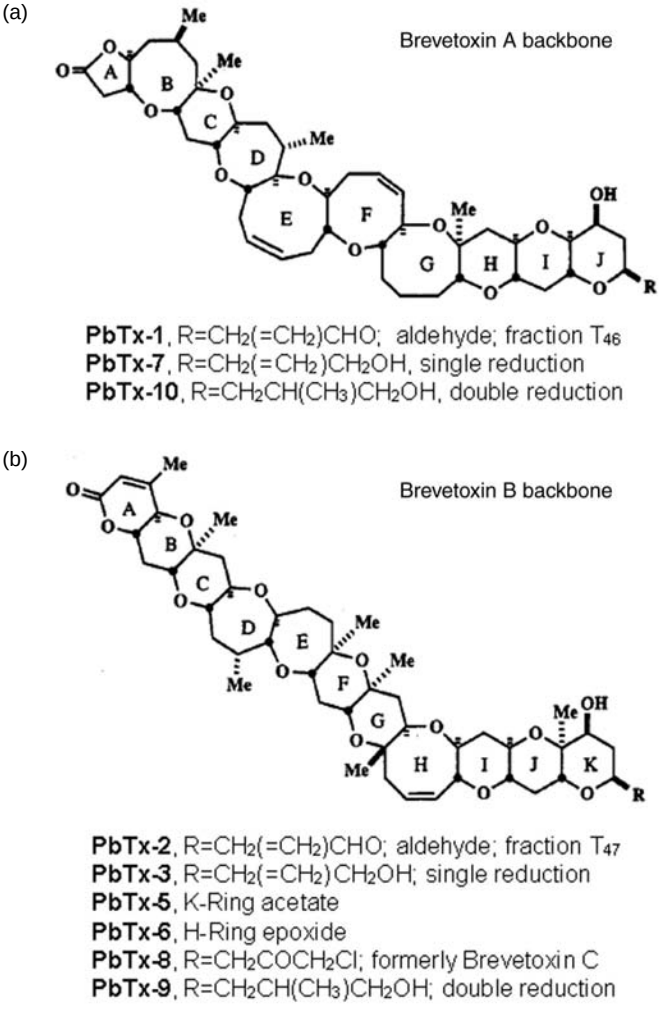

FIGURE 24.1 Structures of algal brevetoxins. Brevetoxins are comprised of either ten fused cyclic ether rings comprising the A-type backbone (a) or 11 fused cyclic ether rings comprising the B-type backbone (b). The A-ring of both molecules contains a terminal lactone. The other end of the molecules contain three six-member fused cyclic ether rings with conserved R-ring substitutions. Proximal to the three six-member rings is an eight-member ring that gives the molecule flexibility to form a boat–chair or crown conformation. The preferred boat–chair conformation yields a relative linear molecule with a length of ~30 Å.

24.3 RAPHIDOPHYTE BREVETOXINS

Three different genera of the family Raphidophyceae (*Chattonella marina, Fibrocapsa japonica, Heterosigma akashiwo*) isolated from Japanese waters and grown in culture have been reported to produce suites of neurotoxins that correspond by thin layer and high performance liquid chromatography (HPLC) to brevetoxin B congeners and, in *Fibrocapsa* and *Heterosigma*, brevetoxin A as well[20] (Table 24.1). In 2002, three brevetoxin B congeners, (PbTx-2, PbTx-3, and PbTx-9) were identified by radioimmunoassay-guided HPLC fractionation from a unialgal fish-killing bloom of *Chattonella cf. verruculosa* from Delaware coastal waters.[21] The abundant PbTx-2 was confirmed by mass spectrometry and proton NMR, providing the first structural confirmation that brevetoxins are produced by raphidophytes.

TABLE 24.1
Brevetoxins Isolated from Raphidophyte Species and Their Correspondence to *Karenia brevis* Brevetoxins

Karenia brevis Culture/Wild	*Chattonella antiqua* Culture	*Fibrocapsa japonica* Culture	*Heterosigma akashiwo* Culture	*Chattonella marina* Culture	*Chattonella verruculosa* Wild
PbTx-1	CaTx-I	FjTx-I			
PbTx-2	CaTx-II	FjTx-II	HaTx-I	CmTx-I	PbTx-2
PbTx-3	CaTx-III	FjTx-IIIb	HaTx-IIb	CmTx-III	PbTx-3
ox-PbTx-2	CaTx-IV		HaTx-III	CmTx-IV	
PbTx-9		FjTx-IIIa	HaTx-IIa	CmTx-II	PbTx-9

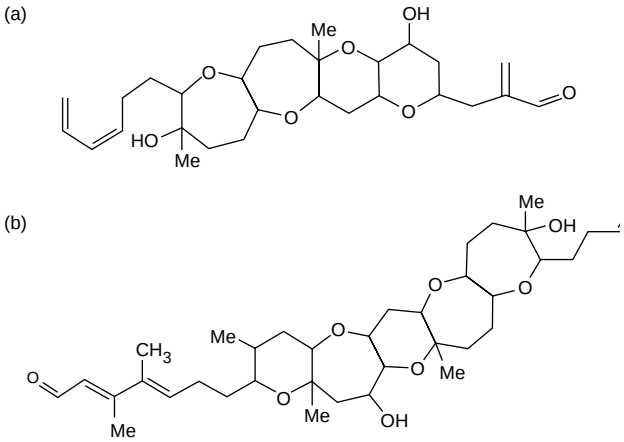

FIGURE 24.2 Structures of hemibrevetoxin and brevenal. The short chained brevetoxins are comprised of either four-fused cyclic ether rings comprising the hemibrevetoxin (a) or five-fused cyclic ether rings comprising the brevenals (b). (Modified from Bourdelais, A. J., Jacocks, H. M., Wright, J. L. et al., *J. Nat. Prod.*, 68, 2, 2005.)

24.4 HEMI-BREVETOXINS AND BREVENALS

K. brevis cultures also produce shorter ring structures, likely to be incomplete products of the brevetoxin biosynthetic pathway. These smaller cyclic ether ring structures exhibit reduced biological activity, yet are still consistent with a site of action common to the brevetoxins (Figure 24.2). Three 4-fused cyclic ether ring (7, 7, 6, 6) structures were described and named hemi-brevetoxins.[22] The hemi-brevetoxins are essentially the four final rings of brevetoxin B. The two other hemi-brevetoxin derivatives include a conjugated aldehyde and the aldehyde with a diene. Two 5-fused cyclic ether rings (6, 7, 6, 7, 7) have also been described and named brevenals.[23] The brevenals share a similar diene side chain from the terminal seven-membered ring as hemi-brevetoxins. The brevenal derivative is an interconvertible dimethyl acetate on the diene side chain that is thought to result from extraction.

24.5 SHELLFISH BREVETOXIN METABOLITES

The incidence of neurotoxic shellfish poisoning of suspected *K. brevis* origin, beginning in December 1992 in New Zealand, led to the identification of several brevetoxin metabolites in shellfish. The first of the shellfish toxins identified was a taurine conjugate of brevetoxin B designated BTX-B1[24]

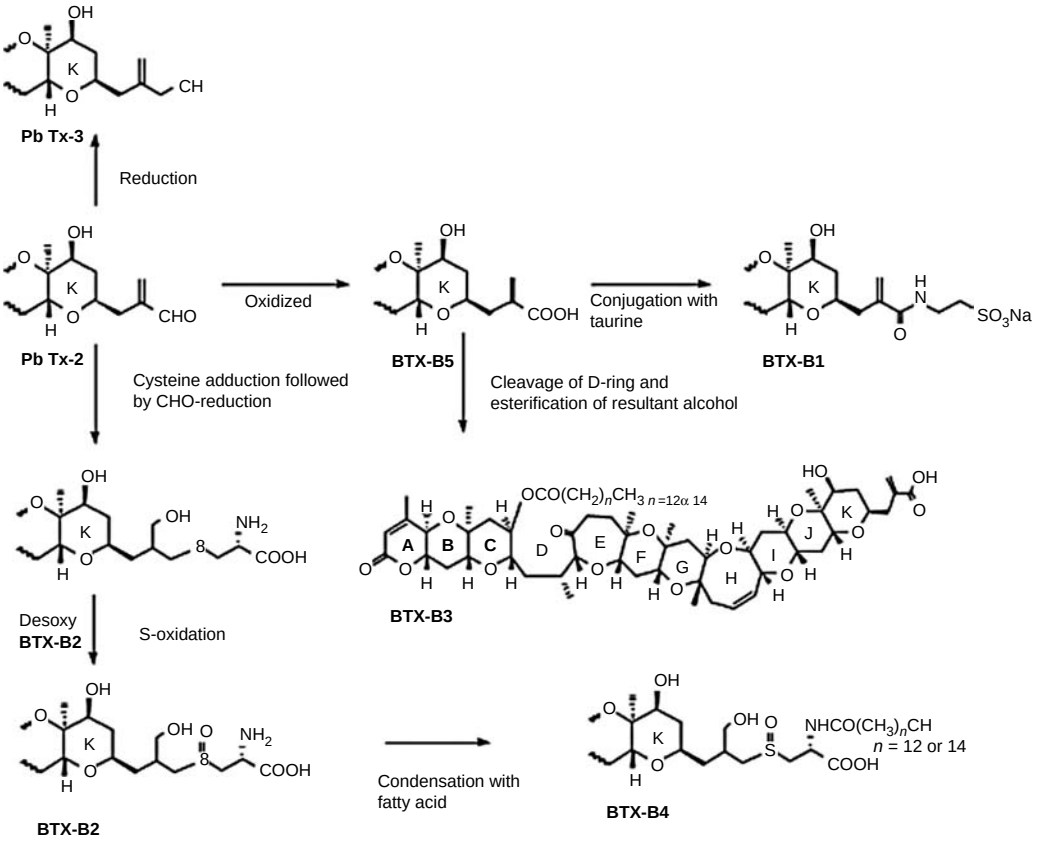

FIGURE 24.3 Structures of shellfish brevetoxin metabolites. Shellfish BTX-B brevetoxins metabolites are comprised of the B-type backbone in which the aldehyde is oxidized or conjugated to cysteine. The oxidized carboxylic acid (BTX-B5) is conjugated with taurine (BTX-B1) or the D-ring is cleaved and esterified (BTX-B3). A desoxy cysteine conjugate with reduced aldehyde is S-oxidized to form (BTX-B2). The terminal amine of BTX-2 can be conjugated with fatty acid to yield BTX-B4. (Modified from Ishida, H., Nozawa, A., Nukaya, H. et al., *Toxicon*, 43, 779, 2004.)

isolated from the New Zealand cockle (*Austrovenus stutchburyi*). Three new brevetoxins, BTX-2 to -4, were isolated from green mussels (*Perna canaliculus*). These toxins include BTX-B2, which has a cysteine adduction to the K-ring followed by S-oxidation and aldehyde reduction[25]; BTX-B3, which has cleavage of the D-ring and esterification of the resultant alcohol[26]; and BTX-B4, which has *N*-myristoyl and *N*-palmitoyl conjugates through the amide linkage of the cysteine conjugate of BTX-B2[27] (Figure 24.3).

The cysteine conjugate and its desoxy derivative were next found in Gulf of Mexico shellfish, as were their corresponding conjugates for the brevetoxin A backbone. The presence of these conjugates were indicated by molecular mass in the Eastern oyster (*Crassostrea virginica*)[28] and whelks (*Busycon contrarium*),[29] and next upheld by tandem mass fragmentation patterns from *C. virginica* exposed to PbTx-2.[30,31] Using field-collected oysters as well as laboratory exposed animals, the cysteine, and desoxy cysteine conjugates, together with their likely precursors glycine-cysteine, glutathione, and gamma-glutamylcysteine, were identified for both the brevetoxin B and brevetoxin A backbone.[31] These derivatives are consistent with the formation of cysteine conjugates from the hydrolysis of glutathione conjugate. This study also identified a larger series of fatty acid-cysteine conjugates of the same amide linkage as BTX-B4.

24.6 TOXIN POTENCY

The initial step in toxicological characterization is determination a toxin's potency, or the concentration of toxin needed to trigger a biological effect. For the purpose of this analysis, brevetoxins' potency will be viewed at two levels of the response spectrum. Brevetoxins' intrinsic potency is the concentration of toxin needed to generate or alter a biological effect in a target cell or organ and is determined using *in vitro* preparations. On the other hand, brevetoxins' integrated potency is the concentration of toxin needed to generate or alter a biological effect in a living organisms and is determined in an intact animal with consideration to the route of exposure. The relative intrinsic and integrated potencies for PbTx-3 and selected natural brevetoxin congeners and metabolites, where they differ substantially from PbTx-3, are described in the following four paragraphs and are depicted in Figure 24.4.

Intrinsic potency of brevetoxin is dependent on two factors. The first is the affinity of the toxin for its target, the alpha subunit of the voltage-gated sodium channel and the second is its efficacy, the capacity to induce an effect on the target cell after binding to the channel. Affinity of brevetoxins has been commonly determined by radioligand competition to sodium channels in rat brain membrane preparations. The development of a tritium radiolabel for brevetoxin by [^{3}H]-sodium borohydrite reduction of PbTx-2 to [^{3}H]-PbTx-3 proved instrumental in determining relative affinities for different brevetoxin analogs.[19] Low concentrations of PbTx-3 bind to an apparent single class of binding sites on the voltage-gated sodium channel with high affinity yielding a dissociation constant of 5 nM at 22°C. The brevetoxin B aldehyde (PbTx-2), the brevetoxin B double reduction (PbTx-9) and the brevetoxin A aldehyde (PbTx-1) compete for binding with similar affinity to PbTx-3.[32] The brevetoxin B epoxide (PbTx-6) has a 10-fold reduction in affinity and parallel reduction relative to PbTx-3 in all downstream biological effects.[32] The brevetoxin cysteine conjugate BTX-2 and its desoxy derivative also have a 10-fold reduction in affinity,[33] whereas the partial ring brevetoxin, brevenal, has a 300-fold reduction in affinity for the sodium channels in brain membranes.[34]

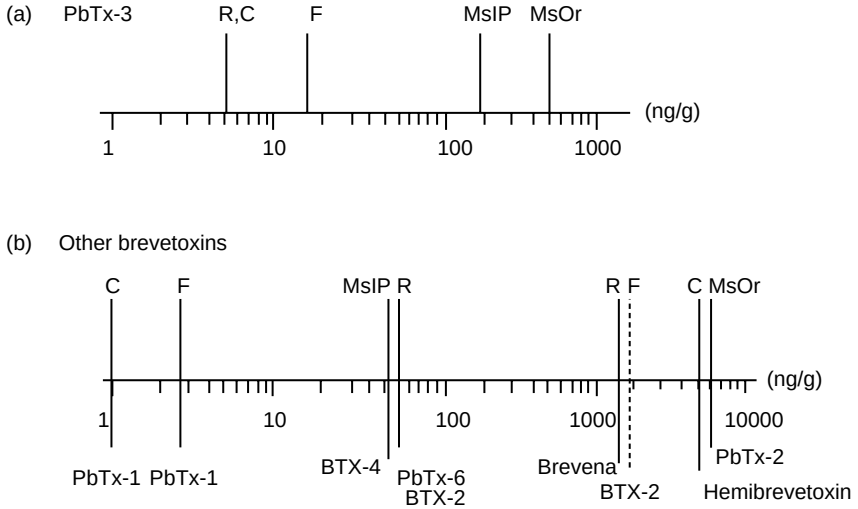

FIGURE 24.4 Brevetoxins potency. Affinity to receptor (R), intrinsic potency to cells (C), integrated potency to small fish by bath application (F) and integrated potency to mice by intraperitoneal injection (MsIP) or oral gavage (MsOr) is shown for PbTx-3 (a) and of selected natural brevetoxin congeners and metabolites that differ substantially from PbTx-3 (b). Values are shown on a logarithmic scale and expressed in ng of toxin/g. Notes: BTX-B2 action on fish is >1.5 µg/g, because of limited toxin. Brevenal binding to receptor does not initiate a biological response (i.e., antagonist), whereas hemibrevetoxin is reported to have intrinsic activity, although receptor affinity was not determined at the time it was purified.

Intrinsic potency of brevetoxins to induce an effect on the target cells after binding to the channel has commonly been determined by a neuroblastoma (N2A) cytotoxicity assay. This assay is based upon brevetoxins' ability to enhance veratridine-induced sodium uptake[7] and has been modified by the inclusion of ouabain to inhibit the Na^+/K^+ ATPase activity, such that the reaction becomes cytotoxic and the MTT tetrazolium salt can be used as a colorimetric endpoint.[35] Precise determination of the ED_{50} for brevetoxin using this cell-based assay can be complicated by the slower time course of action of the brevetoxin A and B, and the vehicle used to introduce the toxin to the cells; however, optimization of the assay indicates that the IC_{50} for the binding site on the channel in rat brain homogenates and ED_{50} value for the cell assay do not significantly differ for PbTx-3.[36] The close correlation between receptor affinity and intrinsic potency for PbTx-3 is also shared for a other cellular endpoints, including squid giant axon depolarization (1.7 nM)[9] and phrenic-diaphragm contraction (5 nM).[37] However, the potency of brevetoxins to induce cytotoxicity via the indirect action glutamate release and activation of *N*-methyl-D-aspartic acid (NMDA) receptors in cerebellar granule cells is about 10-fold less than the above values on N2A cytotoxicity.[38] The intrinsic potency of PbTx-1 in N2A cells is about five times higher than PbTx-3 and PbTx-2 is about five times lower than PbTx-3.[39] The intrinsic potency of the brevetoxin conjugate BTX-2 is about three times lower than PbTx-3, whereas the intrinsic potency of the fatty acid conjugate is about three times higher than PbTx-3.[25,27,33] The short chain brevetoxin, hemi-brevetoxin has a 1000-fold reduction in N2A cytotoxicity relative to PbTx-3. The differences in relative intrinsic potency of the different naturally occurring brevetoxins is likely related to distinctions between their affinity for the voltage-gated sodium channel and their efficacy in disrupting channel-gating transitions, which are discussed in greater detail below.

Integrated potency of brevetoxins has been assessed by bath exposure bioassays using several species of small fish, including zebrafish (*Danio rerio*), mosquito fish (*Gambusia affinis*), and minnow (*Cyprinodon variegatus*). The assay is calibrated by dosage to death at 1 h. The lethal concentration at 50% (LC_{50}) for PbTx-3 is about three times lower than the concentration required to trigger a biological response. PbTx-1 is 5-fold more potent than PbTx-3 and the brevetoxin B epoxide (PbTx-6) is five times less potent than PbTx-3.[5,15,32] These results indicate that the relative potency of the algal brevetoxins for integrated toxicity in the fish assay parallels their intrinsic activity. The partial ring brevetoxin, brevenal, has no toxic effect on fish bioassays at concentrations up to 1 μg/mL; however, pretreatment of fish with brevenal increased survival time of fish subsequently exposed to PbTx-2.[34] This indicates that brevenal has an efficacy of zero or acts as a full antagonist. The shellfish metabolites B1 and B2 having the K ring conjugations are inactive in fish at concentrations up to 1.5 μg/mL; whether this lack of integrated agonist potency is due to poor bioavailability remains to be determined.[24,25]

The integrated potency of brevetoxins has also been assessed by mouse bioassay, using a method originally developed for oysters and then standardized for monitoring of toxic shellfish.[40] The assay endpoint is time of death after i.p. administration of shellfish extracts to mice. One mouse unit is approximately 0.2 mg/kg. Brevetoxin (PbTx-3) potency determined by mouse bioassay is 40 times lower than its intrinsic activity and about ten times lower than its potency determined by fish bioassay. The aldehyde and reduced brevetoxin B are nearly equipotent by intraperitoneal injection (200 and 170 μg/kg, respectively) for a 24 h LD_{50}[41]; however, a mouse LD_{50} value has not been reported for brevetoxin A (PbTx-1). The shellfish brevetoxin conjugates B1, B2, and B4 are also potent in a similar dose range to PbTx-3 (50, 306, and 100 μg/kg, respectively).[24,25,27]

The potency of several brevetoxins has also been examined in mice and other animals using exposure routes designed to reflect common routes of exposure. Of great interest is that oral potency (LD_{50}) of PbTx-2 (6600 μg/kg) is reported to be more than an order of magnitude lower than PbTx-3 (520 μg/kg).[41] At present, the oral potency of brevetoxin conjugates has not been published, yet this remains a critically important issue for the shellfish toxins. Brevetoxin (PbTx-3) has also been administered to rats through nose-only inhalation to reflect respiratory exposure and no biochemical or histologic evidence of toxicity to the respiratory, nervous, or hematopoietic systems has been found

with doses up to 33 μg/kg accumulated over 5 days.[42] However, brevetoxins have been found to have profound effects after respiratory exposure to sheep with a half maximal effect on airway resistance of animals given 20 breaths of 1 pg/mL PbTx-3.[43] PbTx-2 is equipotent to PbTx-3 by respiratory exposure in sheep and brevenal reduced the effects of PbTx-2 and PbTx-3 on nearly an equimolar basis.

Taken together, toxicological studies with brevetoxins indicate that the intrinsic potency of brevetoxin (PbTx-3) is approximately 5 ng/g and accountable largely by affinity for binding to the voltage-gated sodium channel. Integrated toxicity by bath application to fish or interperitoneal injection to mice yields a reduction in potency of 3- and 40-fold, respectively, likely reflective of differences in uptake and disposition of the toxin. Specialized exposures indicate that PbTx-2 is several-fold less potent orally than interperitoneal in mice and highly potent for localized action on airway constriction by aerosolized exposure to sheep but not for systemic effects in mice or rats.

24.7 ROUTES OF EXPOSURE

The effects of red-tide toxins have been known for far longer than the chemical structure of these compounds and result from multiple routes of exposure, including water, air, and food. Reports of fish kills associated with red tides have been recorded as far back as 1530.[44] Taylor also noted a human respiratory irritant associated with red tide events and this association between fish and marine animal kills, red tides, and human respiratory irritation were commonly observed. The direct linkage between the sea and the air was established by Woodcock, who demonstrated that direct misting of ocean water containing *K. brevis* into the nose or mouth of volunteers elicited the same respiratory irritation as experienced on the beach.[2] Concurrently, reports of human poisoning associated with consumption of shellfish during fish kills were ground truthed by McFarren and others via analyses of toxic oysters and clams by mouse bioassay.[3] On the basis of extraction and symptomatology, they determined that the shellfish accumulated a "ciguatera-like" toxin after red-tide events. In an effort to parallel the experiments of Woodcock, McFarren's group also extracted sea water containing *K. brevis* and exposed mice to identify the same "ciguatera-like" symptoms, closing the loop between water, air, and food exposures. Collectively, these environmental exposures identified three distinct environmental scenarios for brevetoxin toxicity: water, air, and food, which are described in greater detail in the next three paragraphs.

Exposure routes for animals in the aqueous environment, include three windows of exposure: cutaneous, respiratory, and oral. Brevetoxin in the water is present within algal cells and in suspension. In the latter case, brevetoxin is likely in a complex equilibrium with various low affinity acceptors of particulate matter and interfaces of detergents leading to a particulate suspension of toxin that is readily available for biologic action. Brevetoxin composition in the water is largely comprised of brevetoxin B aldehyde and its reduced alcohol, and to a lesser extent the corresponding derivates of the brevetoxin A backbones. Substantial amounts of these brevetoxins may also be in the form of an oxidized open A ring.[45] Brevetoxin administered directly to water is acutely toxic to small fish and likely to affect the animals through entry into the gill epithelium. Mullets exposed to *K. brevis* cultures were reported much more sensitive to the toxin when the cells are lysed and can die within minutes of exposure.[46] This is consistent with a respiratory route of exposure of fish to brevetoxins in the water; however, direct evidence of exposure via the gill epithelium remains to be demonstrated. When mullets were exposed to lower levels of *K. brevis*, the uptake of toxin into the blood occurs slowly reaching peak values at 8–10 h.[47] These uptake kinetics into blood are consistent with oral raking of cells or particulates followed by intestinal absorption. Hence, the route of exposure of fish to *K. brevis* in the water may be either through respiratory or oral routes, depending upon the presentation of the toxin. An additional route of aqueous exposure may result from dermal absorption. Although this has not been investigated in aquatic exposures, it has been examined in detail in mammalian skin. [^{3}H]-PbTx-3 uptake has been characterized in explants of human and guinea pig skin using toxin in water, methanol, and dimethysulfoxide.[48] Toxin uptake is approximately 1% in water and methanol and approximately 3-fold higher with dimethyl sulfoxide (DMSO).

A red-tide bloom at Jacksonville Beach on the Atlantic coast in 1999 led to the first definitive quantitation of brevetoxin in aerosols[49] and human event characterization.[50] Pierce and co-workers measured toxin collected on filters from air samplers and identified the primary toxin congeners to be PbTx-2 and PbTx-3 with total concentration values similar to samples collected during earlier events along the Florida west coast and North Carolina coast. Toxin concentration was estimated at 93 and 36 ng/m^3 on high and moderate exposure days, respectively, with upper respiratory symptoms on both days and lower respiratory symptoms predominating on the high exposure day.[50] Subsequent events on the Texas (2000) and Florida (2001 and 2003) coasts of the Gulf of Mexico provided an opportunity to conduct analyses of the aerosols that carry brevetoxins.[51–53] Collected aerosols were found to comprise a predominant population of coarse particles with a median diameter between 6 and 10 μm and in some analyses a lesser population of fine particles with a median diameter between 0.1 and 0.2 μm.[51] Analysis of the different size particles collected in the 2001 and 2003 Florida events, identified brevetoxin in the coarse particle size and with little brevetoxin in fine particles.[52] The association of brevetoxin to the coarse sized particles has substantial significance for exposure estimates. Respiratory exposure based upon the ICRP 66 Lung model estimated that the majority of the coarse aerosol particles (75–84%) deposits in upper (nasal and oral) airway and only a small amount 2–6% in the lower airway. The distribution of the coarse particles to the upper respiratory pathway is consistent anatomically with the common symptoms of throat and nasal irritation as opposed to the lower respiratory symptoms, which include tightness of chest, wheezing, and shortness of breath. On the basis of analysis of these events, effect levels for air concentrations of toxin can be estimated for upper and lower respiratory effects. For example, the 2000 Texas event that had air concentrations of 3.0 ng/m^3 induced upper respiratory effects with an exposure dose in the upper respiratory track of 4–5 ng/h, yet was without effects on the lower respiratory track with corresponding exposure dose of 0.15–20 ng/h. Furthermore, the 2003 Florida event that had air concentrations of 37 ng/m^3 and a corresponding lower respiratory dose of 3.3 ng/h that induced effects on the lower respiratory track. Accordingly, on the basis of toxin associated with coarse aerosol particles, an effect level for air concentrations is estimated at 3 ng/m^3 for upper respiratory symptoms and 10-fold higher for lower respiratory symptoms.

Shellfish represent the most common source of brevetoxin food poisoning to humans, whereas finfish and seagrasses are believed to be the primary vectors for dolphins and manatees, respectively. As described earlier, shellfish toxins include several peptide and lipid conjugates. Finfish are reported to contain the cysteine conjugates, reduction products and aldehyde (the latter particularly in gut contents of planktivorous fish), whereas seagrass is likely to contain the toxins associated with algal cells.[46] Foodstuff is digested under acidic conditions in the stomach, with consideration to buffering capacity of the matrix and physical grinding leading to its likely association with micelles. Entering into the intestinal track, the micelles are mixed with bile acid leading to smaller particles that passively move across the brush border. The toxin is likely absorbed free of the micelle by an active transport mechanism. Upon entry into absorptive cells, the toxin may be packaged into chylomicrons and enter into the lymph or migrate the basolateral surface for entry into the portal blood. The partitioning between these two routes is likely to have a substantial effect on toxin kinetics and metabolism, as the former pathway represents a slower and prolonged entry into the blood supply through the subthoracic vein, and a route that bypasses the first passage to the liver.

24.8 TOXIN DISTRIBUTION

Brevetoxins administered in the form of [^{3}H]-PbTx-3 distribute broadly to multiple tissues after intravenous, oral, and intratracheal administration to laboratory rats. Poli et al. first described the distribution of [^{3}H]-PbTx-3 by intravenous administration to rats.[54] Toxin distribution, reported as body burden, was nearly 70% to skeletal muscle and within 30 min of toxin administration 96% of the toxin distributed to three tissues: skeletal muscle, liver, and intestine. HPLC analysis indicated that the PbTx-3 was intact in muscle; however, multiple products of metabolism were found in the feces.

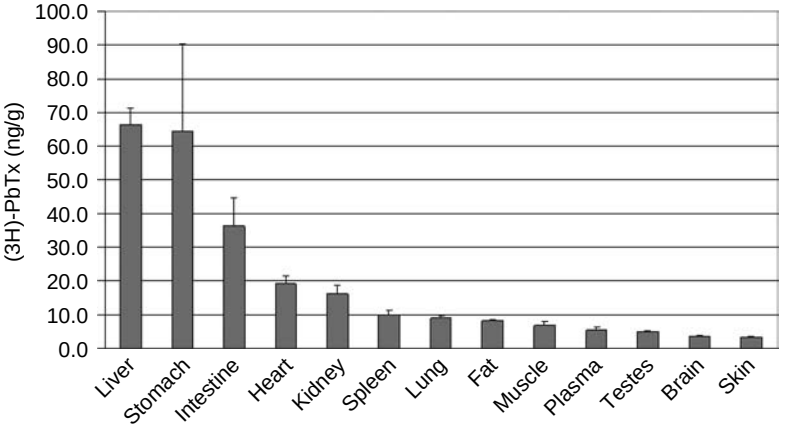

FIGURE 24.5 Distribution of brevetoxin following oral gavage of rats. Brevetoxin [^{3}H]PbTx-3 was administered to rats by oral gavage and tissues analyzed for radioactivity after 6 h. Bars are means + standard errors for three rats. (Chart constructed with data from Cattet, M. and Geraci, J. R., *Toxicon*, 31, 1483, 1993.)

On the basis of these data, Poli suggested that muscle represents a primary storage tissue for PbTx-3, and the liver represents a target for metabolism. Cattet and Geraci conducted an oral gavage of [^{3}H]-PbTx-3. They reported toxin as tissue concentration, rather than body burden, and found that toxin concentrates highest in liver and intestine and to a lesser extent in many other tissues[11] (Figure 24.5). This study suggested that the liver concentrates toxin as a result of both entry from the systemic circulation and re-entry from the portal circulation. Benson et al. conducted an intratracheal exposure of [^{3}H]-PbTx-3.[55] They found that the toxin administered to the lung distributes by body burden predominately to skeletal muscle, liver, and intestines, and by tissue concentration distributes from the lung in rank order to the heart, intestines, kidney, and liver. Brevetoxin was lowest in brain and fat, in agreement with intravenous studies, although the oral studies showed intermediate levels in fat with low levels in brain tissue. The results from intratracheal exposure differed from intravenous and oral exposures in that [^{3}H]-PbTx-3 was found not accumulate in liver over time.

24.9 TOXIN TRANSPORT

The tissue distribution studies are all consistent in that the toxin distributes throughout the body, a process that largely requires blood as the medium for transport. The intravenous study indicated that [^{3}H]-PbTx-3 is rapidly (more than 90% in 1 min) removed from the blood[54]; however, administration to the oral and pulmonary compartments indicated that substantial levels (5 ng/mL) of [^{3}H]-PbTx-3 persists in blood.[11,55] The difference is likely due to the slower time of absorption from the oral and pulmonary compartments. Brevetoxin has been measured in the blood of mice after intraperitoneal exposure to PbTx-3 using biological assays and mass spectrometry and comparable values determined for in blood given the higher dosage used for unlabeled versus radiolabeled PbTx-3.[56] More detailed time course study has found toxin levels of 36 nM at 30 min and maintained levels above 25 nM following maximal nonlethal intraperitoneal dose of 180 μg/kg PbTx-3.[57] Longer duration studies in rats indicate that brevetoxin was measurable in blood for up to 14 days.[58] The role of blood to transport toxin for distribution and elimination has led to questions about its distribution within the blood compartment. Analysis of [^{3}H]PbTx-3 distribution in plasma indicates that 39% of the brevetoxin remains associated with components in mouse plasma after 15 kDa cutoff dialysis.[59] Of this portion, only 6.8% was bound to serum albumin. A large component of brevetoxin in mouse plasma after intraperitoneal exposure to PbTx-3 is associated with high-density lipoproteins (HDL). In human plasma, which unlike other species has higher concentrations of low-density lipoproteins

(LDL) than HDL, [^{3}H]PbTx-3 associates with LDL and very low-density lipoproteins (VLDLs) as well; however, the binding to HDL still predominates both on a basis of per weight and total mass.[60] Given the role of LDLs to distribute cholesterol to tissues, they may also have a common role in distributing brevetoxins to tissues. Likewise, given the role of HDL's to transport cholesterol to liver, they may also have a role to distribute brevetoxins to the liver.

24.10 TRANSFORMATION

The original intravenous distribution study of [^{3}H]-PbTx-3 provided the first insight that brevetoxins are transformed by the liver to undefined polar intermediates eliminated in the feces.[54] An intravenous distribution study of [^{3}H]-PbTx-3 in toadfish reported undefined polar metabolites and found that 40% of the radiolabel is found in the hepatobiliary system.[61] An oral distribution in the study of [^{3}H]-PbTx-3 in the toadfish also found five to six undefined polar intermediates in the bile.[62] Direct effects of brevetoxin on liver have been investigated *in vitro* in liver slice preparations. An initial study indicated that PbTx-3 inhibits oxygen production and increased cellular Na^+/K^+ ratio.[63] A subsequent histochemical study identified ultrastructural changes consistent with detoxification including swelling in smooth endoplasmic reticulum, degranulation of rough endoplasmic reticulum, and deformities and lytic cristae in the mitochondria.[64]

Several studies have examined the effect of brevetoxin on the activity of liver metabolizing enzymes as an indicator of its potential for transformation. An initial study of the effect of PbTx-3 administered orally to the red fish (*Sciaenops ocellatus*) investigated the activity of hepatic P450 regulated enzymes ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-dependentylase and the cytosolic enzyme, glutathione *S*-transferase (GST).[61] This study found a modest increase in EROD activity and concluded that this reflects an induction of the Phase I enzyme P450IA by PbTx-3. However, the lack of GST activity as measured with the 1-chloro-2,4-dinitrobenzene substrate (CDNB) suggested that PbTx-3 did not induce the Phase II enzyme, GST. A second study, this time using oral exposure of PbTx-2 to the striped bass (*Morone saxatilis*), re-examined the effect of brevetoxin on EROD activity as well as GST activity, this time using several different GST substrates.[65] PbTx-2 induced EROD activity in the striped bass, again suggesting that brevetoxin induces P450A1; however, no increase in P450A1 protein was detectable by enzyme-linked immunosorbent assay (ELISA). PbTx-2 was also found to increase GST activity as measured by pi and mu isozyme specific substrates ethacrynic acid and *trans*-phenylbutenone; yet, consistent with the study in red fish, there was no increase in activity using the less-specific substrate CDNB. Taken together, these results again indirectly suggest that brevetoxin induces Phase I and Phase II enzymes in the liver, which are responsible at least in part for its transformation. Washburn and co-workers proposed that the modest effect of PbTx-2 may result from transformation of a smaller amount of this parent toxin to a potentially more potent inducer, the 27, 28 epoxide, PbTx-6.[65] A subsequent study investigated the direct interaction of PbTx-2, PbTx-3, and PbTx-6 to the regulatory cytosolic receptor for P450, the Ah receptor.[66] Gel shift assays indicated that PbTx-2 and PbTx-3 bound poorly to the Ah receptor, whereas PbTx-6 was more effective, nearly equivalent to β-naphthoflavone (BNF). More recently, effects of PbTx-2 and PbTx-6 were examined on gene induction in the liver of intraperitoneal exposed mice.[67] Unexpectedly, the brevetoxins were not found to induce hepatic detoxification enzymes characteristically activated by Ah receptor mediated induction of P450. This indicates that brevetoxins, specifically PbTx-6, increases activity of P450 enzymes by a means other than the classical AhR pathway.

The transformation of brevetoxins has also been examined by liquid chromatography-mass spectrometry (LC-MS) analysis of toxin metabolites after exposure to liver microsomes, purified P450 enzymes, and freshly isolated liver cells. Incubation of PbTx-2 with liver microsomes has been found to yield two brevetoxin metabolites.[68] These metabolites include an A-ring hydrolytic product of PbTx-2 as well as the PbTx-2 reduction product PbTx-3. Incubation of PbTx-2 with purified P450 enzymes has been found to yield additional brevetoxin metabolites.[12] The enzymes appear to fall into two groups with CYP1A2 and CYP2C1 having a slower rate of PbTx-2 metabolism than

CYP2A2, CYP2D1, and CYP3A1. LC-MS analysis of the metabolic products formed by CYP2A2 indicates the presence of the single and double PbTx-2 reduction products, PbTx-3 and PbTx-9 as well as a newly recorded MH+: *m/z* 929 metabolic product. This positive ion mass is consistent with more than one metabolite. One possibility is that it is an A-ring hydrolytic of BTX-B5, the carboxylic derivative of PbTx-2. However, the LC/MS/MS characteristics of retention, fragments, and negative ion differed from this compound. Another candidate is the 27,28-diol of PbTx-2, formed from the 27,28-epoxide (PbTx-6) intermediate. LC-MS analysis of the metabolic products formed by CYP3A1 indicates the presence the same products described above as well as a large amount (65%) of MH+: *m/z* 911. The negative ion mass of this compound (*m/z* 909), indicated that it was the carboxylic derivative of PbTx-2, known as BTX-B5. Both type A and type B brevetoxin metabolites have been investigated by LC-MS analysis after treatment with freshly isolated hepatic cells. The type A brevetoxin (PbTx-1) has been found to form two metabolic products.[68] One product is proposed to be an A-ring hydrolytic product of PbTx-1 and the other an E-ring diol of PbTx-1. The type B brevetoxin (PbTx-2) has been found to form twelve products after incubation with fresh hepatocytes.[12] These products include the PbTx-2 single and double reduction products and the A ring hydrolytic product of each. Two MH+: *m/z* 911 products of different retention times appear to be the carboxylic derivative of PbTx-2 (BTX-B5) and the 27, 28-epoxide (PbTx-6). A MH+: *m/z* 913 product with different LC/MS/MS characteristics from the A ring hydrolytic of PbTx-2, appears to be the 27,28-epoxide of PbTx-3. A MH+: *m/z* 929 and 931 are consistent with 27, 28- diol of PbTx-2 and the 27,28-diol of PbTx-3. In addition, larger metabolites of MH^+: *m/z* 1018, 1204, and 1222 were formed after incubation of PbTx-2 with rat hepatocytes. The first is consistent with the PbTx-3 cysteine conjugate and the latter glutathione conjugates of PbTx-3. Collectively, transformation of brevetoxins includes reduction, oxidation, epoxidation, and conjugation reactions, which is summarized in a proposed metabolic pathway for in Figure 24.6.

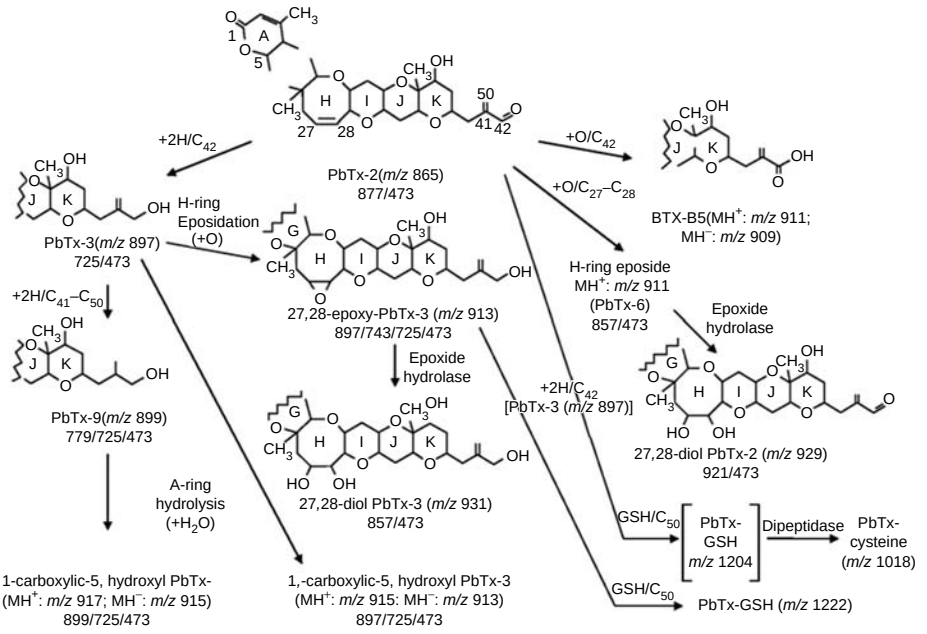

FIGURE 24.6 Proposed liver catalyzed metabolic pathways of PbTx-2 in rat. PbTx-2 is initially metabolized by CYPs to its prominent BTX-B5, PbTx-3, and PbTx-9 conjugates. Additional metabolic reactions, including H-ring epoxidation, yield several biologically active products. Further conjugation steps, mostly with GSH, are essential to trap some of those intermediates into readily eliminated water-soluble glutathione (GSH) and cysteine conjugates. (Reproduced from Radwan, F. F. and Ramsdell, J. S., *Toxicol. Sci*., 89, 57, 2006. With permission.)

24.11 ELIMINATION

Elimination of brevetoxin in mammals occurs largely through feces and urine. The [^{3}H]-PbTx-3 intravenous studies indicate that brevetoxin is eliminated quickly and nearly complete within 3 days.[54] Over the first 2 days, 65% of the toxin was eliminated in the feces and only 10% in the urine. The feces contained little radiolabel in the form of PbTx-3, indicating conversion to metabolites. The [^{3}H]-PbTx-3 oral studies indicate that over the first 2 days, more toxin was eliminated in the feces, whereas more toxin was eliminated in the urine after this time.[11] Brevetoxins have also been measured by radioimmunoassay (RIA) in the urine at levels higher than could be detected in serum of human subjects poisoned by consumption of brevetoxin-contaminated shellfish.[29] LC-mass spectrometric analysis of the brevetoxin in urine was determined to contain both the brevetoxin-B aldehyde and its reduction product (PbTx-3). Rats treated by intraperitoneal administration with brevetoxin-B aldehyde (PbTx-2) or its reduction product (PbTx-3) have been found high concentrations of polar metabolites, particularly in the PbTx-2 treated rats.[58] Concentrations peaked in blood at about 4 h and were eliminated largely via urine during the first 24 h. Assay guided HPLC analysis of urine from PbTx-2 treated rats yielded three major peaks of activity. The first peak was attributed to the two cysteine adducts, cysteine-PbTx sulfoxide and cysteine-PbTx (MH^+: *m/z* 1034 and 1018). The second peak was attributed to the oxidation/reduction products, the oxidized carboxylic acid (BTX-B5) and the single reduction product PbTx-3. Components of the third, more hydrophobic, were not identified. The first two peaks were also found in PbTx-3-treated rats; however, the PbTx-3-treated rats overall had lower levels of the polar metabolites both in blood and urine. Brevetoxin cysteine conjugate and its desoxy derivate contributed nearly three quarters of the brevetoxin in the urine of PbTx-2 treated rats. This study indicated that brevetoxin is rapidly transformed to polar metabolites that appear in blood for up to 4 h and are eliminated in urine within 24 h.

24.12 RECEPTOR INTERACTION

The targeting of brevetoxins to ion channels was implicated in the action of partially purified preparations of *K. brevis* to induce repeated action potential firing in squid giant axon[69] and increased frequency of action potential firing in crayfish ventral nerve cord.[70] At the phrenic–diaphragm junction, partially purified brevetoxins were found to induce mini-endplate potentials, a reflection of depolarization of the presynaptic nerve.[71] Although Westerfield and coworkers originally showed that tetrodotoxin blocked the effects of brevetoxin,[69] Shinnick–Gallagher more thoroughly examined the blockage of these actions by the sodium channel blocker tetrodotoxin and elimination of bath sodium to implicate an action at the voltage dependent sodium channel.[72] The definitive explanation for pharmacologic basis to brevetoxin–receptor interaction was resolved the following year. Using the HPLC fraction T46 was purified by Risk; Caterall was able to provide definitive evidence that brevetoxin A (PbTx-1) activated voltage-gated sodium channels.[7] This seminal study determined that brevetoxin does not cause $^{22}Na^+$ uptake directly, rather it enhanced the action of veratridine, an activator of site 2 of the voltage-dependent sodium channel. Brevetoxin was also found not to interact with pharmacologic sites 1 and 3, as determined by its inability to compete for binding of site-specific radioligands. Taken together, Catterall and Risk deduced that brevetoxin binds to a new receptor site of the sodium channel designated as site 5. However, site 5 is of pharmacologic designation only and this should not be taken to mean that there is a single domain of interaction between brevetoxin and the sodium channel. Brevetoxin is a long molecule that spans the depth of the sodium channel and as described later, it is clear that brevetoxin interacts at many points along the span of the sodium channel. Three years later, ciguatoxin, a related polyether toxin, was also found to enhance $^{22}Na^+$ uptake in the presence of site 2 activators.[73] The development of tritiated-brevetoxin, through reduction of the brevetoxin B aldehyde to form [^{3}H]-PbTx-3 led to direct radioligand characterization of site 5 as a distinct neurotoxin receptor site on the sodium channel for brevetoxins and ciguatoxins.[19,74]

Brevetoxin binding to the voltage-gated sodium channel promotes allosteric interaction with the binding of several other neurotoxins to their receptor sites and this has provided additional insight into the interaction of brevetoxin with the voltage-gated sodium channel. At present, nine distinct pharmacologic receptor sites (designated sites 1–9) have been characterized for neurotoxins, therapeutic drugs, or synthetic pyrethroids on the primary subunit (alpha) of the voltage-gated sodium channel. The alpha subunit is composed of a large polypeptide containing four homologous repeat domains (DI-DIV) each with six transmembrane segments (S1–S6), which aggregate into a pseudotetramer (Figure 24.7). The S5 and S6 of each domain form the inner lining of a central ion-conducting pore. Conformational changes of this pseudotetramer lead to three activity transitions: closed, open, and inactivated. The segments S4 of each domain serve as voltage sensors to open the channel and the intracellular loop between DIII-S6 and DIV-S1 serves as the inactivation gate. Brevetoxins' overall effect on the sodium channel is unique to other nine classes of neurotoxins and therapeutic agents, yet shares some common effects on gating transitions as described later.

Upon recognizing that brevetoxin A bound a unique site on the sodium channel, Catterall and Gainer extended this study to determine that brevetoxin substantially enhanced the binding of radiolabeled batrachotoxin to site 2, consistent with its enhancement of veratridine-induced sodium uptake.[75] Brevetoxin B and ciguatoxin were later found to have the same property to enhance batrachotoxin binding to site 2.[74,76] This allosteric effect to increase batrachotoxin binding to site 2 is also similar to that found for other classes of sodium channels toxins, specifically those that bind to sites 3 (alpha-scorpion) and 6 (pyrethroid).[77,78] This allostery has been interpreted to mean that brevetoxins, like site 3 and 6 ligands, interfere with the gating transitions to stabilize the sodium channel in the open state enhancing binding affinity of batrachotoxin. This action appears to have functional relevance, as a measure of efficacy of brevetoxins for this allosteric effect is reported to parallel the potency of these toxins.[79] As may be expected, other allosteric interactions occur between neurotoxins and therapeutic agents that bind the channel receptor sites. Of note relative to brevetoxin, is that brevetoxin also enhanced the binding of selective ligands to site 4, but not to

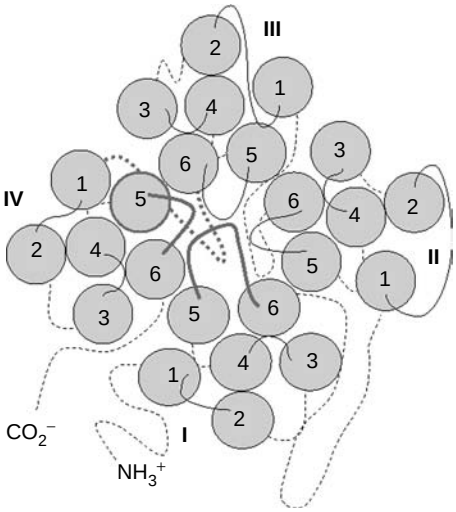

FIGURE 24.7 Schematic diagram of sodium channel subunits and sites of brevetoxin interaction An extracellular view of continuous pseudotetramers (domains I–IV) of six related segments (S1–6) of the voltage-gated sodium channel. Transmembrane segments five and six of each domain line the ion-conducting pore and segments four (S4) of each domain form the voltage sensor. Solid lines represent extracellular loops between transmembrane segments and dashed lines represent intracellular loops between transmembrane segments. Brevetoxin sites of action are shown in bold. They include the S5–6 extracellular loops of domains I and IV, the length of IV-S5 and the intracellular loop of IV-S1 and III-S6.

site 1 or 3; and that site 2 and 6 ligands enhance the binding of brevetoxin to site 5;[76] however, the mechanistic implication of these allosteries have not been determined. Further understanding of the brevetoxin–receptor interaction required other approaches including receptor mapping and analysis of structure–function activity.

Receptor mapping of the sodium channels has utilized two approaches. The first involves photoaffinity labeling, hydrolysis of the protein, and peptide immunoidentification. This provides regional information for toxin binding to susceptible residues within the reach of the photoactivable group. The second mapping approach employs mutagenesis of selected amino acids and assessing neurotoxin binding and functionality of the channel and provides exact residues that bind the toxin. Trainer et al. [80] have mapped the brevetoxin–receptor using p-azidobenzoyl-[^{3}H]-PbTx-3 as a photoaffinity probe to purified alpha subunits of the voltage-gated sodium channel and identified binding to separate peptides; one from DI-S6 and the other from DIV-S5.Photoaffinity labeling a solubilized preparation of the channel and more extensive digestion further defined the peptide fragments within the extracellular region of each of these segments. This finding is consistent with native conformation of the channel having the extracellular loops spanning domain I S5 and S6 and domain IV S5 and S6 in close proximity as may be expected given their position to line the inner wall of sodium channel. The nature of photoaffinity labeling experiments demonstrate only that the photoactive group (*p*-azidobenzoyl linked via carbonyldiimidazole to the terminal hydroxyl of PbTx-3) is within reach of a substrate of the channel, and does not imply that this is necessarily a normal site of interaction between the brevetoxin and the channel. Indeed, as discussed in the following section, brevetoxin is a long plasma membrane-spanning molecule that interacts with several functional group of the channel; hence its interaction with the extracellular loop of the S5 and S6 of domains I and/or IV appears to mediate only part of brevetoxins molecular action. Channel mapping involving mutagenesis of the sodium channel, although conducive to defining some neurotoxin binding sites, has not proven successful for brevetoxins. As an alternative approach, molecular modeling of the preferred conformations brevetoxin A and B backbones has been used to describe the likely orientation of the remainder of the brevetoxin molecule in the binding pocket of the sodium channel. In addition, the analysis of synthetic derivatives for competition of [^{3}H]-PbTx-3 binding to synaptosomes has been successfully used to further predict specific functional groups of brevetoxin required to bind the sodium channel.

The backbone structures of brevetoxin B and A was analyzed by Monte Carlo methods to determine that the eight-membered ring (H-ring for brevetoxin B and G-ring for brevetoxin A) has a strong preference for a boat-chair over a crown rotation.[81,82] This eight-membered ring serves as a flexible hinge for seven conformations of brevetoxin B and 24 conformations of brevetoxin A. Superimposition of the identical three terminal rings of the "tail of the molecule" (IJK for brevetoxin B and HIJ for brevetoxin A) and analysis of the various conformations, yielded several conformers that oriented the lactone A ring of each molecule in nearly the same orientation. These conformers formed a "straight" rather than a "bent" shape, giving rise to the description of the preferred conformation of the brevetoxin molecule to be "cigar-shaped" and approximately 30 Å in length (roughly the thickness of the plasma membrane) (Figure 24.8, bottom three structures). Because of the brevetoxin A and B backbones have similar structure in A ring and three identical terminal rings, these are assumed to be each important for the binding of the toxin to the receptor site. This hypothesis was supported by the first of several structure–receptor studies using synthetic brevetoxin derivatives (Figure 24.9). Rein et al. examined several reduced derivatives of brevetoxin B for receptor-binding activity.[32] Reduction of the A ring C2, C3, and the R-group C41, 43 double bonds had about a 10-fold reduction on receptor-binding affinity; however reduction of the third carbon to carbon double bound (C27, C28) found on the H-ring (Figure 24.9,2,3,27,28-Hexahydro-PbTx-3) caused a greater than three orders of magnitude reduction in receptor binding. Conformational analysis indicated that this reduction favored the crown over the boat–chair configuration, indicating that the latter conformation is indeed the biologically active shape of the molecule (Figure 24.8, top structure).

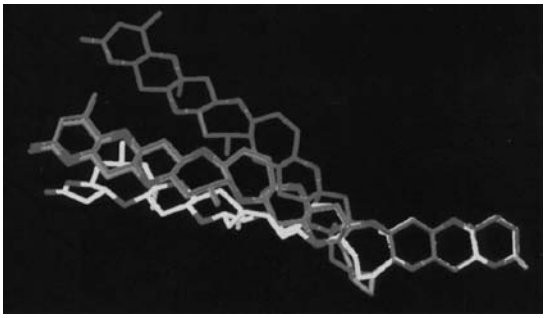

FIGURE 24.8 Minimal aligned structures for brevetoxin. Brevetoxins PbTx-1, PbTx-3, PbTx-6, and the triple reduced brevetoxin B 2,3,27,28-hexahydro-PbTx-3 are aligned by their minimal energy conformations. The strict alignment of the terminal six-member rings aligns the A ring lactone in close proximity for the brevetoxins A and B. The H-ring epoxide of PbTx-6 still aligns the molecule in the boat–chair conformation preferred by PbTx-1 and PbTx-3. However the reduced H-ring of the hexahydor-PbTx-3, prefers the crown orientation and drives the A ring to a strong angle away from the linear brevetoxins. (Reproduced from Rein, K. S., Baden, D. G., and Gawley, R. E., *J. Org. Chem*., 59, 2101, 1994. With permission.)

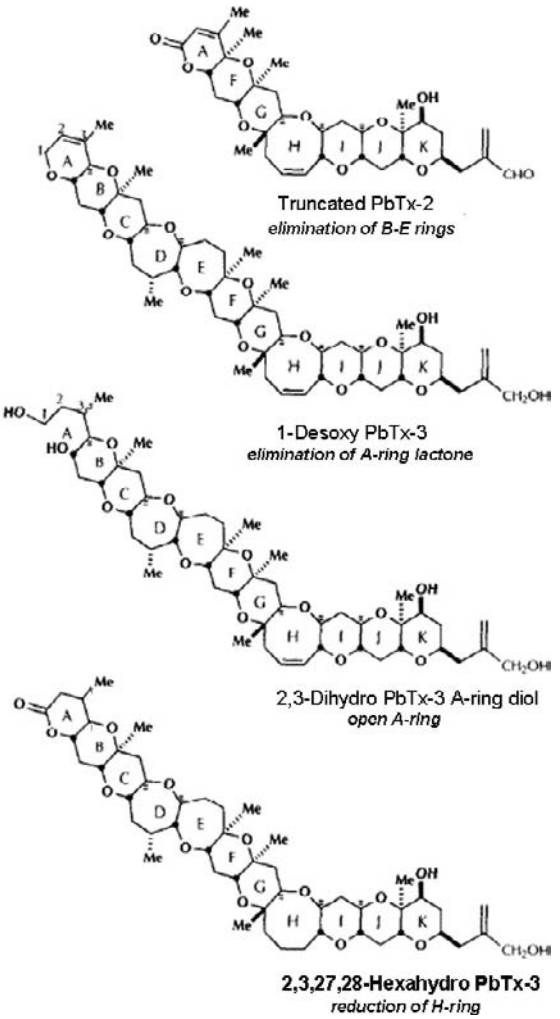

FIGURE 24.9 Synthetic brevetoxin derivatives used to probe structure–function activity relationships with the voltage-gated sodium channel. Synthesis of the structures are described in References 8, 32, and 120.

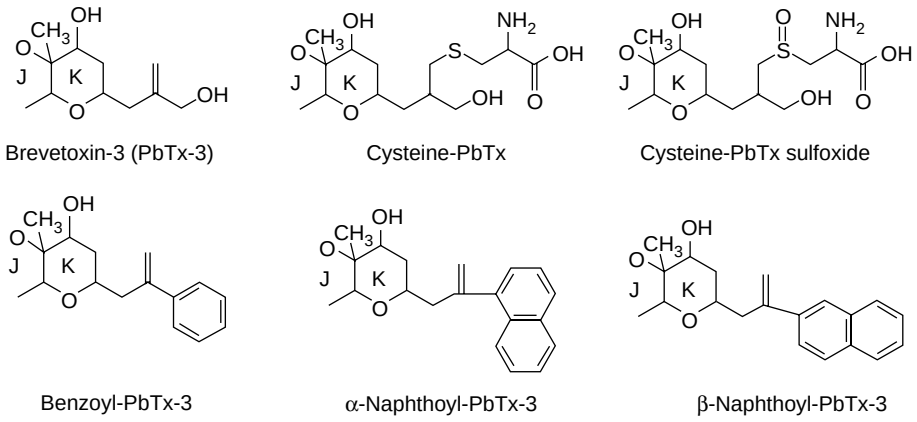

FIGURE 24.10 Synthetic R-group modifications of brevetoxin used to probe structure–activity relationships with the voltage-gated sodium channel. Synthesis of the structures are described in Reference 83.

Two structure–function studies elucidated the role of the A ring for receptor binding. Rein et al.'s above mentioned study[32] determined that opening of the A ring by reductive cleavage to a A-ring diol (Figure 24.9, 2,3, Dihydro PbTx-3) induced loss of binding activity, whereas Gawley and co-workers determined that removal of only the A ring C-1 carbonyl oxygen caused only a nine fold reduction in binding activity (Figure 24.9, 1-Desoxy-PbTx-3). The latter study[8] also determined that a truncated derivative of brevetoxin B (20.4 Å compared with approximately 30 Å) and lacking the BCDE rings (Figure 24.9, truncated PbTx-2), was also devoid of binding activity. These results indicate that an intact A-ring and a full-length cyclic polyether are necessary for brevetoxins' high-affinity binding to the voltage-gated sodium channel.

Although the immunoaffinity studies indicated that brevetoxin binds near the external domain of IVS6, it was unclear as to the role of the K-ring R group in toxin affinity. The addition of three bulky hydrophobic substitutions to the K-ring R group led to (up to 15-fold) increase in apparent affinity for receptor binding[83] (Figure 24.10). By contrast, the presence of polar substitutions to the K ring in the cysteine conjugate BTX-2 and its desoxy derivative led to a 10-fold decrease in apparent affinity for binding.[33] These results indicate that substitutions to the R group can enhance or reduce toxin affinity to the voltage-gated sodium channel, possibly as a function of their size and polarity.

24.13 EFFECTS ON IONIC CONDUCTANCE

At the level of the sodium channel, at least four actions of brevetoxin have been identified leading to the depolarization of excitable cell membranes. These actions on the channel include (1) shift of voltage dependence, (2) inhibition of inactivation, (3) increase of mean open times, and (4) multiple subconductance levels (Figure 24.11). Multiple actions of brevetoxin on the sodium channel are not necessarily surprising, given that its straight conformation of 30 Å length spans the full width of the sodium channel offering multiple points of contact that can modify its open, closed, and inactivated transitions. A summary of the four described effects of brevetoxin on sodium channels is presented in Figure 24.12 and described in greater detail in the following four paragraphs.

24.13.1 Hyperpolarized Shift in Voltage Dependence

A shift in the voltage dependence of voltage-gated sodium channel (VGSC) toward more negative potential is the most commonly observed action of brevetoxin, an effect that leads to channel openings even at the resting membrane potential. Originally described in voltage clamped squid giant axons, Huang and coworkers reported a hyperpolarized shift of 35 mV.[9,84] This shift in the voltage needed for activation of the channel has been described in many other cell preparations including

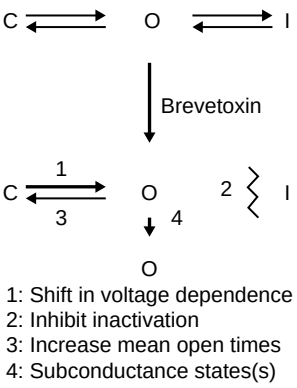

FIGURE 24.11 Brevetoxin actions on transition states of the voltage-gated sodium channel. The voltage-gated sodium channel fluctuates between closed, open, and inactivated. Brevetoxin (1) decreases voltage dependence, (2) inhibits inactivation, (3) increases mean open times, and (4) induces subconductance state(s).

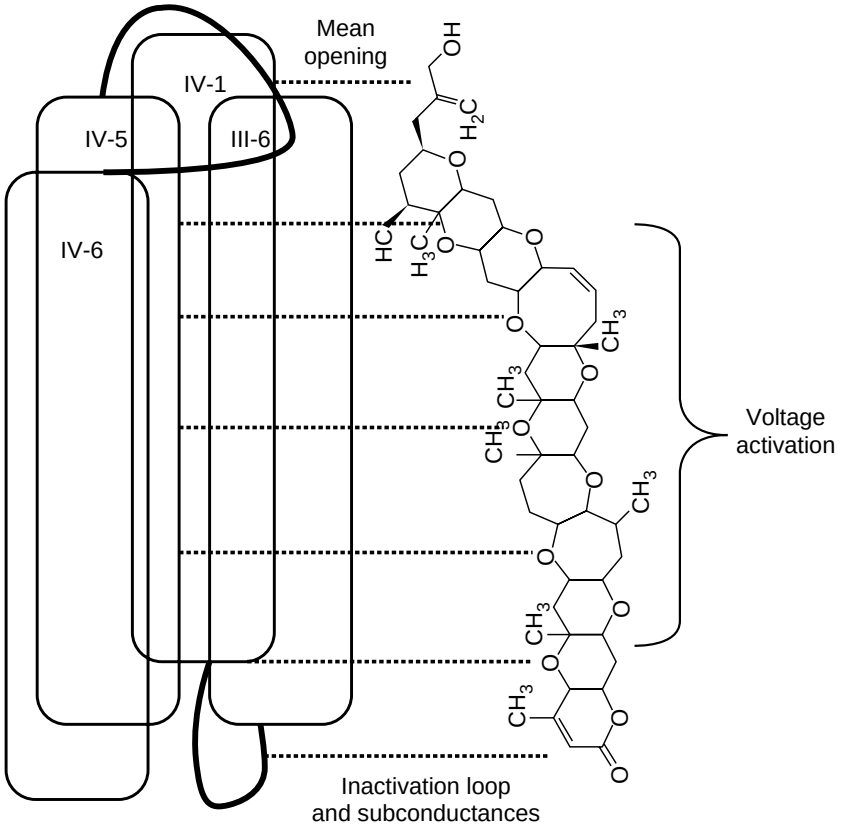

FIGURE 24.12 Schematic diagram of brevetoxin actions on transition states of the voltage-gated sodium channel. A later view of four segments (III-6, IV-1, IV-5, and IV-6) of the voltage-gated sodium channel is depicted with brevetoxin sites of action shown in bold. The K-ring substitutions interfere with the S5–S6 extracellular loops of domains IV and I (later not shown for clarity of viewing other interactions). The length of the fused cyclic polyether backbone interacts with IV-S5 to disrupt its charge interaction with the voltage sensitive IV-S4. The A-ring lactone of brevetoxin interferes with inactivation particle (intracellular loop of IV-S1 and III-S6) to inhibit inactivation and induce subconductance states.

those from the central nervous system (neuroblastoma cells), heart (myocytes), and autonomic nervous system (nodose ganglion cells).[85–89] Structure–function relationship studies show that opening of the A ring, elimination of its lactone, truncation of the BCDE rings or reduction of the H ring still retain activity to shift the activation potential.[8,87] This indicates that the oxygen-rich backbone of brevetoxin interacts with the channel to stabilize the open conformation. The binding of the tail region in the vicinity of extracellular loop spanning DIV-S5 and DI-S6 segment of the sodium channel aligns the oxygen-rich backbone of the brevetoxin in close and parallel orientation with the DIV-S4. The four S4 transmembrane segments each form pillars of positively charged cylindrical alpha helixes, which are in the closed conformation, associated with negatively charged residues from other transmembrane segments. Membrane depolarization is thought to displace the positive charges to rotate and move outward from the contact with the other transmembrane segments to yield a conformation that opens the ion-activating pore. A possible action of brevetoxin is to interfere with the interaction of the S4 segment with the S56 or 6 to reduce the voltage necessary for channel opening.

24.13.2 Inhibition of Inactivation

Inactivation is a basic functional feature of sodium channels, one that occurs within a few milliseconds of channel opening and prohibits unrestrained activation of the channels. Inhibition of channel inactivation is a commonly observed action of brevetoxin, which allows channel openings to occur during maintained depolarization that would normally silence the channels. Originally described by Huang et al.[9] in squid giant axons,this response was reported not to occur in neuroblastoma cells.[85] However, brevetoxin inhibition of channel inactivation has subsequently been characterized in other neuroblastoma cell lines as well as cardiac myocytes and nodose ganglion cells. Structure–function activity studies show that opening of the A ring and elimination of its lactone still retain activity to inhibit channel inactivation; however, truncation of the BCDE rings loses the activity to inhibit channel inactivation.[8,87] This suggests that the overall length of the brevetoxin molecule is critical for channel inactivation. Inactivation of the sodium channel is mediated by an inactivation gate, which occludes the intracellular mouth of the channel pore. This inactivation gate is comprised of hydrophobic amino acids (isoleucine–phenylalanine–methionine) of the cytoplasmic loop (IFM motif) connecting DIII-S6 and DIV-S1. This motif forms hydrophobic interactions with the mouth of the channel pore in a tethered-ball model. A potential action of brevetoxin to align parallel with the DIV-S6 transmembrane domain to reach to the IFM inactivation particle may be sufficient to inhibit this inactivation mechanism.

24.13.3 Increased Mean Open Time

A prolongation of open times of channels by brevetoxin was first described by Schreibmayer and Jeglitsch in cardiac myocytes.[88] Brevetoxin was also found to cause a doubling of mean open times in nodose ganglion cells.[86,87] Structure–function activity studies show that the truncated brevetoxin and 1-desoxy brevetoxin lack the activity to increase mean open times.[8] However, opening of the A-ring can increase the mean open times. This suggests that the A-ring lactone or the diol in the open A ring derivative acts to stabilize the open over the closed or inactivated conformations. Additional structure–function activity relationships involving the other end of the brevetoxin molecule demonstrated that the R-group substitution of the hydrophilic B-naphthoyl group, actually reverses this activity to decrease mean open times, yet permitts reduction of voltage dependency.[83] Given that this is the part of the brevetoxin molecule that was used for photoaffinity labeling, it suggests that they may also interact with the domain IV or III S5 to S6 extracellular loops. Studies have suggested that this loop for each domain needs to fold inside the channels to keep the open configuration stabilized. An action for brevetoxin to increase mean open times by interaction with the extracellular S5 and S6 loops is supported by the action of type 3 neurotoxins to label these extracellular loops lining the ion pore and to increase mean open time of the channel.

24.13.4 Increased Subconductance States

An unexpected finding of brevetoxin was its action to increase the number of subconductance states of the sodium channel. This was first described by Schreibmayer and Jeglitsch in cardiac myocytes[88] who determined that PbTx-3 was unique as a sodium channel modifier in that it does not stabilize a single open state but rather it stabilized up to nine open states of fractional levels of the normal 21 pS conductance. A subsequent study of nodose ganglion cells also demonstrated that PbTx-3 induced subconductance states; this time a single state of half the normal 21 pS conductance.[8,87] These studies indicate that brevetoxin can interfere with the degree of opening of the sodium channel pore. Structure–function activity studies show that the truncated brevetoxin, 1-desoxy brevetoxin lacks the ability to induce the half conductance state in the nodose ganglion cells.[8] This suggested that the A ring may interfere with the inactivation particle causing only a partial inhibition of its closing into the pore. Interestingly the A-ring diol induces not one but additional four subconductance states. This suggests that the lactone is not essential, and that the potential free movement of the hydroxyl groups may impede closure of the inactivation particle to several different extents.

24.14 TRANSLATION OF IONIC EFFECTS OF BREVETOXINS TO PHYSIOLOGICAL RESPONSE

Brevetoxins' most noted action in excitable cells is increased sodium ion conductance through the four molecular actions described earlier leading to depolarization and increased firing of action potentials. However, a substantive complexity to brevetoxin action results from the integration of this action to the physiological response. Three factors that play an important integrating role include distribution of channels along different specialized regions of target cells, progression from excitatory to inhibitory action, and the disruption of higher-level homeostatic pathways. The distribution of distinct voltage-dependent sodium channel subtypes expressed in and within different excitable tissues leads to multiple effects of brevetoxin. For example, brevetoxin can act at the dendritic tree to depolarize the nerve cell, generate action potentials at the hilus, transduce action potential along the axon, and depolarize the presynaptic terminals to release neurotransmitters and depolarize postsynaptic specializations of nerve, skeletal, cardiac, or smooth muscle. A second integrating factor occurs with a reversal of brevetoxins' effect from excitatory to inhibitory. Within a few seconds, transient repetitive neuronal discharges are followed by action potential depression and eventually a complete blockade of neuronal excitability.[9] A higher level of complexity arises *in vivo*, where a third integrating factor results from brevetoxin disruption of the interplay between autonomic pathways at either the level of the peripheral or central nervous systems that serve to maintain the balance of critical physiological processes. Given the multiple levels of brevetoxin effects, a key consideration to evaluate brevetoxin toxicity, which often is reported at a single physiologic condition (cellular, isolated tissues/organs, surgical/conscious animals), is the relative sensitivity of each effect to brevetoxin.

24.15 BREVETOXIN SELECTIVITY FOR SODIUM CHANNEL SUBTYPES

The sodium channel alpha subunit is expressed in at least nine distinct gene products. The Nav1.1, 1.2, 1.3, 1.6 predominate in the central nervous system; Nav1.7, 1.8, and 1.9 in the peripheral nervous system; Nav1.4 in skeletal muscle and Nav1.5 in cardiac muscle. A voltage-dependent sodium channel for smooth muscle has been suggested to be Nav1.7, but this has not been universally accepted. The relative sensitivity of sodium channels has been examined both by examination of native tissue homogenates and expression of specific alpha subunits. Using HEK cell lines, expressing either the heart or skeletal muscle human sodium channel isoforms, type B brevetoxins show

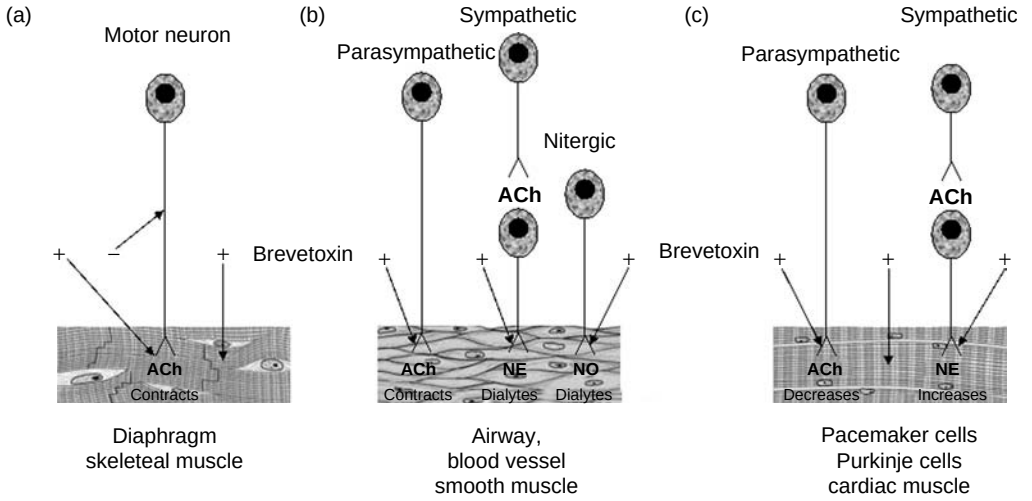

FIGURE 24.13 Schematic diagrams of brevetoxin actions on skeletal, smooth, and cardiac muscle. Brevetoxins' primary action on skeletal muscle is block of nerve conduction with secondary effect to depolarize motor neuron nerve terminals (a). It is much less effective to depolarize skeletal muscle. Brevetoxin causes contraction of airway muscle by depolarization of parasympathetic nerve terminals and smooth muscle of blood vessels and gut by depolarization of sympathetic nerve terminals to release norepinephrine to activate alpha-1 adrenergic receptors on muscle (b). Brevetoxin also dilates smooth muscle by depolarization of nitergic nerve terminals to release nitrous oxide. Dilation of certain smooth muscle in certain tissues is also possible by depolarization of sympthathetic nerve terminals to release neorepinephirne to activate beta adrenergic receptors (not shown). Brevetoxin causes bradycardia and tachydcardia by depolarization of parasympathetic neurons and sympathetic nerve endings, respectively (c). Loss of synchrony between parasympathetic and sympathetic activity leads to arrhythmias. Brevetoxin B is much less effective to depolarize cardiac muscle.

tissue selectivity for binding and activation of the cardiac isoform (Nav1.5) with a lower affinity than that of the skeletal muscle (Nav1.4).[90] This selectivity is not seen for type A brevetoxins. Investigation of sensitivity and selectivity of brevetoxin binding the expressed isoforms of sodium channels from the central and peripheral nervous system has not been examined to date. In native tissue, brevetoxin binding to cardiac tissue is below resolution by radioligand binding, whereas brevetoxin binding to skeletal muscle does not differ from its binding to brain.[91] These results indicate that brevetoxin B, should show a strong preference to nerve over muscle sodium channels in heart, but not necessarily in skeletal muscle. The physiological effects of brevetoxin on preparations of skeletal, smooth and cardiac muscle are described in the following section and the major effects are depicted in Figure 24.13.

24.16 EFFECTS ON SKELETAL MUSCLE

A major site of action for brevetoxin is at the neuromuscular junction, the interface of two potential targets, cholinergic nerve and the highly excitable motor endplate of skeletal muscle membrane. Brevetoxin action at the neuromuscular junction is associated with the symptom of fasciculation or twitching of muscle. However, brevetoxins most severe action on skeletal muscle is on the diaphragm, where it culminates to a flaccid paralysis of the diaphragm and respiratory arrest, the common lethal effects in animals. This action was first described by Borison et al. with a partially purified brevetoxin extract in anesthetized cats in counterpoint with the site 1 channel blocker saxitoxin, which although has an opposing molecular action, also leads to respiratory arrest.[92] The action of brevetoxin has been extensively studied in the rat phrenic nerve diaphragm preparation. Brevetoxin both increases the

frequency of miniature endplate potentials (MEPP), a reflection of depolarization of the presynaptic nerve terminal and directly depolarizes the muscle fiber.[71] However, brevetoxin was found to have a concentration-dependent effect on the frequency of MEPP, depressing the frequency at low doses. This lower dose effect of brevetoxin to depress MEPP frequency correlated with its inhibition of evoked endplate potentials (EPP) at substantially lower concentrations than its effects on MEPP and muscle depolarization. This study indicated that the toxin preferentially depresses muscle contractions by depolarization of the nerve terminal. Brevetoxin was confirmed to inhibit nerve-evoked contraction of diaphragm muscle, yet without a direct effect on the muscle depolarization.[37] In addition, this study also found that the brevetoxin-treated phrenic nerve terminals retained synaptic vesicles, indicating that brevetoxin inhibition of evoked muscle contraction was not due to depletion of neurotransmitter, but rather to persistent nerve depolarization. A later study found that brevetoxin affected action potential conduction in isolated phrenic nerve, initially increasing, but afterwards decreasing the action potentials.[93] Deshpande et al.[94] thoroughly re-examined the effects of brevetoxin on the phrenic-diaphragm preparation and clarified that the toxin preferentially blocks phrenic nerve conduction as its primary action in the diaphragm muscle preparation. Mechanistically, the reduction in voltage dependency in the sodium channel was proposed be sufficient to depolarize sufficient channels to prevent effective conduction of the phrenic nerve action potential.[94]

24.17 EFFECTS ON SMOOTH MUSCLE

A second major site of action for brevetoxin is at smooth muscle, also the interface of two potential targets, cholinergic or adrenergic nerve and the smooth muscle membrane. This action of brevetoxin has been most extensively studied in the canine tracheal smooth muscle preparation. This is of particular significance, because activation of the parasympathetic nerve shuts down airway smooth muscle, a cause of respiratory distress commonly found after exposure to brevetoxin containing aerosols. Brevetoxin induces contraction of canine tracheal smooth muscle strips, and this effect was blocked by the cholinergic antagonist atropine but not by antagonists of histamine (H1), ergot, muscarinic, nicotinic, or alpha adrenergic receptors.[10] This indicated that the action of brevetoxin was inducing the release of acetylcholine from the parasympathetic nerve endings on the smooth muscle. Concurrently, intraperitoneal administration of brevetoxin to ventilated guinea pigs was reported to cause bronchoconstriction, an action also blocked by atropine.[95] A later study with the canine tracheal smooth muscle preparation showed that brevetoxin induced depolarization and contraction in this preparation, but was not effective to induce these responses in cultured airway smooth muscle cells, indicating that like the neuromuscular junction, brevetoxin is substantially more potent on the parasympathetic nerve than on the muscle fiber.[96] The effects of brevetoxin have also been examined in lower (bronchial) airway smooth muscle. Brevetoxin induced atropine-sensitive contraction of both the canine and human bronchial smooth muscle.[97,98] Brevetoxin's effect on airway smooth muscle shows a rapidly decreasing response, or tachyphylaxis, after the first administration.[97,99] During brevetoxin-induced tachyphylaxis, the smooth muscle responses to acetylcholine and sodium channel site 2 and 3 activators remain intact[99]; yet, are reduced by the prostaglandin inhibitor indomethacin and enhanced by prostaglandins of the E-series, indicating the downstream involvement of these local mediators in this response.[97] The airway response to brevetoxins has more recently been examined using sheep model in which the toxin is administered by forced inhalation of toxin laden aerosols.[43] Brevetoxin caused a significant bronchoconstriction in both nonallergic and allergic sheep (exposed to *Ascaris suum* as allergen). Further analysis of this response in the allergic sheep indicated that it was blocked by atropine, and contrary to the studies with canine tracheal smooth muscle explants, also blocked by a histamine HI blocker diphenhydramine. Diphenhydramine furthered the inhibition of atropine and a stabilizer of mast cells, the cellular source of histamine, also reduced the brevetoxin induced bronchoconstriction. This response in asthmatic sheep indicates that brevetoxin effect *in vivo*, involves both an effect on the parasympathetic nerve enervating smooth muscle and a parallel action on mast cells.

Brevetoxin on smooth muscle includes action on sympathetic as well as parasympathetic nerves. A comparison of the effects of brevetoxin on guinea pig ileum and rabbit aorta muscle strips indicated contractions were selectively inhibited by either atropine or the alpha-adrenergic antagonist phentolamine.[100] The effect of brevetoxin has been characterized on a rat vas deferens smooth muscle preparation and also found to cause contractions selectively sensitive to alpha-adrenergic antagonists.[101] Brevetoxin contractions were blocked by the alpha-1 selective antagonist prazosin, but not by antagonists to muscarinic, nicotinic, histaminergic, ergot, or beta-adrenergic receptors. Brevetoxin contractions were also prevented by pretreating the rats with reserpine, a compound that depletes the synaptic content of sympathetic neurotransmitters. Together these results indicate that brevetoxin stimulates sympathetic nerves to release norepinephrine, causing contraction of smooth muscle.

Finally, brevetoxin effects on smooth muscle also involve noncholinergic and nonadrenergic nerves, known as nitrergic because of their release of nitric oxide as a neurotransmitter. Nitrergic nerves relax smooth muscle serving to dilate to increase blood flow or maintain muscle tone. Brevetoxin has also been found to induce relaxation of smooth muscle in rabbit corpus cavernosum preparations.[102] Brevetoxin caused a slowly induced relaxation of contracted muscle, which were reduced by nitric oxide synthesis inhibitor and restored by the nitric oxide precursor L-arginine. This suggested that brevetoxins action on these sympathetic nerves lead to the production of nitric oxide. The action of brevetoxin was also inhibited by a guanyl cyclase inhibitor and potentiated by a phosphodiesterase inhibitor consistent with activation of the nitric oxide-GMP pathway characteristic of nitrergic-smooth muscle relaxation.

24.18 EFFECTS ON CARDIAC MUSCLE

Brevetoxin causes both inotropic and arrhythmic effects on heart tissue. Brevetoxin actions on heart have been characterized in isolated heart preparation of the rat and guinea pig.[103] Brevetoxin increases the strength of muscular contractions in the rat and to a lesser extent in the guinea pig. These actions were only partially blocked by beta-adrenergic blocker or catecholamine depletion, suggested a combined effect of direct action on cardiac muscle and indirect action via sympathetic nerve endings. A primary action on nerve versus muscle cells is supported by the findings that brevetoxin B has a sevenfold higher affinity for binding and activating nerve over heart sodium channels.[90] Brevetoxins effects on the electrical conduction system were manifest in ventricular tachycardia and atrio-ventricular block.[103] Although the tachycardia was blocked fully by the beta-blocker and reserpine, indicating an indirect action on sympathetic innervation, the heart block persisted suggesting an indirect action on parasympathetic vagal innervation. Brevetoxin's effects on the electrical conduction in heart have been well characterized under different physiological conditions in several mammalian species. Owing to the interplay between homeostatic mechanisms, the effects of brevetoxin on cardiovascular function are complex. The initial action of brevetoxin on anesthetized dogs and cats is a reduction in heart rate, that is reversed by cholinergic inhibitors or bilateral vagotomy.[13,104] This bradycardic action of brevetoxin was proposed not to be a result from stimulation of the vagal ganglia or nerve endings, but rather by activation of peripheral sensors that regulate the overall tone of vagal activity. In anesthetized and ventilated dogs, vagotomy unmasks a tachycardic action of brevetoxin, resulting from activation of sympathetic cardiac nerves.[104] However, in nonanesthetized rats, no effects of brevetoxin are noted on heart rate[105], suggesting compensatory interplay between sympathetic and parasympathetic innervation on pacemaker cells. Nonetheless the adverse effect of brevetoxin on the conduction system of the heart are evident in several forms of ventricular arrhythmias culminating in heart block, indicative of a complete block of conduction through the Purkinje system. The mechanism for these arrhythmias is most likely the result of a combined and discordant activation of parasympathetic and sympathetic autonomic input to the conduction system of the heart disrupting the synchronization of cardiac muscle contraction.

24.19 EFFECTS ON AUTONOMIC CONTROL OF BREATHING AND BODY TEMPERATURE

The most common lethal effect of brevetoxin is respiratory arrest. Although brevetoxin has well defined effects to block conduction of the phrenic nerve, the toxin effect *in vivo* is associated with abnormal breathing patterns, normally indicative of damage to the midbrain. A series of elegant pharmacophysiological experiments by Ellis and colleagues conducted in anesthetized cats and dogs have elucidated unique effects of brevetoxins on the autonomic control of breathing.[13,92,104] These studies indicated a preference for low doses of brevetoxin to disrupt breathing via an autonomic reflex pathway and for higher doses to disrupt breathing by a direct action on the central nervous system. The action of brevetoxin to disrupt breathing, occurred without disruption of motor control of diaphragm contraction through stimulation of the phrenic nerve, indicating that *in vivo* blockage of the phrenic nerve may not be prerequisite to brevetoxin disruption of breathing. The autonomic reflex to brevetoxin is referred to, as the Bezold-Jarish reflex is triad response that includes hypotension, bradycardia, as well as slowing of breathing (bradypnea) (Figure 24.14). This response is mediated by carotid, aortic, and pulmonary sensor activation of vagal afferents of the nodose

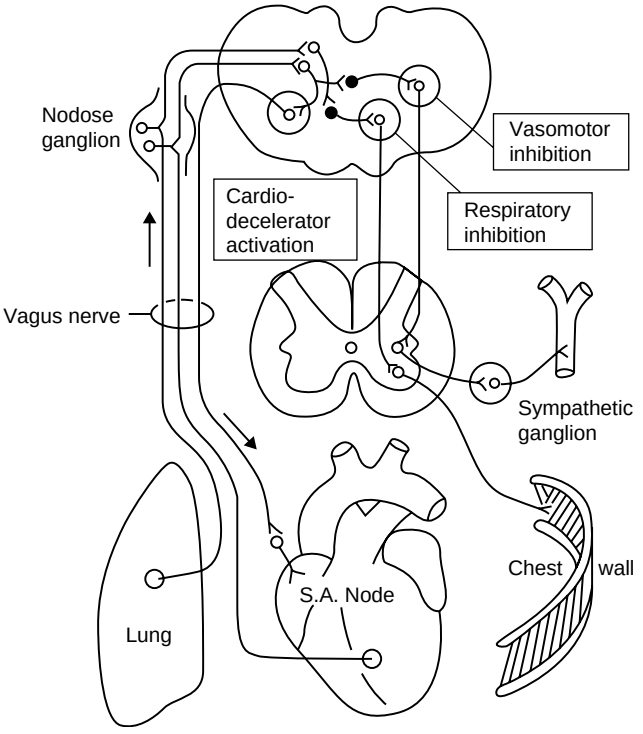

FIGURE 24.14 Neuropathways for brevetoxin activation of autonomic pathways controlling cardiopulmonary function. Nodose ganglion atrial afferents carried by the vagus nerve sense heart rate and project to the nucleus solitarius, which sends interneurons to the nucleus ambiguous (cariodecelarator), which activate parasympathetic efferents via the vagus nerve to slow heart rate. Nodose ganglion pulmonary afferents carried by the vagus nerve sense low oxygen and project to the nucleus solitarius, which sends inhibitory interneurons to the respiratory center in the midbrain, which activate descending pathways to the spinal cord to activate parasympathetic efferents via the phrenic nerve to the diaphragm (not shown) and motor nerves to the chest wall. Nodose ganglion atrial afferents carried by the vagus nerve sense heart rate and project to the nucleus solitarius, which sends inhibitory interneurons to the vasomotor inhibition center in the midbrain, which activate descending pathways to the spinal cord to activate pre- and post-sympathetic efferents to dilate vascular smooth muscle. (Reproduced from Borison, H. L., McCarthy, L. E., and Ellis, S., *Toxicon*, 23, 517, 1985. With permission.)

ganglion that project to nucleus solitarius of the midbrain. For control of breathing, relay neurons project from the nucleus solitarius to a respiratory center, which send descending pathways to spinal column, which activate motor neurons of the phrenic nerve to control diaphragm movements. The inhibition of heart rate is mediated by motor neurons of the vagus nerve and lowering of blood pressure by activation of sympathetic ganglion. The sensitivity of this autonomic reflex to brevetoxin is supported by sensitive effects of brevetoxin to induce ionic conductances in nodose ganglion cells.[8,87] In addition, systemically administered brevetoxin induces neuroexcitatory effects, as assessed by Fos immunostaining, in the nucleus of the solitary tract, which is consistent with brevetoxin activation of peripheral sensory vagal afferents (nodose ganglion cells) terminating in the brain stem.[106] By contrast, additional doses of brevetoxin administered to anesthetized cats lead to abnormal deep gasping patterns of breathing, and is associated with reversal of cardiovascular symptoms to hypertension, tachycardia.[13] These effects were also seen at lower doses that were preferentially directed to the brain, by intracerebroventricular or intracartid infusion with blockage of anastomotic flow back to the periphery indicating a direct action on the central nervous system. Higher doses of brevetoxin, lead to respiratory arrest; however, under respiratory support, the neuromuscular reflexes remained intact.

Templeton and co-workers approached brevetoxin toxicity with a complementary, yet somewhat different perspective. Using purified toxin and nonanesthetized animals (rats), they examined the effect of a 1 h infusion of four dosages of toxin as opposed to repeated bolus injections used for the anesthetized cat studies.[105] They next sought to distinguish central versus peripheral actions of brevetoxin through selective reduction of the peripheral actions of brevetoxin by infusion of antibrevetoxin serum, which would not readily penetrate the central nervous system.[107] Brevetoxin caused a dose-dependent reduction of breathing rate within minutes with effects at all doses. Heart rate was reduced at only the two highest doses and blood pressure was reduced only near death of the animals. Cardiovascular functions of the Bezold–Jarish reflex in the nonanesthetized rats may have remained more resilient to brevetoxin rats due to compensatory mechanisms to maintain blood gasses. Antibrevetoxin serum, given after the completion of the brevetoxin infusion readily reversed the depression of breathing rate and dramatically increased survival rate. This suggested that the action of brevetoxin on respiratory depression and respiratory failure is mediated primarily by a peripheral action, either directly or via the central nervous system. The observation that cutting the vagus does not alter the effect of aerosolized brevetoxin to reduce breathing rate in guinea pigs,[95] indicates that the afferent connection between chemosensors and nodose ganglion cells is not necessarily required to be intact. However, it is also possible that brevetoxin may short circuit this loop without activation of vagal afferent endings. Given that the nucleus of the solitarius is proximal to the area postrema, a circumventricular region that is not protected by the blood–brain barrier, this region of the central nervous system would be accessible to protection by the antibrevetoxin antibody. The area postrema is highly sensitive to toxins, including brevetoxin.[106] This organ has connections to the nucleus solitarius and its activation is reported to decrease breathing rate and cause respiratory arrest.[108] Thus, the proximal target for brevetoxin induced respiratory arrest is not certain; it would still appear likely that this effect is mediated by respiratory center of the midbrain.

Other than respiratory depression, the most prominent effect of brevetoxin on conscious rats is a reduction in core body temperature.[105] This effect occurs within minutes of brevetoxin infusion and is insensitive to reversal by infusion of antibrevetoxin, suggesting a direct action of brevetoxin on the central nervous system.[107] Further analysis of brevetoxin-induced hypothermia indicates that the toxin causes a forced rather than a regulated hypothermia, indicative of altered the central nervous system control of body temperature.[109] A central nervous system action of brevetoxin is further supported by the finding that brevetoxin induces Fos expression in neurons of the medial preoptic nucleus of the hypothalamus, component of the hypothalamus' thermoregulatory region.[106] The hypothermic response to toxins is limited to smaller mammals with a low surface to mass ratio; however, a delayed effect of brevetoxin is the induction of fever, indicating a more disruptive effect on the thermoregulatory region of the hypothalamus, which may potentially occur in larger animals well.[109]

24.20 SOMATOMOTOR DYSFUNCTION

Brevetoxin treatment to rats leads to several levels of disruption of motor control.[105] Gasping abdominal movements, loss of righting flexes, and uncontrolled muscle movements, including head bobbing and convulsive movements of hindquarters are all indications of dysfunction of central nervous system pathways descending through the spinal cord. They may occur at the level of the cerebellum, which coordinates the fine motor movement or at the junction of descending fibers to the spinal ventral root. Somatomotor seizures have also been reported after extended dosing in cats.[13] These effects have prompted a study of brevetoxin on the hippocampal slice preparation in guinea pig.[110] Addition of brevetoxin to the bathing solution of the slice preparation induced transient excitability of pyramidal cells, which leads to progressive depression of evoked responses. Brevetoxin also depressed antidromic responses; however at somewhat higher concentrations. These effects of brevetoxin were very similar to the effects of brevetoxin on the phrenic nerve-diaphragm preparation discussed earlier. However, the main objective of this study was to determine if brevetoxin induced seizure-activity in the hippocampus. Although brevetoxin, induced additional discharges after evoked responses, it did not produce the spontaneous synchronous epileptiform activity seen with the glutamatergic agonist NMDA. Although this might have been related to the presence of voltage-gated sodium channels on inhibitory neurons as well as the pyramidal cells, the fact that depolarization by elevation of potassium caused epileptiform activity whereas depolarization by brevetoxin did not indicate that conductance properties of the sodium channels activated by brevetoxin do not support induction of seizure activity in the hippocampus.

24.21 STRUCTURAL EFFECTS ON NERVE CELLS

The action of brevetoxins has been characterized in cerebellar granule cells, the feedforward excitatory interneurons that connect input to the cerebellum to Purkinje cell output to deep cerebellar nuclei controlling fine motor control. This region of the brain is also reported to show selective uptake of [^{3}H]-PbTx-3.[34] Cerebellar granular cells are commonly utilized as dispersed cell populations because of their relative homogeneity and great abundance for investigation of excitatory amino acids. Brevetoxin has been demonstrated to induce granule cell cytotoxicity, via an action that is mediated by the release of glutamate and activation of NMDA receptors.[38] The cytotoxic action of brevetoxin is the result of indirect activation of the well-characterized Ca^{2+} overload mechanism for excitatory neurotransmitters.[111] Cellular actions of brevetoxin have also been examined in a second primary culture of embryonic neurons, neocortical cells.[112] In this case, brevetoxin increased glutamate-mediated calcium oscillations; however, it did not induce excitotoxicity of these cells, which like cerebellar granule cells are sensitive to NMDA-mediated cytotoxicity. Hence, the brevetoxin-induced cytotoxicity *in vitro* may not necessarily occur simply upon the release of glutamate and activation of NMDA receptors. Factors such as subpopulations of inhibitory interneurons may restrict the indirect cytotoxic effects of brevetoxins. More recently, brevetoxin administered by repeated inhalation exposure has been reported to cause cell degeneration in the posterior cingulate/retrosplenial cortex as assessed by cupric silver and fluoro-jade B histochemistry.[113] Interestingly, cell death was not found in the either cerebellum or elsewhere in the brain. Posterior cingulate/retrosplenial cortex is a cortical brain region highly sensitive to glutamatergic excitotoxicity. Collectively these findings indicate that brevetoxin has the potential to induce excitotoxic neuronal injury, but under very selective conditions.

Brevetoxin also causes structural effects in peripheral nerve through depolarization induced osmotic changes that lead to cell swelling in regions of high channel density. This swelling can take place at nerve endings and might be associated with paresthesias associated with toxin exposure.[114] Brevetoxin also induces axonal membrane swelling at the Nodes of Ranvier, where it may contribute to nerve conduction deficits.[115]

24.22 POTENTIAL FOR DEVELOPMENTAL TOXICITY

Brevetoxin exposure of pregnant rats leads to distribution of the toxin to the fetuses.[116] Using both airway and parenteral exposure, brevetoxin accumulated in fetus of GD 21 mice reaching tissue concentrations close to those achieved in other tissues and within a factor of 0.3 of its concentration in blood. Although the amount of toxin (84 fg/g tissue) projected to reach a fetus from human exposure to aerosols is well below effect level, the amount that may reach the fetus during neurotoxic shellfish poisoning may be substantial. The potential effects of brevetoxin early in mammalian development has only been examined in cultured postnatal neurons where the intrinsic potency of brevetoxins are about 10-fold lower than that observed in rat brain synaptosomes and other cellular systems[38]; however, the effects of brevetoxin A and B have been characterized in Medaka embryos. Microinjection of PbTx-1 into the yolk sac of freshly fertilized embryos causes pronounced muscular activity (hyperkinesis) after embryonic day 4.[117] As the animals mature toward hatching, lateral curvature of the spinal column is a common observation in the larvae. Although the observed twitching response may be due to direct activation of motor nerves, spinal defects may be the result of secondary responses to persistent activation of skeletal muscle by the toxin. PbTx-3 treated embryos developed hyperkinetic twitches in the form of sustained convulsions suggestive of a central nervous system effect, but overall develop fewer spinal defects.[118] A delayed bradycardic response was observed on day 6 in the PbTx-1-treated fish whereas embryos treated with the PbTx-3 developed a sustained tachycardia. The opposing effects on heart rate of the two brevetoxin classes suggest differential activation of the sympathetic and parasympathetic nervous system by each class of toxin. The acute effects of brevetoxin on Medaka embryos are similar to those observed in adult mammalian species, indicating that brevetoxin can have comparable effects on adult and fetal somatomotor and autonomic functions in early in development.

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25 Detection of Brevetoxins in the Twenty-First Century

Mark A. Poli

CONTENTS

25.1 INTRODUCTION

Blooms of toxic dinoflagellates resulting in mass mortalities of inshore fishes have been reported in the Gulf of Mexico since the mid-nineteenth century [1]. These "red tides," characterized by dead and dying fish and discolored water, have occurred on a semiregular basis since that time and remain problematic to the coastal seafood and tourism industries of the Gulf states. In addition to fish kills, these blooms have been associated with morbidity and mortality of invertebrates and birds [2], bottlenose dolphins [3,4], and Florida manatees [5–7]. Human intoxications, known as neurotoxic shellfish poisoning (NSP), can occur by ingestion of either filter-feeding bivalve mollusks such as oysters and clams [8,9] or, less commonly, molluscivorous whelks [10]. Near-shore aerosolization of toxins from wind and wave action can also cause respiratory irritation in beachgoers [11,12].

In 1947, Davis [13] identified the causative organism of these toxic blooms as the unarmored dinoflagellate *Gymnodinium brevis* (later *Ptychodiscus brevis,* now *G. breve*), and a decade later, Starr [14] suggested that a lethal toxin elucidated by *G. breve* was the cause of the fish kills. With this knowledge came the need for detection methods to better understand the ecological and public health implications of brevetoxins in the environment.

For the next several decades, detection of brevetoxins was dependent on *in vivo* bioassays such as the mouse bioassay [15] or the fish bioassay [16]. These assays were based on exposure of living organisms through either natural routes, such as exposing fish by adding toxin to the water, or unnatural routes such as intraperitoneal injection of mice. In either case, the test organism was then monitored for a reaction to the administered toxin. Death was usually the end point, but important information could be gleaned through careful observation of symptoms and time to death. Death curves could be constructed and, when performed properly, quantitation could be quite good.

A major drawback to *in vivo* assays derives from their lack of specificity. Even with the information provided by extraction method, symptomatology, and death times, they could at best only define classes of compounds sharing a similar mechanism of action. Even so, they enjoyed wide use throughout the latter part of the twentieth century and are still in use today. During the late 1980s and throughout the 1990s, however, America experienced a groundswell of popular support for the idea of minimizing the use of animals in research. This, along with the resulting increase in regulation and rising cost of animal experimentation, was the driving force behind efforts to phase out

in vivo assays. In addition, new studies indicated a large number of brevetoxin congeners and metabolites, thus increasing the complexity of the scientific endeavor and making the differentiation of similar compounds at low concentrations increasingly necessary. The final decade of the twentieth century saw the rapid evolution of brevetoxin detection methods along two parallel lines: methods based on molecular pharmacology and methods based on molecular structure.

25.2 PHARMACOLOGICAL METHODS

Pharmacological methods are based on binding of the toxin to the endogenous sodium channel receptor. The simplest of these is the direct receptor-binding assay [17–19], which was made possible by the chemical synthesis of tritium-labeled brevetoxin by Dan Baden at the University of Miami [17]. This assay directly measured binding of radiolabeled brevetoxin to receptor sites in rat brain membranes. Set up in a competition format, it measured unlabeled toxin in unknown samples by detecting the decrease in bound radioactivity resulting from competition with labeled toxin for the available receptor sites. This assay was simple, straightforward, and reproducible. Sensitivity was less than 1 ng/mL, far superior to that of the mouse bioassay. Matrix effects, although variable, were not problematic. Later modified into a 96-well format for rapid screening [20], this assay has been extremely useful in both the research and regulatory environments. This usefulness was aided in part by its cross-reactivity with ciguatoxins, another group of polyether marine neurotoxins, and it has been invaluable for activity-based screening for new toxin congeners and metabolites [10,21,22].

Although the receptor binding assay utilizing direct detection of a radiolabeled probe bound to rat brain membranes is the simplest and most robust format of the pharmacological assays, other variations have also been developed. Trainer et al. [23] detected brevetoxins bound to purified sodium channels isolated from rat brain membranes and reconstituted into phospholipid vesicles. Although analogous to the membrane binding assay, time and labor costs associated with channel isolation and reconstitution precluded it from being used as a routine assay. Finally, receptor binding assays have been developed using membranes from animals other than rats [24,25]. These differ primarily in the details of membrane preparation and channel affinities between species. Although these variations have proven useful for evaluating ecological effects of brevetoxins in the environment, the receptor assay based on rat brain membranes has largely predominated in the research and regulatory science arenas.

While receptor binding assays measure only the binding event, other assays that measure various physiological events occurring downstream from receptor binding have been developed. Manger et al. [26,27] used cultured neuroblastoma cells as the sodium channel receptor source and measured cell viability as the assay end point. In this assay, channels were treated with veratridine in the presence of ouabain to inhibit sodium efflux through the Na^+–K^+ ATPase. Binding of brevetoxin led to sodium influx through the allosterically activated channels and reduced cell viability. Dose-dependent enhancement of cell death was measured by colorimetrically monitoring the cells' ability to metabolize a tetrazolium compound to a colored product. The reported detection limit was 25 ng/mL, and the 96-well format allowed for high sample throughput in a time frame of 4–6 h. Another advantage was flexibility; with minor modifications, the assay could test for sodium channel blockers such as saxitoxin and tetrodotoxin as well as activators such as brevetoxins and ciguatoxins. However, the cell-based assay also suffers from serious disadvantages. Highly trained operators are required, and the cell culture process requires a great deal of support in the form of feeding, splitting, and timing of cell cultures. More importantly, analysis of common samples indicated poor agreement with other assay methods for some toxins [28,29]. These differences have not been fully explained, but may result from artifacts inherent to the use of immortal cell lines that differ in their cellular physiology from normal cells.

Another version of a cell-based assay was the reporter gene assay [30,31]. This assay used a *c-fos*-luciferase construct transfected into neuroblastoma cells as a "reporter" of brevetoxin interaction.

The assay end point was light generated through the luciferin/luciferase pathway. Although the signal was modest, the authors reported a useful detection limit of about 3 ng/mL [31]. The primary drawback to the assay, along with the previously mentioned issues of cell culture, was the lack of specificity inherent to using a reporter of nonspecific cellular stress to generate the assay end point. The resulting wide range of potential matrix effects likely precluded this assay from being widely accepted.

Kerr et al. [32] developed an assay based on an *in vitro* neurophysiological model. This assay measured changes in antidromic and orthodromic spike amplitudes and afferent action potential volley in rat hippocampus slices after toxin exposure. The authors felt they could differentiate brevetoxins, saxitoxin, and domoic acid from characteristic signatures based on these parameters. The assay was relatively rapid and was also demonstrated to be useful in the evaluation of shellfish extracts. However, the use of animals, plus the complexities of tissue preparation and the need for expensive equipment and highly trained operators severely limited the usefulness of this assay.

In general, assays based on the pharmacological action of the toxins have the advantage of physiological relevance, but suffer from the disadvantage of reduced specificity. They are limited to detecting toxins of like pharmacology, and thus can differentiate only to the level of compounds binding to the same receptor or eliciting the same physiologic response. They provide a measure of total toxic potential of the sample, but are unable to differentiate individual congeners. Composite biological activity is useful for regulatory screening, but can be misleading if samples contain vastly different toxin profiles. This is especially true if toxin congeners are metabolized to more toxic compounds *in vivo*.

25.3 METHODS BASED ON TOXIN STRUCTURE

Structurally based methods are of two types: those based on recognition of structural components of the target by antibodies (immunoassays), and those based on chemical interactions of the molecules in a physical environment (analytical methods).

25.3.1 Immunoassays

Immunoassays for brevetoxins began with the first production of antibodies by Baden et al. [33]. Their immunogen was a PbTx-3/bovine serum albumin (BSA) conjugate injected intramuscularly into a goat. Using [^{3}H] PbTx-3 as the labeled probe, this serum was shown to be specific for the PbTx-2-type backbone structure, with minimal cross-reactivity to the PbTx-1-type backbone. No binding affinity was measured, but competition curves with unlabeled toxin suggested a detection limit around 3–4 ng/mL. No assay optimization or verification data were included, but this remains the first brevetoxin immunoassay, and it was significant because it improved detection sensitivity by at least two orders of magnitude over the mouse bioassay.

Poli and Hewetson [34] also produced polyclonal antibodies in goats by immunization with an analogous immunogen. These antibodies were of high affinity (Kd = 0.8 nM) and were neutralizing *in vivo* in rat model [35]. From this serum, they developed, optimized, and characterized a specific and sensitive radioimmunoassay (RIA) specific for the PbTx-2-type brevetoxins. Again, cross reactivity with the PbTx-1-type toxins was minimal, and the lower limit of detection was 0.3 ng/mL. The assay was optimized for shellfish extracts as well as human urine, which is an important matrix for human exposure. It was later used to detect brevetoxins, as well as brevetoxin metabolites, in the urine of NSP victims [10,18]. Levine and Shimizu [36] elicited anti-PbTx antibodies in rabbits, also using a PbTx/BSA conjugate. As with the goat sera described above, specificity was to the PbTx-2-type brevetoxins, with minimal recognition of PbTx-1. Although the binding affinity of the serum was very high (Kd = 8–12 pM), the titer was low, and the serum was never fully developed and validated as a functional assay.

RIAs are rapid, sensitive, and reproducible, but not well suited for field use owing to the requirement of radioisotopes for signal generation. To address this problem, as well as those associated

with handling and disposal of radioactive toxins, several research groups began developing colorimetric or other assay formats. The first of these was that of Trainer and Baden [37], who developed a direct enzyme-linked immunosorbent assay (ELISA) to detect brevetoxins in seawater and/or cell cultures. In this assay, a sandwich was formed between brevetoxin adsorbed to the surface of 96-well microtiter plates, a goat antibrevetoxin antibody, and a rabbit-anti-goat antibody conjugated to horseradish peroxidase (HRP). When used to evaluate *G. breve* cell cultures, a linear increase in color development was seen over the range of 1–100 cells, with an assay time of 5–6 h.

Garthwaite et al. [29] developed a competitive ELISA based on rabbit antibodies and a brevetoxin/peroxidase conjugate. In this assay, antibodies were coated on the plate, and free toxin in the sample competed with peroxidase-labeled toxin for antibody binding. Upon addition of enzyme substrate, a concentration-dependent reduction in color development was observed. Initial assay performance was modest, but careful optimization of assay parameters later resulted in reduced assay time (1–2 h) and a working range of approximately 0.5–17 ng/mL. This assay has been used extensively in New Zealand to evaluate toxin production in *G. breve* cell cultures as well as regulatory screening of shellfish.

Naar et al. [38] developed a competitive ELISA using goat polyclonal antibodies and a three-step signal amplification process. This amplification process reduced nonspecific binding associated with complex matrices. More importantly, the assay utilized a simple aqueous extract of shellfish and was also optimized for seawater as well as human serum and urine. Their reported detection limit in oysters was 2.5 μg/100 g shellfish meat. The working range of the assay was 0.2–2 ng/mL, and analysis was performed without dilution in seawater, serum, or urine. This assay is notable in that it eliminated the principal barrier to high-throughput toxin testing, which is the sample preparation time. Their simple aqueous homogenates abrogated the need for laborious solvent extraction of these lipophilic toxins. This assay is currently commercially available, and is in the process of undergoing the rigorous multilaboratory validation procedure required to receive Official Method of Analysis status from the Association of Official Analytical Chemists (AOAC).

Finally, Poli et al. [39] developed a competitive electrochemiluminescence (ECL)-based immunoassay for the PbTx-2-type brevetoxins. This assay is analogous to RIA, except that the probe consisted of brevetoxin conjugated to an electrochemiluminescent ruthenium chelate rather than [^{3}H] to produce the desired assay signal. Also developed using a polyclonal goat antibody, this assay could accurately detect brevetoxins in organic extracts of shellfish tissues, as well as aqueous homogenates and human clinical samples. The assay was rapid (~2 h), simple (only two additions, one incubation period, and no wash steps), and sensitive (LOQ = 50 pg/mL, or 1 ng/g tissue). Formatted in 96-well plates and accurate in analyzing simple aqueous extracts of shellfish, it is amenable to rapid-throughput screening. Further, it is sufficiently sensitive to support pharmacokinetic studies in animals and clinical evaluation of NSP victims.

While immunoassays achieve impressive sensitivity with a modest requirement for high-tech instrumentation and technician training, they are not without their drawbacks. Like specific receptors, antibodies are specific only for toxin structural components. Different congeners and metabolites are often recognized with equal or nearly equal affinity. Unlike the receptor-based assays, there is no connection to physiological events, so antibody recognition is not a measure of toxicity; congeners of vastly different toxicity can be recognized equally. Although very useful for screening for the presence of brevetoxins as a class, they cannot be used to estimate toxicity in a mixed sample, generate toxin profiles, or follow environmental or metabolic conversions. These issues are best addressed by analytical methods.

25.3.2 Analytical Methods

Early isolation of brevetoxins from cell cultures relied heavily on thin-layer chromatography, flash chromatography, and high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection [8]. These methods can simultaneously separate and detect toxins in a mixture, but can suffer from

interferences from coeluting peaks. In addition, they provide insufficient sensitivity for monitoring seafood products or environmental samples. While HPLC or some other chromatographic method remained necessary to separate toxins from other sample constituents, detection of toxin peaks soon evolved towards more sensitive methods. Dickey et al. [40] derivatized the brevetoxin PbTx-3 with 7-diethylaminocoumarin-3-carbonyl azide to produce the diethylamino-coumarin-carbamate derivative, which they then detected by fast atom bombardment mass spectrometry (FAB-MS). Although this method was not amenable for routine screening because of the necessity of conjugating and prepurifying the toxin, it pointed out the potential usefulness of MS detection.

Shea [41] developed a method that separated the toxins with micellar electrokinetic capillary chromatography, followed by detection with laser-induced fluorescence. This method was sufficiently sensitive to follow food chain transfer of toxins from *K. brevis* cells in culture to planktivorous copepods and from the copepods to finfish [42]. However, the requirements for derivatization of the sample and assay complexity again worked against common acceptance of this method.

Lewis et al. [43] optimized conditions for electrospray ionization mass spectrometry (ESI-MS), and reported excellent results for a range of marine toxins, including brevetoxins, using pure standards. Hua et al. [44,45] coupled HPLC with ESI-MS to separate and detect at least six brevetoxins from cell culture. Using standard solutions of brevetoxins, the detection limits appeared to be in the high ng/mL range. Quilliam [46] also developed an HPLC-ESI-MS method for brevetoxins, which he reported could detect as little as 10 pg brevetoxin when using selected ion monitoring of the $[M+H]^+$ ions. He also detected all known brevetoxin constituents from these cultures using this method.

This early developmental work led to MS being routinely used in brevetoxin analysis in a range of applications. Hua and Cole [47,48] used ESI-MS to evaluate the solution reactivity of brevetoxins, as well as to derive structural information on the protonated forms. Bourdelais et al. [49] unequivocally linked brevetoxins to a bloom of *Chattonella* cf. *verruculosa* in Delaware Bay, expanding the known range of their environmental impacts. However, while the coupling of reverse-phase HPLC and ESI-MS worked well in seawater solutions or other clean buffers, extracts of shellfish tissue using solvents consistent with the lipophilic nature of the toxins were problematic due to suppression of ionization by lipid constituents in the extract. This required sample extraction and solid-phase clean-up steps before liquid chromatography mass spectrometry (LC-MS) analysis, but otherwise did not present a significant obstacle. Once these procedures were optimized [50,51], LC-MS detection of brevetoxins became an indispensable method for the protection of seafood safety. It is currently routinely used to regulate brevetoxins in shellfish in New Zealand and the United States, as well as to evaluate potential problems stemming from brevetoxins in the environment [50,52]. Appropriately, an LC-MS method for identification of brevetoxins in shellfish, jointly developed by the United States and New Zealand, is currently being evaluated for AOAC certification as an Official Method of Analysis.

In addition to detection of individual known brevetoxins, LC-MS has been instrumental in the discovery of new brevetoxin derivatives. Compounds identified as conjugated metabolites of brevetoxins have been isolated and identified from the cockle (*Austrovenus stutchburyi*), Pacific oyster (*Crassostrea gigas*), and greenshell mussel (*Perna canaliculus*) in New Zealand [53–55]; the eastern oyster (*Crassostrea virginica*) in the Gulf of Mexico [56]; and whelks (*Busycon contrarium*) and clams (*Chione cancellata* and *Mercenaria* spp.) from Sarasota Bay, Florida [10]. It appears that different species of shellfish metabolize brevetoxins in different ways and that different metabolite markers may be required for optimal surveillance of the fisheries. The identification of a series of conjugated brevetoxin metabolites within the last decade and the resulting interest in metabolism and trophic transfer attest to the ability of increased analytical capability to add new dimensions to a research field.

25.4 SUMMARY

As we enter the twenty-first century, we now possess a reasonable arsenal of detection methods to support both the regulatory and the research communities. Immunological methods can provide

sensitive screening capability and high-throughput capacity. These allow rapid and reliable evaluation of the presence of brevetoxins as a class in seawater, seafood stocks, or environmental or clinical samples. Receptor-binding assays can be used as confirmation for immunologically based screening assays, or independently if toxicity is the desired end point measurement.

When identification of individual toxins is desired, LC-MS has evolved as the analytical method of choice. Capable of high-level resolution and selectivity, it is ideal for complex matrices or mixtures of analytes. A new generation of lower-cost and user-friendly instruments has brought the cost and complexity within the reach of most laboratories. Fully automated high-throughput instruments are ideal for the regulatory laboratory, and sensitivities are well within the needs of the research community. The strength of this instrument lies in its ability to separate toxins in a mixture while providing high-confidence identity based on mass.

The challenge for the future lies in extending our capabilities outside of the laboratory. Innovative technologies are needed to bring the sensitivity and selectivity of our laboratory instruments to the dockside, the beach, or the home. Hand-held instruments or simple test kits need to be available to the fisherman, recreational harvester, or consumer. Ideally, these kits will have minimal requirements for organic solvents, they will be multiplexed to include several potential toxin hazards, and they will be validated and certified as reliable. Smaller, less expensive LC-MS instruments capable of operating from boats, docks, or vehicles are needed for rapid field testing and analysis. This will aid event response teams and help provide important and timely information to program managers for better event management and protection of public health. Finally, identification of new and/or relevant metabolites among different fisheries species will allow easier and more reliable regulation of seafood products and better protection to the consumer.

DISCLAIMER

The views, opinions, and/or findings contained herein are those of the author and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

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Part VIIID

Cyclic Imines

26 Cyclic Imine Toxins: Chemistry, Biogeography, Biosynthesis, and Pharmacology

Allan Cembella and Bernd Krock

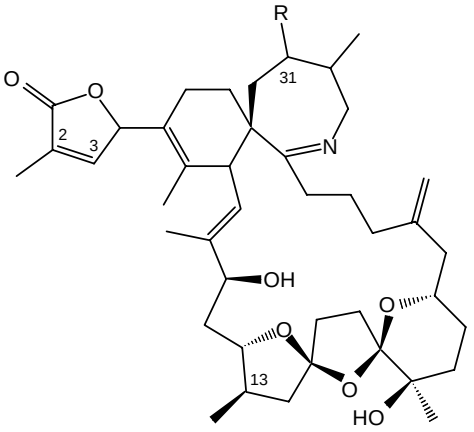
CONTENTS

26.1 INTRODUCTION

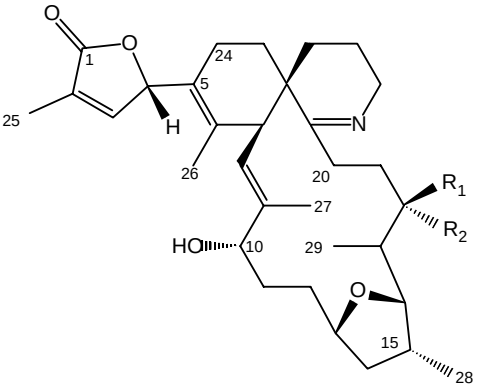
The cyclic imine toxins are a heterogeneous group of marine natural products sharing common macrocyclic features and the presence of an imine moiety, which is presumed to be the bioactive pharmacophore. This toxin group is known to comprise gymnodimines [1], pinnatoxins [2], prorocentrolides [3,4], pteriatoxins [5], spirolides [6], and spiro-prorocentrimine [7]. Comparison of the skeletons of the six cyclic imine toxin subclasses shows a high degree of structural similarity (e.g., ~70% homology between pinnatoxins and spirolides) (Figure 26.1).

Some cyclic imines, such as pinnatoxins and pteriatoxins, have been found exclusively in shellfish, although it is generally assumed that their presence in shellfish represents dietary incorporation and not *de novo* synthesis. This conclusion is supported by evidence that all of the other known cyclic imine toxins (gymnodimine, spirolides, spiro-prorocentramine, and prorocentrolides) originate in benthic or pelagic species of marine dinoflagellates.

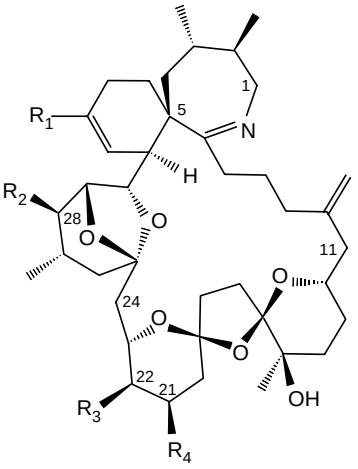
The cyclic imine toxins are usually relegated to the category of miscellaneous "emerging toxins" of uncertain human health concern. This is at least partly due to their relatively recent discovery



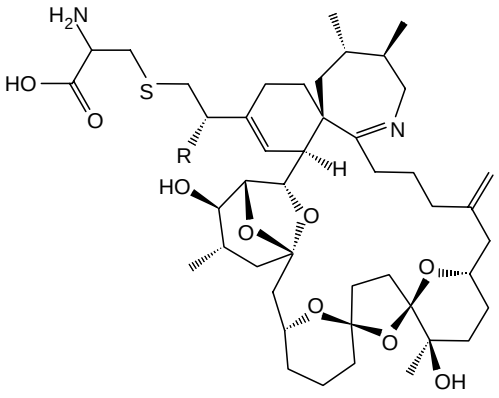
Spirolides



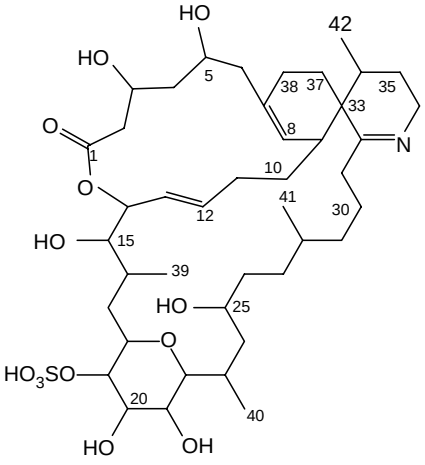
Gymnodimines



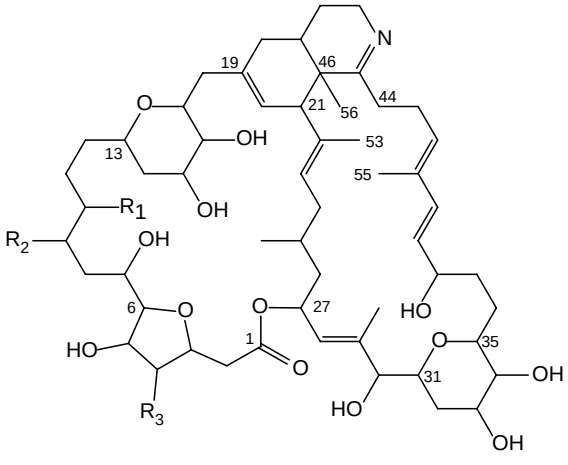
Pinnatoxins



Pteriatoxins



Spiro-prorocentrimine



Prorocentrolides

FIGURE 26.1 Major structural features of the cyclic imine toxins.

within the last two decades, but also to the delayed recognition of the imino group as the common pharmacophore among these compounds. In all cases, the biological activity of undescribed cyclic imine toxins was first recognized via their "fast acting toxicity" with an acute threshold response ("all or nothing") in mammalian bioassays. This acute reaction has been exploited in bioassay-guided fractionation schemes for structural elucidation of new macrocyclic imines by advanced liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) technologies. Unfortunately, the known structural homologies and thus presumably functional activity and mode of action have not led to a consequent synthesis of the toxicological implications. Absence or paucity of standards and reference material for analysis of most macrocyclic imines, lack (until recently) of high-resolution analytical methods, and shortage of purified material for conducting toxicological trials have impeded this synthesis. Reluctance of regulatory authorities to engage in complex studies of the acute and chronic effects of new "toxins" in the absence of demonstrable evidence of human cases of intoxication has also been a factor. Consequently, in spite of these unresolved issues, no regulatory limits have been established for any of the macrocyclic imines and they are assumed to be "safe for human consumption" until proven otherwise.

26.2 BIOLOGICAL ORIGIN AND BIOGEOGRAPHY

With the exception of the spirolides, which appear to have a global distributional range (Figure 26.2), other macrocyclic imines have been found only in a few locations and in a taxonomically limited group of vector species. This restricted biogeographical distribution may be expected to expand with increasing vigilance for biotoxins monitoring and advances in confirmatory analytical methodology. Nevertheless, to date, the pinnatoxins and pteriatoxins have been found only in the bivalve shellfish *Pinna muricata* [2,8,9] and *Pteria penguin* [5], respectively, from Okinawa in the Western Pacific. A multiple human poisoning incident in Guangdong, China, caused by the consumption of *Pinna attenuata* was first circumstantially linked to the presence of neurotoxins (e.g., pinnatoxins) [10], but later the cause was more plausibly attributed to the pathogenic bacterium *Vibrio parahaemolyticus* (T. Yasumoto, pers. comm.). The biological origin is unknown, but these toxins are strongly

FIGURE 26.2 Global distribution of spirolides in populations of the toxigenic dinoflagellate *Alexandrium ostenfeldii* and where the occurrence of these toxins in shellfish is proven or circumstantially associated with this dinoflagellate.

suspected to be of marine dinoflagellate origin, based upon their high degree of structural similarity and common features of the polyketide biosynthetic pathways for spirolides and the linear polyether dinophysistoxins [11,12].

The gymnodimines first came to scientific attention after a neurotoxic shellfish toxicity incident in New Zealand in 1992–1993 [13]. In the following year, acetone soluble extracts of oysters caused aberrant symptoms (rapid death accompanied by jumping, convulsions, and then paralysis) in the intraperitoneal mouse bioassay for lipophilic toxins [1]. These novel macrocyclic imines called gymnodimines were ultimately absolved of responsibility for the cases of human poisoning. The culprit species of gymnodimine toxicity was first attributed as the marine dinoflagellate *Gymnodinium* sp. [1], and described as closely related morphologically to *G. mikimotoi* (now *Karenia mikimotoi*), which is more commonly known for ichthyotoxic incidents, particularly in the eastern Pacific and northern Europe. Subsequent taxonomic revision assigned the gymnodimine producer from New Zealand to a new dinoflagellate species *K. selliformis* [14] (formerly *G. selliforme*). Gymnodimines are widely found in low concentrations or trace levels in shellfish from New Zealand [15], but toxicological experiments have indicated that these compounds are of low risk to humans by oral ingestion, and they are therefore not subject to a regulatory limit. Recently, gymnodimines have been detected in shellfish from the Moreton Bay region in Australia and also appear to be associated with *K. selliformis*, as in New Zealand.

As the names suggest, the prorocentrolides and spiro-prorocentrimine are produced by members of the dinoflagellate genus *Prorocentrum*. To date, the occurrence of these macrocyclic imines is restricted to primarily benthic or epiphytic *Prorocentrum* species, which tend not to form dense blooms unlike their pelagic counterparts. The taxonomic status of the benthic *Prorocentrum* group is currently in a state of flux, but nevertheless the assignment of prorocentrolide [3] and prorocentrolide B [4] to *P. lima* and *P. maculosum* (formerly attributed as *P. concavum*) [16] is probably correct. Spiro-prorocentrimine is produced by a yet undetermined benthic epiphytic species of the genus *Prorocentrum* [7]. The biogeographical distribution of these compounds in shellfish or causative dinoflagellates is virtually unknown because most work has focused on the isolation and structural elucidation of these compounds from a few cultured isolates, primarily from the subtropics. The benthic habitat and relatively low cell concentration may account for the typically low or absent concentrations of these metabolites in shellfish. Although prorocentrolides and spiro-prorocentrimine exhibit the classic "fast acting toxicity" [4] and low toxic threshold response in intraperitoneal administration to laboratory mice, they are normally considered as bioactive natural products rather than as phycotoxins with associated acute human health risk. No incidents of human toxicity have been linked to these compounds, and therefore, they are not subject to regulatory controls.

The spirolides are perhaps the best-studied subgroup of macrocyclic imine toxins. Detection of spirolide toxicity was first revealed in the course of routine intraperitoneal mouse bioassays for the lipophilic diarrhetic shellfish poisoning (DSP) toxins. Beginning in 1991, cultured blue mussels (*Mytilus edulis*) and sea scallops (*Placopecten magellanicus*) from coastal embayments in Nova Scotia, Atlantic Canada, elicited an unusual seasonal toxicity response in laboratory mice—characterized as rapid death, preceded by arching of the back, tail whirling, hyperactivity, and convulsions. Although anecdotal reports of symptoms (tachycardia and digestive discomfort) in humans who consumed shellfish from locations in Nova Scotia at periods during which spirolide concentrations were at seasonal maxima (typically May–June) are acknowledged, no causative link to spirolides was established. Since the initial toxicity reports and structural identification of spirolides from Nova Scotia in the 1990s, these compounds have been detected at widely dispersed locations around the globe. Spirolides have been detected in shellfish and/or in plankton fractions from the North Atlantic at multiple locations in Atlantic Canada, the Gulf of Maine, the United States [17], and in fjords in Denmark and Norway [18], the Bay of Biscay, France, and along the south coast of Ireland and western Scotland [19]. Perhaps more unusually, spirolides have also been found in Mediterranean shellfish from the Adriatic coast, in plankton isolated from the Gulf of Bothnia,

Baltic Sea, and harvested by adsorption from the water column from aquaculture sites on the Catalan coast (Ebro Delta) [20]. Spirolides in Chilean mussels have not yet been clearly associated with a specific planktonic source. For many years, New Zealand was considered to be "spirolide-free" and no spirolides were found in natural populations or isolates of the putative causative organism [21], but now these cyclic imines have been detected in shellfish and in a few cultured isolates [22].

Following the identification of spirolides in shellfish, related analogues were found in plankton size fractions dominated by presence of "golden balls" of mean diameter 40–45 μm [23]. These were later identified as athecate forms (pellicular cysts) of gonyaulacoid dinoflagellates. Clonal isolation and culture of these cells revealed the dinoflagellate *Alexandrium ostenfeldii* as the proximal source of spirolides [24]. Subsequent field and culture studies on *A. ostenfeldii* from Nova Scotia, Denmark, the Gulf of Maine, the United States, Ireland (Bantry Bay), the Gulf of Bothnia, and New Zealand have confirmed this species as the primary (perhaps exclusive) origin of spirolides.

26.3 ISOLATION AND PURIFICATION OF CYCLIC IMINES

Isolation and purification of all cyclic imine toxins may be subdivided into three basic operations, as depicted in Figure 26.3 and described as follows:

1. Extraction with intermediate polar organic solvents, such as aqueous methanol, aqueous ethanol, or acetone
2. Liquid–liquid partitioning, first against *n*-hexane for lipid removal and then with slightly more polar solvents, such as ethyl acetate, dichloromethane, chloroform, or 1-butanol

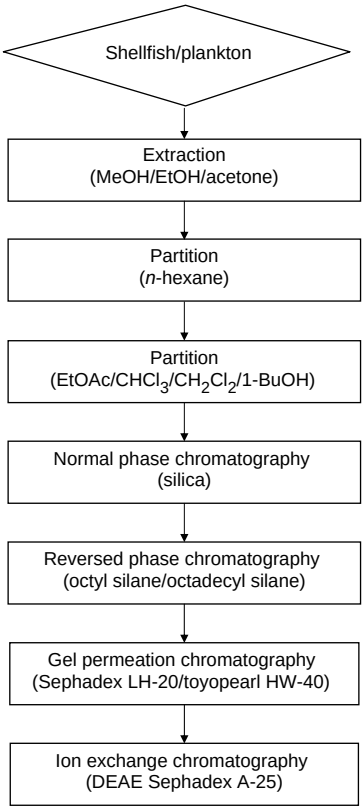


FIGURE 26.3 Purification and isolation scheme for cyclic imine toxins.

3. Chromatographic separations performed on normal phases, reversed phases, size-exclusion gels and/or ion-exchange resins.

The chromatographic techniques and their order of application vary among laboratories and the respective cyclic imine toxin type. Four to five subsequent separation procedures are typically employed for final isolation of single toxins.

Uemura and coworkers [2,9] isolated pinnatoxins by methanolic extraction and partition against ethyl acetate. The residue of the aqueous phase was twice separated by gel-permeation chromatography (GPC) on different polymers, ion exchange chromatography (IEC), reversed-phase middle-pressure liquid chromatography (RP-MPLC), and finally RP-HPLC. Takada et al. [5] used a slightly different order of chromatographic steps for the isolation of pteriatoxins: they exchanged the second GPC step with IEC and used an octyl silane (OS) instead of an octadecyl silane (ODS) phase for the final purification.

Gymnodimines were isolated from shellfish tissue by Seki et al. [25] by extraction with acetone. The dry residue was taken up in 80% methanol and partitioned with *n*-hexane and subsequently against ethyl acetate. The organic fraction was chromatographed twice on OS, ion-exchange resin and finally by GPC. The same group isolated gymnodimines from dinoflagellates by applying liquid–liquid chromatography and GPC after the two partition steps. A different approach was adopted by Miles et al. [26] for isolation of gymnodimines from dinoflagellates, taking advantage of the amphoteric nature of the target compounds. The methanolic (80% MeOH) cell extract was adjusted to pH 8.5 and then partitioned against dichloromethane. The organic fraction was evoporated dryness and then taken up in 0.5% acetic acid and partitioned against diethyl ether. The aqueous fraction was again adjusted to pH 8.5 and subsequently extracted with dichloromethane and the residue chromatographed on ODS.

Prorocentrolide was extracted by Torigoe et al. [3] from *P. lima* with methanol and acetone; dried extracts were reconstituted in water and partitioned against diethyl ether. After extracting prorocentrolide with 1-butanol, the fraction was then chromatographed over silica and ODS. Prorocentrolide B was isolated by Hu et al. [4] from the related species *P. maculosum* by extraction with methanol. The aqueous methanol extract was partitioned against *n*-hexane, diethyl ether, which contained the DSP toxin fraction, and finally 1-butanol for the extraction of prorocentrolide B. This fraction was further purified by GPC and two separation steps on ODS.

Spiro-prorocentrimine was isolated from *Prorocentrum* cells by Lu et al. [7] by methanolic extraction and subsequent partition against hexane, chloroform, and 1-butanol. Combined chloroform and butanol phases were subjected to flash chromatography on silica, RP-HPLC on ODS, GPC, again silica flash chromatography, and RP-HPLC on ODS.

Spirolides were first isolated from shellfish by Hu et al. [6] using 80% aqueous methanol for extraction. The second partition was performed against chloroform and the residue was subsequently chromatographed on silica, ODS, Sephadex LH-20, and again ODS. This procedure was followed by Aasen [18], who isolated spirolide G from shellfish and plankton. In the isolation of spirolide G and didesmethyl spirolide C from plankton McKinnon et al. [27] omitted the partition against hexane and chromatographed the residue on Sephadex LH-20, and subsequently twice on ODS. Sleno et al. [28,29] used fewer steps, but they only purified planktonic extract for structural analysis by LC-MS/MS and did not intend to isolate single compounds. For the same purpose, Ciminiello et al. [30] purified methanolic extracts only by solid phase extraction (SPE) on ODS.

26.4 COMPARATIVE STRUCTURES AND CHEMICAL PROPERTIES

26.4.1 General Characteristics

All the macrocyclic imines possess several characteristics in common: (1) a six- or seven-membered imino ring; (2) a cyclohexenyl ring with spiro linkage to the cyclo-imine (except for the prorocentrolides with condensed rings); (3) para-substitution on the cyclohexenyl ring; (4) a macrocycle

TABLE 26.1
Diagnostic Structural Features of the Major Macrocyclic Imine Subgroups

	Gymnodimines	Spirolides	Spiro-Prorocentrimine	Pinna-/Pteriatoxins	Prorocentrolides
Lactone	Yes	Yes	Yes	No	Yes
Cyclic imino ring size	6	7	6	7	6
Atom size of macrocycle	16	23/24	25	27	26
Ether bridges in macrocycle	1	3	1	5	1
Hydroxy functions on macrocycle	1/2	2	4	2	4
Number of unsaturations in macrocycle	2	2	1	1	4

comprising 16–27 carbon atoms; and (5) five- or six-membered cycloether entities in the macrocycle. Some additional chemical functions are summarized in Table 26.1.

The carboxy function at the end of the side chain of the cyclohexenyl ring is a very noteworthy feature to be compared among the cyclic imines. In the case of gymnodimines and spirolides, the side chain is very short (C4) and the carboxy function is condensed into a lactone (3′-methyl-5′ H-furan-2-onyl unit). Prorocentrolides and spiro-prorocentrimine have far longer side chains, but they are also condensed to the macrocycle by a lactone bridge. Prorocentrolides are the only group of cyclic imine toxins that have ether bridges not only in the macrocycle, but also in the side chain. Only pinnatoxins and pteriatoxins have free side chains. In the case of pinnatoxins, this occurs as a free carboxylic acid with a varying chain length from one (pinnatoxin-A), two (pinnatoxin-B and pinnatoxin-C) to four carbon atoms (pinnatoxin-D).

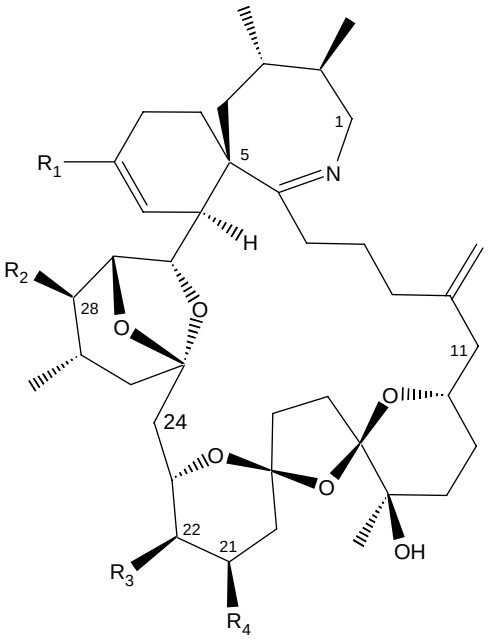
Biotransformation products must also be considered in comparing structural analogues within and among cyclic imine groups, particularly when the components are identified in matrices from vector organisms. Many shellfish are capable of cleaving lactones to the free acids. For example, several species of the dinoflagellate genera *Dinophysis* and *Protoperidinium* produce the lactone pectenotoxin-2 as the main toxin, whereas in shellfish, pectenotoxin-2 seco acid, the hydrolysis product, is often found. Analogous biotransformations are also to be expected among the macrocyclic imines.

26.4.2 Group Specific Features

26.4.2.1 Pinnatoxins

Pinnatoxins are the cyclic imines most closely related in structure to spirolides (Figure 26.4). They differ slightly in the polyether ring system (6-5-6) and in an additional bicyclic ether moiety in the macrocycle, which is absent in spirolides. Pinnatoxin variants differ in the length of their cyclohexenyl side chains: pinnatoxin-A has just a C1 carboxylic group, the enantiomeric diastereoisomers pinnatoxin-B and -C possess a C2 entity consisting of a 2-amino acetic acid function, and pinnatoxin-D includes a C4 γ-ketobutyric acid moiety.

Together with pteriatoxins, pinnatoxins are the only cyclic imines that have a free carboxylic group at the cyclohexenyl side chain, whereas all other cyclic imino toxins form lactones. It is therefore very likely that pinnatoxins are not directly produced by microalgae, but rather that they are shellfish metabolites of related phycotoxins. Further evidence for this hypothesis can be seen from the fact that pinnatoxins occur with different side chains lengths, which may represent different stages of degradation of the parent compounds. Pinnatoxins are amphoteric due to their free carboxy group and the imino function, and they are soluble in ethanol and ethyl acetate at neutral pH.



	R1	R2	R3	R4	MW	Sum formula
Pinnatoxin-A	COOH	OH	H	H	711.4	$C_{41}H_{61}NO_9$
Pinnatoxin-B	$CHNH_2$—COOH (R)	OH	H	H	740.5	$C_{42}H_{64}N_2O_9$
Pinnatoxin-C	$CHNH_2$—COOH (S)	OH	H	H	740.5	$C_{42}H_{64}N_2O_9$
Pinnatoxin-D	CO-CH_2—CH_2-COOH	OH	OH	CH_3	781.5	$C_{45}H_{67}NO_{10}$

FIGURE 26.4 Structural variants of pinnatoxins isolated from bivalve shellfish.

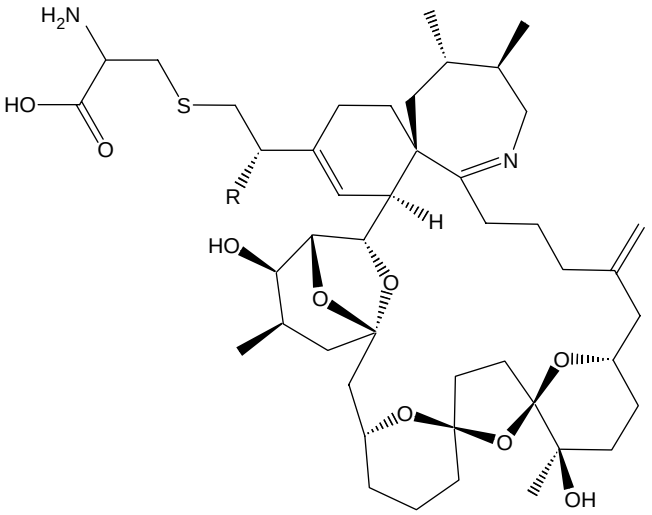
26.4.2.2 Pteriatoxins

Pteriatoxins are almost structurally identical to pinnatoxins, except for their cyclohexenyl side chain, which ends in a cysteine moiety (Figure 26.5). Like pinnatoxins, pteriatoxins have only been detected in shellfish. The cysteine terminus is not found in any other phycotoxins; therefore, the proximal origin of pteriatoxins is very likely to be via metabolic conversion of an algal precursor in shellfish. Owing to the structural similarity of the toxins and the geographical overlap of the shellfish species, *Pteria penguin* and *Pinna muricata* may accumulate the same toxin precursors from a common microalgal source (most probably a dinoflagellate), but metabolize the toxins in slightly different ways.

Pteriatoxin-A has a hydroxy substitution at the 6′-position of the cyclohexenyl side chain whereas the enantiomeric pair pteriatoxin-B and -C have a hydroxymethyl at the same position. Pteriatoxins possess one acidic and two basic functional groups and therefore have zwitterionic properties. They are water soluble at both low and high pH and similar to pinnatoxins, they are soluble in ethanol and ethyl acetate at neutral pH.

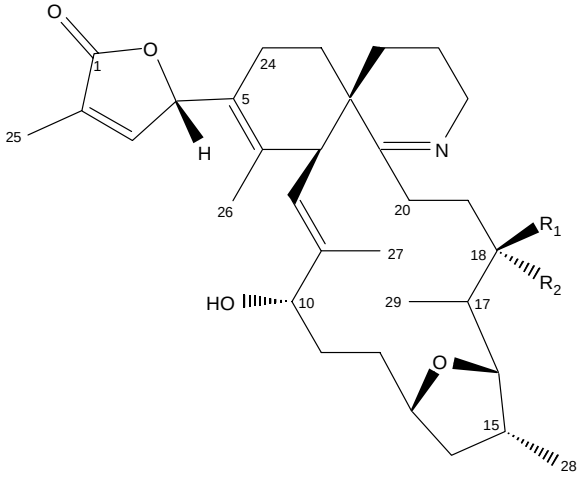
26.4.2.3 Gymnodimines

Gymnodimines, with a molecular weight around 500 Da, are the smallest molecules among the cyclic imine toxins (Figure 26.6). All members of the gymnodimine group have a six-membered



	R	MW	Sum formula
Pteriatoxin-A	OH	830.5	$C_{45}H_{70}N_2O_{10}S$
Pteriatoxin-B	CH_2—OH (R or S)	844.5	$C_{46}H_{72}N_2O_{10}S$
Pteriatoxin-C	CH_2—OH (S or R)	844.5	$C_{46}H_{72}N_2O_{10}S$

FIGURE 26.5 Structural variants of pteriatoxins isolated from bivalve shellfish.



	R1	R2	δ	Sum formula
Gymnodimine-A	H	No	17–18	$C_{32}H_{45}NO_4$
Gymnodimine-B	H	OH	17–29	$C_{32}H_{45}NO_5$
Gymnodimine-C	OH	H	17–29	$C_{32}H_{45}NO_5$

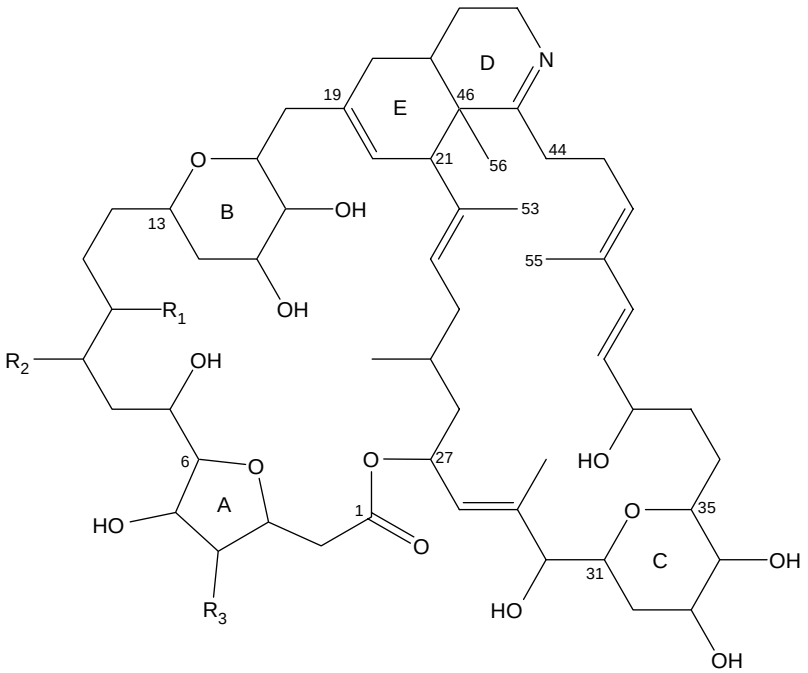
FIGURE 26.6 Structural variants of gymnodimine found among marine dinoflagellates.

imino ring and their macrocycle is the smallest of all the known cyclic imines, consisting of only 16 carbon units. The macrocycle has only one ether bridge. The parent compound gymnodimine has an endocyclic unsaturation at the 17 position, whereas gymnodimines B and C both have an exocyclic double bond and an additional hydroxy substitution at the 18 position. Gymnodimines are soluble in acetone and ethyl acetate.

26.4.2.4 Prorocentrolides

Prorocentrolides are the largest of the cyclic imine toxins, with molecular masses around 1 kDa (Figure 26.7). The prorocentrolides, although often found together with derivatives of okadaic acid including diol and sulfated esters in the same dinoflagellate (*P. lima*), are readily distinguished from the polyethers associated with DSP by both structure and mode of toxic action. These compounds also differ from all other cyclic imines by the condensed link of the cyclic imino and cyclohexenyl ring, whereas all other members of this group have a spiro-link. A common feature shared with spiro-prorocentrimine is the single ether bridge in the macrocycle. Prorocentrolides have the longest cyclohexenyl side chain, with 18 carbon atoms including two ether bridges in this part of the molecule. Like all other cyclic imine toxins of algal origin, the side-chain is condensed to a lactone in this case the macrocycle at position 27.

Prorocentrolides are soluble in methanol, 1-butanol, and chloroform and belong to the more polar fraction of the lipophilic toxins. The variant prorocentrolide B is distinguished by the presence of a sulfate group [4], but both prorocentrolides have an ultraviolet (UV) absorption maximum at



	R1	R2	R3	MW	Sum formula
Prorocentrolide	H	CH_2	H	979.6	$C_{56}H_{85}NO_{13}$
Prorocentrolide-B	CH_2	H	OSO_3H	1075.6	$C_{56}H_{85}NO_{17}S$

FIGURE 26.7 Structural variants of prorocentrolides isolated from marine dinoflagellates.

235 nm. Further spectroscopic data are reported for prorocentrolides by Torigoe et al. [3], and for prorocentrolide B by Hu et al. [4].

26.4.2.5 Spiro-Prorocentrimine

Spiro-prorocentrimine is thus far the only representative of this group. The spiro-prorocentrimine structure (Figure 26.1) has a molecular formula of $C_{42}H_{69}NO_{13}S$, yielding a molecular weight of 827.5 Da. Spiro-prorocentrimine has only a six-membered imino ring, but in addition to the basic imino function, includes an acidic function in the form of a sulfate substituent on the macrocycle. The molecule is therefore zwitterionic under neutral conditions. In a strict sense, spiro-prorocentrimine is not a polyether toxin, unlike all other cyclic imino toxins, because it possesses only a single ether bridge. This compound is soluble in methanol, 1-butanol, and chloroform and the UV absorption maximum lies at 214 nm. Further IR- and NMR-spectroscopic as well as crystallographic data are given by Lu et al. [7].

26.4.2.6 Spirolides

Spirolides are the largest of the known cyclic imine subgroups, comprising nine structurally distinct variants found among various strains of marine dinoflagellates (Figure 26.8). More spirolide derivatives whose structures have not yet been elucidated have been detected in dinoflagellate strains; this list of spirolide variants is therefore expected to lengthen in the near future.

Spirolides consist of two structural types. Type A, comprising spirolides A, B, C, D, and their desmethyl variants, has a 6-5-5 polyether ring system and a hydroxy group at the 10 position. Spirolides A and B have one methyl group on the cyclo-imine ring, whereas spirolides C and D are doubly methylated. Spirolides A and C are 2,3-unsaturated, whereas spirolides B and D are the saturated counterparts. The Type G spirolides, in contrast to Type A, have a 6-6-5 polyether ring system and a hydroxy group shifted from the 10 to the 17 position.

All spirolides are soluble in methanol and chloroform and therefore are readily extracted into lipophilic fractions of shellfish prepared for mouse bioassay. All of the above spirolides A–G possess a closed imine ring and are highly biologically active. Opening of the imine ring via thermal treatment or oxidation deactivates the compounds. In shellfish, spirolides E and F, derivatives with the opened cyclic imine ring, are occasionally found [31]. There is strong evidence that these spirolides E and F are exclusively metabolites produced in shellfish, because they have never been detected in spirolide-producing dinoflagellates in culture or in natural field populations.

It is instructive to consider the geographical and population level differences in spirolide toxin profiles among cultured isolates and natural blooms of *A. ostenfeldii*. In general, typical spirolide profiles of this species from the northeast Atlantic region often contain primarily desmethyl C, didesmethyl C and 20-methyl G spirolides, but this is accompanied by considerable inter- and intrapopulation heterogeneity. The spirolide profile from plankton size fractions containing *A. ostenfeldii*, from Ship Harbor, Nova Scotia, were dominated by spirolides A, B, C, and desmethyl C, whereas those from Graves Shoal, less than 100 km south, were remarkably different, with spirolides B, D, and isomer D2 as the major components [32]. A clonal cultured isolate (AOSH1) from Ship Harbor yielded a similar profile to the natural mixed population, that is, dominated by 13-des-methyl C, [24, 33], but another isolate AOSH2 isolated simultaneously from the same water mass produces primarily spirolide C and 20-methyl G [34]. Multiple clonal isolate of *A. ostenfeldii* and natural populations from the Gulf of Maine, the United States, revealed a complex pattern of major spirolide toxin phenotypes, with highly different mixtures of spirolides A, B, C, C2, desmethyl C, D, and D2 expressed among different isolates [17]. The spirolide 20-methyl G was identified in mussels from Norway and associated plankton [18], whereas the major components of the toxin profile of cultured *A. ostenfeldii* from Limfjord, Denmark, were des-methyl C and two recently identified derivatives spirolide G and 13,19-didesmethyl spirolide G [27]. Spirolides 13-desmethyl C

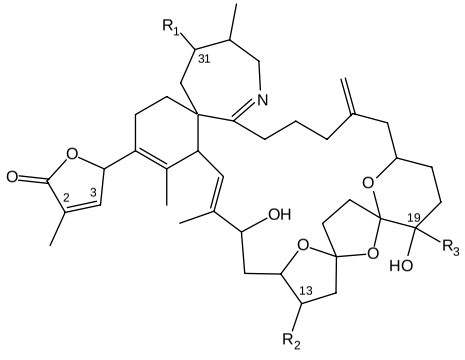
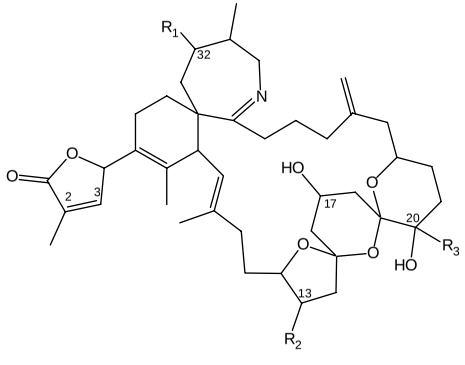
	Spirolide	R1	R2	R3	Δ^{2-3}	MW
	A	H	CH_3	CH_3	√	691,5
	B	H	CH_3	CH_3	—	693,5
	C	CH_3	CH_3	CH_3	√	705,5
	13-desMe-C	CH_3	H	CH_3	√	691,5
	13,19-didesMe-C	CH_3	H	H	√	677,5
	D	CH_3	CH_3	CH_3	—	707,5
	13-desMe-D	CH_3	H	CH_3	—	693,5
	G	CH_3	H	H	√	691,5
	20-Me-G	CH_3	H	CH_3	√	705.5

FIGURE 26.8 Structural variants of spirolides found among marine dinoflagellates

and 13-desmethyl D, plus an unidentified spirolide component, dominate the toxin profile in cultured *A. ostenfeldii* from Stewart Island, South Island, New Zealand [22]. Mussels and associated *A. ostenfeldii* blooms were determined to contain only desmethyl C as a major component [35] but this apparently restricted profile appears to be a function of the multiple reaction monitoring (MRM) LC-MS method, as subsequent investigation has revealed that another dominant spirolide is present (B. Krock unpublished observation).

26.5 CHEMICAL DETECTION AND ANALYSIS

26.5.1 LC-MS Methods

Cyclic imine toxins lack chromophores; therefore, optical detection methods for these compounds suffer from very unspecific UV spectra and very low sensitivity. In contrast, mass spectrometric detection is both highly sensitive and specific, especially because the imino function is a very good proton acceptor, resulting in very high ion yields and hence correspondingly high sensitivity. As a consequence, all detection methods for cyclic imine toxins are based on mass spectrometry. Among the cyclic imines, spirolides have been most intensively studied with respect to analytical method development, and several alternatives have been recently described for detection in various matrices [24,30,36–38]. Two major alternative approaches are described here in detail for analysis of cyclic imine toxins, with specific reference to spirolides. The first approach focuses on optimal separation and detection of spirolides, to yield specific information on unusual and undescribed variants. The second approach is a so-called multimethod for detection of not only cyclic imine toxins, such as gymnodimines and spirolides, but also for the secondary amino acid toxin domoic acid, and lipophilic polyether phycotoxins, such as okadaic acid and dinophysistoxins, pectenotoxins, azaspiracids, and yessotoxins.

26.5.1.1 Spirolide-Fractionation Method

Most of the methods described for spirolides have been developed for rapid monitoring of these toxins in phytoplankton or shellfish matrices. Due to mass selectivity, a baseline separation of the different spirolides is not achieved in most cases. For research and toxin profiling purposes, a comprehensive separation method for a vast suite of spirolides may be of interest. Recently, several isobaric spirolides, which coelute and thus remain unidentified under standard chromatographic conditions, were detected (Krock et al., unpublished data). Only the attempt at a baseline-separation of all compounds revealed these variants. This was achieved by the following liquid chromatographic conditions for fractionation of spirolides:

Column: Phenomenex Luna C18 150 × 3 mm, 3 µm, 100 Å
Eluents:
A: 2 mM ammonium acetate and 50 mM formic acid in water
B: 2 mM ammonium acetate and 50 mM formic acid in acetonitrile/water (95:5 [v/v])
Gradient: linear from initial 22% to 65% B at 50 min
Flow rate: 200 µL/min
Column temperature: 35°C

An MRM chromatogram with a baseline separation of all spirolides in a Nova Scotia strain of *A. ostenfeldii* (AOSH2) is shown in Figure 26.9.

26.5.1.2 Multitoxin Screening Method

An elegant multitoxin method has been developed for comprehensive phycotoxin monitoring for which details are given by Vershinin et al. [39]. This multimethod consists of three periods, that is, three chromatographic time-windows, in which different mass spectrometric parameters are applied to achieve maximum sensitivity for each toxin group. The first period from 0 to 8.75 min covers

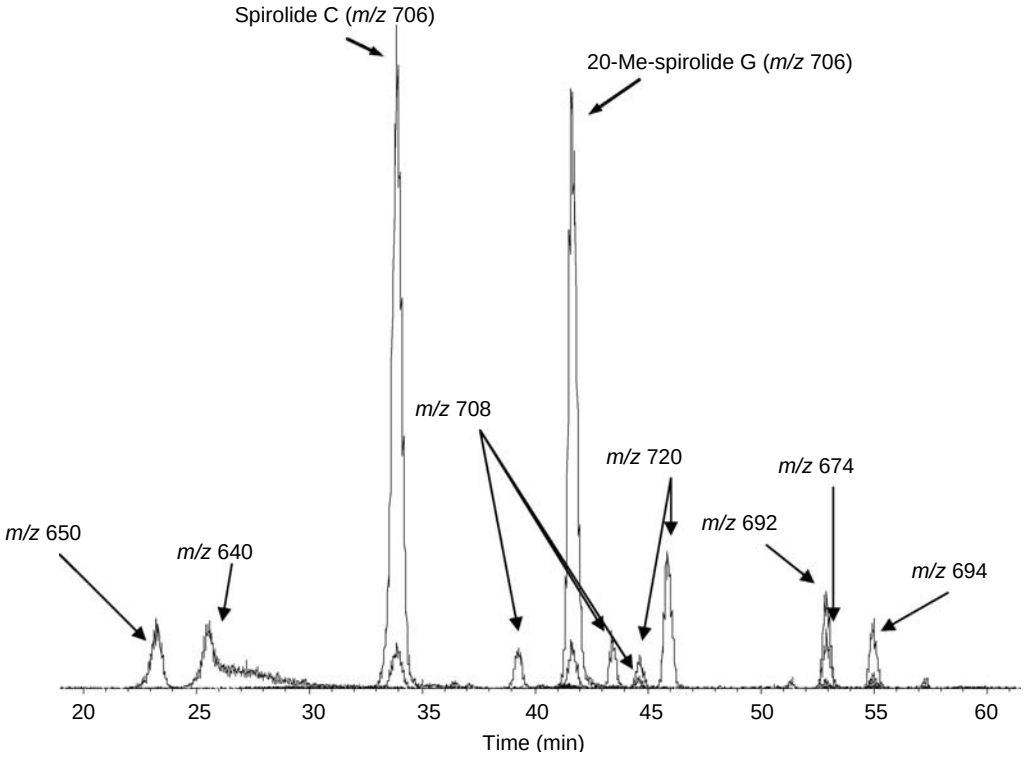


FIGURE 26.9 A multiple reaction monitoring (MRM) LC-MS chromatogram with a baseline-separation of all spirolides in a Nova Scotian strain of *Alexandrium ostenfeldii* (AOSH2).

domoic acid, whereas the second period from 8.75 to 11.2 min yields the cyclic imine toxins, such as gymnodimine and spirolides A, B, C, D, desmethyl C, and desmethyl D. The third period from 11.2 to 16 min is optimized for detection of okadaic acid and dinophysistoxins, pectenotoxins, azaspiracids, and yessotoxins. Figure 26.10 shows a multi-phycotoxin chromatogram of a field sample from Alfacs Bay, Catalonia, Spain.

26.6 BIOSYNTHESIS OF CYCLIC IMINE TOXINS

All of the cyclic imine toxins (except spiro-prorocentrimine) may be structurally classified as macrocyclic polyethers. Although the elucidation of biosynthetic pathways for polyether dinoflagellates toxins is limited to the ladder frame polyether brevetoxins [40], the linear polyether dinophysistoxins DTX4 and DTX5 [11,41] and the macrocyclic imine des-methyl spirolide C [12], they all exhibit features characteristic of polyketide biosynthesis. A hypothetical pathway for pinnatoxins (origin unknown) has also been recently proposed [42] and this follows the common dinoflagellate scheme.

Biosynthetic origins of the 13-desmethyl spirolide C were established by supplementing cultures of the producing dinoflagellate *A. ostenfeldii* with stable isotope labeled precursors [1,2-$^{13}C_2$] acetate, [1-^{13}C] acetate, [2-$^{13}CD_3$] acetate, and [1,2-$^{13}C_2$,^{15}N] glycine (Figure 26.11), and measuring the incorporation patterns by ^{13}C NMR spectroscopy [12]. Despite partial scrambling of the acetate labels, most carbons of the macrocycle were determined to be polyketide-derived. Glycine is incorporated as an intact unit into the cyclic imine moiety.

If spirolides and other cyclic imines are derived via polyketide biosynthetic pathways, synthesis is almost certainly mediated by polyketide synthase (PKS) genes. An ongoing genomic

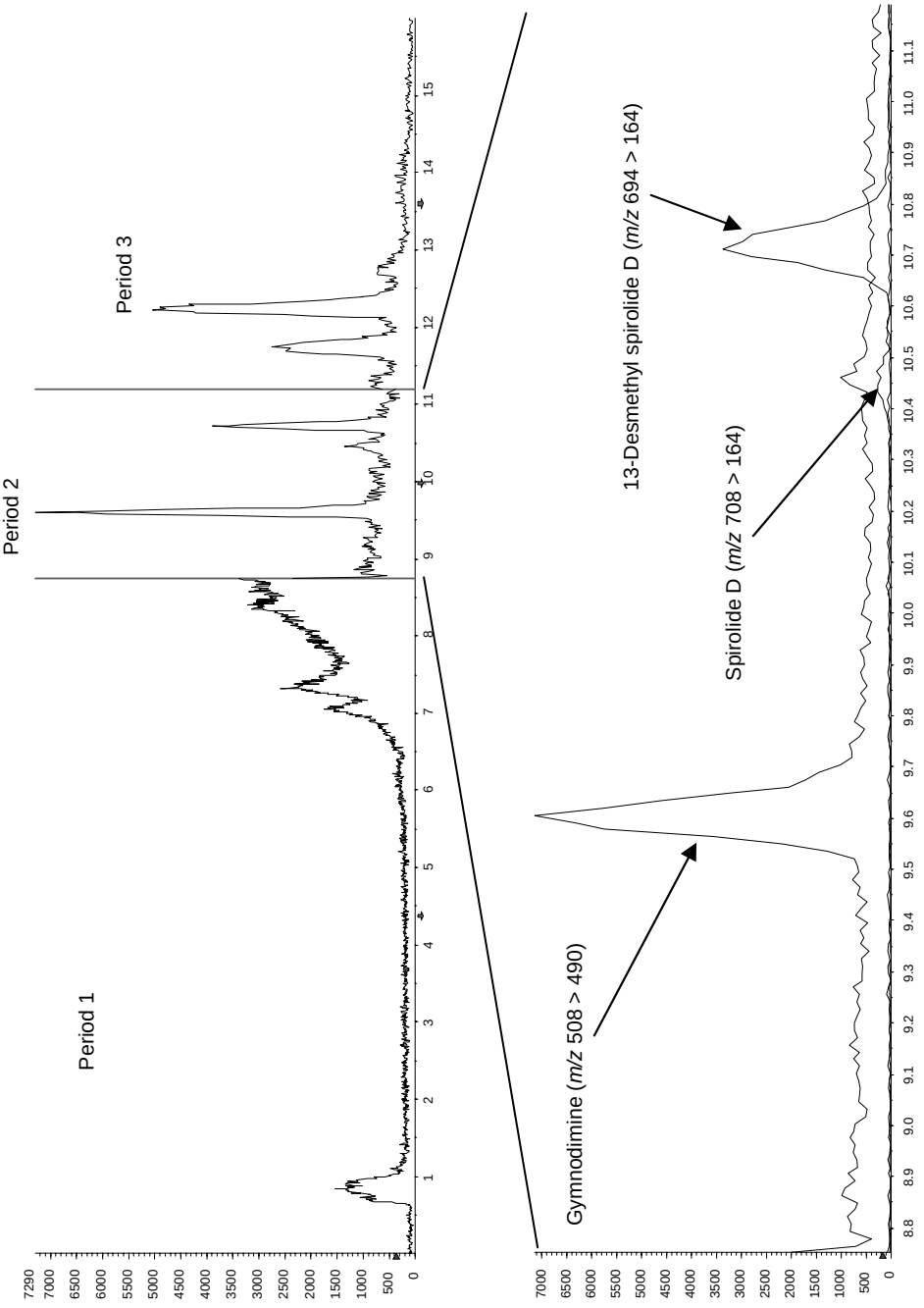


FIGURE 26.10 Multiphycotoxin chromatogram of a field sample from the Alfacs Bay, Catalonia, Spain. Other lipophilic toxins are okadaic acid, pectenotoxin-2, and pectenotoxin-2 seco acid.

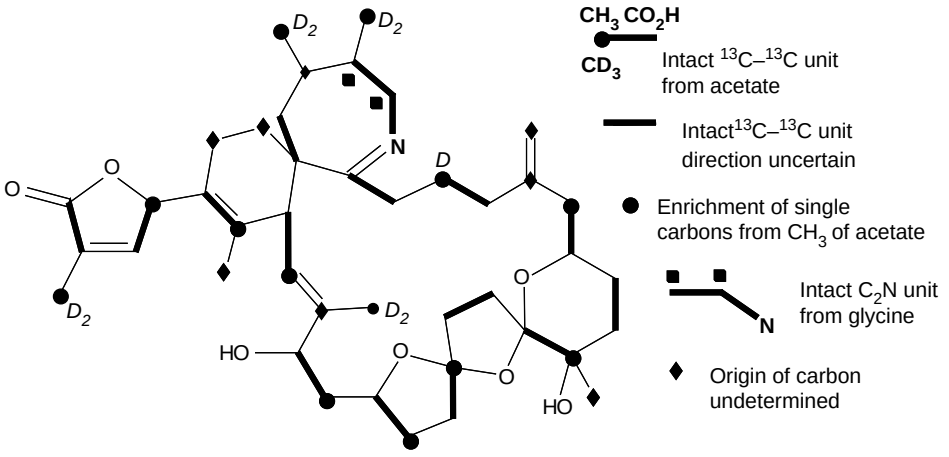


FIGURE 26.11 Structure of 13-desmethyl spirolide C **1**, showing incorporation of stable isotope labels from [1,2-$^{13}C_2$] acetate, [1-^{13}C] acetate, [2-$^{13}CD_3$] acetate, and [2,3-$^{13}C_2$, ^{15}N] glycine. (Adapted from MacKinnon, S.L., et al., *J. Org. Chem.* 71, 8724–8731, 2006.)

characterization of a spirolide-producing strain of *A. ostenfeldii*, conducted by generating an expressed sequence tag (EST) data bank based on a normalized cDNA library has revealed several putative PKS genes in this species [43]. Phylogenomic analysis has associated these PKS sequences most closely with related sequences of the apicomplexan *Cryptosporidium parvum*—a close relative of the dinoflagellates. Further evidence suggests that these are modular Type I PKSs, large multifunctional enzymes with several catalytic domains located on a single protein, which are also found among other protists [44].

26.7 BIOLOGICAL ACTIVITY AND MODE OF ACTION

Detailed analysis of the toxicological effects of cyclic imines is outside the scope of this review and has been dealt with in other literature [45], including the subsequent Chapter 27 of this volume. In summary, the intact cyclic imine group is the pharmacophore as evidenced by the dramatic decrease in biological activity after cleavage of the imine ring. For the spirolides, inactive variants E and F formed by keto amine hydrolysis are occasionally found within shellfish [31]. In the gymnodimine group, naturally occurring inactive variants are unknown but the secondary amine reduction product also has greatly reduced potency [46]. Spiro-prorocentrimine occurs only rarely in shellfish extracts and is an order of magnitude less toxic in intraperitoneal mouse bioassays than the other macrolide toxins.

A common mode of action is strongly suspected for the cyclic imines given the similarity in the symptomology following administration of these compounds to laboratory mice by intraperitoneal injection. There is some evidence to suggest that spirolides at least may affect Ca^{++} channels [31]. The potency of gymnodimine to activate Na^+ channels is much weaker than that of brevetoxins [1]; thus, it is doubtful that the cyclic imine toxins are behaving as classic ion channel effectors such as brevetoxins, tetrodotoxin, or saxitoxin. A rather detailed study of the pharmacological effects of administering a battery of therapeutants (atropine, physostigmine, propanolol, and epinephrine, etc.) followed by a spirolide challenge to laboratory mice [47] indicated at least one plausible mode of action. Circumstantial evidence implicated an effect on the muscarinic acetylcholine receptors in mammalian systems.

Evidence from transcriptional and histological analysis of brain tissues [48] indicated that muscarinic and nicotinic subreceptors were all upregulated after exposure to spirolides, suggesting that these receptors might be targets for spirolide action. This is supported by studies showing damage

to astrocytes in the hippocampus region and affects on *c-fos* activity [49]. Application of purified desmethyl spirolide C to neuroblastoma cells (N2a) caused blebbing and ultimately lysis of cells in a dosage-dependant manner [50], providing further strength to this hypothesis as the primary mode of action.

26.8 SUMMARY AND FUTURE PERSPECTIVES

In addition to the aforementioned regulatory and toxicological considerations, there remain numerous challenges with respect to understanding the biogeography, biosynthesis, genetic regulation, and ecological function (if any) of macrocyclic imine toxins. Knowledge of the biosynthetic pathways of macrocyclic imine toxins is thus far limited to desmethyl spirolides C and a hypothetical scheme for pinnatoxin. Gene expression studies should help resolve the unknown factors regulating macrocyclic imine synthesis through the cell cycle in the producing organisms. At present, there have been no definitive attributions of specific PKS genes to the biosynthetic pathways of any macrocyclic imine, although such Type 1 PKS genes are known to exist in dinoflagellates. Recent developments of fosmid libraries and DNA microarrays for gene expression studies should soon begin to rectify this gap in knowledge.

The environmental effects on production of macrocyclic imines are also poorly understood in the race to discover yet more new derivatives. Current evidence suggests that these compounds are constitutively produced, that is, a given strain is either toxigenic or not—these are not classic stress metabolites that can be induced by nutrient or light deprivation. Nevertheless, most of these conclusions are based upon relatively few studies, mostly on spirolides, and these comparative investigations must be extended to the other toxin groups.

The biogeography of the cyclic imine toxins is complex and shows highly heterogeneous patterns. On a global scale, it is only possible to draw inferences based on the spirolides, but it seems likely that the distributional range and profile complexity of the other toxins in this group will undergo a similar "apparent expansion" as more isolates and natural population are placed under study and more efforts are extended to multitoxin screening of shellfish extracts. Preliminary conclusions for the spirolides suggest that the toxin profile is fixed genetically within a given isolate and hence is propagated and maintained in culture. Yet, natural populations contain a mix of toxin phenotypes yielding only an averaged profile from natural plankton samples. Some geographical affinities are apparent, but radically different spirolide composition can be expressed even over short distances and even within a given population. Knowledge of the phenotypic and genotypic patterns of toxin expression awaits further exploration of geographically disjunct populations. This will be important to confirm or refute suggestions regarding the introduction of "new" toxins via invasive mechanisms or transfer of exogenous species.

The mode of action of cyclic imine toxins is still vague. Why are they so acutely lethal and so fast-acting in mammalian bioassays? It is not clear if they all share a common mode of action that is modulated merely by configurational differences among various derivatives and toxin types.

The sources of pinnatoxins and pteriatoxins remain unknown. It would be fascinating in terms of chemical prediction and not unimportant from a regulatory perspective to know if these are indeed dinoflagellate-borne toxins. Effort should be expended to elucidate the source in cases where these toxins appear in shellfish.

Finally, the oral toxicity and risks to human health posed by macrocyclic imine toxins has not yet been resolved. There have been no long-term trials on the affects of chronic sublethal exposure to cyclic imines in any animal model or even any concerted attempt to predict such consequences based upon pharmacological first principles reported in the literature. Development of a rational and precautionary strategy for human health protection will require more comprehensive knowledge on the toxicology and pharmacological implications of these compounds than is currently accessible.

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27 Toxicology of Cyclic Imines: Gymnodimine, Spirolides, Pinnatoxins, Pteriatoxins, Prorocentrolide, Spiro-Prorocentrimine, and Symbioimines

Rex Munday

CONTENTS

27.1 OCCURRENCE OF CYCLIC IMINES

27.1.1 Gymnodimine

In 1993, extracts of oysters (*Tiostrea chilensis*) dredged from the Foveaux Strait, New Zealand, showed unusual toxicity in routine bioassays for lipophilic toxins [1,2]. At the same time, a bloom of a dinoflagellate was observed in the affected area, which was classified as *Gymnodinium* cf. *mikimotoi* [1]. This organism was later renamed *Karenia selliformis* [3].

Chemical analysis of contaminated oysters revealed the presence of a new toxin, gymnodimine, which was shown to be a cyclic imine [4–6]. This same substance was obtained from cultures of *K. selliformis*, confirming that this organism is a source of the toxin in New Zealand [4,5]. No other gymnodimine-producing organisms have yet been identified.

Further analytical studies have revealed the presence of gymnodimine not only in New Zealand oysters but also in Greenshell mussels (*Perna canaliculus*), blue mussels (*Mytilus edulis*), scallops (*Pecten novaezelandiae*), pipis (*Paphies australis*), and paua (*Haliotis iris*) [7,8]. More recently, gymnodimine has been identified in clams (*Ruditapes decussatus*) from Tunisia [9], and the presence of gymnodimine in Canadian waters has also been reported [10].

27.1.2 Spirolides

In the early 1990s, routine bioassays detected the presence of a fast-acting toxin in extracts of shellfish from sites along the southeastern coast of Nova Scotia, Canada [11]. Two new toxins of the cyclic imine group, named spirolide B and spirolide D, were isolated from the viscera of scallops (*Placopecten magellanicus*) and mussels (*M. edulis*) and their structures determined [12]. Subsequently, spirolides A, C, E, and F were described, along with 13-desmethyl spirolide C [13,14].

Alexandrium ostenfeldii was identified as the primary and possibly exclusive source of spirolides in Canada [11,15,16]. The spirolide derivatives present in this organism showed considerable variation between locations [16] and among different strains [14]. Many new spirolide congeners have recently been isolated, not all of which have been fully characterized [17–20]. *A. ostenfeldii* is well known for producing paralytic shellfish poisons (PSP), but there is no evidence for production of PSP toxins in Canadian strains that produce spirolides [15].

A. ostenfeldii is of widespread distribution, and spirolide-producing strains of this organism have now been identified in Norway [21], Denmark [15,20,22], Italy [18], the United States [23], New Zealand [24], and Scotland [25–28]. In many cases, the spirolide derivatives present in the organisms from these countries were found to be quite different from those recorded in Canada, and it was shown that certain populations of *A. ostenfeldii* found in Scandinavia produce both spirolides and PSP toxins [15].

Apart from Canada, spirolide contamination of shellfish has been identified in Norway [21,29] and Spain [30], and with the widespread distribution of spirolide-producing strains of *A. ostenfeldii*, these substances are likely to be present in shellfish from other locations. Some of the spirolides found in shellfish are not present in *A. ostenfeldii*, and it is believed that these result from metabolism of the parent spirolides within the shellfish. In this category are spirolides E and F, found in Canadian shellfish, which are keto-amines formed by hydrolytic cleavage of the imine function of spirolides A and B [13] and the fatty acid esters of 20-methyl spirolide G, recently discovered in Norwegian mussels [29].

27.1.3 Pinnatoxins

Pinnatoxins A and D have been isolated from the Pen Shell, *Pinna muricata*, from Japan [31–33]. Pinnatoxins B and C, which are stereoisomers, were isolated as a 1:1 mixture from the same source [34]. A substance designated "pinnatoxin" was reported to be present in *Pinna attenuata* from China [35], although how well this material was characterized is unclear.

The source of the pinnatoxin in these shellfish has not been determined, and its presence in *Pinna* species appears to be confined to South East Asia.

27.1.4 Pteriatoxins

Pteriatoxin A and a 1:1 mixture of the stereoisomeric pteriatoxins B and C have been isolated from the pearl oyster *Pteria penguin* from Japan [36]. Although *Pteria* species are widely distributed, these toxins have not, as yet, been reported in any other parts of the world. Their origin is unknown.

27.1.5 Prorocentrolide, Spiro-Prorocentrimine, and Symbioimines

Prorocentrolide has been isolated from Japanese and Taiwanese strains of the dinoflagellate *Prorocentrum lima* [37,38]. A congener, prorocentrolide B, was found in a Japanese strain of *Prorocentrum maculosum* [39]. Spiro-prorocentrimine was extracted from an unidentified benthic *Prorocentrum* species from Taiwan [37], while symbioimine and neosymbioimine were found in a symbiotic marine dinoflagellate of the species *Symbiodinium* in Japan [40,41].

27.2 TOXICITY OF CYCLIC IMINES TO CULTURED CELLS AND TO ISOLATED TISSUE *IN VITRO*

27.2.1 Gymnodimine

Gymnodimine did not lyse mouse erythrocytes at a concentration of 10 μM, or cause toxic effects in mouse neuroblastoma NB41 cells or mouse leukemia P388 cells *in vitro* at the same concentration [4]. Gymnodimine gave small and inconsistent decreases in cell viability in a second type of mouse neuroblastoma cell (Neuro2a) at 10 μM, but at this concentration, it significantly sensitized the cells to the cytotoxicity of okadaic acid. Similar effects were seen with gymnodamine, the reduced form of gymnodimine lacking the imino function, and with gymnodimine acetate and gymnodimine methylcarbonate [42]. Gymnodimine was reported [4] to be a weak activator of sodium channels, although no details of the experimental conditions were given.

27.2.2 Spirolides

Spirolides B and D were shown to have no effect on *N*-methyl-D-aspartic acid (NMDA), α-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA), or kainate receptors *in vitro*, and they did not inhibit protein phosphatases PP-1 or PP-2A. They have no effect on voltage-dependent sodium channels, but are reported to be weak activators of type L calcium channels [12].

27.2.3 Pinnatoxins

The pinnatoxins show cytotoxicity against the P388 mouse leukemia cell line. The most toxic congener was pinnatoxin D, with an IC_{50} of approximately 3 μM [43].

An extract of *P. attenuata*, described as pinnatoxin, was reported to increase the contractility of strips of rabbit aorta and guinea pig ileum, and it was suggested that this substance was a calcium channel activator [35]. As mentioned previously, however, the identity of the substance described as pinnatoxin in this work is uncertain.

27.2.4 Pteriatoxins

No reports of *in vitro* studies on pteriatoxins have been found.

27.2.5 Prorocentrolide, Spiro-Prorocentrimine, and Symbioimines

Prorocentrolide was reported to be toxic to the murine leukemia cell line LC-1210 at micromolar concentrations, but this substance showed no activity against *Aspergillus niger*, *Candida rugosa*, or *Staphylococcus aureus* [38].

Symbioimine inhibited osteoclastogenesis in the murine monocytic cell line RAW264, with an EC_{50} of approximately 120 μM, but showed no cytotoxicity to these cells at 265 μM [40]. Significant inhibition of purified cyclooxygenase 2, but not cyclooxygenase 1, was recorded at a concentration of 10 μM [41].

27.3 ACUTE TOXICITY OF CYCLIC IMINES TO ANIMALS

27.3.1 Gymnodimine

27.3.1.1 Acute Toxicity by Intraperitoneal Injection

Data on the acute intraperitoneal (i.p.) toxicity of gymnodimine and gymnodamine are summarized in Table 27.1. Seki et al. [4,5] and Stewart et al. [6] reported minimum lethal doses (MLD) of gymnodimine by intraperitoneal injection of 450 μg/kg and 700 μg/kg, respectively. A later study, using a fully characterized pure sample of the toxin, gave an LD_{50} of 96 μg/kg [44]. In a study performed with extracts of naturally contaminated clams, Biré [45] found that the LD_{50} of gymnodimine lay between 97 μg/kg, at which no deaths were recorded, and 110 μg/kg, at which all the test mice died within a few minutes.

Immediately after intraperitoneal injection of lethal doses of gymnodimine, mice became hyperactive. After 1 min, however, movement became slower, and the animals walked with a rolling gait. Soon after, the hind legs became paralyzed and extended. The mice subsequently became completely immobile and unresponsive to stimuli. Respiratory distress was apparent, with marked abdominal breathing. The respiratory rate progressively decreased, until respiration ceased altogether. Pronounced exophthalmia was observed shortly before death, which invariably occurred within 15 min of injection. No macroscopic abnormalities were recorded at necropsy. At toxic, but sublethal, dose levels, prostration and respiratory distress were recorded, but the mice recovered within 30 min to an apparently normal state, and no adverse effects were observed during a subsequent 21-day observation period [44].

TABLE 27.1
Acute Toxicity of Gymnodimine and Gymnodamine by Intraperitoneal Injection in Mice

Compound	Mouse Strain	Sex	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
Gymnodimine	?	?	MLD	450	[4,5]
Gymnodimine	?	?	MLD	700	[6]
Gymnodimine	Swiss albino	Female	LD_{50}	96 (79–118)*	[44]
Gymnodimine	?	Female	LD_{50}	Between 97 and 110	[45]
Gymnodamine	?	?	MLD	>4040**	[6]

* Figures in brackets indicate 95% confidence limits.
** No effects were observed at this dose level.

TABLE 27.2
Acute Toxicity of Gymnodimine by Oral Administration to Mice

Compound	Method of Administration	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
Gymnodimine	Gavage	LD_{50}	755 (600–945)*	[44]
Gymnodimine	Gavage	MLD	>1225**	[45]
Gymnodimine	Feeding	MLD	>7500**	[44]

* Figures in brackets indicate 95% confidence limits.
** No effects were observed at these dose levels.

Histological examination of the spleen, liver, kidneys, thymus, and brain of mice receiving a sublethal dose of gymnodimine intraperitoneally revealed no changes attributable to the test substance [45].

Reduction of the imine function, yielding gymnodamine, greatly decreased the acute toxicity of gymnodimine. No toxic effects were observed when the reduced compound was injected into mice at 4040 μg/kg [6].

The short-acting cholinesterase inhibitors, neostigmine and physostigmine, protected mice against an intraperitoneal lethal dose of gymnodimine [44].

27.3.1.2 Acute Toxicity by Oral Administration

Gymnodimine was less toxic when administered orally by gavage, with an LD_{50} of 755 μg/kg (Table 27.2). When fed to fasted mice on a small piece of mouse food, it was even less toxic. The gymnodimine, absorbed into the mouse food, was consumed in less than 10 min, but no effects were observed at the highest dose employed, 7500 μg/kg. Furthermore, the subsequent body weight gains of the mice given this dose of gymnodimine were similar to those of control mice, and no lesions or changes in organ weights were recorded at necropsy [44]. No deaths or symptoms of toxicity were recorded in mice given gymnodimine in extracts of naturally contaminated mussels at up to 1225 μg/kg when administered by gavage [45].

The symptoms of intoxication observed after administration of gymnodimine to mice by gavage were the same as those seen after injection, although the time to death was extended [44].

Histological examination of the spleen, liver, kidneys, thymus, and brain of mice receiving a sublethal oral dose of gymnodimine (1225 μg/kg) revealed no changes attributable to the test substance [45].

Gymnodimine was lethal to the freshwater fish, *Tanichthys albonubes*, at a concentration of 20 nM in the aquarium water [4].

27.3.2 Spirolides

27.3.2.1 Acute Toxicity by Intraperitoneal Injection

Data on the acute i.p. toxicity of spirolides are summarized in Table 27.3. By intraperitoneal injection, spirolides B and D are of similar toxic potential [12]. In contrast, spirolides E and F, in which the cyclic imine moiety is destroyed, are much less toxic [13]. The LD_{50} of a mixture of spirolides, containing predominantly 13-desmethyl spirolide C, was reported as 40 μg/kg after intraperitoneal injection in mice [46]. The toxicities of pure samples of desmethyl spirolide C, spirolide C, and 20-methyl spirolide G were much higher, however, with LD_{50} values between 6.5 and 8.0 μg/kg [47]. The acute toxicity of desmethyl spirolide C by intraperitoneal injection was the same in fasted

TABLE 27.3
Acute Toxicity of Spirolides by Intraperitoneal Injection in Mice

Compound	Mouse Strain	Sex	State of Alimentation	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
Spirolide B	?	?	?	LD_{100}	250	[12]
Spirolide D	?	?	?	LD_{100}	250	[12]
Spirolide E	?	?	?	MLD	>1000*	[13]
Spirolide F	?	?	?	MLD	>1000*	[13]
Spirolide mixture	?	Female	?	LD_{50}	40**	[46]
Desmethyl spirolide C	Swiss albino	Female	Fed	LD_{50}	6.9 (5–8)**	[47]
Desmethyl spirolide C	Swiss albino	Female	Fasted	LD_{50}	6.9 (5–8)**	[47]
Spirolide C	Swiss albino	Female	Fed	LD_{50}	8.0 (4.6–16.2)**	[47]
20-Methyl spirolide G	Swiss albino	Female	Fed	LD_{50}	8.0 (3.9–14.1)**	]47]
Dihydrospirolide B	?	?	?	MLD	>1000*	[12]

* Figures in brackets indicate 95% confidence limits.
** No effects were observed at these dose levels.

mice as in animals allowed free access to food [47]. Dihydrospirolide B, in which the imine moiety is reduced, showed no toxicity at 1000 μg/kg [12].

Gill et al. [48] reported that mice given lethal doses of desmethyl spirolide C became hunched and lethargic, with piloerection. They were uncoordinated in their movements, and showed jerky locomotion. The hind limbs became splayed, and lacrimation, exophthalmia, and abdominal breathing were observed. Arching of the tail toward the head also occurred, with mouth breathing, followed by respiratory arrest. Similar effects were recorded in a subsequent study, although in these experiments tail arching was not a prominent feature of the intoxication [47]. Mice receiving lethal doses of simple spirolides died between 3 and 20 min after dosing. If the animals survived for more than 20 min, they recovered fully, and their subsequent appearance and behavior were normal [46–48]. In contrast, the onset of symptoms was delayed in mice dosed with fatty acid esters of 20-methyl spirolide G, and deaths occurred 45–50 min after dosing [29].

No macroscopic changes were seen in mice after lethal doses of desmethyl spirolide C [47–49]. No histological changes were recorded in the retina, skeletal muscle, peripheral nerves, heart, liver, kidney, spleen, lungs, adrenals, or gastrointestinal tract of mice receiving lethal doses of desmethyl spirolide C. No histological changes were observed in the brains of rats dosed with this substance, but widespread neuronal damage was seen in mouse brains, particularly in the brain stem and hippocampus [48,49].

Transcriptional analysis of animals dosed with desmethyl spirolide C showed major changes in the brains of rats, but not in those of mice. In the brain stem and cerebellum of rats, there was an increase in the early-injury markers HSP-72 and c-jun, and certain subtypes of muscarinic (mAChR1, mAChR4, and mAChR5) and nicotinic (nAChRα2 and nAChRβ4) acetylcholine receptors were upregulated [48]. Other markers, such as acetylcholinesterase and the glutamate receptors *N*-methyl-D-aspartate (NMDAR1) and kainate (KA2) were unchanged [48].

The time to death of animals given a mixture of spirolides was increased after pretreatment with physostigmine [46]. Conversely, the time to death was decreased when atropine or other acetylcholine antagonists were given before administration of spirolide [46].

27.3.2.2 Acute Toxicity by Oral Administration

The spirolides are less toxic by oral dosing than by injection (Table 27.4). The aforementioned spirolide mixture had an LD_{50} of 1000 μg/kg after dosing by gavage [46], while pure desmethyl

TABLE 27.4
Acute Toxicity of Spirolides by Oral Administration to Mice

Compound	Method of Administration	State of Alimentation	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
Spirolide mixture	Gavage	?	LD_{50}	1000	[46]
Desmethyl spirolide C	Gavage	Fed	LD_{50}	157 (123–198)†	[47]
Desmethyl spirolide C	Gavage	Fasted	LD_{50}	125 (87–166)†	[47]
Spirolide C	Gavage	Fed	LD_{50}	176 (ND) ‡	[47]
Spirolide C	Gavage	Fasted	LD_{50}	53 (50–63) †	[47]
20-Methyl spirolide G	Gavage	Fed	LD_{50}	157 (ND) ‡	[47]
20-Methyl spirolide G	Gavage	Fasted	LD_{50}	88 (27–120) †	[47]
Desmethyl spirolide C	Feeding, method 1*	Fasted	LD_{50}	625 (547-829) †	[47]
Desmethyl spirolide C	Feeding, method 2*	Fasted	LD_{50}	591 (500–625) †	[47]
Desmethyl spirolide C	Feeding, method 3*	Fed	LD_{50}	1005 (861–1290) †	[47]
Desmethyl spirolide C	Feeding, method 3*	Fed	LD_0	780	[47]
Desmethyl spirolide C	Feeding, method 3*	Fasted	LD_{50}	500 (381–707) †	[47]
Desmethyl spirolide C	Feeding, method 3*	Fasted	LD_0	400	[47]
Spirolide C	Feeding, method 3*	Fed	LD_{50}	780 (ND) ‡	[47]
Spirolide C	Feeding, method 3*	Fed	LD_0	625	[47]
Spirolide C	Feeding, method 3*	Fasted	LD_{50}	500 (353–657) †	[47]
Spirolide C	Feeding, method 3*	Fasted	LD_0	400	[47]
20-Methyl spirolide G	Feeding, method 3*	Fed	LD_{50}	625 (476–882) †	[47]
20-Methyl spirolide G	Feeding, method 3*	Fed	LD_0	500	[47]
20-Methyl spirolide G	Feeding, method 3*	Fasted	LD_{50}	500 (381–707) †	[47]
20-Methyl spirolide G	Feeding, method 3*	Fasted	LD_0	400	[47]

1. A solution of pure toxin was added to a small piece of dry mousefood, and fed to a mouse that had been fasted overnight.
2. A solution of pure toxin was fed to mice mixed with a pellet of moist mousefood.
3. A solution of pure toxin was fed to mice mixed with a pellet of cream cheese.

* Feeding methods (for details see text):
† Figures in brackets indicate 95% confidence intervals.
‡ Not determined. In these cases, the pattern of deaths were such that the AOT programme [54] was unable to calculate confidence intervals.

spirolide C gave an LD_{50} of 157 μg/kg when administered to fed mice by this technique. The oral toxicities of spirolide C and 20-methyl spirolide G by gavage were very similar to that of desmethyl spirolide C, with LD_{50} values of 176 and 157 μg/kg being recorded [47]. The toxicities of these spirolides by gavage were increased by subjecting the mice to an overnight fast. In fasted mice, spirolide toxicity was higher by a factor of between 1.3 and 3.3 than that in mice with unrestricted access to food.

The symptoms of spirolide poisoning by the oral route were the same as those seen after intraperitoneal injection [47].

The spirolides were even less toxic when administered to mice as a mixture with food. In initial studies, fasted mice were found to be reluctant to eat dry mouse food containing desmethyl spirolide C, but enough mice consumed the food within a reasonable period of time (<10 min) to establish an LD_{50}. This was 625 μg/kg [47]. Other feeding techniques were also employed in order to disguise the presence of the test substance and thereby ensure its rapid consumption by the mice. Desmethyl spirolide C was mixed into powdered mouse food (~150 mg), which was then made into a paste by addition of water. This was rolled into a pellet and fed to a mouse that had been fasted

TABLE 27.5
Acute Toxicity of Pinnatoxins by Intraperitoneal Injection in Mice

Compound	Strain of Mouse	Sex	Parameter	Acute toxicity (μg/kg Body Weight)	Reference
(+)-Pinnatoxin A	?	?	LD_{99}	180	[31]
(+)-Pinnatoxin A	?	?	LD_{99}	135	[50]
(−)-Pinnatoxin A	?	?	MLD	>5000*	[50]
Pinnatoxins B and C†	?	?	LD_{99}	22	[34]
Pinnatoxin D	?	?	LD_{99}	400	[33]

* No effects were observed at this dose level.
† 1:1 mixture of B and C. These compounds are stereoisomers.

overnight. This was eaten within 2–3 min, and gave an estimate of the LD_{50} (591 μg/kg) similar to that obtained with dry mouse food. Spirolide was also fed to fasted mice mixed with a pellet (~300 mg) of cream cheese, which was eaten in less than 2 min, again giving a similar estimate of the LD_{50} (500 μg/kg). Because of the similarity between the acute toxicity values, it was concluded that the vehicle in which the spirolide was administered was without significant effect on toxicity. In studies with spirolide C and 20-methyl spirolide G, acute toxicity by feeding was determined using only cream cheese as the carrier. The acute toxicities of these substances in fasted mice (500 μg/kg) were the same as that determined for desmethyl spirolide C [47].

Because of the avidity of the mice for cream cheese, the spirolides could also be fed in this matrix to mice without the need for fasting. The acute toxicities of desmethyl spirolide C, spirolide C, and 20-methyl spirolide G were higher in fasted mice than those in fed mice by factors of between 1.3 and 2.0 (Table 27.4).

27.3.3 Pinnatoxins

27.3.3.1 Acute Toxicity by Intraperitoneal Injection

Data on the acute i.p. toxicity of pinnatoxin derivatives to mice are summarized in Table 27.5. The LD_{99} of natural (+)-pinnatoxin A was reported as 180 [31] and 135 μg/kg [50]. In contrast, synthetic (–)-pinnatoxin A was without toxic effect at an intraperitoneal dose of 5000 μg/kg [50]. Pinnatoxin D was less toxic than pinnatoxin A [33], but a mixture of pinnatoxins B and C, which are stereoisomers, was much more toxic to mice, with an LD_{99} of only 22 μg/kg [34]. No information on the symptoms of intoxication by the pinnatoxins or on the histology of animals dosed with pinnatoxin derivatives has been found.

27.3.3.2 Acute Toxicity by Oral Administration

No data on the oral toxicity of pinnatoxins have been found.

27.3.4 Pteriatoxins

27.3.4.1 Acute Toxicity by Intraperitoneal Injection

Data on the acute i.p. toxicity of pteriatoxin derivatives to mice are summarized in Table 27.6. The LD_{99} of pteriatoxin A was 100 μg/kg when administered by intraperitoneal injection to mice. A 1:1 mixture of pteriatoxins B and C was much more toxic, with an LD_{99} of 8 μg/kg [36]. No information on the symptoms of intoxication by the pteriatoxins or on the histology of animals dosed with pteriatoxin derivatives has been found.

TABLE 27.6
Acute Toxicity of Pteriatoxins by Intraperitoneal Injection in Mice

Compound	Strain of Mouse	Sex	Parameter	Acute Toxicity (μg/kg Body Weight)	Reference
Pteriatoxin A	?	?	LD_{99}	100	[36]
Pteriatoxins B and C*	?	?	LD_{99}	8	[36]

* 1:1 mixture of B and C. These compounds are stereoisomers.

27.3.4.2 Acute Toxicity by Oral Administration

No data on the oral toxicity of pteriatoxins have been found.

27.3.5 Prorocentrolide, Spiro-Prorocentrimine, and Symbioimines

27.3.5.1 Acute Toxicity by Intraperitoneal Injection

No details of the acute i.p. toxicity of prorocentrolide are available. Torigoe et al. [38] reported that the "lethality" of this substance was 400 μg/kg in mice, though whether this figure relates to the MLD or to a particular proportion of deaths in treated animals was not stated. It is reported that prorocentrolide is a fast-acting toxin, with deaths occurring within minutes of intraperitoneal injection. At sublethal doses, the animals recovered completely [39]. No information on the clinical signs or macroscopic pathology associated with administration of this substance is available, and no information on the histology of mice dosed with prorocentrolide has been found.

Spiro-prorocentrimine appears to be much less toxic than other cyclic imines. Lu et al. [37] reported an intraperitoneal LD_{99} of 2500 μg/kg in mice. No information on the symptoms of intoxication with spiro-prorocentrimine has been found or on pathological changes induced by this substance.

No data on the acute toxicity of symbioimines by intraperitoneal injection have been found.

27.3.5.2 Acute Toxicity by Oral Administration

No data on the oral toxicity of prorocentrolide, spiro-prorocentrimine, or symbioimines have been found.

27.4 CHRONIC TOXICITY OF CYCLIC IMINES

Little information is available on the effects of repeated doses of the cyclic imines to animals.

A small pilot study has been conducted in mice in order to assess the effect of multiple sublethal injections of spirolide [51]. 13-Desmethyl spirolide C was dosed intraperitoneally to mice at 12.5, 25, and 35 μg/kg at 0, 7.5, and 21.5 h. A lethal dose (75 μg/kg) was then given to each mouse at 25 h. No ill effects were recorded following the initial, second, or third dose of the test material, except for one mouse that died 14 min after receiving the second dose of 35 μg/kg. All the mice died within 6 min after the lethal dose. All animals were subjected to macroscopic examination and samples of brain and internal organs were processed for histology. No macroscopic or histological changes were observed in any of these mice when compared to untreated controls.

27.5 EVALUATION AND RECOMMENDATIONS FOR FUTURE STUDIES

There is presently no evidence for human intoxication by any of the cyclic imines. Anecdotal reports from New Zealand indicate that no adverse effects are seen in humans after consumption of shellfish

contaminated with gymnodimine [2,44]. Furthermore, although contamination of clams in Tunisia has been reported [9], no case of poisoning has been shown to be associated with the presence of this substance [52]. Episodes of toxicity, involving rather nonspecific symptoms such as gastric distress and tachycardia, were recorded in individuals in Nova Scotia consuming shellfish during times when spirolides were known to be present, but these could not definitively be ascribed to spirolides [46]. Indeed, if the effects of spirolides in humans reflect those in animals, such minor effects are not to be expected, but rather paralysis and death. Pinnatoxins have been isolated from shellfish of the genus *Pinna*. Several outbreaks of poisoning have been reported in Japan and China among individuals consuming *Pinna* species [31,33]. An association between toxicity of the shellfish and the presence of the pinnatoxins therein has not, however, been established. Indeed, the poisoning incidents were initially ascribed to contamination of the shellfish by *Vibrio* spp. [31].

Cyclic imines are, however, toxic to experimental animals, and the possibility of harmful effects in humans who consume foodstuffs contaminated with these substances must therefore be evaluated.

Cyclic imines are not particularly toxic to isolated cells *in vitro*. Some cytotoxicity has been recorded, but the relevance of this to *in vivo* toxicity is questionable. Pinnatoxin D was more toxic to P388 cells than other pinnatoxins [43], although its toxicity *in vivo* is less. Similarly, the *in vitro* effect of gymnodamine in Neuro2a cells was as great as that of gymnodimine [42], although high doses of the former compound caused no toxic effects in mice.

Data are available, with a greater or lesser degree of detail, on the acute intraperitoneal toxicity of gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocentrolide, and spiro-prorocentrimine in mice. No data are available on the symptoms of intoxication by the pinnatoxins or spiro-prorocentrimine, but all the other cyclic imines are fast-acting toxins, with death occurring in a matter of minutes after administration of lethal doses. These materials must therefore be rapidly absorbed from the peritoneum after injection. Equally, they must be rapidly detoxified or excreted, since although animals may show severe toxic effects when given sublethal doses of the toxins, they recover quickly and completely [39,44,46,47]. For those compounds for which data are available, the symptoms of intoxication are remarkably similar, with death following respiratory arrest [44,47–49].

Among the spirolide derivatives that have been studied in detail (spirolide C, desmethyl spirolide C and 20-methyl spirolide G), there is little difference in acute toxicity, either by injection or by oral administration. In pinnatoxin and pteriatoxin derivatives, however, large differences in toxicity are seen among compounds of different structure. Pinnatoxins A–C and pteriatoxins A–C differ only in the nature of the substituent at position 33. In both, compounds B and C are stereoisomers. Pinnatoxin A, which has a carboxyl group at C-33, is approximately 14 times less toxic than a mixture of pinnatoxins B and C, which have a glycine residue at this site. The situation with the pteriatoxins is even more remarkable. These compounds may be considered as derivatives of 3-(2-hydroxyethylthio)-2-aminopropanoic acid, $HOCH_2CH_2SCH_2CH(NH_2)COOH$, in which the macrocycle is substituted in the hydroxyethylthio chain. The only difference between pteriatoxin A and pteriatoxins B and C is that in the former the macrocycle is attached at the 2-position of the hydroxyethylthio moiety and in the latter it is attached at the 1-position. Yet pteriatoxins B and C are 12.5 times more toxic than pteriatoxin A [34]. No explanation can presently be offered for these pronounced structure–activity relationships.

No deaths were observed in mice given twice the LD_{50} of gymnodimine after pretreatment of the animals with physostigmine or neostigmine. Under these conditions, control animals died within a few minutes [44]. Similarly, physostigmine increased the time to death of animals given a lethal dose of spirolide [46]. Physostigmine and neostigmine are short-acting acetylcholine inhibitors, which increase acetylcholine concentrations in the synaptic cleft of nicotinic receptors, thereby competitively inhibiting binding of foreign compounds to the receptor. The results of Gill et al. [48], showing upregulation of both muscarinic and nicotinic acetylcholine receptors in mice given lethal doses of desmethyl spirolide C, are also consistent with a mechanism of toxicity involving acetylcholinergic receptors. These receptors are widely distributed in tissues, and are involved in

central nervous system, autonomic ganglia, and neuromuscular transmission. It is feasible, therefore, that these compounds act at multiple sites. The work of Gill et al. [48] suggests a central toxic effect, although other factors and mechanisms cannot be ruled out. Interestingly, the symptoms of intoxication by the cyclic imines are very similar to those of tubocurarine, a known neuromuscular blocking agent, suggesting that the neuromuscular junction may be a site of action for this group of compounds [44]. At present, there is limited information on the specific sites and mechanisms of action of the cyclic imines, and further work in this area is required.

The imine function of the cyclic imines is of paramount importance for their toxicity. When the imine group is reduced, as in gymnodamine and dihydrospirolide B, or destroyed by ring-opening, as in spirolides E and F, the toxicity is greatly decreased [6,13]. Indeed, there are no data on the acute toxicity of derivatives devoid of the imine function, with no effects being reported at the highest dose levels employed (up to 4040 μg/kg).

Oxalic acid at 60°C in aqueous tetrahydrofuran (THF) mediates ring-opening of spirolides A and B, although spirolides C and D were not affected by this treatment [14]. Under milder conditions (aqueous hydrochloric acid at pH 3), however, neither spirolide B nor spirolide D were degraded [46]. The latter conditions are similar to those pertaining to the human stomach, suggesting that the spirolides may not be detoxified by ring opening in the stomach. However, the fact that shellfish metabolize spirolides A and B to the ring-opened products [13] suggests that ring opening may be enzymatically mediated, and a study of the metabolism of cyclic imines both in tissue homogenates or cultured cells *in vitro* and in the whole animal would be of considerable value.

For risk assessment, oral toxicity data are particularly relevant, since the shellfish contaminants are eaten by humans. Unfortunately, relatively little information on oral toxicity is available, but what comparative data are available indicate that cyclic imines are less toxic by oral administration than by injection. When administered by gavage, gymnodimine was approximately 8 times less toxic and desmethyl spirolide C, spirolide C, and 20-methyl spirolide G approximately 20 times less toxic than when injected [44,47]. It must be borne in mind, however, that the way in which materials are administered orally may have a pronounced effect on their toxicity [53]. In humans, ingested material immediately becomes mixed with the liquid contents of the stomach, and toxins are diluted throughout the mass of contents. The diluted material is then gradually released into the small intestine, where absorption may occur. The situation is different in mice, however, since the contents of their stomachs are not liquid, but semisolid. By administration of materials by gavage to mice, the test material may flow around the stomach contents and rapidly enter the duodenum at high concentration. Dosing by gavage, therefore, may give an artifactually high estimate of acute toxicity. The situation in humans may be simulated in mice by feeding the materials admixed with a small amount of food, when the test material then becomes dispersed throughout the mass of material in the stomach, and will be slowly released into the small intestine. Results with the spirolide derivatives in fed and fasted mice also support the conclusion that rate of transfer from the stomach into the small intestine is important to toxicity. With both gavage and feeding, mice whose stomachs contained relatively little food after an overnight fast were more susceptible than mice allowed continued free access to food. The effect of fasting did not reflect a change in rate of detoxification or excretion, since fasting had absolutely no effect on the intraperitoneal toxicity of desmethyl spirolide C. It may be argued, therefore, that administration of material by feeding gives a more realistic estimate of acute oral toxicity for use in risk assessment than by gavage [53].

No data on the oral toxicity of pinnatoxins, prorocentrolide, spiro-prorocentrimine, or symbioimines are available, and information is required before a valid risk assessment can be made. Since the pinnatoxins have been found in shellfish consumed by humans, and they have been suggested to be involved in human intoxication, priority should be given to these substances. For gymnodimine and some spirolides, oral acute toxicity values are available, permitting comment on the acute reference doses of these compounds and guidance levels for acceptable degrees of contamination in shellfish.

In the case of gymnodimine, the acute toxicity by feeding was >7500 μg/kg. By application of a safety factor of 100, the acute reference dose would be 75 μg/kg body weight, equivalent to a dose

of 4.5 mg for a 60 kg human. Taking a serving size of 250 g, the guidance level would be 18 mg/kg shellfish flesh. This high guidance level, together with the lack of evidence of human intoxication by gymnodimine, suggests that regulation against gymnodimine is not required.

In contrast, spirolide C, desmethyl spirolide C, and 20-methyl spirolide G were much more toxic than gymnodimine, and showed high toxicity even when fed. The no observable adverse effect level (LD_0) of these compounds, from acute toxicity studies by feeding in both fed and fasted mice, was approximately 500 µg/kg [47]. Because of the high toxicity of these substances, a safety factor of 300 is recommended, giving an acute reference dose of 1.67 µg/kg and a guidance level of 400 µg/kg shellfish flesh. Regulations on spirolides should exclude analogues that lack the imino function.

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Part VIIIE

Polycavernosides and Gambierol

28 Polycavernosides and Gambierol: Chemistry, Pharmacology, Toxicology, and Detection

M. Carmen Louzao Ojeda, Eva Cagide Otero, Mari Yotsu-Yamashita, and Makoto Sasaki

CONTENTS

28.1 POLYCAVERNOSIDES

28.1.1 Chemistry

28.1.1.1 Introduction

Polycavernoside A (PA, Figure 28.1) was isolated by Yasumoto and his colleagues with a minor analog polycavernoside B (PB) as the causative toxin of the human fatal poisoning occurred in Guam in 1991, resulting from ingestion of the red alga *Gracilaria edulis*.[1,2] Out of 13 patients, 3 were killed. PA was also identified in the same alga that caused the poisoning, killing 8 out of 36 patients in Philippines in 2002–2003.[3] The planar structures of PA[2] and four minor analogs, polycavernoside A2 (PA2), A3 (PA3), B (PB), and B2 (PB2) (Figure 28.2) were determined.[4] All these polycavernosides possess the same macrolide aglycon containing a five-member cyclohemiacetal adjacent to a ketone at C9. Structural variation among these analogs are in the conjugated diene (PB, PB2) or triene (PA, PA2, PA3) side chain at C15, and in *O*-methylated or *O*-acetylated L-fucosyl-D-xylose sugar unit at C5. Yotsu-Yamashita et al. also isolated other two minor analogs, polycavernoside C (PC) and polycavernoside C2 (PC2), from *G. edulis* collected in Guam in 1991–1992, and their structures were recently determined (Figure 28.1).[5] PC and PC2 have a common aglycon structure, that is distinctly different from that of PA and other analogs.

28.1.1.2 Chemical Total Synthesis of Polycavernoside

28.1.1.2.1 Isolation

As it was previously pointed out, polycavernoside A (PA, 0.4 mg) was isolated from *G. edulis* collected on June 4, 1991 (2.6 kg) at Tanguisson Beach, Guam, together with polycavernoside B (PB, 0.2 mg).[2] *G. edulis* was extracted with acetone three times, and the solvent was evaporated *in vacuo*. The residue was partitioned between H_2O and CH_2Cl_2, and the residue from CH_2Cl_2 layer was applied to column chromatography on silica gel 60 using CH_2Cl_2–MeOH [1:0, 99:1 (PB), and 9:1 (PA)]. Each residue from the eluate with CH_2Cl_2–MeOH 99:1, and 9:1 was purified by successive chromatography on ODS-Q3 (H_2O–MeCN 15:85), Develosil ODS-7 (H_2O–MeCN 1:4), and Cosmosil 5C18AR with H_2O–MeCN 1:4. Similarly, PA (0.4 mg), PA2 (0.1 mg), PA3 (0.4 mg), and PB2 (0.1 mg) were isolated from *G. edulis* (2 kg) collected at the same beach in Guam on June 11, 1992.[4] *G. edulis* was extracted with CH_2Cl_2–MeOH 2:1 three times. After solvent evaporating, the residue was successively partitioned between H_2O–MeOH 1:4 and hexane (toxins in the H_2O–MeOH 1:4) and then between CH_2Cl_2 and H_2O. The residue from CH_2Cl_2 phase was applied to column chromatography on ODS-Q3 (H_2O–MeCN 1:3) and then on Develosil ODS-5 (H_2O–MeCN 1:3). PA2, PB2, PA, and PA3 were sequentially eluted from the last column in this order and further purified on the same column by gradient elution (H_2O–MeCN from 35:65 to 0:100).[4] Throughout the purification, elution of polycavernosides was monitored by mouse assays and with a diode array ultraviolet (UV) detector.

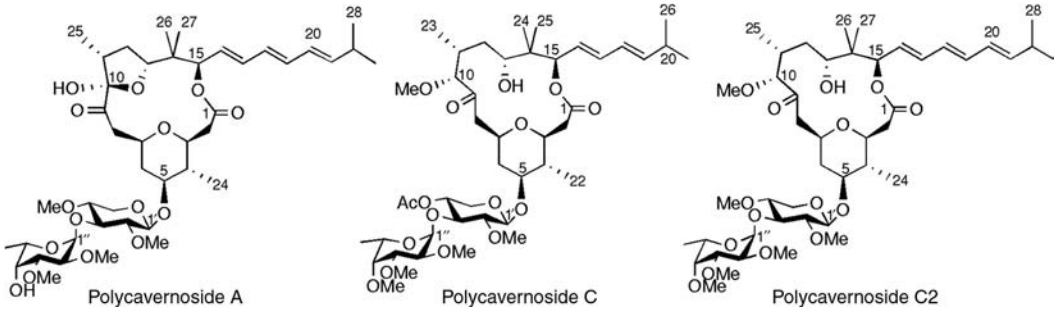
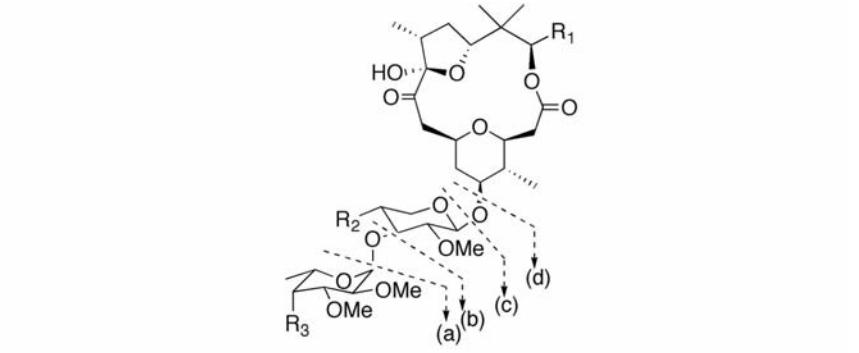


FIGURE 28.1 The structures of polycavernoside A (PA), C (PC), and C2 (PC2). (Reprinted from Yotsu-Yamashita et al., *Tetrahedron Lett.*, 48, 2255, 2007. With permission.)



	R_1	R_2	R_3	UV λ_{max} (nm)	MNa^+	MNa^+-fucose	MNa^+-fucosylxylose
PA		OMe	OH	259, 269, 280	847	673 (a)	513 (c), 497 (d)
PA2		OH	OH	259, 269, 280	833	657 (-2H) (a)	513 (c)
PA3		OMe	OMe	259, 269, 280	861	685 (+MeOH-H_2O-2H) (a) 673 (a), 675 (b)	513, 495 (-H_2O) (c) 497 (d)
PB		OAc	OMe	231	863	Not determined	Not determined
PB2		OH	OMe	231	821	633 (a)	83 (-2H) (c)

FIGURE 28.2 The structures, UV λ_{max} wave lengths, MNa^+ ions, and the prominent fragmentations by ESI-MS/MS of polycavernosides (PA, PA2, PA3, and PB2). (Modified from Yotsu-Yamashita, M. et al., *Chem. Res. Toxicol*., 17, 1265, 2004.)

28.1.1.2.2 Chemical Properties, Structure Determination, and Total Synthesis

The molecular formula $C_{43}H_{68}O_{15}$ for PA was determined by HR-FABMS ($[M+Na]^+$ *m/z* 847.4483 Calcd for $[C_{43}H_{68}O_{15}Na]^+$ *m/z* 847.4455).[2] The data of UV_{max} (MeCN) 256 (ε 25000), 270 (ε 32000), 280 (ε 26000) nm suggested the presence of conjugated triene. *E, E, E* geometry was determined by the $^3J_{HH}$ value (15 Hz). IR (film) absorption at 1630, 1730, 1738 cm^{-1} suggested the presence of double bond and carbonyl groups. The ^{13}C NMR spectrum (CD_3CN) confirmed the presence of a ketone (δ 207.4) and an ester (δ 172.1). On the basis of the detailed analyses of ^1H–^1H COSY, TOCSY, HMQC, HMBC, ^{13}C, NOESY, ROESY, NOE difference spectra, the planar structure of PA was determined.[2] The relative configuration of PA was determined by Fujiwara et al. by synthesis of the disaccharide with both enantioisomers of the tetrahydropyran rings.[6] Then, finally, the absolute structure of PA was established by agreement of the circular dichroism (CD) spectrum of the totally synthesized PA by Fujiwara et al.[7] to that of the natural PA.

The total synthesis of (−)-PA was achieved by three groups: Fujiwara et al. in 1998,[7] Paquette et al. in 1999,[8,9] and White et al. in 2001.[10,11] Further synthetic efforts on tetrahydropyran subunit of PA were recently reported by Perez-Balado and Marko[12,13] and Barry et al.[14]

28.1.1.3 Structure–Activity Relationship Studies on Polycavernosides

Barriault et al.[15] synthesized the four derivatives **3–6** of PA (Figure 28.3), which possess different structures, β-styrenyl (**3**), isopropyl dienes (**4**), isopropyl substituted enynene (**5**), and cyclohexyl dienes (**6**), in the C-15 side chain instead of isopropyl triene of PA. In addition, vinyl iodide (**7**) and PA aglycone (**8**) synthesized by Fujiwara et al.[7] were also subjected to bioassay in mice (Figure 28.3). The toxicities of these analogs to male mice (ddY strain, 12–15 g body weight) were determined by intraperitoneal (i.p). injection and the symptoms in mice were observed until 24 h after injection. The minimal lethal dose (MLD) values were shown in Figure 28.3. The analogs **3**, **6**, and **7** showed significantly low activities compared with other analogs. In addition, the aglycone of PA (**8**) still

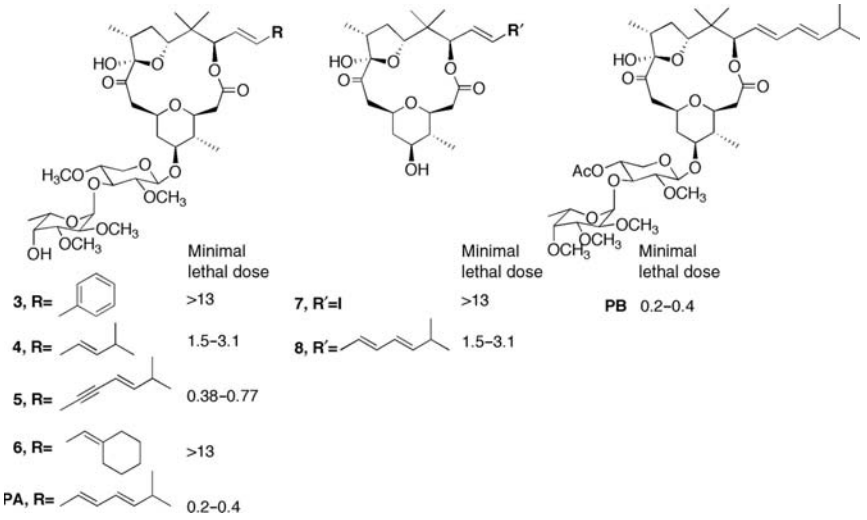


FIGURE 28.3 Structure–activity relationships of polycavernosides. (Modified from Barriault, L. et al., *Bioorg. Med. Chem. Lett*., 9, 2069, 1999.)

retained reduced activity. However, the symptoms caused in mice by **8** were partially different from those of PA, suggesting that macrocyclic core and triene side chain are required for toxicity. The analogs, which possess an isopropyl group in the side chain (**4**, **5**, and **8**), showed high-level toxicity. Analog **4** and PB have close structures sharing the same aglycone including the C15 side chain, so the differences in the toxicity levels of **4** and PB may arise because of the availability of a free hydroxyl substituent in the sugar component of **4**.

28.1.2 Pharmacology

Pharmacological actions of polycavernosides were poorly investigated due to the relative novelty and lack of material. The complete mechanism of action and molecular targets of these new phycotoxins are still unknown. In fact, only little information is available about the polycavernosides effects.

Taking into account the neurological symptoms observed in mice, Louzao et al.[16] used analog **5** (Figure 28.3) of polycavernoside A synthesized by Barriault et al.[15] in order to detect a possible toxin-induced change in membrane potential. They used the fluorescent dye bis(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$), bis(oxonol),[17–20] and tested 4 μg/ml of analog **5** on a suspension of human neuroblastoma cell line BE(2)M-17. No change in membrane potential was observed, even when the cells were preincubated with veratridine (Figure 28.4), an allosteric activator of sodium channels that increases the depolarization produced by other sodium channel activators.[21,22] Following those experiments, Cagide et al.[23] continued studying the mechanism of action of polycavernosides by using the same analog **5** as was done previously. In this case, a different approach was chosen; variations in membrane potential were detected in plated cells with an imaging system. In addition to bis(oxonol), fura-2 was used to monitor intracellular calcium. They found that 12 μM (10 μg/ml) of the synthetic analog **5** (a higher concentration than the one tested before) was able to depolarize neuroblastoma cells and produce an increase in intracellular calcium levels.[23] Depolarization seems to be a calcium-dependent secondary effect induced by the toxin, since it was abolished in a Ca^{2+}-free medium (Figure 28.5a). They also observed that in this Ca^{2+}-free solution, intracellular calcium increment was also annulled (Figure 28.5b), indicating that the toxin induced a calcium influx as the first effect, lately confirmed by using nickel, an unspecific calcium channels blocker. The lack of inhibitory effect of nifedipine, a commonly used L-type voltage-gated calcium channels blocker, indicated that those channels are not involved in the polycavernoside-induced calcium entry.[23]

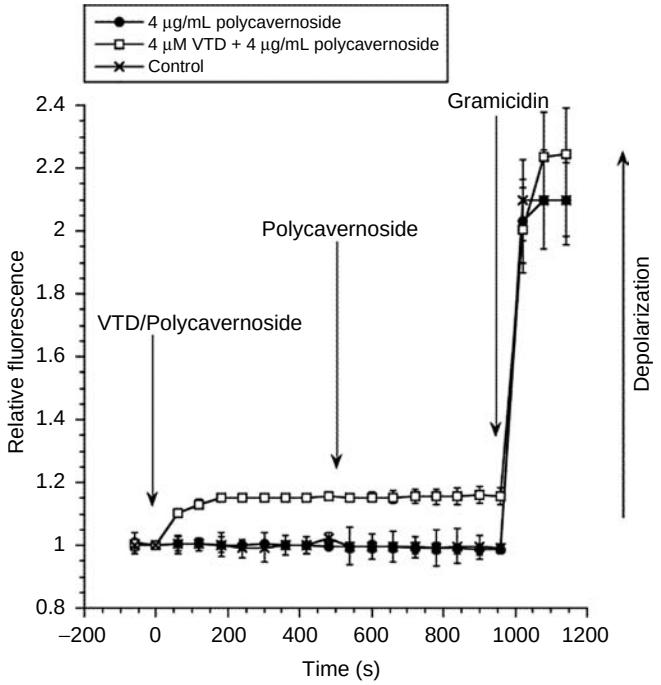


FIGURE 28.4 Human neuroblastoma cells loaded with bis(oxonol). Lack of changes in membrane potential induced by addition of 4 µg/mL polycavernoside (circles). Depolarization stimulated with 40 µM VTD first and then no effect after addition of polycavernoside (squares). At the end of the experiment, 10 µg/mL gramicidin was added to induce complete cell depolarization. (Reprinted from Louzao et al., *Chem. Res. Toxicol*., 19, 788, 2006. With permission from American Chemical Society (ACS).)

Some of the symptoms observed in mice (hypersalivation, lacrymation, etc.) are similar to a stimulation of the parasympathetic nervous system. However, based on the fact that atropine, a muscarinic receptors antagonist, did not modify the polycavernoside-induced calcium increase, these symptoms are not related with the calcium increase induced by the toxin.[23]

In order to check if the toxin was able to exhibit activity toward the cytoskeleton, filamentous actin (F-actin) of the cells was stained with Oregon Green® 514 Phalloidin and detected with laser-scanning cytometer (LSC) and confocal microscopy. Neuroblastoma cells incubated with 4 µg/ml of analog **5** at 37°C for long time periods, until 24 h, did not show modifications in the amount of F-actin or its distribution indicating a cellular integrity in treated cells (Figure 28.6).

Yotsu-Yamashita and Taya are testing cytotoxicity of natural polycavernoside A, and above synthetic analogs, **3**, **4**, **5**, and **6** (Figure 28.3)**,** in mouse neuroblastoma cells, Neuro-2a.[24] Owing to the limitation of availability of the compounds, the cytotoxicities were tested only at 12 µM by counting the viable cells treated with toxins for 24 h. As the results, **5** and **6** executed more than 90% death responses, whereas PA only approximately 20% death responses, and **3** and **4** did not show cytotoxicities at this level, suggesting that cytotoxicity is not comparable with the toxicity to mice. They proved that **5** at 12 µM for 24 h induced apoptosis in Neuro-2a by activation of caspase-3/7, nucleosomal DNA fragmentation, and TUNEL (TdT-mediated dUTP-biotin nick end labeling) staining.[25]

28.1.3 Toxicology

The first reported case of human intoxication was in Guam in 1991, resulting from the ingestion of the widely consumed red alga *G. edulis (Polycavernosa tsudai)*. In this incident, 13 people

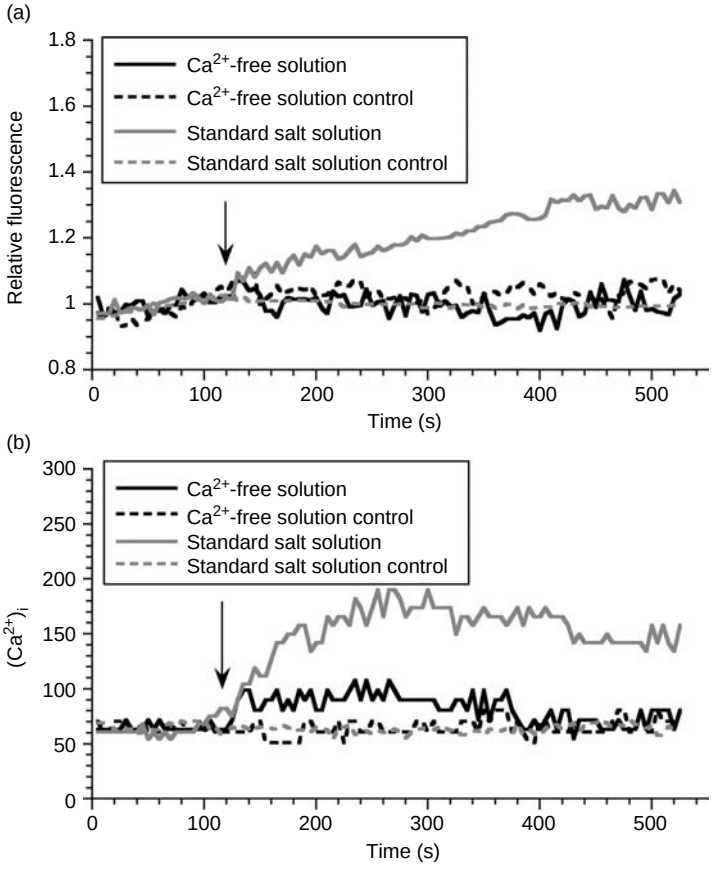


FIGURE 28.5 Representative time-course showing the depolarizing effect (a) and intracellular calcium increase (b) induced by 10 μg/mL polycavernoside (arrow) in a Ca^{2+}-containing solution (gray lines). Those effects were abolished in a Ca^{2+}-free solution (black lines).

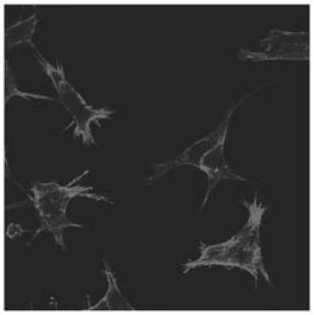
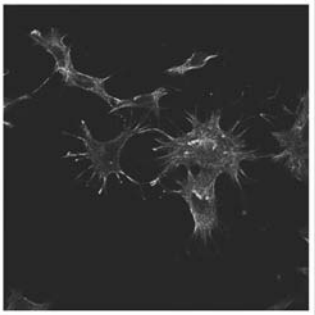
		(a) Control	(b) Polycavernoside A analog 5
Neuroblastoma cells	% Actin cytoskeleton	100 ± 5.04	101.17 ± 7.15
	Fluorescence images		

FIGURE 28.6 Percentage of fluorescence associated to F-actin versus control after 24 h incubation with 4 μg/mL polycavernoside A analog 5. Representative images show actin cytoskeleton of untreated cells (a) and cells incubated with 4 μg/mL analog 5 (b).

were intoxicated and three of them died.[2] PA and PB were the first ones identified as responsible for this poisoning, and the LD_{99} in mice by i.p. injection was estimated to be 200–400 μg/kg. Both toxins induced gastrointestinal and neurological disorders in experimental animals, causing diarrhea, hypersalivation, lacrymation, muscle spasms, and cyanosis, these symptoms being similar to those observed in the human patients, according to Dr. R. Roos, Guam Memorial Hospital.[2]

Outbreaks of poisoning due to polycavernosides were not reported again until 2002–2003 in Philippines. Three fatal human intoxications occurred from ingestion of the same red alga as in Guam case, *G. edulis*, beside another one, *Acanthophora specifera*. In these cases, 8 of 36 patients died, and the symptoms observed were also similar to the Guam's incident.[3] Although the toxicity of the Philippines' alga to mice was seven times lower than that of Guam, the number of deaths and patients were quite similar to each other. On the basis of this high fatality, Yotsu-Yamashita et al.[3] speculate that the toxicity of polycavernoside A to human by oral administration is much higher than toxicity to mice by i.p. administration.

28.1.4 Detection

28.1.4.1 *In Vivo* Bioassays

In order to avoid food intoxications due to the consumptions of these red algae, the mouse bioassay is a simple method for being used as a practical test to monitoring the level of toxicity. The test consists of intraperitoneally injecting toxic extracts into mice and observing the symptoms over 24 h, until death occurs. Although in this case, for polycavernosides assays, the low sensitivity of i.p. injection to mouse must be taken into account.[3] In order to improve the sample preparation process for the mouse bioassay, lyophilization of seaweed is recommended before extraction with organic solvent. This process would reduce the amount of inorganic salt in the extracts. In addition, after extraction, the organic layer ($CHCl_3$ layer, for example) containing toxins should be washed with water or MeOH–water 2:3 (v/v) to remove the salts. These processes are very important, because inorganic salt from seaweed can kill mice at relatively low doses when injected intraperitoneally.

28.1.4.2 *In Vitro* Assays

Recent advances in pharmacology of polycavernosides are turning out this toxin as a cytotoxic compound against neuroblastoma cells. Taya and Yotsu-Yamashita[24] first discovered this effect with several analogs of P.A on the neuroblastoma cell line Neuro-2a, and Cagide et al.[26] seem to confirm this effect with analog 5 on a different neuroblastoma cell line, BE(2)M-17. Taking these preliminary experiments into account, a cytotoxic assay for detection of polycavernosides could be developed.

28.1.4.3 Chemical Assays

28.1.4.3.1 Semipurification of the Toxic Fraction from G. edulis

G. edulis (1.9 kg, wet weight) collected in Philippines on December 2, 2002, was extracted with MeOH (2 L) thrice, and the extract was filtered through cellulose filter. The filtrate was concentrated by evaporation and partitioned between hexane and aqueous 80% MeOH. The aqueous 80% MeOH layer was concentrated by evaporation and partitioned between aqueous 40% MeOH and $CHCl_3$. The toxicity in the $CHCl_3$ layer was estimated as 150 MU, when the toxicity to mice was expressed in mouse units (MU): one MU temporally denotes the amount of toxin, which kills one male mouse (ddY strain) of 15 g body weight in 24 h. A half of the $CHCl_3$ layer (75 MU) was concentrated by evaporation, and applied to a silica gel column (Wakogel C100, 2 × 10 cm Wako, Osaka, Japan).

The column was sequentially eluted with $CHCl_3$ (<20 MU), $CHCl_3$-MeOH 99:1 (<20 MU), 9:1 (>20 MU), and 1:1 (<20 MU) (each 100 ml). The $CHCl_3$–MeOH (9:1) fraction was concentrated by evaporation and applied to a reversed phase column, Develosil LOP ODS (1 × 35 cm, Nomura Chemicals, Seto, Japan) with aqueous solution containing 80% MeCN. The column was eluted stepwise with aqueous 80%, 90%, and 100% MeCN. The fraction eluted in the first 40–60 ml portion of aqueous 80% MeCN was toxic to mice (30–60 MU). A half of this fraction was concentrated by evaporation and applied to another reversed phase column, Cosmosil 5C18-AR-II (1 × 25 cm, Nakarai Tesque, Kyoto, Japan). The column was eluted by gradient increasing MeCN concentration in water from 70% to 100%. The toxic fraction (10–20 MU) was eluted with aqueous 70–80% MeCN. Aliquots of this fraction were applied to liquid chromatography-diode array detection (LC-DAD), LC/electrospray ionization-mass spectrometry (LC/ESI-MS), and LC/ESI-MS/MS for analysis of polycavernosides.

28.1.4.3.2 LC-DAD

The LC system consisted of a Jasco 880 PU pump (JASCO, Tokyo, Japan) for the mobile phase, a Rheodyne Model 7125 injector with a 25 μL loop, and a 0.2 × 15 cm Mightysil RP-18 GP (5 μm) column (Kanto Chemical, Tokyo, Japan). LC was performed using an aqueous solution containing 80% MeCN as the mobile phase at a flow rate of 0.2 ml/min at 26°C. The column was connected to L-7455 diode array detector (Hitachi, Tokyo, Japan) with a flow cell (17.7 μL, 10 mm), and Hitachi D-7000 advanced high performance liquid chromatography (HPLC) system manager was utilized for data acquisition.

The LC-DAD chromatogram of the standard mixture of polycavernosides (PA, PA2, PA3, PB2) and that of the semipurified toxic fraction obtained from the causative *G. edulis* are shown in Figure 28.7. The retention times of the standard PA, PA2, PA3, and PB2 were 9.60, 7.38, 15.9, and 7.77 min, respectively (Figure 28.7a). The relationship between the applied amount of standard PA and its peak area in the sliced chromatogram at 269 nm, as shown above the 3D chromatogram in Figure 28.7a,b, was examined at four levels ranging from 0.4 pmol to 11 pmol. The obtained calibration curve showed a good linearity ($r^2 = 0.99$), suggesting that the peak area is applicable to quantify purified PA. The estimated detection limit was 0.4 pmol (signal to noise ratio:3). On the LC-DAD chromatogram of the semipurified toxic fraction from *G. edulis* (Figure 28.7b), a peak possessing typical UV absorption for a conjugated triene at 259, 269, and 280 nm appeared at 9.51 min, indicating the presence of PA (Figure 28.2). The total amount of PA in the semipurified toxic fraction from the *G. edulis* (0.65 kg, wet alga) was estimated to be 55 nmol (84 nmol/kg wet alga) by using the above calibration curve. Since the reported toxicity of PA to mice (LD_{99}, i.p. 200–400 μg/kg)[2] can be converted to 3–6 μg/MU for a 15 g body weight mouse, the above estimated amount of PA (55 nmol) was calculated to 8–15 MU, which roughly agreed with the experimentally determined toxicity to mice (10–20 MU).

28.1.4.3.3 LC/ESI-MS

The LC system used for LC/ESI-MS was almost the same as that for LC-DAD except for a Hitachi L-6000 pump being used for the mobile phase instead of a Jasco 880 PU pump. The column was connected to the ion interface of the mass spectrometer through a fused-silica capillary without splitting. All LC/ESI-MS, LC/ESI-MS/MS, and flow injection ESI-MS/MS experiments were recorded on a TSQ700 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a ESI source with an ICIS II data system in the positive-ion mode. ESI was effected by a spray voltage of +4.8 eV, and the heated capillary temperature was maintained at 250°C. Nitrogen served both as the sheath gas at an operating pressure of 60 psi and as the auxiliary gas at a flow rate of approximately 3 l/min. In the positive ESI-MS of the authentic polycavernosides measured by flow injection, the ions corresponding to $[MNa\text{-}H_2O]^+$, MNa^+, and MK^+ were observed. Among them, the ions of MNa^+ were most prominent, facilitating the use of MNa^+ ions at *m/z* 847, 833, 861, and 821, for PA, PA2, PA3, and PB2, respectively, in selected ion monitoring (SIM) mode (Figure 28.8a). The retention times recorded for these compounds were 9.80, 7.04, 16.5, and 7.80 min, respectively. The detection limit for PA on LC/ESI-MS in SIM mode was estimated to be 2 pmol (signal to noise

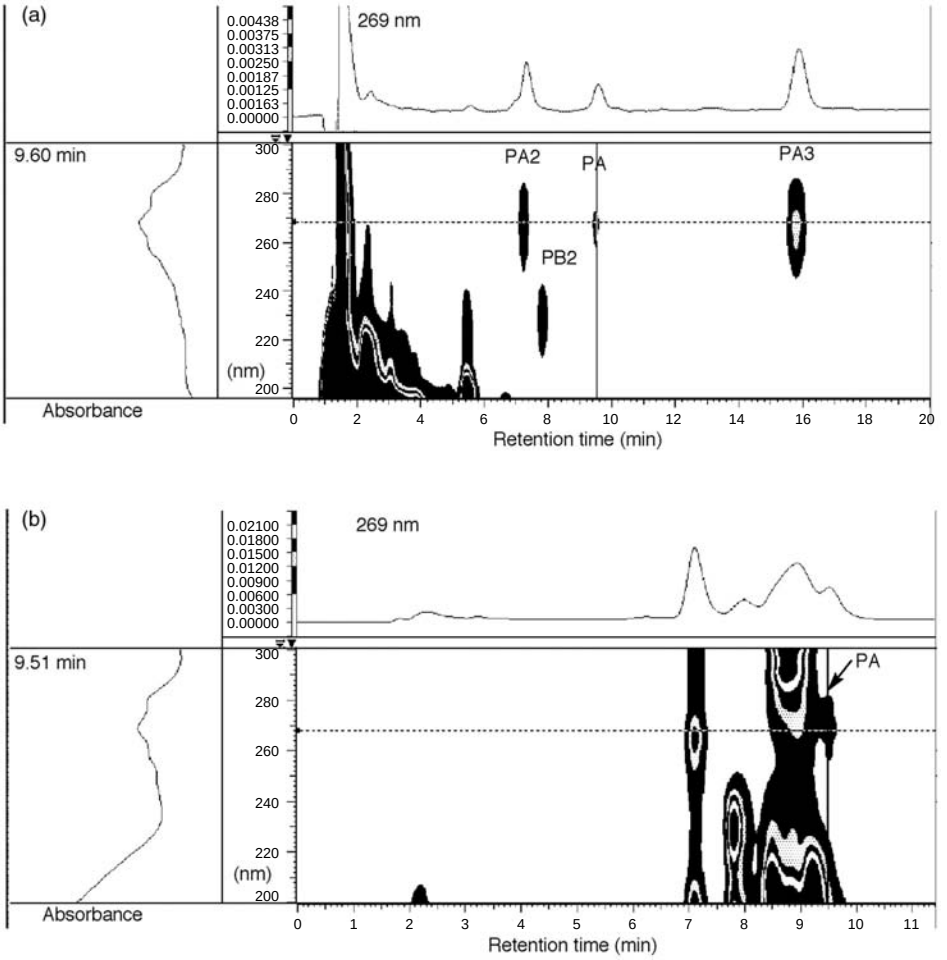


FIGURE 28.7 LC-DAD chromatograms of authentic polycavernosides (a) and the semipurified toxic fraction from *G. edulis* (b).[3] The chromatographic conditions: 0.2 × 15 cm Mightysil RP-18GP 5 μm, aqueous MeCN 80%, 0.2 mL/min, 26°C.

ratio: 2), which was approximately five times higher than that by LC-DAD (0.4 pmol). The relationship between the applied amount of standard PA and its peak area on the SIM mass chromatogram at *m/z* 847 was examined at four amounts of PA over the range of 3–54 pmol. The obtained calibration curve showed a good linearity ($r^2 = 0.99$). The mass chromatogram of the semipurified toxic fraction obtained from the causative *G. edulis* (Figure 28.8b) monitored at *m/z* 847, clearly showed a peak corresponding to PA at 10.0 min. The total amount of PA in this fraction from 0.65 kg of wet *G. edulis* was estimated to be 47 nmol (72 nmol/kg wet alga) by using the above calibration curve. The value was nearly compatible with that estimated by LC-DAD (84 nmol/kg wet alga).

28.1.4.3.4 ESI-MS/MS

The MS/MS measurements were based on collision-induced dissociation (CID) occurring in the collision cell (Q_2) of the triple quadrupole at a collision energy of 49 eV. Argon was used as the target gas at a target thickness of 1.5×10^{14} molecules/cm^2. Each 100–200 pmol PA, PA2, PA3, and PB2 (5 μL in MeOH) was introduced into the mass spectrometer by flow injection with MeOH at a flow rate of 0.1 ml/min. The fragment ion spectra of the MNa^+ ions for standard PA, PA2, PA3, and PB2 were measured by choosing the MNa^+ ions at *m/z* 847, 833, 861 and 821, respectively, as the

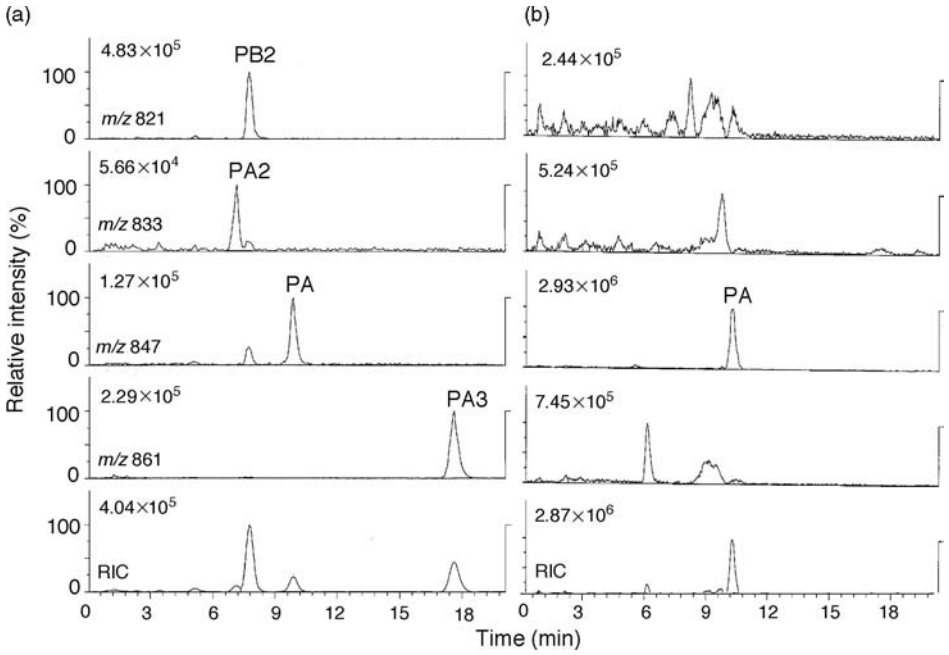


FIGURE 28.8 LC-ESI/MS SIM chromatograms of authentic polycavernosides (a) and the semipurified toxic fraction from *G. edulis* (b). The chromatographic condition was the same as that in Figure 28.7. (Reprinted from Yotsu-Yamashita et al., *Chem. Res. Toxicol*., 17, 1265, 2004. With permission from American Chemical Society (ACS).)

precursor ions by the positive-ion ESI-MS. As summarized in Figure 28.2, the prominent fragment ions were interpreted as those corresponding to sequential loss of the methylated fucosylxylose residues.

28.1.4.3.5 LC/ESI-MS/MS

The LC/ESI-MS/MS was performed under the same conditions as those used for the LC/ESI-MS and for the MS/MS experiments already described above. The characteristic fragmentation patterns of polycavernosides summarized in Figure 28.2 were applied to LC-ESI/MS/MS. First, the solution containing authentic PA, PA2, PA3, and PB2 were applied to LC-ESI/MS/MS in selected reaction monitoring (SRM) mode by monitoring at *m/z* 847-513, 833-513, 861-513, and 821-483 for PA, PA2, PA3, and PB2, respectively, as parent and daughter ion pairs (Figure 28.9a). The semipurified toxic fraction obtained from *G. edulis* containing approximately 780 pmol of PA quantified by LC-DAD (Figure 28.7b) was applied to LC/MS/MS in the SRM mode by monitoring at *m/z* 847-513 and 847-673 for PA (Figure 28.9b). The peaks corresponding to PA were clearly shown on both mass chromatograms, supporting the identification of PA as the cause of poisoning of *G. edulis* in Philippines.

28.2 GAMBIEROL

28.2.1 Chemistry

28.2.1.1 Introduction

Gambierol (**1**, Figure 28.10) was isolated as a toxic constituent from the cultured cells of the ciguatera causative dinoflagellate, *Gambierdiscus toxicus*, by Yasumoto and coworkers in 1993.[27]

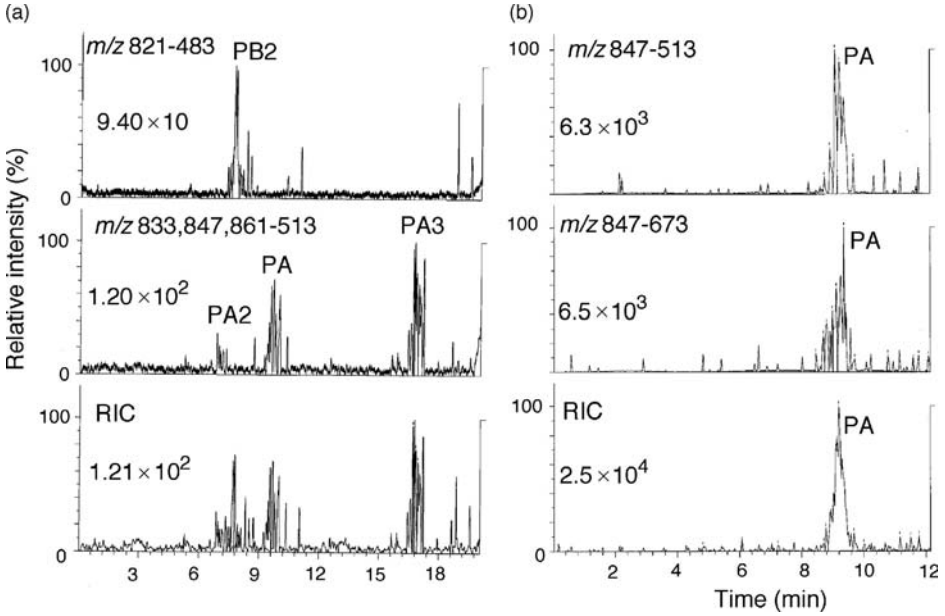


FIGURE 28.9 LC-ESI/MS/MS SRM chromatograms of authentic polycavernosides (a) and the semipurified toxic fraction from *G. edulis* (b).[3] The chromatographic condition was the same as that in Figure 28.7.

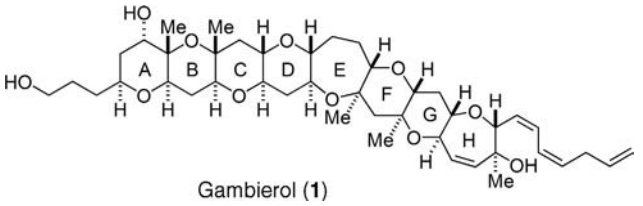
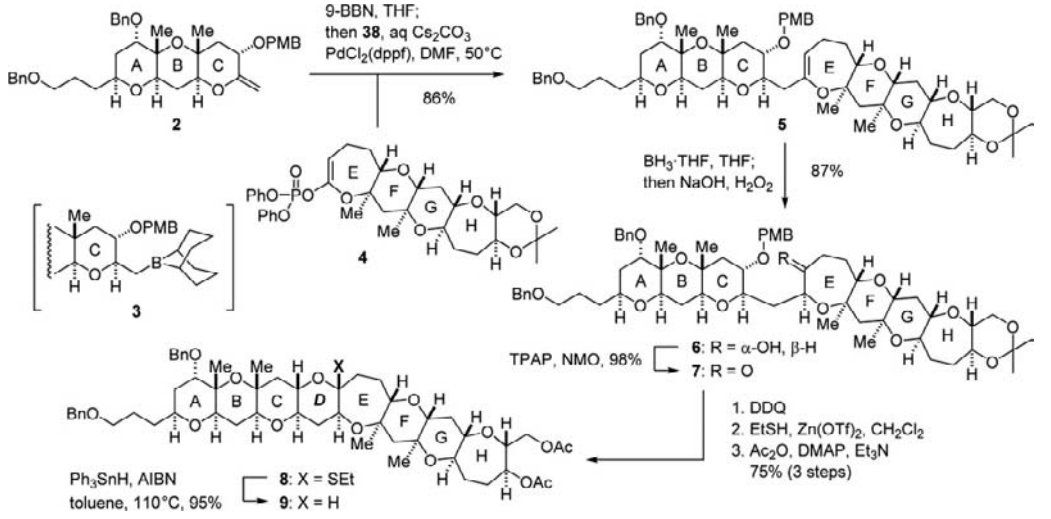


FIGURE 28.10 Structure of gambierol.

The chemical structure, including the relative stereochemistry, was established by extensive 2D-NMR experiments.[27] The absolute configuration was subsequently determined by derivatization and application of the modified Mosher's method.[28] Structurally, the toxin molecule consists of a *trans*-fused octacyclic polyether skeleton containing 18 stereogenic centers and a partially skipped triene side chain including a conjugated (*Z*,*Z*)-diene system. Gambierol exhibits potent lethal neurotoxicity against mice (minimum lethal dose = 50 μg/kg, i.p.), and the symptoms resemble those caused by ciguatoxins, implying that gambierol is also involved in ciguatera seafood poisoning. However, as is often the case with other polycyclic ether marine toxins, the extremely limited availability of gambierol from natural sources has hampered detailed biological studies so far. In 2002, Sasaki and coworkers accomplished the first total synthesis of gambierol,[29,30] which was based on their developed Suzuki–Miyaura coupling chemistry.[31–36] This chemical synthesis enabled for the first time extensive biological studies of this toxin. Shortly thereafter, the Yamamoto/Kadota group completed the second total synthesis using their intramolecular allylstannane cyclization chemistry.[37,38] More recently, Rainier and coworkers also described their own total synthesis.[39–41] In this chapter, we present the completed total synthesis of gambierol by Sasaki et al. and other two groups.



SCHEME 28.1 Sasaki's total synthesis of gambierol.

28.2.1.2 Chemical Total Synthesis of Gambierol

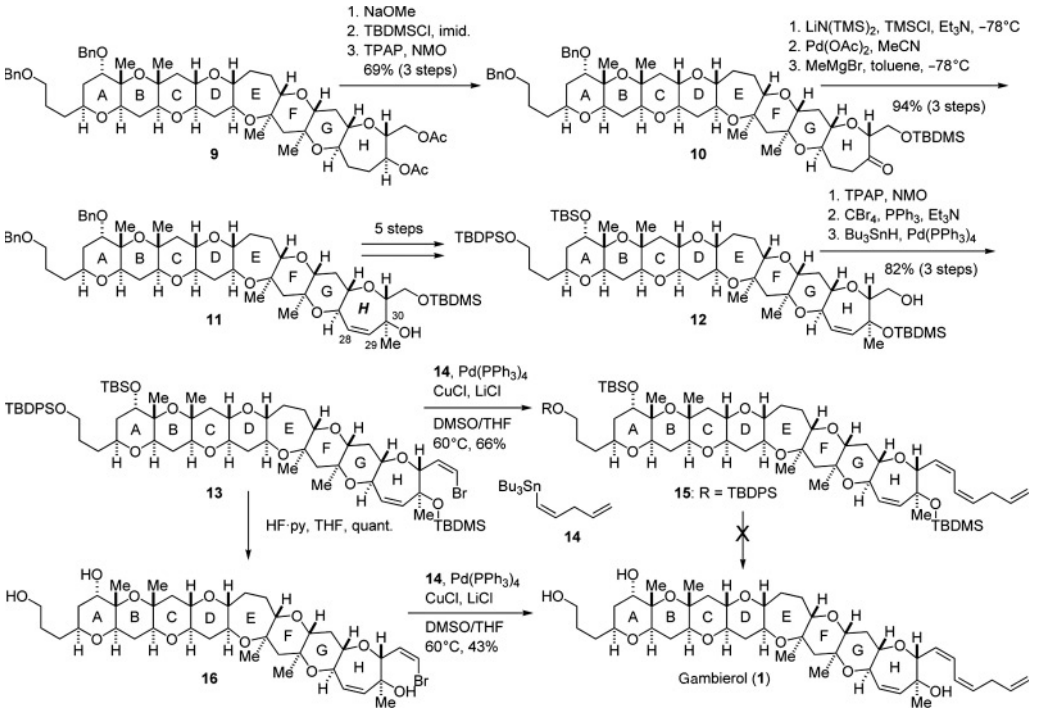
28.2.1.2.1 Sasaki Total Synthesis

In 2002, the first chemical total synthesis of gambierol was achieved by Sasaki group.[29,30] The synthesis features (i) convergent union of the ABC- and EFGH-ring fragments to construct the octacyclic polyether skeleton based on their developed Suzuki–Miyaura coupling strategy and (ii) stereoselective introduction of the labile triene side chain by $Pd(PPh_3)_4$/CuCl/LiCl-promoted modified Stille coupling at the final stage of the synthesis.

Hydroboration of the ABC-ring exocyclic enol ether **2**[30,42] with 9-borabicyclo[3.3.1]nonane (9-BBN) generated the intermediate alkylborane **3**, which was *in situ* reacted with the EFGH-ring ketene acetal phosphate **4**[30,42] in the presence of aqueous Cs_2CO_3 and a catalytic amount of $PdCl_2$(dppf) (dppf = diphenylphosphinoferrocene) in THF/DMF at 50°C (Scheme 28.1). The desired cross-coupled product **5** was obtained in a gratifying 86% yield. Subsequent hydroboration of the endocyclic enol ether with BH_3·THF led stereoselectively to the desired alcohol **6**, which was then oxidized with tetra-*n*-propylammonium perruthenate (TPAP)[43] and 4-methylmorpholine *N*-oxide (NMO) to afford ketone **7**. Oxidative removal of the *p*-methoxybenzyl (PMB) group from **7** followed by treatment with ethanethiol and zinc trifluoromethansulfonate ($Zn(OTf)_2$) effected cyclization of the D-ring as the mixed thioacetal to yield, after acetylation, **8**. Finally, desulfurization under radical reduction conditions (triphenylstannane, 2,2′-azobisisobutyronitrile (AIBN), toluene, 110°C)[44] proceeded cleanly to furnish the octacyclic polyether skeleton **9** in excellent yield.

For the functionalization of the seven-membered H-ring, octacycle **9** was converted to ketone **10** in a three-step sequence (Scheme 28.2). Incorporation of the C28–C29 double bond was performed by the Ito–Saegusa protocol.[45] The resulting enone was subsequently treated with methylmagnesium bromide (toluene, −78°C)[46] to give tertiary alcohol **11** in 94% yield (three steps) as a single stereoisomer. Standard protective group manipulations, performed on **11**, provided primary alcohol **12**. Oxidation to the aldehyde and Corey–Fuchs reaction,[47] followed by stereoselective reduction of the resulting dibromoolefin by the Uenishi et al. protocol,[48] led to (*Z*)-vinyl bromide **13** in good overall yield.

The final steps to complete the total synthesis required stereoselective construction of the triene side chain and global deprotection. After extensive experimentation, it was found that the $Pd(PPh_3)_4$/CuCl/LiCl-promoted modified Stille coupling conditions[49] were quite suitable for the construction



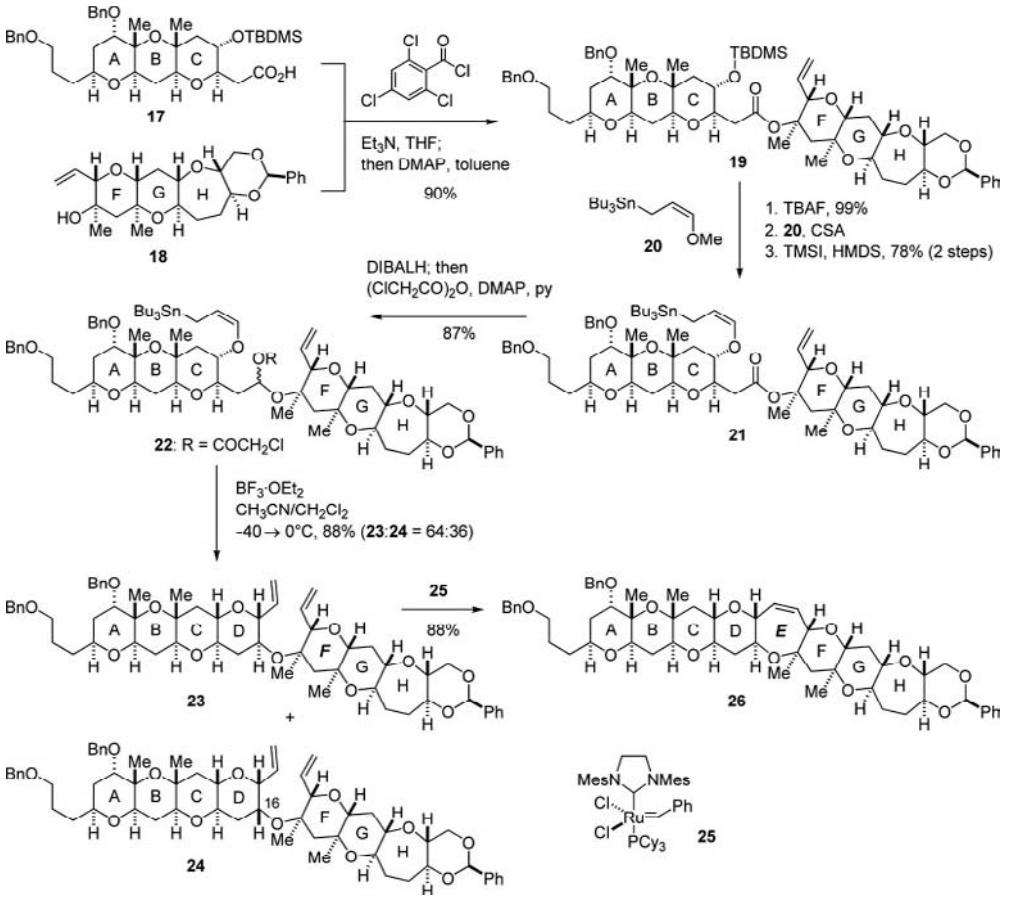
SCHEME 28.2 Sasaki's total synthesis of gambierol (continued).

of the side chain. Under the optimal conditions, the Stille coupling of **13** with (*Z*)-vinyl stannane **14**[50,51] furnished fully protected gambierol **15** in 66% yield (82% yield based on recovered **13**). However, all attempts to remove the silyl protecting groups from **15** under various conditions were unsuccessful because of the labile nature of the triene side chain. This critical issue was finally overcome by executing global deprotection before introduction of the side chain. Exposure of (*Z*)-vinyl bromide **13** to excess HF·pyridine facilitated clean deprotection of the three silyl groups, giving triol **16** in quantitative yield. Finally, the Stille coupling of unprotected **16** with **14** under the established conditions furnished (–)-gambierol (**1**) in 43% isolated yield. The spectroscopic data (^{1}H and ^{13}C NMR, HRMS, and CD) and mice lethality of the synthetic gambierol were completely identical to those of the natural sample, confirming the structure of gambierol including the absolute configuration. Thus, the first total synthesis of gambierol (**1**) was completed in 0.57% overall yield over a 71-step longest linear sequence. The present synthesis clearly demonstrated the utility of the Suzuki–Miyaura coupling strategy for the convergent synthesis of polycyclic ether natural products and allowed, for the first time, supply of ample quantities of gambierol for extensive biological studies.[52–55]

28.2.1.2.2 Yamamoto/Kadota Total Synthesis

Shortly after the first total synthesis was published by Sasaki, Kadota et al. they completed the second total synthesis of gambierol (**1**) in 2003.[37,38] The Yamamoto/Kadota group developed a convergent strategy for the synthesis of polycyclic ethers based on an intramolecular allylation of α-acetoxy ether followed by ring-closing metathesis reaction.[56–58] This convergent strategy was successfully applied to the union of the ABC- and FGH-ring fragments, leading to the total synthesis of gambierol.

The ABC-ring carboxylic acid **17** and the FGH-ring alcohol **18** were connected under Yamaguchi conditions to give ester **19** (Scheme 28.3). Desilylation with tetra-*n*-butylammonium Fluoride (TBAF), acid-catalyzed acetal formation with γ-methoxyallylstannane **20**, and acetal cleavage with iodotrimethylsilane/hexamethyldisilazane produced (γ-alkoxyallyl)stannane **21**. The ester **21** was

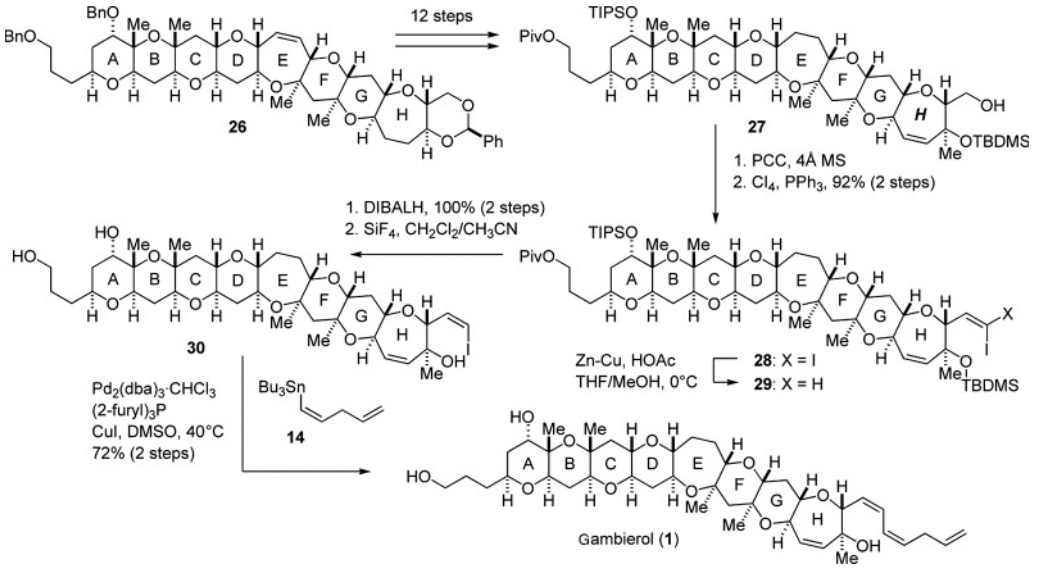


SCHEME 28.3 Yamamoto/Kadota's total synthesis of gambierol.

then converted into α-chloroacetoxy ether **22** following the Rychnovsky's protocol.[59–61] Thus, partial reduction of ester **21** with diisobutylaluminum hydride (DIBALH), followed by *in situ* acylation of the intermediate hemiacetal with chloroacetic anhydride/4-dimethylaminopyridine (DMAP)/pyridine, produced **22** as a mixture of diastereomers in 88% yield. Treatment of **22** with boron trifluoride etherate in CH_3CN/CH_2Cl_2 generated the desired **23** and the diastereomer **24** in a ratio of 64:36 (87% combined yield). RCM reaction of diene **23** with second-generation Grubbs catalyst **25**[62] furnished the octacyclic polyether **26** in high yield.

Construction of the fully functionalized H-ring was carried out from **26** in a 12-step sequence similar to the Sasaki's synthesis, giving primary alcohol **27** (Scheme 28.4). For the stereoselective construction of the triene side chain, a simple and practical method for the synthesis of (*Z*)-iodoolefin was developed.[63] Thus, oxidation of **27** with pyridinium chlorochromate (PCC) was followed by treatment with CI_4 and PPh_3 to yield diiodoolefin **28**, which was reduced with zinc–copper couple in the presence of acetic acid to afford the desired (*Z*)-iodoolefin **29**. After a two-step deprotection, the modified Stille coupling of the resulting triol **30** with **14** under the influence of $Pd_2(dba)_3{\cdot}CHCl_3/P(2\text{-furyl})_3/CuI$ (dba = dibenzylideneacetone) furnished gambierol (**1**) in 72% yield over the four steps. Overall, the synthesis required 66 steps (longest linear sequence, 102 total steps) and 1.2% overall yield.

Similar transformations starting from the isomer **24** provided 16-*epi*-gambierol (**31**), a non-natural analogue (Figure 28.11). Interestingly, the isomer **31** exhibited no toxicity against mice at higher dose (14 mg/kg, i.p.). This result indicates that the *trans*-fused polycyclic ether framework is important for the toxicity.[38]



SCHEME 28.4 Yamamoto/Kadota's total synthesis of gambierol (continued).

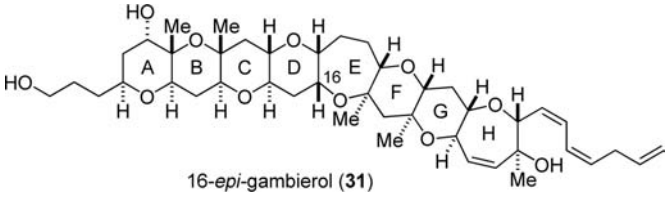


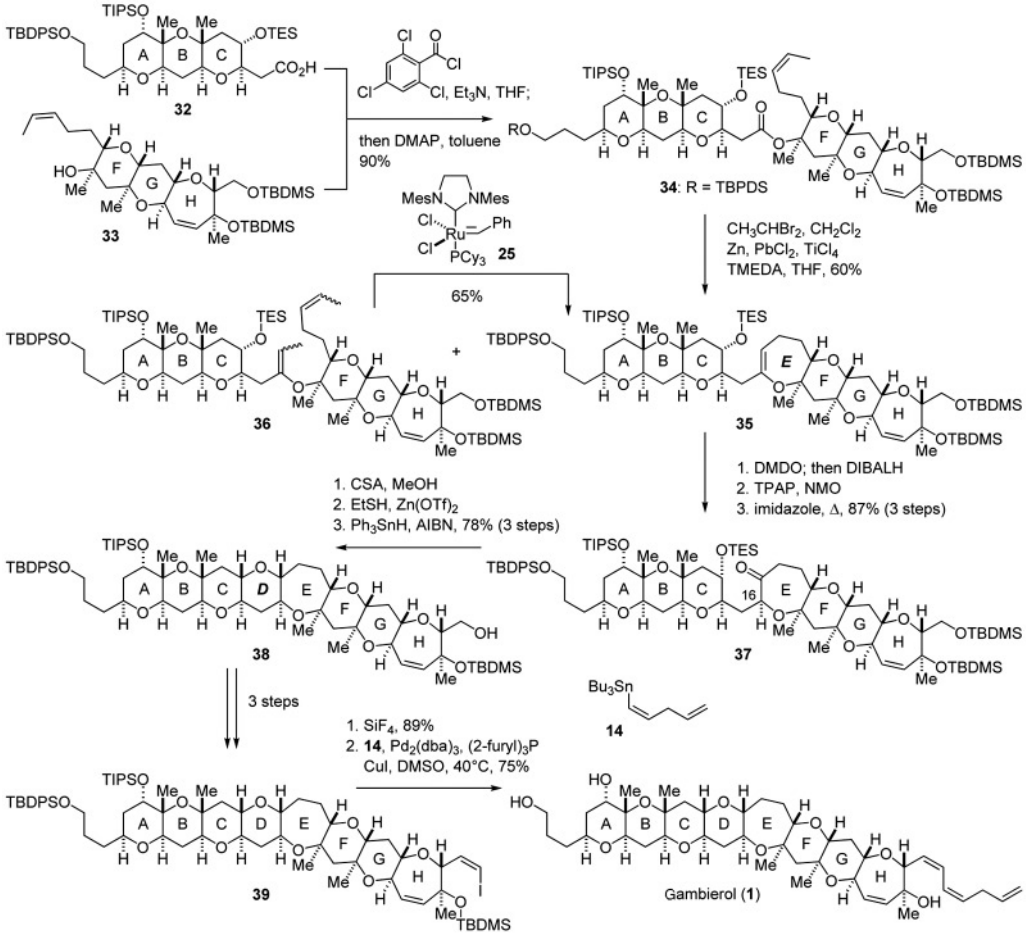
FIGURE 28.11 Structure of 16-*epi*-gambierol.

28.2.1.2.3 Rainier Total Synthesis

Most recently, Rainier and coworkers reported a more convergent total synthesis of gambierol (**1**).[39,41] Their synthesis involves as a key feature enol ether-olefin ring-closing metathesis for the iterative synthesis of the ABC- and FGH-rings and the fragment coupling to form the E-ring.

Convergent union of the ABC- and FGH-ring fragments (**32** and **33**, respectively) through esterification under the careful Yamaguchi conditions to provide **34** (Scheme 28.5). Initial attempts to generate acyclic enol ether metathesis precursor from **34** using the Takai-Utimoto titanium methylidene protocol[64,65] were unsuccessful. After considerable experimentation, they found effective conditions for the cyclization of the E-ring. Thus, reaction of **34** with the titanium ethylidene reagent, generated from 1,1-dibromoethane instead of dibromomethane, led to the formation of seven-membered enol ether **35** in 60% yield, along with the corresponding acylic enol ether **36** (30% yield). The latter compound could be converted to **35** in 65% yield by treating with the second generation Grubbs catalyst **25** in the presence of ethylene at elevated temperature.

Oxidation of cyclic enol ether **35** with dimethyl dioxirane (DMDO) followed by *in situ* reduction of the intermediate epoxide with DIBALH gave secondary alcohol as a 10:1 mixture of diastereomers. Oxidation of these alcohols with TPAP/NMO afforded a 10:1 mixture of ketone **37** and its C16 epimer. The isomers were separated and the minor isomer was recycled to a 4:1 mixture of isomers by treatment with imidazole. Subsequent construction of the D-ring was performed by radical reduction of mixed thioacetal in the same way as that adopted by the Sasaki group, leading to octacycle **38**. Stereoselective installation of the triene side chain was then carried out via (*Z*)-vinyl iodide **39**



SCHEME 28.5 Rainier's total synthesis of gambierol.

according to the Yamamoto's protocol to complete the total synthesis of gambierol (**1**). The high convergency of the Rainier's synthesis is noteworthy in light of the use of fragment **33** furnished with the H-ring functionality, thus, the longest linear sequence to **1** was 44 steps from D-glucal with 1.5 % overall yield.

28.2.1.3 Structure–Activity Relationship Studies on Gambierol

There exist only a few reports concerning structure–activity relationships (SAR) of marine polycyclic ether toxins, mainly due to (i) extremely limited availability of these toxins from natural sources and (ii) difficulties of derivatization of the highly complex natural products themselves.[66–68] In the case of gambierol, the first problem is being solved by the efficient total synthesis achieved, which allows the preparation of ample quantities of this natural toxin. On the other hand, the second problem would be overcome by "diverted total synthesis."[69] The synthetic route is diverted at the stage of an advanced intermediate for further chemical manipulation. Fuwa/Sasaki selected octacyclic polyether **9** as a branching point for diverted total synthesis of gambierol analogues not accessible from the natural product itself. Thus, a series of structural analogues were synthesized starting from **9** and investigated for their toxicity against mice, thus, allowing for the first time the systematic studies of SAR of this complex marine toxin.[70,71] These SAR studies revealed that the structural

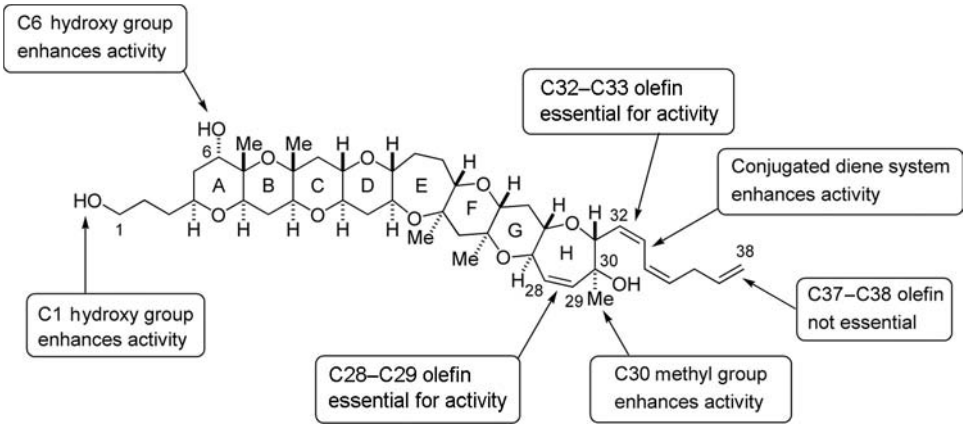


FIGURE 28.12 Structure–activity relationships of gambierol.

elements of gambierol, indispensable for exhibiting potent toxicity are (i) the C28–C29 double bond within the H-ring and (ii) the unsaturated side chain of specific length (Figure 28.12). In contrast to these important structural elements, the C1 and C6 hydroxy groups, the C30 methyl group, and the C37–C38 double bond are not essential but are preferred functional groups for toxicity. The present results will allow rational design of biotinylated or fluorescently labeled molecular probes useful for detailed biological studies and detection of gambierol.

28.2.2 Pharmacology

Information on the mechanism of action of gambierol is scarce. Recent pharmacological studies on this toxin led to clarification of its possible targets. Gambierol could be affecting two distinct voltage-gated ion channels: sodium[54,72] and potassium.[53,55]

28.2.2.1 Sodium Channels as a Target for Gambierol

Voltage-gated sodium channels are critical elements for generation and propagation of electrical signals in most excitable cells.[73–75] These channels are targets for a great number of drugs, such as local anesthetic, insecticides, and neurotoxins like ciguatoxins (CTXs) and brevetoxins (PbTXs).[21,22,76–80] These lipid-soluble polycyclic ethers bind specifically to receptor site 5 of the α-subunit of the sodium channels. The binding alters channels function by changing the activation voltage for channel-opening to more negative values,[81,82] extending the mean open time, inhibiting the inactivation of opened channels resulting in a persistent activation, and also inducing subconductance states.[79,83]

Researchers have determined the drugs binding site on the voltage-gated sodium channel by using a variety of ion fluxes and radiolabeled neurotoxin-binding studies. Following this methodology, Inoue et al. tested the behavior of some polycyclic ethers.[72] Ciguatoxins are not available as radiolabeled analogues for direct investigation of their specific binding, therefore, tritiated dihydrobrevetoxin-B ([^{3}H]PbTx-3) has been used in homologous and heterologous displacement experiments to define site 5 toxins.[78,80,84,85] Inoue et al.[72] checked if gambierol, gambieric acid-A, ciguatoxins and yessotoxins were able to inhibit the binding of PbTx-3 to site 5 of the voltage-gated sodium channel.All those compounds share as common structural feature, a long semirigid architecture with *trans*/*syn*-fused ether rings of various sizes. Inoue found that all compounds tested except yessotoxin were able to displace [^{3}H]PbTx-3 from site 5 (Figure 28.13). Gambierol inhibited the binding of [^{3}H]PbTx-3 in the micromolar range, indicating a common target with brevetoxins. They suggested that the

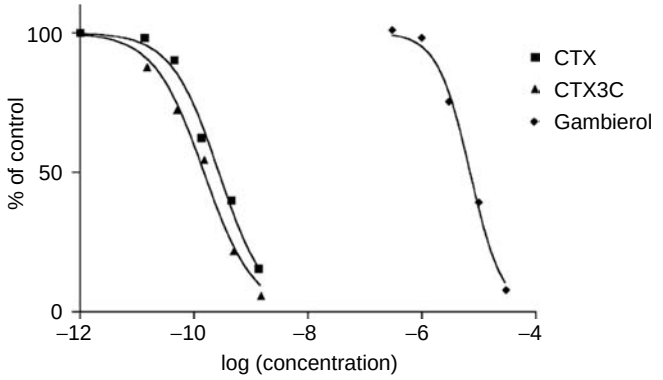


FIGURE 28.13 Inhibition of the binding of [^{3}H]PbTx-3 to site 5 of the voltage-gated sodium channels by polyethers. (Reprinted from Inoue et al., *Toxicon*, 41, 469, 2003. With permission from Elsevier.)

gambierol could bind to site 5, or at least overlap it. Although they do not exclude the possibility that gambierol may be allosterically modulating the sodium channels in order to displace [^{3}H]PbTx-3.

They also considered the relationship between the sizes of the polycyclic backbones and the binding affinities of the active molecules and concluded that there was a linear relationship. Therefore, the accessible surface area of the polycyclic region could contribute to the binding abilities. This way, they observed that gambierol, having less fused rings than CTXs, has a lower free energy of binding.

Louzao et al.,[54] by using another methodology, microscopical detection of fluorescent dyes, reached to the conclusion that gambierol is a partial agonist of sodium channels in human neuroblastoma cells. They made a set of experiments in these excitable cells in order to correlate the gambierol effect with the neurological symptoms observed in mice. The technique used was a potentiometric probe based on the fluorescent dye *bis*(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$), bis(oxonol).[17–20] Results showed that 30 μM gambierol induced a sodium-dependent depolarizing effect (Figure 28.14). Moreover, confirmation that sodium channels are involved in this toxin effect was obtained with neosaxitoxin, a known specific inhibitor of the voltage-gated sodium channel.[86–88]

On the basis of the depolarization evoked by gambierol, Louzao et al.[21] suggest that gambierol could be acting as ciguatoxins on the site 5 of the α subunit of the voltage-gated sodium channel. It is well known that neurotoxins receptor sites at the sodium channel are allosterically modulated.In this way, depolarization induced by toxins binding to the receptor site 2, such as the alkaloid veratridine, is enhanced by CTXs or PbTXs acting on the receptor site 5 of the sodium channel complex.[20,22] Louzao et al. found that veratridine increased its depolarizing effect after preincubating the cells with gambierol. Also in a competitive assay gambierol was able to reduce depolarization evoked by CTX3, which agrees with the previous binding assay carried out by Inoue et al.[72] Louzao et al.[54] conclude that gambierol is acting as a low-efficacy partial agonist at neurotoxin site 5 opening the sodium channel.

In addition, they used another fluorescent dye, Fura-2 acetoxymethyl ester (Fura-2, AM) in order to test cytosolic calcium changes induced by gambierol.[89] They detect a calcium increase depending on extracellular sodium and abolished in a Ca^{2+}-free solution (Figure 28.15). Then they reach to the conclusion that gambierol induced a sodium-dependent calcium entry. Inhibitors such as dichlorobenzamil, a Na^+–Ca^{2+} exchanger blocker,[90–92] nifedipine, a L-type calcium channel blocker,[93–97] and nitrendipine, a L- and N-type calcium channels blocker,[95,98] all of them reduced in some manner the calcium entry. Therefore, the calcium increment induced by gambierol was secondary to the depolarization and due to voltage-gated calcium channels and to the Na^+–Ca^{2+} exchanger acting in reverse mode.

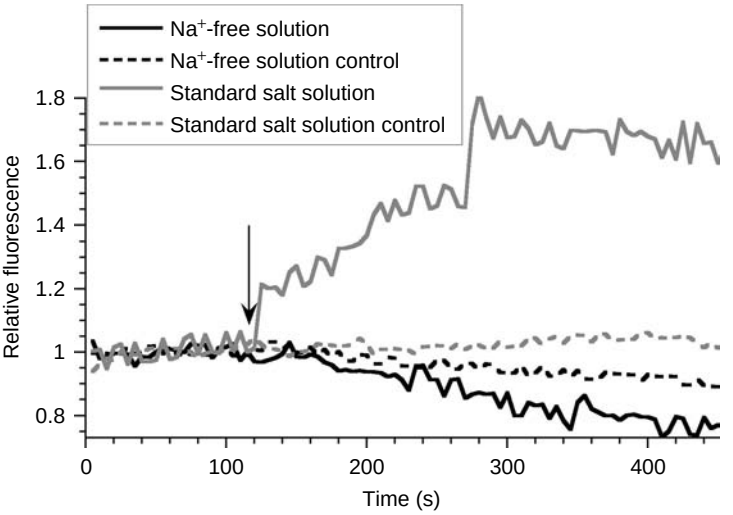


FIGURE 28.14 Influence of extracellular Na^+ on the depolarizing effect induced by 30 μM gambierol (arrow) in human neuroblastoma cells. This effect was measured in a Na^+-free (black lines) and Na^+-containing (gray lines) solution. Depolarization resulted abolished in the Na^+-free solution (black continuous line). Representative time course.

In order to check if the toxin was able to exhibit activity toward cytoskeleton of neuroblastoma cells, Louzao et al.[99] stained filamentous actin (F-actin) of the cells with Oregon Green® 514 Phalloidin and detected the fluorescence with LSC and confocal microscopy. Neuroblastoma cells incubated with 30 μM gambierol for 4 h, did not show modifications in the amount of F-actin or its distribution, indicating a cellular integrity in treated cells (Figure 28.16). Same results were observed with CTX3.

They also tested cytotoxicity of gambierol and CTX3 for long periods, until 72 h. Gambierol seems to induce a small reduction in the activity of the cells starting at 36 h that does not appear in cells treated with CTX3, which may indicate a difference between both toxins.[99]

28.2.2.2 Potassium Channels as Another Possible Target for Gambierol

Ghiaroni et al.[53] described that gambierol could be acting as a voltage-gated potassium channels blocker. These channels take part in membrane excitability, being responsible for setting the resting potential and repolarizing the cellular membranes during action potentials.[100–102]

They tested the effect of gambierol on taste buds from mouse vallate papilla by using the patch-clamp technique to monitor membrane ion currents. These excitable cells are believed to be sensory cells able to detect food chemicals, so alterations on the ionic currents produced by the toxin could affect the signal transduction and processing.

Pharmacology of ciguatoxins is characterized by their ability to cause persistent activation of voltage-gated sodium channels and increase neuronal excitability.[80,82,103,104] On the basis of the similar chemical structure and biogenetic origin, it was likely that gambierol and ciguatoxins share the voltage-gated sodium channel as a common target. Unexpectedly, Ghiaroni et al. did not find significant alterations on the voltage-gated sodium current (I_{Na}) induced by gambierol. Surprisingly, they found that concentrations from 1 nM to 1 μM gambierol during 5 min as maximum time inhibited the potassium currents (I_K) with a IC_{50}= 1.8 nM. They found some differences with the inhibitory effect induced by tetraethylammonium (TEA). The first one was that only TEA induced a complete abolishment of I_K, on average they observed just a approximately 60% reduction of I_K induced by 100 nM gambierol. And the other difference is that after washing out TEA, I_K recovered to control

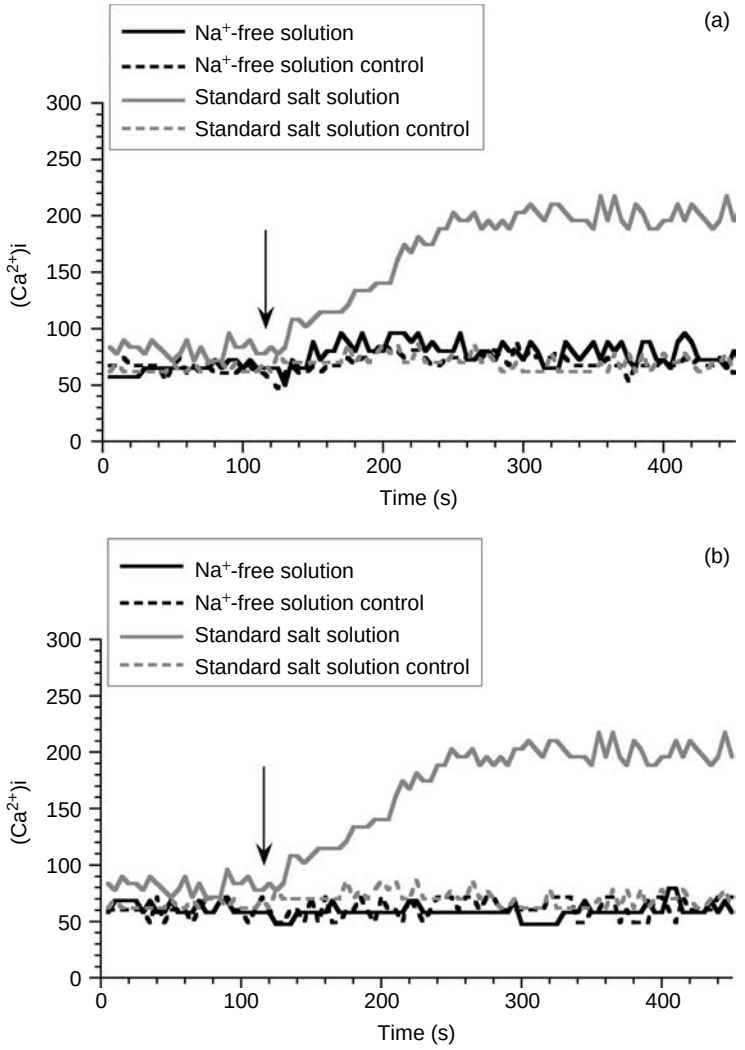


FIGURE 28.15 Influence of extracellular ions on the effect of 30 μM gambierol (arrow) in intracellular calcium. This effect was measured in human neuroblastoma cells (a) in a Na^+-free and Na^+-containing solution and (b) in a Ca^{2+}-free and Ca^{2+}-containing solution. Intracellular calcium increase triggered by 30 μM gambierol resulted annulled in the Na^+-free and in the Ca^{2+}-free solutions. Representative time course.

values, whereas gambierol effect was irreversible. On the other hand, among repolarizing currents (I_K and I_{Cl}) occurring in mouse taste cells,[105] they found that gambierol affected only I_K.

Recently, it has been reported that CTX-1, the main ciguatoxin involved in ciguatera fish poisoning[84,106] also has a blocking effect on voltage-gated potassium channels.[107,108] Hidalgo et al., by using electrophysiological recordings on cultured rat myotubes from skeletal muscle cells, explore the effects of CTX-1 on potassium currents, observing a blockade of the net K^+ currents at concentration 5 nM and even 2 nM. In this work, they conclude that CTX-1 besides interacting with the sodium channels also interacts with potassium channels.[107]

Birinyi-Strachan et al. used patch-clamp recording techniques on rat dorsal root ganglion neurons in order to study the blockage of voltage-gated potassium channels by CTX-1, specifically delayed-rectifier potassium (K_{DR}) channels and transient A-type potassium (K_A) channels. They observed that in addition to cause a depolarization of the resting membrane potential and a significant increase

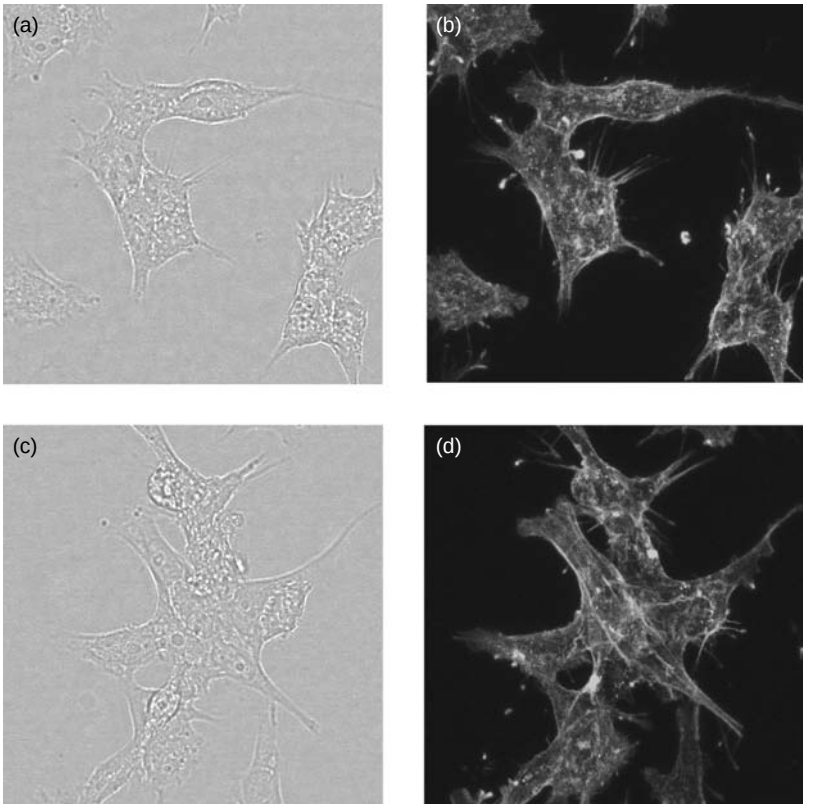


FIGURE 28.16 Representative images showing morphology and actin cytoskeletons of control cells (a,b) and neuroblastoma cells incubated with 30 μM gambierol (c,d).

in mammalian action potential and after hyperpolarization duration, 20 nM P-CTX-1 also blocks I_K. This blockage was comparable to the inhibition induced by TEA or 4-aminopyridine, known blockers of the potassium channels, although the effects of the toxin could not be reverted even after prolonged washout in toxin-free solution.[108] They suggested that CTX-1 may either slow the inactivation of the voltage-gated sodium channels (maintaining them in an open state) or may cause a significant block of voltage-gated potassium channels with IC_{50} values around 20 nM.

Recently, Molgó group provided another effect evoked by gambierol. They used isolated frog cutaneous pectoris and mouse phrenic-hemidiaphragm nerve–muscle preparations, finding that gambierol increased in a great manner the quantal acetylcholine (Ach) release.[109] This modulating effect discriminate this toxin from CTXs.

28.2.3 Toxicology

Since gambierol was first discovered by the Yasumoto group,[27] this marine toxin became a really interesting molecule because of its complex chemical structure and potency.[37,70,71] Nevertheless, the study of this particularly interesting compound has been slowed down, mainly due to the scarcity of this toxin from natural sources. In spite of it, Ito et al. were able to determine the MLD in mice,[52] resulting 50–80 μg/kg by i.p. injection and 150 μg/kg by per oral (p.o.) administration. The effective dose level is quite close, which may be indicative of an efficient toxin absorption from the intestine.[52]

Ciguatera poisoning is due to consumption of ciguatoxins,[110,111] a group of lipid-soluble, polyether toxins accumulated in the muscles of certain subtropical and tropical marine finfish.

Although apparition of gambierol in the flesh of ciguateric fish has never been reported, its pathological effects in mice resemble those evoked by ciguatoxins.[52]

Symptoms of ciguatera include a variety of gastrointestinal, neurological, and cardiovascular disturbances, with a diffuse pain syndrome and an onset inversely related to the quantity of toxin consumed, ranging from 30 min until 24 h or occasionally 48 h, and lasting for few days, weeks or even months.[112] Initially, poisoning is characterized by the appearance of gastrointestinal symptoms, like nausea, vomiting, diarrhea, and abdominal pain. Frequently, but not always, those gastrointestinal symptoms are accompanied by neurological disturbances including numbness of the lips, hands and feet, reversal of temperature perception (dry ice phenomenon), itching of the skin, heightened nociception, dysgeusiae (taste alterations), headache, muscle, joint, and, less frequently, teeth aches, and in severe cases blurred vision, lack of coordination, and paralysis.[113–115] Cardiovascular disorders, such as bradycardia and hypotension, are relatively slightly reported, though death may occur as a result of cardiac dysrhythmias, hypotension, shock or cerebral edema, being these cases the fewer ones.[116] In any case, the mortality rate is very low.

Ito et al. studied pathological effects on experimental mice after administrations of gambierol at 60–150 μg/kg,[52] and Terao et al. tested pathological changes induced in mice receiving 0.7 μg/kg of ciguatoxins.[117] After i.p. administration of gambierol, the organs affected were lung and secondary heart. In the case of ciguatoxins, the main pathological changes were also found in heart, medulla of adrenal glands and autonomic nerves. All the symptoms observed suggest that mice treated with gambierol died mainly due to dyspnea, as well as the ciguatoxin treated ones.

In both cases, after administration of the toxins ultrastructural changes in heart and lung were characteristic (Figure 28.17). Treated mice showed a marked congestion of the heart with the right ventricles dilated, and with the cardiac muscle fibers separated each other because of the edema and congestion. In addition, the more damaged mice showed a marked edema of the lungs. Alveolar spaces and bronchioles of the ciguatoxin-treated mice were filled with plasma, and the alveolar septum and vessels showed prominent congestion. In the same way, alveolar lumens of gambierol-treated mice were filled with flocculent and/or fine granular substances in addition to surfactant. In those lumens were also frequently found alveolar macrophages, neutrophils and lymphocytes, maybe mobilized to remove gambierol from the alveolar lumen.

Another organ damaged by gambierol was the liver (Figure 28.18), which evidenced congestion resulting in fatty changes around the central vein, and the kidney, which got a prominent congestion at the medulla. Ciguatoxins also induced marked congestion in the liver, with several thrombi in the main trunks of the hepatic veins.

Another common characteristic observed after the administrations of both toxins was congested or bleeding penises in some of the mice.

Opposite to ciguatoxin, gambierol did not induce diarrhea. However, after gambierol treatment stomachs showed sporadically ulceration and erosion of mucosa and edema at submucosa. Minor changes were observed in the small intestine although became dilated, containing gas and liquid (as well as the stomach).

28.2.4 Detection

In 1993, Yasumoto and coworkers reported gambierol isolation from the cultured *G. toxicus* (GI-1 strain) collected at Rangiroa Atoll, Tuamotu Archipelago, French Polynesia.[27,30] They cultured dinoflagellates in seawater medium enriched with ES-1 nutrient at 25°C during 21 days. They obtained that 1100 L of fermentation broth resulted in 1.2 mg of gambierol, so it is difficult to get significant quantities of this toxin from the natural source.[41] However, this toxin could be a precursor of any ciguatoxin. In fact, ciguatoxins originally derive from precursors found in *Gambierdiscus* spp. These dinoflagellates produce toxins less polar and less potent than ciguatoxins (known as gambiertoxins) that are biotransformed into ciguatoxins in the liver of fish by oxidative metabolism.[106,118]

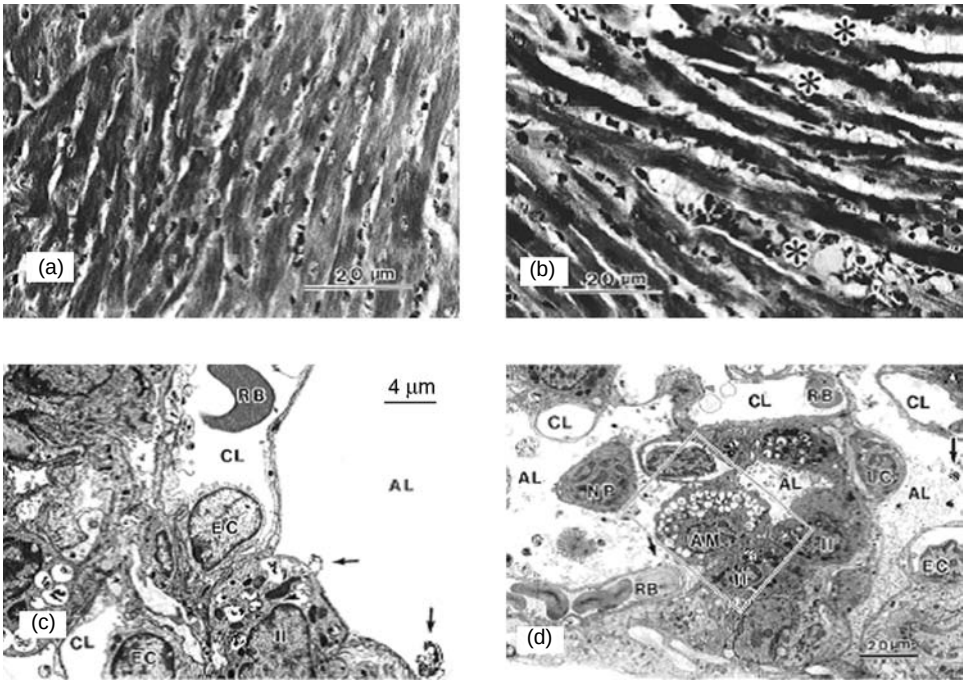


FIGURE 28.17 (a) The heart of control from nontreated mice and (b) 2 h after p.o. administration of 140 μg/kg gambierol. The myocardium showed separation of muscle fibers by edema (*) and congestion. (c) The lung of control shows a wide alveolar lumen (AL) and surfactant (arrows) can be seen on the epithelial surface. (d) Low-magnification image of the alveoli of a mouse in serious condition 40 min after p.o. administration of gambierol at 140 μg/kg. Note the narrow alveolar lumens (ALs) are filled with materials including surfactant (arrows). AM: alveolar macrophase in the alveolar lumen of the treated mice, II: type II alveolar cells, CL: capillary lumen EC: endothelial cell, NP: neutrophil, RB: erythrocyte. (Reprinted from Ito et al., *Toxicon*, 42, 733, 2003. With permission from Elsevier.)

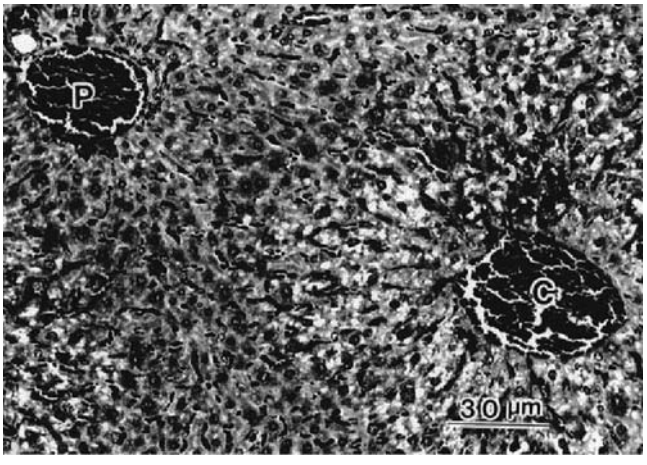


FIGURE 28.18 Mouse liver 2 h after p.o. administration of 140 μg/kg gambierol. Prominent congestions of sinusoidal capillaries appeared at the periphery and fatty changes (white) appeared in the central area. P: portal veins, C: central vein. (Reprinted from Ito et al. *Toxicon*, 42, 733, 2003. With permission from Elsevier.)

On the basis of the structural characteristics, toxicological common effects, similar targets, and possible biotransformation between them, gambierol and ciguatoxins could share methods of detection.

The diagnosis in humans depends only on clinical signs associated to a recent ingestion of fish and therefore, ciguatera poisoning remains largely underdiagnosed and underreported.[119,120] In the European Union , Council Directive 91/493/EEC lays down the health conditions for the production and the placing on the market of fishery products, stating: "The placing on the market of the following products shall be forbidden: poisonous fish of the following families: Tetraodontidae, Molidae, Diodontidae, Canthigasteridae; and fishery products containing biotoxins such as ciguatera toxins or muscle-paralyzing toxins," and "In order to allow the import conditions to be fixed, and in order to verify the conditions of production, storage, and dispatch of fishery products for consignment to the Community, inspections may be carried out on the spot by experts from the Commission and the Member States."[121] In May 1994, the European Commission adopted an additional regulation, which made it mandatory to impose more exact rules for the application of the "own health checks" (term used instead of HACCP, Hazard Analysis and Critical Control Point).[122] In endemic areas it is recommended to avoid large fish that are on the top of the food chain and may have concentrated the toxin.[123]

Several detection methods have been developed to screen for ciguatoxins; however, there is still no widely generalized diagnostic test for ciguatera poisoning.

28.2.4.1 *In Vivo* Bioassays

The bioassays are semiquantitative and sensitive because ciguatoxins induce characteristic signs of toxicity, but the use of some animal species can be problematic in terms of cost and ethical difficulties. As gambierol induces similar symptoms than ciguatoxins to mice, the mouse bioassay could be used for detecting it.

The mouse bioassay is presently the most widely used assay for detection of ciguatoxins in fish. Toxic extracts are intraperitoneally injected into mice in duplicate, and the symptoms are observed over at least 24 h.[124,125]

A number of other animal assays have been reported for the detection of ciguatoxins, such as chicken assay, mongoose and cat assay, brine shrimp assay, mosquito assay, or diptera larvae assay. These bioassays are in use only in a few laboratories, but just the mouse bioassay has been validated.[126]

28.2.4.2 *In Vitro* Assays

28.2.4.2.1 Sodium Channel Binding Assays

Ciguatoxins, same as brevetoxins, bind to site 5 on sodium channels, so toxins can be detected based on their ability to selectively inhibit the binding of [^{3}H]-brevetoxin to sodium channels in rat brain synaptosomes.[80,85,125] Inoue et al. published that gambierol was able to displace [^{3}H] - brevetoxin from its binding site in the same conditions as ciguatoxins, though at higher concentration.[72]

Ciguatoxins bind to sodium channels and open them at normal cell resting membrane potentials. This results in an influx of sodium ions, cell depolarization, and the appearance of spontaneous action potentials in excitable cells. Toxins that activate the sodium channels through an allosteric mechanism can enhance this influx of sodium ions. Louzao et al.[16,20] and Manger et al.[127,128] proposed to use this effect in order to develop an assay that is sensitive to ciguatoxins and other sodium channel activator toxins, in this case the gambierol.

28.2.4.2.2 Antibody Assays

Immunoassays are also used to marine toxin detection. Hokama et al. first developed a test for detecting ciguatoxin directly from natural sources by radioimmunoassay (RIA).[129] In this assay,

CTX conjugated to human serum albumin was injected into sheep, which produced antibodies. The sheep antibody to CTX was purified and coupled to ^{125}I as a label, and then used in the RIA. Those assays resulted to be too complex and expensive, as well as reporting cross-reactivity with other polyether compounds such as brevetoxin and okadaic acid.[124]

Hokama et al. developed an enzyme immunoassay (EIA) that replaced the RIA.[130] The procedure incorporated horseradish peroxidase coupled to the sheep anticiguatoxin, and was similar in efficacy to the earlier RIA, but less expensive and more practical. However, trying to further simplify the enzymatic procedure, Hokama incorporated a coating on the bamboo sticks used as test tools, so they can adsorb the toxins and then be mixed with reagents.[131] This method was proved successful in separating toxic from nontoxic fish.

Those efforts culminated in a rapid stick-enzyme immunoassay using horseradish peroxidase-labeled sheep anticiguatera toxin antibody developed by Hawaii Chemtect International (Cigua-Check®) for detecting ciguatera toxins and toxins associated with diarrheic shellfish poisoning. The Cigua-Check® test can only be used as a general screening method to select samples for further analysis, because the lack of ciguatoxins standards has hampered the determination of relative cross reactivity with various derivatives.

In 1990, a solid-phase immunobead assay (SPIA), with colored polystyrene particles coated with anti-CTX monoclonal antibody (MAb-CTX) began to be used for direct detection of ciguatoxins adsorbed on bamboo paddles coated with organic correction fluid.[132] The membrane immunobead assay (MIA) presented by in 1998 Hokama et al.[133] is based on the immunological principles used to develop the SPIA, using a MAb-CTX coated onto colored polystyrene beads. The polyether toxins extracted from a piece of fish tissue bind to the hydrophobic membrane on a plastic support (membrane stick), and can be detected with the MAb-CTX coated onto the colored polystyrene beads. The color intensity of the membrane is related to the concentration of the toxin bound to the membrane.

Since then, many improvements have been made on the format and antibodies used in the test in order to minimize error,[134–136] however, there does still exist some variations and nonspecific binding for different toxins.

Based on the possibility that gambierol could be a precursor of ciguatoxins and the cross-reactivity of the previous antibodies with other structurally related toxins, these antibody assays could become an alternative for detection of gambierol. Nevertheless, cross-reactivity studies with existing antibodies must be done, or design a more specific one.

28.2.4.3 Chemical Assays

28.2.4.3.1 Liquid Chromatography

High performance liquid chromatography has been reported to provide a highly sensitive potential to detect natural levels of ciguatoxins in crude extracts from fish flesh and wild *G. toxicus*.[84,118,125]

G. toxicus was collected at Rangiroa Atoll and cultured in seawater medium. Cultured cells were extracted with MeOH, and the extract was partitioned between CH_2Cl_2 and aqueous MeOH (6:4). Then, the CH_2Cl_2 extract was successively chromatographed over a Florisil column using Me_2CO/MeOH (9:1), a Toyopearl HW-40 column using MeOH, and an Asahipak ODP-50 column using MeCN/H_20 (45:55) as eluent, λ 210 nm.

HPLC coupled to selective-ion monitoring ion spray mass spectrometry (ISMS) is an alternative to fluorescence detection of ciguatoxin in LC eluants, since ISMS is a sensitive method capable of the determination of the molecular weight of polyether toxins such as ciguatoxins, brevetoxins, and maitotoxin.[137]

28.2.4.3.2 NMR/MS

Although applicable to a wide variety of toxins, conventional HPLC with UV detection is limited by low sensitivity of many toxins and from interference of naturally occurring biochemicals with similar

retention times as the toxins. The problem of low sensitivity can be improved by derivatization of the toxin to a fluorescent molecule. Electrochemical oxidation also has provided an alternative to chemical oxidation. Replacing a single-wavelength UV detector with a diode array detector (DAD) also provides better selectivity for target compounds among interfering compounds. However, a major advancement in HPLC technology has been coupling of the liquid chromatography column with a mass spectrometer (LC-MS), which provides far superior selectivity and sensitivity over UV detection.

Nuclear magnetic resonance (NMR) and/or MS techniques have been used to characterize ciguatoxins present in fish[84,126,138] and to characterize gambiertoxins (included gambierol) in wild and cultured *G. toxicus* extracts.[27,138]

Cultured cells were extracted as for the LC system, and NMR spectra were recorded on a JEOL GSX-400 spectrometer (400 MHz) in MeCN or C_5D_5N. The molecular formula $C_{43}H_{64}O_{11}$ for gambierol was determined by HR-FABMS (high-resolution fast atom bombardment mass spectra) $[M + Na]^+$ *m/z* 779.4348 calcd for $[C_{43}H_{64}O_{11}Na]^+$ *m/z* 779.4346; UV_{max} (MeOH) 237 nm (ε 15800). The ^1H and ^{13}C NMR spectra showed five single methyls, three hydroxyls, and four olefins (two conjugated). Coupling constants of angular protons (10 Hz) also supported transfusion of rings, being the ring system of gambierol 6/6/6/6/7/6/6/7.

On the basis of that, Satake et al. concluded that the molecular size, chromatographic properties and symptoms caused in mice make gambierol and ciguatoxin to be similar compounds causative of ciguatera.[27]

Presently, there is no generalized screen for ciguateric fish, just alternative approaches to monitoring that can reduce the health risk associated with ciguateric fish. A positive finding in a fish would remove that animal from sale. In some cases, restrictions are placed on the sale of fish of certain species or size from a given area, with no testing of the toxin. The larger and older the fish is, more toxin has probably accumulated. Reef carnivores, considered regular ciguatoxin carriers, are often banned from sale as a matter of principle. The hazard is linked to the accumulation in the food chain of the toxins, which in case of fishes is impossible to link with algal blooms. Formal risk assessments of the marine biotoxins should be performed by recognized international bodies, such as the Joint FAO/WHO Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA).

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Part IX

Palytoxin, Ostreocin, Ovatatoxin

29 Palytoxin and Analogues: Ecobiology and Origin, Chemistry, Metabolism, and Chemical Analysis

Panagiota Katikou

CONTENTS

29.1 INTRODUCTION

Palytoxin is one of the most poisonous nonprotein substances known to date. The lethal doses of palytoxin in rats, mice, guinea pigs, rabbits, dogs, and monkeys range between 0.03 and 0.45 μg/kg by intravenous administration [1]. By extrapolation, a toxic dose in a human would be between 2.3 and 31.5 μg [2]. This toxic compound was first isolated in 1971 from marine soft corals of the genus *Palythoa* [3]. At the time, an initial estimation was a molecular weight of about 3300 and a chemical formula of $C_{145}H_{264}N_4O_{78.}$ However, almost 10 years later, in 1981, the gross structure of palytoxin was elucidated by two groups independently, the one led by Professor Hirata at Nagoya in Japan [4] and the other by Professor Moore at Honolulu in the United States [5].

Palytoxin is a large, very complex molecule with both lipophilic and hydrophilic areas, and has the longest chain of continuous carbon atoms known to exist in a natural product. Its chemical formula is $C_{129}H_{223}N_3O_{54}$ with 115 of the 129 carbons being in a continuous chain. Another unusual structural feature of palytoxin is that it contains 64 stereogenic centers, which means that palytoxin can have 2^{64} stereogenic isomers. Moreover, there are eight double bonds able to exhibit cis/trans isomerism, which means that palytoxin can have more than 10^{21} (one sextillion) stereoisomers [6]. Despite this huge amount of isomers, in 1989, the group of Professor Kishi from Harvard University achieved the monumental task of complete chemical synthesis of the correct isomer of palytoxin in its carboxylic acid form [7].

The present chapter is divided into three sections. The first one will be dealing with the ecobiology and origin of this fascinating molecule, palytoxin, and will also emphasize on the most important available data regarding its chemistry and metabolism. The second section will be focused on the available data with regard to ecobiology, origin, and chemistry of certain naturally occurring palytoxin analogues, commonly known as ostreocins. The third and final one will refer to the up-to-date reported chemical analysis methods for this group of compounds.

29.2 PALYTOXIN

29.2.1 Ecobiology and Origin

Palytoxin has been isolated to date in numerous areas of the world both from zoanthids belonging to the genus *Palythoa* and in other species. Moore and Scheuer [3] first reported the isolation of palytoxin from the Hawaiian zoanthid *Palythoa toxica*, while 1 year later, it was also isolated from *Palythoa tuberculosa* in tropical Pacific waters of Okinawa, Japan [8]. Since then, palytoxin and its congeners have been found in *P. vestitus* from Hawaii [9], *P. mammilosa* [10] and *P. caribaeorum* [11] from the islands of West Indies such as Jamaica, Puerto Rico, and the Bahamas, unidentified *Palythoa* spp. from Tahiti [5,12] and Okinawa [13], and recently in *Palythoa* aff. *margaritae* from Nakanoshima Island in Japan [14]. Spreading of palytoxin in the organisms living close to the zoanthid colonies has also been reported [15,16].

The origin of palytoxin in the *Palythoa* zoanthids has been a matter of speculation for long due to the significant seasonal and regional fluctuations observed in its contents. For this reason, a bacterial origin has been suggested, but this has never been experimentally confirmed [17]. Uemura et al. [13] supported the theory of palytoxin biosynthesis by symbiotic microorganisms, after isolation and structural elucidation of at least four minor toxins coexisting with palytoxin in *P. tuberculosa*. The location of the structural differences between palytoxin, homo-palytoxin, and bishomo-palytoxin is in the ω-aminoalcohol moiety, which is often observed in natural products produced by microorganisms. The ability of the bacterial genera *Aeromonas* and *Vibrio* for production of compounds antigenically related to palytoxin has also been reported recently [18].

Symbiotic algae, which are able to synthesize secondary products similar to palytoxin [19] and which live in large masses in the mesogloea of the Zoantharia, have also been considered as potential producers. In this context, the detection of palytoxin or a closely related compound in the red alga *Chondria crispus* was reported [20]. The lack of correlation between algae (as expressed by chlorophyll *a* content) and palytoxin content, however, appears to contradict their involvement in toxin synthesis [21]. Kimura et al. [8] reported a correlation between the presence of eggs in *Palythoa* polyps from the Pacific and their palytoxin content. In support of this view, Uemura [22] reported that between April and June *P. tuberculosa* coelentrates produce eggs and have the strongest toxicity. However, this was not confirmed in *Palythoa* species from the Caribbean Sea. Considerable concentrations of palytoxin were measured even in sterile polyps; on the other hand, some egg-bearing polyps were entirely free from palytoxin [21].

Dinoflagellates belonging to the genus *Ostreopsis* have been proposed as possible biogenetic origins of palytoxin in Japan [23–25]. This theory is further supported because of the implication of *Ostreopsis siamensis* in a case of clupeotoxism in Madagascar where the causative agent was found to be palytoxin or one of its analogues [26]. Furthermore, numerous recent investigations have identified palytoxin analogues in *Ostreopsis mascarenensis* from the Indian Ocean [27] and in *Ostreopsis ovata* and *O.* cf. *siamensis* from the Mediterranean Sea [28–31; unpublished data of NRLMB Greece].

Palytoxin is one of the most potent natural nonprotein compounds exhibiting extreme toxicity in mammals with an intravenous LD_{50} ranging between 10 and 100 ng/kg [1]. Its toxic dose in humans has obviously not been experimentally determined; however, extrapolation of the available animal toxicity data will give a toxic dose in humans of about 4 μg [32] or between 2.3 and 31.5 μg [2]. Despite its high lethality in terrestrial animals, palytoxin has also been detected in crabs from the Philippines [33] and Singapore [34], in various fish such as *Alutera scripta* filefish from Okinawa, Japan [35], *Melichtys vidua* trigger fish from Ponape, Micronesia [36,37], *Decapterus macrosoma* mackerel from Hawaii [38], in the sea anemone *Radianthus macrodactylus* from the Seychelles [39], as well as in sponges, mussels, and soft corals [16] without causing any deleterious effects. This resistance of marine animals to palytoxin enables its sequestration and accumulation in the food chain [16] as these have been repeatedly implicated in numerous cases of human poisoning and lethality [40–44]. One common feature of the marine species implicated in such cases though is that they are bottom-feeders. The fact that both *Palythoa* spp. and *Ostreopsis* spp., the possible sources of palytoxin and analogues, grow on the sea bottom strengthens the hypothesis of their involvement in palytoxin biogenesis.

29.2.2 Chemistry

29.2.2.1 Properties

Palytoxin is a white, amorphous, hydroscopic solid, which has not been crystallized to date. It is insoluble in nonpolar solvents such as chloroform, ether, and acetone, sparingly soluble in methanol and ethanol and soluble in pyridine, dimethyl sulfoxide and, water. The partition coefficient for the distribution of palytoxin between 1-butanol and water is 0.21 at 25°C based on comparison of the absorbance at 263 nm for the two layers. Palytoxin foams on agitation, like a steroidal saponin, when in aqueous solutions, probably because of its amphipathic nature. The toxin shows no definite melting point and is heat resistant but chars at 300°C. It is an optically active compound, having a specific rotation of +26° ± 2° in water. The optical rotatory dispersion curve of palytoxin exhibits a positive Cotton effect with $[\alpha]_{250}$ being +700° and $[\alpha]_{215}$ being +600° [1,45].

29.2.2.2 Structure

Excluding the naturally occurring polymers, palytoxin could be considered as one of the most complicated and largest molecules. The basic molecule consists of a long, partially unsaturated

(with eight double bonds) aliphatic backbone with spaced cyclic ethers, 64 chiral centers, and 40–42 hydroxyl and 2 amide groups [13,45]. The third nitrogen present as a primary amino group at the C-115 end of the molecule accounts for the basicity of palytoxin [12]. Moore and Bartolini [5] reported that both molecular weight and molecular formula of palytoxins differ depending on the species from which they are obtained and that certain species contain mixtures of different isomers. These subtle differences are attributed to structural differences in the hemiketal ring. Fragmentation by sodium periodate oxidation and ozonolysis and subsequent ordering of the fragments, together with the use of mass spectrometry (MS) and nuclear magnetic resonance (NMR) have been employed during the determination of the gross structures of palytoxins [4,5,12,46–48].

Palytoxin from the Tahitian *Palythoa* has a molecular formula of $C_{129}H_{221}N_3O_{54}$ (M_r 2659). On the other hand, palytoxin from *P. toxica* has a molecular formula of $C_{129}H_{223}N_3O_{54}$ (M_r 2677) with two possible structures (Figure 29.1), which differ from the former in the C-55 hemiketal ring. Palytoxin from the Hawaiian *P. tuberculosa* is a mixture of the three palytoxins from the Tahitian *Palythoa* and *P. toxica* [5]. The two components in palytoxin from the Okinawan *P. tuberculosa* with a M_r 2681 [49] may be the related C-54 ketal and hemiketal [5]. The palytoxin from *P. caribaeorum* has a molecular mass of 2680 while the palytoxin-like substance isolated from *Lophozozymus pictor* crab, which showed a very similar positive ion MS profile was estimated to possess a molecular mass of 2681.0 Da [34].

The length of the palytoxin molecule has been recently calculated by small-angle-x-ray scattering to be 50, while its molecular weight has been calculated to be 5700. In an aqueous solution, palytoxin was found to exist as a dimmer, while by using a low resolution model simulation, it was

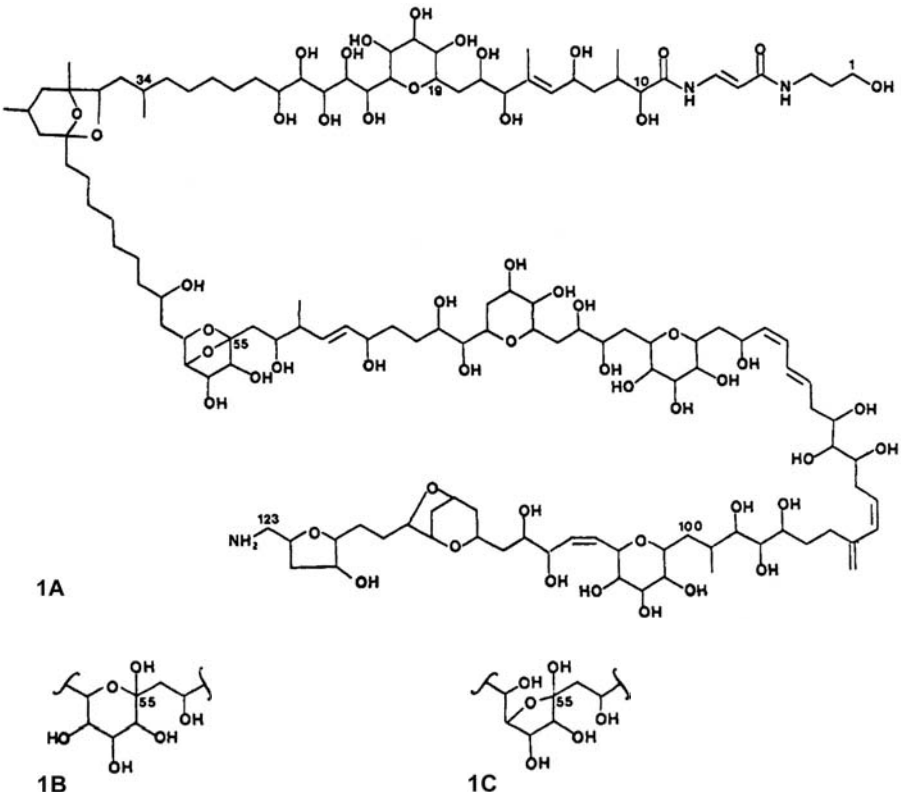

FIGURE 29.1 Proposed structure of palytoxin from a Tahitian *Palythoa* sp. (**1A**). The two palytoxins from Hawaiian *P. toxica* are the related C-55 hemiketals (partial structures **1B** and **1C**). (Reprinted from Moore, R.E. and Bartolini, G., *Journal of the American Chemical Society*, 103, 2491–2494, 1981. © American Chemical Society. With permission.)

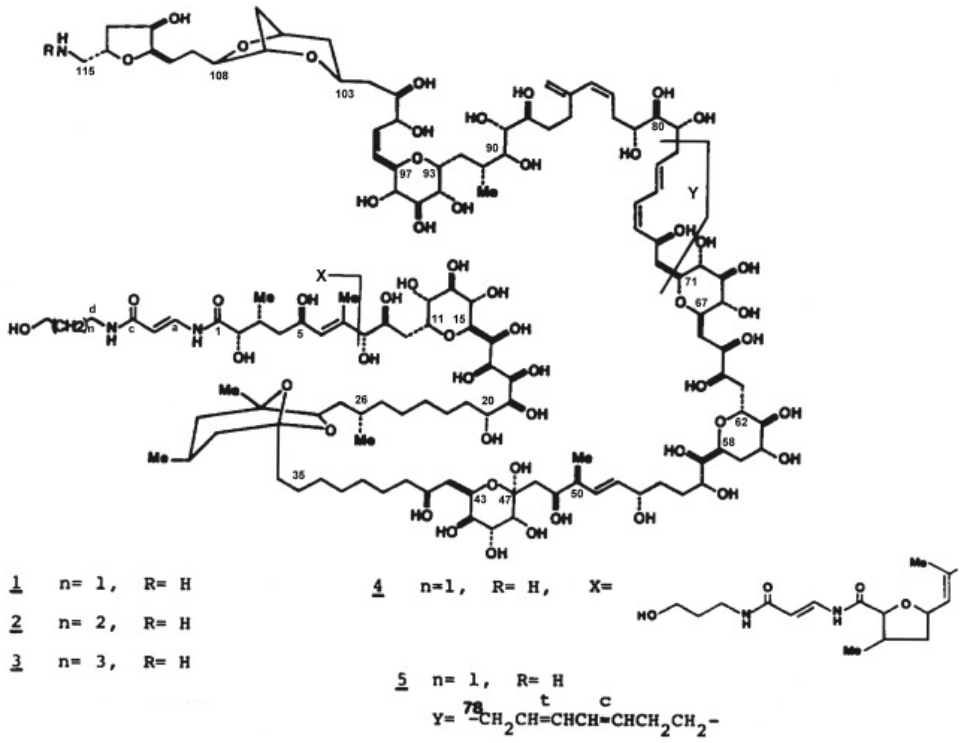

FIGURE 29.2 Complete structure of the major palytoxin from *P. tuberculosa* (1) and of minor palytoxins: (2) homopalytoxin, (3) bishomopalytoxin, (4) neopalytoxin and (5) deoxypalytoxin. (Reprinted from Uemura, D., Hirata, Y., Iwashita, T., and Naoki, H., *Tetrahedron*, 41, 1007–1017, 1985. © Elsevier.)

also found that the dimmer of palytoxin consists of two "⊃"-shaped molecules overlapping each other to give a figure-eight shape [22].

Four other minor toxins have been found to co-occur with palytoxin in *P. tuberculosa,* namely homopalytoxin, bishomopalytoxin, neopalytoxin, and deoxypalytoxin. These have been structurally characterized as palytoxin analogues, having minor differences from the major palytoxin. For instance, the structural difference among homo-, bishomo-, and palytoxin is in the proximity of the C-a proton (Figure 29.2) while molecular weights of homo- and bishomopalytoxinis are larger than palytoxin by 14 and 28 mass units, respectively. The same major and minor toxins have been isolated from another unclassified *Palythoa* species in Ishigaki Island, but the contents of homo-, bishomo-, and deoxypalytoxins were higher than that in *P. tuberculosa* [13].

The absolute stereochemistry of palytoxins from both *P. toxica* and *P. tuberculosa* (Figure 29.2) has been assigned to all 64 chiral centers [50,51] employing a long series of degradation experiments with the use of x-ray crystallography, NMR, and circular dichroic spectroscopy together with organic synthesis [52–54].

29.2.2.3 Mass Spectrometry

Mass spectrometry has been largely employed with regard to detection of palytoxin's presence, as well as for molecular weight determination and elucidation of the structural differences between palytoxins from various origins. For example, the loss of numerous water molecules during MS analysis indicated that palytoxins possess a large number of hydroxyl and/or ether moieties [27,34]. On the other hand, the presence of a fragment ion at or near $m/z = 327$ in the bivalent and trivalent positive ion MS/MS spectra is indicative of palytoxin-like substances. This fragment ion could arise from cleavage between carbons 8 and 9 of palytoxin (see Figure 29.3) and the additional loss of one

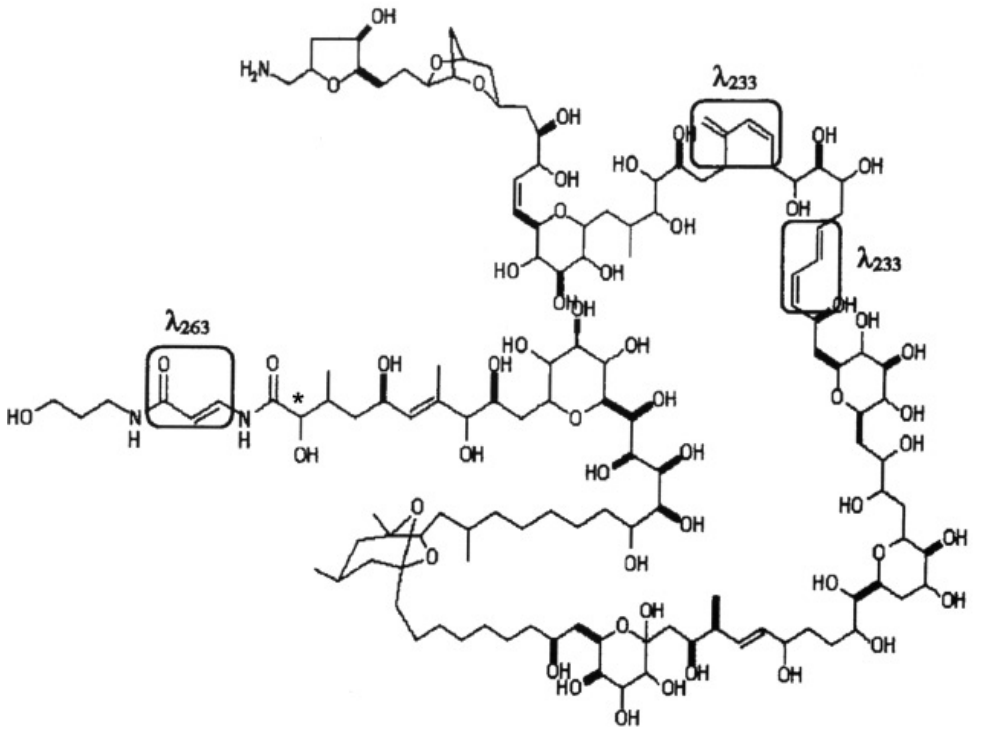

FIGURE 29.3 Planar structure of reference Pacific palytoxin (P-PTX) showing the UV chromophores. Asterisk denotes carbon eight. (Reprinted from Lenoir, S., Ten-Hage, L., Turquet, J., Quod, J.-P., Bernard, C., and Hennion, M.C., *Journal of Phycology*, 40, 1042–1051, 2004. © Blackwell Publishing.)

water molecule [13,27]. Presence of the $m/z = 327$ fragment ion has been confirmed in the palytoxin from *P. tuberculosa* [13,29], *P. caribaeorum* [34], and *P. toxica* [27]. The absence of this fragment ion, however, does not necessarily exclude the possibility of a palytoxin-like compound, but could be attributed to structural differences at this end of the molecule, as in the case of *L. pictor* toxin or minor *P. tuberculosa* toxins [13,34].

29.2.2.4 Ultraviolet Spectrum

Palytoxin exhibits an ultraviolet (UV) absorption spectrum with λ_{max} at 233 (ε 47000) and 263 nm (ε 28000) [55], attributed to the presence of respective chromophores (Figures 29.3 and 29.4). The reported ratio of UV absorbances between 233 and 263 nm is 1.71 [3].

This spectrum is similar for all palytoxins, regardless of subtle differences present in their proton magnetic resonance (PMR; ^{1}H-NMR) and carbon magnetic resonance (CMR; ^{13}C-NMR) spectra, as in the case of palytoxins from *P. toxica* from Hawaii, *P. mammilosa* from Jamaica and *Palythoa* sp. from Tahiti [11,56]. This characteristic UV absorption profile of palytoxin is another parameter indicative of the presence of this toxin and/or its analogues [45].

The 233-nm absorption maximum is reported to occur due to the presence of two chromophores, a conclusion deduced from the respective value of the extinction coefficient [46]. ^{1}H NMR studies suggest that both these chromophores are conjugated dienes [57].

The λ 263 chromophore is a *N*-(3′-hydroxypropyl)-*trans*-3-amidoacrylamide moiety [56]. This moiety accounts for the positive response of palytoxin to the ninhydrin test, while its destruction is connected to loss of toxicity, accompanied by a negative ninhydrin test [46]. The 263-nm chromophore is sensitive to methanolic 0.05 M HCl or aqueous 0.05 M NaOH, disappearing with a half-life of 85 min and 55 min, respectively. Neutralization within 2 min, however, regenerates palytoxin

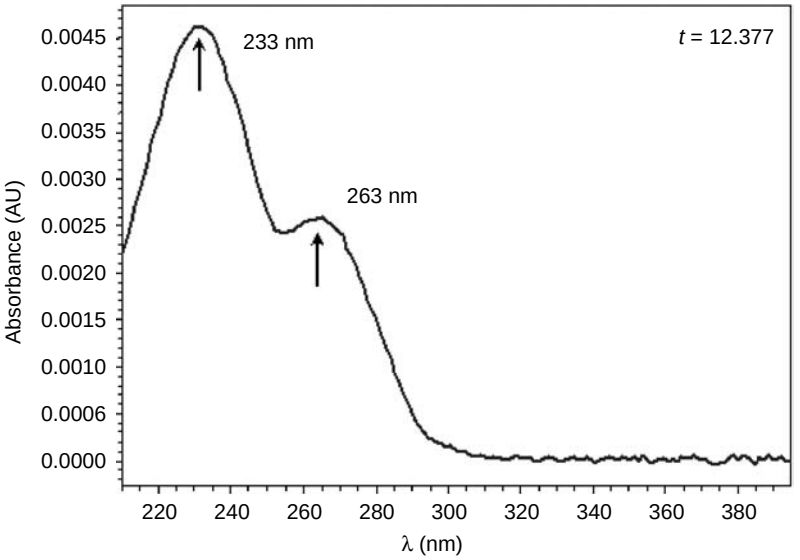

FIGURE 29.4 Ultraviolet spectrum of reference palytoxin from *Palythoa tuberculosa* at 25 μg/mL in water.

with no apparent loss in toxicity [3]. Exposure of palytoxin to both visible and ultraviolet light results in structural changes in both the 263 and 233 chromophores and can reduce its toxicity at least 20-fold in only 5 min in UV and 30 min in visible light [58]. This connection is further supported by the 100-fold weaker toxicity of *N*-acetylpalytoxin compared to palytoxin, as *N*-acetylpalytoxin possesses only a slight structural difference around the N-terminal part of palytoxin, where the 263 chromophore is located [59].

29.2.2.5 Infrared Spectrum

The infrared (IR) spectrum of purified palytoxin or its congener shows a band at 1670/cm, which is due to the presence of an α,β-unsaturated amide carbonyl group [3]. Similar IR data have also been obtained for purified palytoxin from *P. caribaeorum* [11], from the sea anemone *R. macrodactylus* [39] and from the xanthid crab *L. pictor* [45].

29.2.2.6 Nuclear Magnetic Resonance Spectra

Structural elucidation of palytoxin has been largely based on data derived from NMR determinations on various degradation products from periodate oxidation or ozonolysis [5,17,50]. Kan et al. [59] have reported the complete chemical shift assignment of ^{1}H and ^{13}C NMR signals of the whole *P. tuberculosa* palytoxin molecule, which is presented in Table 29.1. A detailed description of the NMR spectra of palytoxin is beyond the aims of this chapter. It is however interesting to note that deuterated methanol (CD_3OD or CD_3OH) gave much sharper signals in the ^{1}H NMR spectrum than deuterated water (D_2O), probably owing to a fast averaged conformation or a monomer [59]. However, Oku et al. [14] also obtained satisfactory ^{1}H NMR spectra of reference palytoxin and *P.* aff. *margaritae* palytoxin by using D_2O with 0.2% acetic acid-d_4.

29.2.2.7 Chemical Synthesis

In 1989, despite the huge number of possible stereoisomers, a research group led by Professor Kishi managed to achieve the total chemical synthesis of a fully protected palytoxin carboxylic acid [7]. This was subsequently converted to palytoxin carboxylic acid and palytoxin amide without the use of protecting groups [60]. By comparison of biological activity, chromatographic behavior, and

TABLE 29.1
^{1}H and ^{13}C NMR Chemical Shift Data of *P. tuberculosa* Palytoxin in CD_3OD or CD_3OH with One Drop of Deuterium Oxide

No.	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)	No.	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)
1	175.92	s	—		**60**	70.18	d	3.85	
2	75.70	d	4.09		**61**	76.57	d	3.15	
3	34.73	d	2.17		**62**	73.11	d	3.74	
3-Me	13.99	q	0.88		**63**	36.77	t	1.96	1.70
4	41.73	t	1.77	1.40	**64**	71.77	d	3.68	
5	66.62	d	4.50		**65**	72.20	d	3.76	
6	131.85	d	5.49		**66**	37.01	t	2.04	1.53
7	138.28	s	—		**67**	77.22	d	3.44	
7-Me	13.17	q	1.72		**68**	76.04	d	3.12	
8	80.91	d	3.92		**69**	79.74	d	3.36	
9	72.34	d	3.81		**70**	75.85	d	3.09	
10	29.23	t	2.12	1.73	**71**	77.08	d	3.44	
11	76.19	d	4.18		**72**	41.51	t	2.04	1.43
12	73.88	d	3.64		**73**	64.99	d	4.84	
13	75.17	d	3.54		**74**	133.47	d	5.37	
14	71.68	d	3.60		**75**	130.04	d	6.00	
15	72.91	d	3.62		**76**	128.87	d	6.46	
16	71.28*[1]	d	4.03		**77**	133.88	d	5.78	
17	71.68*[1]	d	4.04		**78**	38.64	t	2.42	
18	73.27	d	3.54		**79**	71.20	d	3.93	
19	71.35	d	3.79		**80**	76.29	d	3.27	
20	71.11	d	3.87		**81**	73.04	d	3.63	
21	27.38	t	1.48	1.39	**82**	34.35	t	2.75	2.39
22	26.93	t	1.47	1.35	**83**	130.18	d	5.69	
23	35.03	t	1.64	1.55	**84**	132.64	d	5.95	
24	28.44	t	1.36		**85**	146.73	s	—	
25	39.72	t	1.26		**85′**	114.86	t	5.07	4.94
26	29.70	d	1.67		**86**	34.30	t	2.34	2.25
26-Me	19.30	q	0.92		**87**	33.13	t	1.72	1.59
27	40.78	t	1.47	0.91	**88**	74.19	d	3.71	
28	80.17	d	3.97		**89**	74.02	d	3.50	
29	82.31	s	—		**90**	77.82	d	3.35	
29-Me	21.01	q	1.18		**91**	33.00	d	1.89	
30	45.74	t	1.70	1.14	**91-Me**	15.65	q	0.91	

31	25.55	d	2.04		**92**	27.86	t	2.21	1.30
31-Me	21.89	q	0.91		**93**	74.83	d	4.03	
32	43.74	t	1.67	1.09	**94**	73.04	d	3.65	
33	109.23	s	—		**95**	74.73	d	3.61	
34	38.64	t	1.60		**96**	76.01	d	3.15	
35	23.98	t	1.41		**97**	69.71	d	4.32	
36	30.98*[2]	t	1.31		**98**	132.43	d	5.55	
37	30.93*[2]	t	1.31		**99**	135.28	d	5.71	
38	30.81*[2]	t	1.31		**100**	71.90	d	4.36	
39	31.29	t	1.36		**101**	71.77	d	3.68	
40	39.20	t	1.48		**102**	40.21	t	1.58	
41	69.26	d	3.80		**103**	68.39	d	4.22	
42	39.37	t	1.86	1.44	**104**	40.53	t	1.74	1.38
43	64.86	d	4.39		**105**	76.14	d	4.51	
44	73.88	d	3.65		**106**	36.83	t	1.84	1.78
45	74.28	d	3.95		**107**	79.62	d	4.21	
46	68.25	d	3.67		**108**	82.74	d	4.35	
47	101.24	s	—		**109**	26.59	t	1.78	1.67
48	41.95	d(t)	1.83		**110**	32.30	t	1.47	
49	72.40	d	3.94		**111**	83.81	d	3.89	
50	44.07	d	2.26		**112**	73.27	d	4.27	
50-Me	16.58	q	1.03		**113**	39.78	t	2.10	1.86
51	134.46	d	5.62		**114**	75.31	d	4.36	
52	134.74	d	5.51		**115**	45.13	t	2.99	2.87
53	74.06	d	4.05		**a**	134.82	d	7.79	
54	34.93	t	1.77	1.61	**b**	106.82	d	5.95	
55	27.79	t	1.69	1.46	**c**	169.66	s	—	
56	73.11	d	3.74		**d**	37.42	t	3.33	
57	72.81	d	3.85		**e**	33.28	t	1.74	
58	74.19	d	3.87		**f**	60.40	t	3.60	
59	33.05	t	2.27	1.66					

Source: Reprinted from Kan, Y., Uemura, D., Hirata, Y., Ishiguro, M. and Iwashita, T., *Tetrahedron Letter*, 42(18), 3197–3202, 2001. Copyright (2001), with permission from Elsevier.

Note: TMS was used as an internal or external chemical shift reference of 0 ppm in proton NMR spectra. The solvent peak was used as an internal chemical shift reference of 49.00 ppm in carbon-13 NMR spectra. The "mult" column shows a number of proton attached to the carbon as a multiplicity of peak (s: singlet, d: doublet, t: triplet, q: quartet). The marked rows from *1 to *2 show a group of carbons which are exchangeable with each other.

spectroscopic data (MS, 1D and 2D ^{1}H NMR, ^{13}C NMR) it was found that the synthetic products were identical to their naturally occurring counterparts [60,61]. Few years later, this work was completed by conversion of palytoxin carboxylic acid to palytoxin identical to the natural product from *P. tuberculosa* [62], with a yield of 100%. It is, however, unlikely that chemical synthesis of palytoxin will be of practical use, as the whole procedure involves approximately 65 steps [63].

29.2.3 Metabolism

Very few data are available with regard to metabolism of palytoxin in the animal/human body and its connection with toxicity. The main mode of palytoxin action (which is presented in detail in Chapter 34) is related to disruption of the mammalian cell sodium pump functionality. Targeting the Na^{+},K^{+}-adenosine triphosphatase (ATPase) pump, palytoxin binds to the ATPase and converts the pump into a nonspecific ion channel, thereby, short-circuiting membrane function of the cell and eventually causing cell lysis [64].

Despite the fact that to date there is no experimental data to support the opposite, it seems that in order to be toxic palytoxin will bind to the ATPase in its intact form, as even minor structural alterations in the palytoxin molecule often result in loss of toxicity. For example, Moore et al. [56] have reported that acid hydrolysis of palytoxin with refluxing 2 N HCl for 4 h results in destruction of the λ 263 chromophore, which is connected to loss of toxicity. Major loss of toxicity was also observed after treatment of palytoxin with 0.1 N NaOH at room temperature for 50 min or 0.1 N acetic acid at 80°C for 24 h or by catalytic hydrogenation [55]. Similarly, Wiles et al. [1] reported deactivation of 5 LD_{50}'s of palytoxin with solutions of 1–5 N HCl or 0.5–5 N NaOH in a 5-min contact period, whereas acetic acid was found to be totally ineffective. One could therefore suppose that such effects could probably be one of the underlying reasons for the major reduction in toxicity observed when palytoxin is administered orally, compared to intravenous or intraperitoneal administration [1], as the pH of the alimentary tract is strongly acidic in the stomach and strongly alkaline in the small intestine. This speculation is also in agreement with the views expressed in the first meeting of the Working Group on Toxicology of Marine Biotoxins held in 2005 in Cesenatico, Italy (Luis Botana, pers. comm.).

With regard to other effects of palytoxin on metabolism, it is interesting to mention that palytoxin has been reported to stimulate both metabolism of arachidonic acid and prostaglandin production [65]. These properties are thought to be related with palytoxin's tumor-promoting activity, as palytoxin is a known non-TPA-type tumor promoter [66].

29.3 PALYTOXIN ANALOGUES FROM *OSTREOPSIS* SPP. (OSTREOCINS)

Although some of the toxins produced from dinoflagellates belonging to the genus *Ostreopsis* (Dinophyceae) are named after the producing species, the term "ostreocins" is frequently used to describe such toxins. The palytoxin-like character of the major ostreocin from *O. siamensis* was first established by Usami et al. [23], indicating that this species could be one of the biogenetic sources of palytoxin. Since then, numerous researchers have reported the presence of palytoxin-like compounds in *Ostreopsis* spp.

Ostreopsis species are mainly benthic and epiphytic dinoflagellates with a worldwide distribution. They are considered as important components of subtropical and tropical marine coral reef–lagoonal environments and are also thought to be potential progenitors of toxins associated with ciguatera fish poisoning [67–69]. During the past decade, however, *Ostreopsis* spp. are regularly isolated in temperate areas of the world, mostly during summer time. Presence of potentially toxic *Ostreopsis* spp. has been reported in Mediterranean countries such as Spain, Italy, Greece, and Tunisia [29,70–73]. In the south of Spain and certain parts of Greece, their presence was accompanied by the incidence of relevant toxicity in shellfish (L. Botana, personal communication; unpublished

TABLE 29.2
Toxic *Ostreopsis* Species and Summary of Reported Data on Properties of Respective Toxins Produced

Producing Species	Toxin	Considered a Palytoxin Analogue?	Molecular Weight	Chemical Formula	Mouse Lethality (LD_{50} i.p.)	Reference
O. siamensis	Unnamed					[67]
	Ostreocin-D	Yes	~2635	$C_{127}H_{219}N_3O_{53}$	0.75 μg/kg	[23,24,85]
O. mascarenensis	Unnamed		~2500–2535	n.d. [a]		[69]
	Mascarenotoxins-A and -B	Yes			0.9 mg/kg	[27,93]
O. ovata	Unnamed	Yes	n.d.	n.d.	n.d.	[67]
						[29]
O. lenticularis	Ostreotoxin	Not known	n.d.	n.d.	32.1 mg/kg	[68]
	Ostreotoxin 1					[76]
	Ostreotoxin 3					[77]
O. heptagona	Unnamed	Not known	n.d.	n.d.	n.d.	[78]

[a] n.d.: not determined.

data of NRLMB Greece). Nine different *Ostreopsis* species have been described to date. Five of them, namely *O. siamensis*, *O. ovata, O. mascarenensis, Ostreopsis Lenticularis, and Ostreopsis heptagona* have been reported as producers of toxic substances (Table 29.2). No data is available to date as regards toxin production ability of the remaining four species*, Ostreopsis labens, Ostreopsis marinus, Ostreopsis belizeanus*, and *Ostreopsis carribeanus* [74,75].

This section will focus on the available chemical data concerning only toxic substances produced by *Ostreopsis* spp., shown to possess palytoxin characteristics. For reasons of convenience, toxins will be presented according to producing species. Palytoxin-like compounds have been reported for *O. siamensis*, *O. ovata*, and *O. mascarenensis.* The neurotoxins ostreotoxin-1 and -3 produced by *O. lenticularis* have not been characterized as palytoxin analogues until today by use of analytical methods, despite their reported mouse lethality and possible connection to ciguatera [68,76,77]. Regarding the last of the toxic species, the only available information is that methanol extracts of clonal cultures of *O. heptagona* isolated from Knight Key, Florida, were weakly toxic to mice [78]. To the best of our knowledge, no published data are available concerning metabolism of palytoxin analogues until today.

29.3.1 *O. siamensis* Schmidt (1901)—Ostreocins

29.3.1.1 Ecobiology, Origin, Distribution, and Toxin Production

O. siamensis was first isolated by Schmidt in the Gulf of Siam (Thailand) in 1901 [79]. This dinoflagellate occurs, mainly as epiphytic and less frequently as planktonic, in many tropical and subtropical areas of the world, and also in temperate areas during summertime. Until today, the presence of *O. siamensis* has been reported in the coastal waters of Japan [80], New Zealand [81,82], Tasmania [83], Spain, Italy [29,70], Greece (*O.* cf. *siamensis*; [73]), and Tunisia [72].

Nakajima et al. [67] were the first to characterize *O. siamensis* as a toxin producer. The lethality and hemolytic activity of the *O. siamensis* toxins was reported some years later [80,84]. Usami et al. [23] first elucidated the structure of the major ostreocin produced by *O. siamensis* (strain SOA 1 from Aka island, Okinawa, Japan) and pointed out its structural and chemical properties' resemblance to palytoxin. This major constituent, accounting for 90% of the total extracts' toxicity,

was named ostreocin-D. None of the other minor ostreocins present in the *O. siamensis* extracts (more than ten) were shown to be identical to palytoxin, as initially indicated by ESI-MS [24,85]. New Zealand *O. siamensis* isolates have also been reported to produce toxins exhibiting strong hemolytic activity and mouse lethality [82,86]. The presence of toxins with strong delayed hemolytic activity in *O.* cf. *siamensis* from the NW Mediterranean Sea was recently reported [29]. This hemolytic activity was inhibited by the palytoxin antagonist ouabain, indicating the palytoxin-like nature of these toxins.

Detailed studies regarding chemistry of *O. siamensis* toxins have been reported until today only for ostreocin-D.

29.3.1.2 Chemistry of Ostreocin-D

Ostreocin-D is a colorless amorphous solid, positive to ninhydrin. This compound possesses optical activity with a specific rotation of +16.6 in water (*c* 0.12, $T = 23°C$), as well as an UV absorption spectrum exhibiting two maxima, at 234 (ε 35000) and 263 nm (ε 22000). The UV absorptions together with NMR spectra indicate that ostreocin-D has conjugated diene and ketone functionality analogous with palytoxin [23].

Structure elucidation of ostreocin-D involved the use of MS and NMR spectra (Table 29.3). Ostreocin-D displayed cluster ions having a centroid at *m/z* 2636.51 in the high mass range of the fast-atom bombardment mass spectrometry (FABMS). The ion distribution pointed to a composition of $C_{127}H_{219}N_3O_{53}$, with the MH^+ calculated to be 2636.47. This difference in composition (C_2H_4O) between palytoxin and ostreocin-D was initially attributed to the substitution of two methyls and one hydroxyl of the former with protons in the latter. The substitution of methyls is located at C3 and C26 (Figure 29.5). Partial structures around methyls and double bonds were identified by comparison of NMR spectra of palytoxin and ostreocin-D in 0.2% CD_3COOD/D_2O. Due to severe congestion of the NMR signals, reduction of the molecule size was required for further studies. Degradation of the molecule by ozonolysis proved useful for dissolving the NMR signals and for clarification of the entire structure. Finally, it was concluded that in ostreocin-D two hydroxyls (positions C19 and C44) are substituted by protons while an extra hydroxyl is present at C42, compared to palytoxin. Ostreocin-D was therefore deduced to be 42-hydroxy-3,26-didemethyl-19,44-dideoxypalytoxin [23,24], a structure that was also confirmed by using negative ion FABMS [85]. It should be noted that the observed small structural differences between ostreocin-D and palytoxin have barely an effect on the mouse lethality of the former [LD_{50} (i.p.): 0.75 μg/kg and 0.50 μg/kg, respectively], but cause significant reduction in cytotoxicity and hemolytic potency [23,24].

29.3.2 *Ostreopsis ovata* Fukuyo (1981)

29.3.2.1 Ecobiology, Origin, Distribution, and Toxin Production

O. ovata is the smallest species in the genus *Ostreopsis*. It is distributed in numerous areas of the world such as the Pacific Ocean [80,87], the Caribbean Sea [68], the Brazilian coasts in the Atlantic Ocean [88], and the Mediterranean Sea [29,71,73,89].

The production of a butanol-soluble compound exhibiting mouse lethality was reported for *O. ovata* from Okinawa, Japan, [19]. This was later confirmed by Yasumoto et al. [80], who also detected slight hemolytic activity in the *O. ovata* cell extracts. Crude methanol extracts of *O. ovata* from the Virgin Islands, however, were found to be nontoxic to mice [68]. Recently, summer blooms of *O. ovata* in the Italian coasts have been suspected to be the cause of respiratory problems in swimmers and sunbathers, most probably through inhalation of toxic aerosols [71,90,91]; such problems could possibly arise from inhalation of a palytoxin-like substance [92]. Extracts of *O. ovata* from both Brazil and the Mediterranean Sea were reported to contain certain substances exhibiting strong delayed hemolysis, which was inhibited by ouabain and mouse lethality with symptoms typical of palytoxin [28,29,88].

TABLE 29.3
NMR Assignments of Ostreocin-D in 0.2% CD_3COOD/D_2O

Position	^1H (δ)	^1H (δ)	^{13}C (δ)	Position	^1H (δ)	^1H (δ)	^{13}C (δ)
1	—		175.5	**60**	3.93		68.5
2	4.31		71.2	**61**	3.21		75.1
3	1.69	1.88	29.8	**62**	3.68		71.4
3-Me	—		—	**63**	1.56	2.09	35.5
4	1.59	1.75	31.8	**64**	3.65		70.3
5	4.51		67.3	**65**	3.77		70.4
6	5.46		129.9	**66**	1.49	2.09	35.6
7	—		137.9	**67**	3.48		75.9
7-Me	1.71		12.1	**68**	3.51		75.7
8	3.96		79.7	**69**	3.21		74.0
9	3.86		70.5	**70**	3.22		74.1
10	1.72	2.13	27.9	**71**	3.49		77.4
11	4.23		74.5	**72**	1.55	2.12	39.2
12	3.72		71.2	**73**	4.86		63.8
13	3.64		73.2	**74**	5.43		131.2
14	3.62		70.1	**75**	6.14		130.1
15	3.67		72.6	**76**	6.49		127.7
16	3.62		74.9	**77**	5.86		133.4
17	4.02		68.7	**78**	2.42		36.9
18	4.01		67.3	**79**	3.94		69.9
19	1.50	1.80	40.6	**80**	3.41		75.2
20	3.85		68.2	**81**	3.79		71.5
21	1.52		24.9	**82**	2.48	2.66	32.1
22	1.35		29.0	**83**	5.69		128.8
23	1.35		29.0	**84**	6.04		132.1
24	1.35		29.0	**85**	—		145.1
25	1.35		29.0	**CH_2=**	5.00	5.14	114.4
26	1.35		29.0	**86**	2.27	2.32	32.7
26-Me	—		—	**87**	1.61	1.75	31.3
27	1.34		31.8	**88**	3.76		72.6
28	3.95		81.6	**89**	3.60		72.9
29	—		81.9	**90**	3.47		76.1
29-Me	1.24		19.9	**91**	1.84		31.7

Continued

TABLE 29.3 (Continued)

Position	^{1}H (δ)	^{1}H (δ)	^{13}C (δ)	Position	^{1}H (δ)	^{1}H (δ)	^{13}C (δ)
30	1.19	1.77	43.8	**91-Me**	0.92		14.3
31	2.00		23.9	**92**	1.34	2.16	25.9
31-Me	0.92		20.8	**93**	4.17		73.6
32	1.16	1.74	41.9	**94**	3.78		71.2
33	—		108.5	**95**	3.70		72.6
34	1.66		36.8	**96**	3.32		74.3
35	1.40		22.3	**97**	4.29		68.2
36	1.35		29.0	**98**	5.62		130.9
37	1.35		29.0	**99**	5.78		134.8
38	1.35		29.0	**100**	4.35		70.9
39	1.35		29.0	**101**	3.66		70.3
40	1.50		37.4	**102**	1.58	1.62	38.5
41	3.72		71.1	**103**	4.21		67.4
42	3.40		75.1	**104**	1.48	1.79	38.5
43	4.39		63.4	**105**	4.64		75.1
44	1.78	2.05	33.5	**106**	1.83	1.93	35.3
45	4.25		68.9	**107**	4.33		78.4
46	3.51		69.7	**108**	4.42		81.6
47	—		100.0	**109**	1.52		30.4
48	1.85	1.92	40.5	**110**	1.66	1.76	24.8
49	3.94		71.0	**111**	3.94		82.3
50	2.34		42.3	**112**	4.37		71.9
50-Me	1.04		15.4	**113**	1.98	2.21	37.9
51	5.62		134.5	**114**	4.45		73.4
52	5.56		132.6	**115**	3.05	3.16	43.2
53	4.14		72.8	**a**	7.71		133.4
54	1.62	1.77	33.1	**b**	5.95		106.5
55	1.44	1.74	32.4	**c**	—		168.9
56	3.83		71.0	**d**	3.34		36.4
57	4.04		71.7	**e**	1.79		31.2
58	3.84		71.9	**f**	3.68		59.3
59	1.72	2.30	31.8				

Source: Ukena et al. (2001), Copyright (2001), with permission from the Japan Society for Bioscience Biotechnology and Agrochemistry.

Note: CH_3COOD was used as an internal reference at 2.06 ppm for ^{1}H and at 21.0 ppm for ^{13}C.

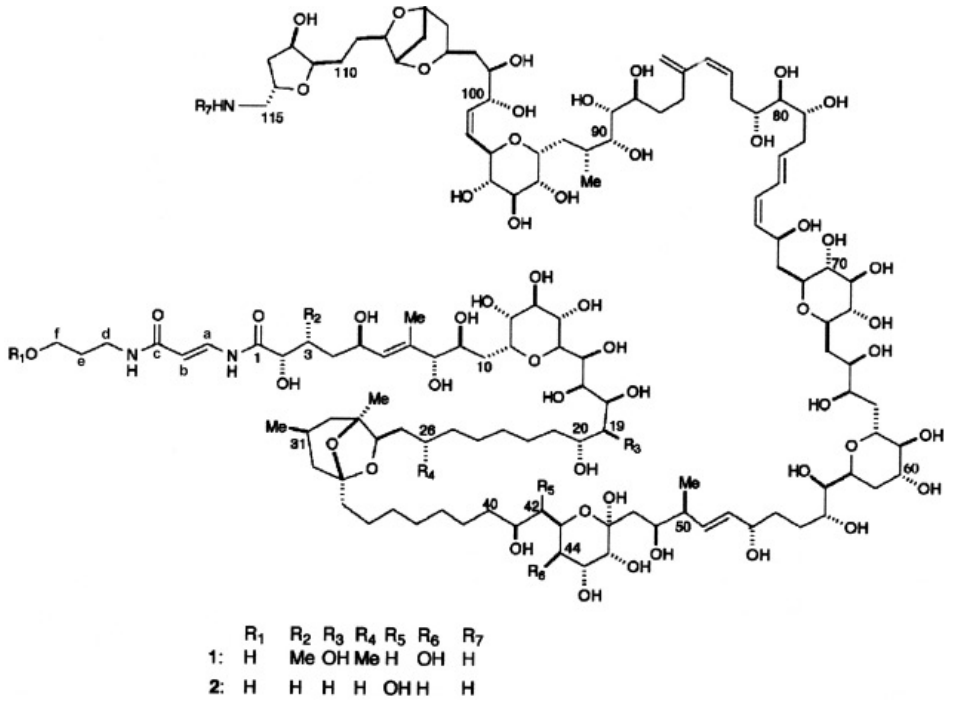

FIGURE 29.5 Structures of palytoxin (**1**) and ostreocin-D (**2**). (Reprinted from Ukena, T., Satake, M., Usami, M., Oshima, Y., Fujita, T., Naoki, H., and Yasumoto, T., *Rapid Communications in Mass Spectrometry*, 16, 2387–2393, 2002. © John Wiley & Sons.)

29.3.2.2 Chemistry of *O. ovata* Toxins

Limited data are available regarding the chemistry of *O. ovata* toxins. The extracts from all tested strains of *O. ovata* from Brazil and the Mediterranean Sea (Italy and Spain) exhibited strong delayed hemolytic activity, which was neutralized by ouabain [28,29]. Subsequent high performance liquid chromatography with ultraviolet detection (HPLC-UV) analysis of the extract from one of these strains (*O. ovata* OS06BR) showed a peak at the same retention time (at min 9) as standard palytoxin from *P. tuberculosa*. UV spectrum of this peak exhibited two absorbance maxima at 230 nm and 263 nm, indicating the palytoxin-like character of the contained compound (Figure 29.6a). MS analysis of this characteristic peak from the *O. ovata* extract revealed a spectrum identical to that of the palytoxin standard, showing a positive ion spray with bi-charged ions at *m/z* 1346.7 and a fragment at *m/z* 327 from a thermic fragmentation process (Figure 29.6b). This *m/z* 327 fragment is characteristic of thermally fragmented palytoxin [13] and, combined with the *m/z* 1346.7 of the molecule, is indicative of compounds structurally similar to palytoxin [93].

29.3.3 *O. mascarenensis* Quod (1994)—Mascarenotoxins-1 and -2

29.3.3.1 Ecobiology, Origin, Distribution, and Toxin Production

O. mascarenensis is the largest species of the genus and has been first identified in shallow (2–5 m) barrier reef environments and coral reefs in the Southwest Indian Ocean [69,94]. This species was found in low numbers as an epiphyte on macroalgae (*Turbinaria* spp., *Galaxaura* spp.) and dead corals and sediments at Mayotte and Reunion islands and in high numbers at Rodrigues island [95]. Crude methanol extracts of this species were toxic to mice [69, 93]. During a monospecific bloom of *O. mascarenensis* in 1996 in Rodrigues island (Mascareignes Archipelago, SW Indian Ocean) both

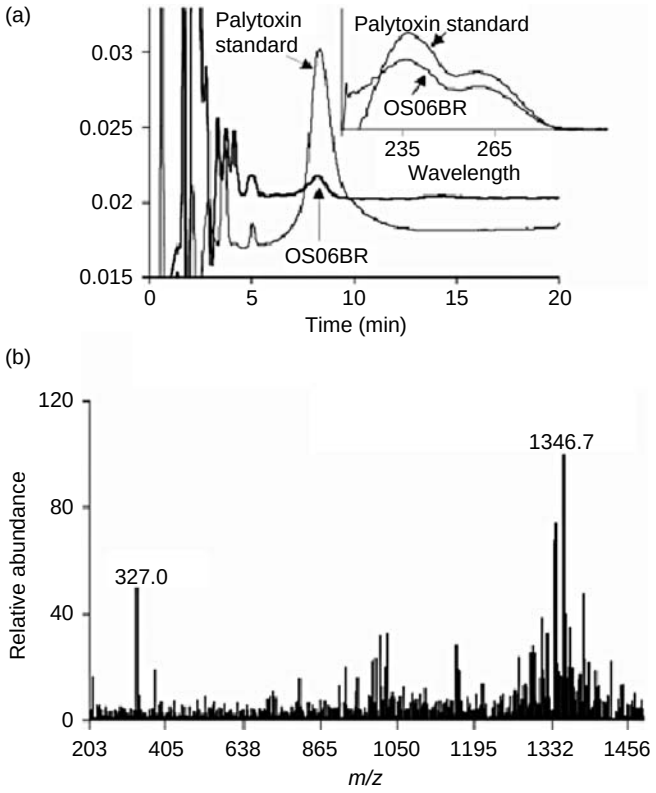

FIGURE 29.6 (a) Overlapped UV-photodiode array detector chromatograms of palytoxin standard and an extract of *Ostreopsis ovata* IEO-OS06BR showing peaks at the same retention time (min 9) with a similar UV spectrum shown in the upper right window. (b) MS spectrum of palytoxin and an extract of *O. ovata* IEO-OS06BR showing bi-charged ion *m/z* 1346.7 and characteristic fragment *m/z* 327. (Reprinted from Penna, A., Vila, M., Fraga, S., Giacobbe, M.G., Andreoni, F., Riobó, P., and Vernesi, C., *Journal of Phycology*, 41, 212–225, 2005. © Blackwell Publishing.)

crude methanol extracts (CME) and their polar *n*-butanol soluble fractions (BSF) showed mouse lethality ($LD_{50} \approx 0.9$ mg/kg) with symptoms similar to those induced by palytoxin but without diarrhea. Both CME and BSF also exhibited delayed hemolytic activity, typical of palytoxin. Most of the mouse toxicity and hemolytic potency were found in the BSF and subsequent analyses were carried out to identify the nature of the toxic compounds [27,93].

29.3.3.2 Chemistry of Mascarenotoxins-A and -B

HPLC-DAD in comparison to reference palytoxin from *P. toxica* was carried out for further analysis of the BSF [27]. The method employed a mobile phase of water acidified to pH 2.5 with trifluoroacetic acid (solvent A) and pure acetonitrile (solvent B). A linear gradient was applied from 30% to 70% of solvent B over 45 min (see also Table 29.4). The BSF HPLC screening revealed two distinct peaks, which were eluted at approximately 38% acetonitrile with retention times very close to that of reference palytoxin. Both peaks showed the two characteristic UV absorption maxima, at 233 and 263 nm, while the ratio between their absorbance (233 versus 263 nm) was identical to that calculated for reference palytoxin. The peaks were collected separately and the toxic compounds purified from the BSF were called mascarenotoxin-A (McTx-A) and mascarenotoxin-B (McTx-B) [27].

Further analyses of both McTx-A and McTx-B in comparison to reference palytoxin were carried out by nano-electrospray ionization quadrupole time-of-flight (nano-ESI-Q-TOF) and LC-ESI MS/MS. Fragmentation patterns of both McTx-A and McTx-B showed a serial dehydration process, indicating the presence of numerous hydroxyls. Furthermore, the fragment ion $m/z = 327.2$ was abundantly obtained for both McTx-A and McTx-B by selecting the bi- and tri-charged ions. The same typical fragment ion $m/z = 327.2$ was also detected in reference palytoxin [27], as described before for Caribbean palytoxin [13,34].

The MS profiles of McTx-A and McTx-B were both very similar to the respective profile of reference palytoxin. The estimated molecular masses of McTx-A and McTx-B were found to range between 2500 Da and 2535 Da and were lower than that of reference palytoxin (2680 Da) or other palytoxins and ostreocin-D. Nevertheless, the MS profile and fragmentation patterns of McTx-A and McTx-B together with mouse bioassay symptomatology and delayed hemolytic activity were enough, confirm the palytoxin-like character of these compounds. Quantitative differences in the hemolytic action and mouse lethality, as well as minor deviations in the MS spectra and retention times could arise from structural variations between mascarenotoxins and the reference palytoxin [27]. This is also supported by Usami et al. [23] who showed that small changes in the structure of palytoxin analogues can have an impact on mouse toxicity, hemolytic potency, and cytotoxicity.

The SW Indian Ocean is a known clupeotoxism endemic zone [94] and *Ostreopsis* spp. have been suspected to be a source of palytoxin in certain cases of clupeotoxism [26, 96]. It is therefore highly possible that *O. mascarenensis*, which is largely distributed in the western Indian Ocean, is involved in regional clupeotoxism incidents.

29.4 CHEMICAL ANALYSIS METHODS

29.4.1 Extraction And Purification Of Palytoxin And Analogues

Ethanol and methanol are the most common solvents used to extract palytoxin, as the toxin is quite soluble in water or other water-miscible solvents. The first published protocol for isolation and purification of palytoxin from Hawaiian *Palythoa* [3] involved the use of 70% aqueous ethanol during the steps of extraction of the polyps for 2–3 h, blending and washing of the ground polyps' residue. The combined extracts were evaporated under reduced pressure at 50°C to remove ethanol and the concentrate was defatted by triplicate extraction with benzene and duplicate extraction with portions of 1-butanol saturated with water. The 1-butanol extracts were backwashed thrice with water saturated with 1-butanol. The combined aqueous portion, after removal of the dissolved 1-butanol under reduced pressure at 50°C, was desalted and further purified with ion-exchange chromatography. In the following years, a variety of modified procedures, employing aqueous methanol or ethanol and different methods of liquid chromatography (LC), thin-layer chromatography (TLC), or electrophoresis, were followed for the extraction and purification of palytoxin from zoanthids or other marine species [8,11,13,14,26,33,39,42,55,97]. Fukui et al. [36] on the other hand reported an isolation protocol employing acetone as the initial extraction solvent and subsequent extraction with diethyl ether and 1-butanol followed by column LC and HPLC.

A completely different procedure for the isolation of palytoxin from *L. pictor* crab, involving hot aqueous extraction and acidification followed by column LC, was established by Teh and Gardiner [98]. This procedure was later applied by Lau et al. [97,99] in purpose of toxin extraction from the same crab species, following a documented outbreak of human poisoning after ingestion of crab soup. The toxin was found to be heat resistant under the boiling conditions employed [45]. However, a subsequent modification using aqueous ethanol for extraction instead of heat resulted in higher yields and an increase in specific activities [97].

Similar procedures are employed with regard to isolation and purification of palytoxin analogues derived from *Ostreopsis* spp. cell cultures for subsequent toxicity studies or chemical analysis.

TABLE 29.4
Comparative Table of Most Important Quantitative Chemical Analysis Methods for Palytoxin and Analogues

Method	Toxin	Matrix	Column Details	Mobile Phase
LC-MS				
Reversed phase LC-ESI-MS/MS	PLT, OST	Reference PLT + *Ostreopsis* cultures	5 µm Gemini C18 (150 × 2 mm) Temperature: Ambient	A: Water + 30 mM acetic acid B: 95% Acetonitrile/water + 30 mM acetic acid
LC-FLD-MS	PLT, OST	Reference PLT + *O. ovata* and *O.* cf. *siamensis* (derivatized and non-derivatized with 6-aminoquinolyl-*N*-hyroxysuccinimidyl carbamate, Acc-Q)	5 µm Xterra C18 (150 × 2.1 mm) Temperature: 35°C	A: MeOH B: 2 mM aqueous ammonium acetate pH = 5.8
LC-ESI-MS	PLT, OST	Reference PLT + *O. ovata* cultures	5 µm Xterra C18 (150 × 2.1 mm) Temperature: 35°C	A: MeOH B: [MeOH: 0.1 M ammonium acetate, pH = 4.0 (58:42)]
Micro-LC-ESI-MS/MS	PLT, OST (McTx-A & McTx-B)	Reference PLT + *O. mascarenensis* cultures	5 µm RP Equisil BDS C18 silica (100 × 1 mm) or 5 µm RP Hypersil C18 (250 × 1 mm) Temperature: not reported	A: Water + trifluoroacetic acid (pH 2.5) B: Acetonitrile
HPLC-UV				
HPLC-UV-DAD	PLT, OST	Reference PLT + *O. ovata* cultures	5 µm Xterra C18 (150 × 4.6 mm) Temperature: 35° C	A: MeOH B: [MeOH: 0.1 M ammonium acetate, pH=4.0 (58:42)]

Flow Rate	Detection Details	Limit of Detection (LOD)	Injection Volume	Retention Time (min)	Reference
0.2 mL/min, Gradient (20–100% B over 10 min and hold 4 min)	Positive ion mode, SIM (*m/z* 1340 & 912) and MRM (*m/z* 1340 → 327 & 912 → 327), Bi-charged and tri-charged ions	**PLT standard:** SIM: 40 ng/mL MRM: 25 ng/mL (MeOH/H_2O 1:1) 39 ng/mL (butanol extract) 38 ng/mL (pellet extract)	5 µL	**PLT standard:** SIM: 6.45 MRM: 6.40 **OST (pellet and butanol extract):** SIM: 6.45 MRM: 6.45	[30]
0.3 ml/min, Gradient (Initial: 20% A; linear rise over 5 min to 60% A; linear rise from 5–15 min to 80% A; 15–25 min steady 80% A; linear decrease from 80% to 20% A between 25–27 min and from 27–30 min steady 20% A)	Fluorescence: Excitation 250 nm, emission 395 nm Positive ion mode, mass range *m/z* 100–2000. Ionization parameters: capillary temperature 300±1°C, spray voltage 4.5 kV, sheath gas 20 mL/min, aux. gas 5 mL/min. Detection of bi-charged ion *m/z* 1362.7 $[M+2Na]^{2+}$ Loss of *m/z* 327 fragment	**PLT standard:** 7.5 ng LOQ = 20 ng	Not reported	Not reported	[31]
0.1 mL/min Gradient (Initial: 100% B for 16 min; 16–18 min linear rise to 100% A; 18–22 min steady 100% A; 22–25 min linear decrease to 100% B; 25–30 min 100% B)	Positive ion mode, mass range *m/z* 200–1500. Ionization parameters: capillary temperature 300±1°C, spray voltage 4.5 kV, sheath gas 20 ml/min, aux. gas 5 ml/min	Not reported	Not reported	Not reported	[29]
0.05 mL/min, Gradient (30–70% B over 45 min)	Positive ion mode, SRM (*m/z* 912 → 327), Tri-charged ions. Collision energy: 100 eV	Not reported	1 µL	**PLT standard:** Not reported **McTx-A:** SRM (*m/z* 327): 9.9 **McTx-b:** SRM (*m/z* 327): 10.25	[27]
0.75 mL/min Gradient (Initial: 100% B for 16 min; 16–18 min linear rise to 100% A; 18–22 min steady 100% A; 22–25 min linear decrease to 100% B; 25–30 min 100% B)	UV at 230 and 263 nm	1–2 µg injected quantity	Injected quantity 1.25 µg PLT standard (50 µL from 25 µg/mL)	**PLT standard & *O. ovata* toxin:** ≅ 9	[29,31, Riobó P. (pers. comm.]

Continued

TABLE 29.4
(Continued)

Method	Toxin	Matrix	Column Details	Mobile Phase
HPLC-UV				
HPLC-UV-DAD	PLT, OST (McTx-A & McTx-B)	Reference PLT + *O. mascarenensis* cultures	5 µm Hypersil ODS C18 (250 × 4.6 mm) Temperature: not reported	A: Water + trifluoroacetic acid (pH 2.5) B: Acetonitrile
HPLC-UV	PLT	Reference PLT, toxin extracted from *Palythoa* aff. *margaritae*	Cosmosil 5PE (250 × 4.6 mm) Temperature: not reported	25% aqueous MeCN with 0.1% AcOH
HPLC-UV	PLT	Reference PLT	BIO-SIL 5 ODS (250 × 4 mm) Temperature: not reported	52:48 water:acetonitrile with 0.1% trifluoroacetic acid
HPLC-UV	PLT	Reference PLT, toxins extracted from *L. pictor* and *D. alcalai* crabs	(i) ERC ODS-1282 (250 × 6 mm) (ii) ERC ODS-1282 (250 × 6 mm) (iii) TSK G3000SW (600 × 7.5 mm)	(i) MeOH:0.1 N acetic acid (8:2) (ii) MeOH:0.1 N acetic acid (5:5) (iii) 0.03 N acetic acid
HPLC-FLD				
HPLC-FLD	PLT, OST	Reference PLT + *O. ovata* and *O.* cf. *siamensis* cultures	5 µm Xterra C18 (150 × 4.6 mm) Temperature: 35° C	A: MeOH B: 2 mM aqueous ammonium acetate pH=5.8
HPCE				
HPCE-UV	PLT	Reference PLT and toxin extracted from *L. pictor* crab	50 cm × 50 µm, uncoated Voltage: 15 kV cross 50 cm Temperature: not reported	Electrolyte solution: 25 mM borate buffer at pH 8.5
HPCE-UV	PLT	Reference PLT	50 cm × 75 µm Voltage: 15 kV cross 50 cm Temperature: 25°C	Electrolyte solution: 25 mM borate buffer at pH 8.5

Flow Rate	Detection Details	Limit of Detection	Injection Volume	Retention Time (min)	Reference
1 mL/min, Gradient (linear 30–70% B over 45 min)	UV at 230 nm	Not reported	Injected dose 1.25 µg	**PLT standard:** 8.5 **McTx-A:** 8.3 **McTx-B:** 8.8	[27]
1 ml/min Isocratic	UV at 263 nm	**PLT standard & toxin *P.* aff *margaritae*:** Aliquots of 7.5 mg sample	Not reported	**PLT standard:** 8 min 55 sec **Toxin *P.* aff *margaritae*:** 8 min 58 sec	[14]
1 ml/min, Isocratic	UV at 230 nm	125 ng/ injection (≅ 2.5 µg/mL)	50 µL from 2.5 µg/mL	**PLT standard:** 9.8	[103]
0.9 ml/min, Isocratic in all (i)–(iii)	UV at 263 nm	Not reported	Not reported	**PLT standard and crab toxins:** (i) ≈ 13–14 (ii) ≈ 5–6 (iii) ≈ 17–18	[33]
0.75 ml/min Gradient (Initial: 100% B for 16 min; 16–18 min linear rise to 100% A; 18–22 min steady 100% A; 22–25 min linear decrease to 100% B; 25–30 min 100% B)	Derivatization with 6-aminoquinolyl-*N*-hyroxysuccinimidyl-carbamate SPE clean-up Fluorescence: Excitation 250 nm, emission 395 nm	**PLT standard (derivatized):** 0.75 ng Recovery: 95.13 ± 7.80 % (S.D.) LOQ = 2 ng	Not reported	**PLT standard:** ≈13.5 **OST (derivatized extract):** ≈13.5	[31]
—	UV at 230 and 263 nm	Not reported	**PLT standard:** 16720 MU/ml ***L. pictor* toxin:** 14400 MU/mL Inj. pressure: 20 psi/sec	**PLT standard & *L. pictor* toxin:** ≅3.5	[99]
—	UV at 230 and 263 nm	0.5 pg/ injection (≅ 100 ng/ml)	5 nL from 100 ng/mL	**PLT standard:** 9.0	[103]

PLT = palytoxin, OST = ostreocins, LC-MS = Liquid Chromatography-Mass Spectrometry, HPLC = high performance liquid chromatography, HPCE = high performance capillary electrophoresis.

Cells are harvested either by filtering or centrifugation and extracted subsequently with pure or aqueous methanol [23,25,27,29–31,82] or aqueous ethanol [28], with or without mild sonication. After removal of the cell pellet by centrifugation, the steps of defatting the supernatant with diethyl ether, hexane dichloromethane, or chloroform and partitioning between water and 1-butanol usually follow. On the basis of mouse toxicity and chromatographic separation and identification, some researchers report that palytoxin analogues are detected in the 1-butanol fraction [23,25,27,30], while others have detected these substances in the aqueous fraction [28]. In certain procedures, the butanol/water partitioning step is excluded and the methanolic culture extracts produced after defatting are used for analyses, either with [31] or without [29] subsequent solid phase extraction (SPE) cleanup.

29.4.2 Detection and Quantification

Palytoxin and its analogues are nowadays considered as one of the emerging toxin groups due to their recently observed occurrence in areas of the world with temperate climate, such as the Mediterranean where it was not considered as a threat earlier. The European Union Commission has initially recognized the necessity for regulation of this toxin group and in this context, a provisional limit of 250 μg/kg fish was proposed during the first meeting of the Working Group on Toxicology, held in Cesenatico, Italy, in 2005 (Report on Toxicology Working Group Meeting, Cesenatico, Italy, 24–25 October 2005).

It is necessary that appropriate methods for detection and quantification be in place not only to achieve regulatory monitoring for this toxin group but also to check purity of the extracted toxin in research. Detection and quantification of palytoxin in biological samples can be accomplished by both analytical and biological methods (see Chapter 34), and it is often necessary to use a combination of methods in order to positively confirm the presence of palytoxin. The simplest way to detect palytoxin and one of the most sensitive ones in terms of detection limit is the mouse bioassay, which involves intraperitoneal injection of properly prepared contaminated samples to mice and recording of symptomatology and death times. Lethal potency is expressed as mouse units (MU), where 1 MU is the amount of toxin that kills a 20 g mouse in 24 h. On the basis of the reported LD_{50} value for palytoxin of 450 ng/kg, 1 MU is presumed to be 9 ng of palytoxin [26]. Mice injected with palytoxin exhibit several characteristic symptoms before death, namely, sudden jerks and convulsions. Despite this, however, the mouse bioassay is not able to individuate the nature of the causative agent unequivocally. Alternative assays with very favorable detection limits have been developed, which take advantage of certain palytoxin functional properties. Such methods include *in vitro* cytotoxicity [100], delayed hemolysis [101], and monoclonal antibody-based enzyme-linked immunoassays [102]. All these assays are extremely sensitive, but in most regulatory situations, positive results require further confirmation by instrumental methods.

Several chemical analysis methods have been developed for the determination of palytoxin and/or palytoxin analogues, based on chemical properties characteristic and intrinsic to the toxin. Such methods include (1) infrared spectrometry, (2) ultraviolet spectrometry, (3) mass spectrometry, (4) high-performance capillary electrophoresis, (5) thin-layer chromatography, and (6) liquid chromatography. A comparative table of the most important quantitative chemical analysis methods summarizes the main features of each method and could assist in selection of the most "fit-for-purpose" analysis method depending on available equipment and sensitivity required (Table 29.4).

29.4.2.1 Infrared Spectrometry

The IR spectrum of palytoxin from *P. toxica* shows a band at 1670/cm, which has been assigned to the presence of an α,β-unsaturated amide carbonyl group [3], while in the case of *P. tuberculosa* palytoxin in KBr, this band has been observed at 1655/cm [55]. Similar IR spectra have also been obtained for purified palytoxin from *P. caribaeorum* [11], from the sea anemone *R. macrodactylus* [39] and from the xanthid crab *L. pictor* [45].

29.4.2.2 Ultraviolet Spectrometry

The UV spectrum of palytoxin, as discussed earlier, shows two characteristic absorption peaks at 233 and 263 nm, contributed by the respective chromophores (Figure 29.4). The ratio of their absorbance (233 versus 263 nm), which is approximately 1.7 [3], is characteristic and indicative of the toxin's presence. The absorptions at either wavelength have been reported to be linearly related to palytoxin concentration in the range of 5–20 μg/mL. However, the disadvantage of this method is its detection limit, as the minimum detectable concentration has been reported to be 5 μg/mL (palytoxin standard in water), while toxicological and physiological effects have been observed with concentrations as low as 0.05–0.1 μg/mL [103], which limits its suitability as a regulatory analysis method.

29.4.2.3 Mass Spectrometry

Details with regard to MS data, which have been used for determination of molecular weights and elucidation of the structural differences between palytoxins from various origins, have been presented earlier in the present chapter. Ion-spray MS is usually employed for the detection of palytoxin and/or its analogues, while certain MS data can be strongly indicative of their presence in a matrix: (1) the presence of a fragment ion at or near $m/z = 327$ in the bivalent and trivalent positive ion MS/MS spectra; (2) the presence of peaks at or near $m/z = 1340$ and $m/z = 912$, corresponding to the bi-charged ion $[M + 2H]^{2+}$ and the tri-charged ion $[M + 2H + NH_4]^{3+}$, which can be used as precursor ions in multiple reaction monitoring (MRM) experiments yielding the product ion $m/z = 327$ (transitions m/z 1340→327 and 912→327); (3) multiple losses of water molecules from the $[M + 2H]^{2+}$ ion corresponding to the large number of hydroxyl moieties contained in palytoxin and palytoxin-like substances; and (4) a molecular mass around 2600 Da [27,29,30]. As pointed out earlier, the absence of the $m/z = 327$ fragment ion does not exclude the possibility of a palytoxin-like substance, but could indicate a structural isomer differing at the respective end of the molecule responsible for the $m/z = 327$ fragment ion [34].

29.4.2.4 High-Performance Capillary Electrophoresis

High-performance capillary electrophoresis (HPCE) is a very sensitive method for the identification and detection of palytoxin (Table 29.4). Mereish et al. [103] have carried out HPCE with UV detection at 230 nm and 263 nm using an open-capillary and applying a voltage of 15 kV across a 50 cm × 75 μm column. The column temperature was maintained at 25°C and the conducting buffer solution used was 25 mM sodium borate at pH 8.5 (Figure 29.7). The detection limit was 0.5 pg of palytoxin, and the detection sensitivity of palytoxin at 230 versus 263 nm was 2-fold. A similar method was used by Lau et al. [99] for palytoxin extracted from *L. pictor* crab. HPCE has the advantage that it allows measurement of palytoxin at low concentrations and in small volumes and can be applied to determine palytoxin in biological fluids [103].

29.4.2.5 Thin-Layer Chromatography

Thin-layer chromatography can be used both for detection of palytoxin as well as in homogeneity examinations of extracted palytoxin [11,99,104]. Béress et al. [11] have used silica gel plates developed with pyridine–water–*n*-butanol–acetic acid in the ratio 10:12:15:13, and the toxin was stained purple with ninhydrin in ethanol with an R_f of 0.55, while Habermann et al. [104] used the same solvent mixture with cellulose plates and observed two close ninhydrin positive spots (R_f 0.67 for the major and R_f 0.75 for the minor spot). In another work [33] comparing pure palytoxin with toxins extracted from the crabs *L. pictor* and *Demania alcalai*, TLC was carried out on Silica gel 60 and NH_2F_{254}s plates with two different solvent systems: (A) pyridine–water–*n*-butanol–acetic acid (10:12:15:13) and (B) 1-pentanol–pyridine–water (7:7:6). The toxin on the silica gel plates

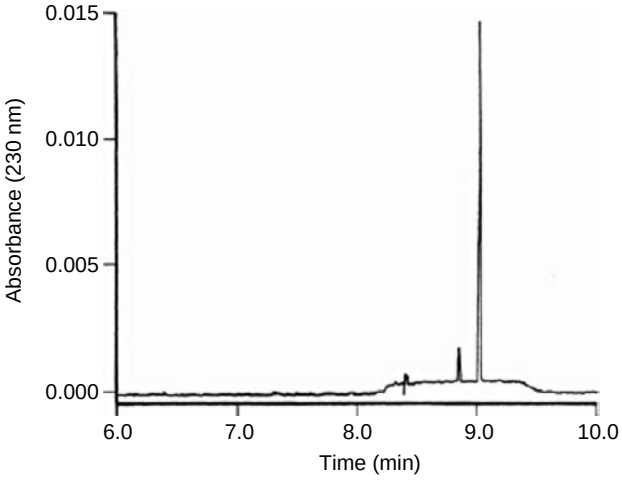

FIGURE 29.7 Electrogram of palytoxin. (Reprinted from Mereish, K.A., Morris, S., Mc Cullers, G., Taylor, T.J., and Bunner, D.L, *Journal of Liquid Chromatography*, 14, 1025–1031, 1991. © Taylor and Francis Inc.)

was detected by heating the plates after spraying with H_2SO_4 while that on the NH_2F_{254}s plates by exposure to UV light (254 nm). The R_fs recorded for pure palytoxin for Silica gel 60 plates were 0.50 with solvent A and 0.35 and 0.60 (two spots) for solvent B while the respective R_fs for the NH_2F_{254}s plates were 0.76 with solvent A and 0.19 and 0.20 for solvent B. The toxins extracted from both crab species were found to have identical R_fs with pure palytoxin regardless of plate type and solvent mixture used. A similar but slightly modified method was followed by Fukui et al. [36] for comparison of the toxin extracted from the trigger fish *M. vidua*. The same types of plates were used in combination with solvent A and a modified solvent B (1-propanol–pyridine–water, 7:7:6) and detection by spraying the plates with a sulfuric acid:methanol (1:1) solution and charring at 150°C while measured R_fs were slightly different. During isolation of a fluorescent palytoxin congener [99], two-dimensional TLC has been used with *n*-butanol–acetic acid–water (9:3:8) followed by *n*-propanol–water (7:3) in the first dimension and then *n*-propanol–25% ammonia in the second dimension. A fluorescent spot was observed, which also stained yellow with exposure to iodine vapor in a chamber and purple with ninhydrin [45].

29.4.2.6 Liquid Chromatography

As mentioned earlier in the present chapter, LC has been broadly utilized for purification of palytoxin extracted from its sources. With regard to identification, detection, and quantification of palytoxin, numerous LC methods have been developed through the years. The vast majority of methods employ HPLC-UV, taking advantage of the characteristic UV-spectrum (230 nm and 263 nm) of palytoxin. Very often, however, especially in the case of analyzing biological matrices (e.g., phytoplankton cells, marine animal tissues), the presence of interferences notably diminishes sensitivity of HPLC-UV methods, resulting in poor performance characteristics and/or high detection limits. Such factors reduce the possibility for utilization of these methods for regulatory purposes. In order to overcome problems with poor sensitivity, LC methods with fluorescence detection (HPLC-FLD) or employing MS detection (LC-MS) have been developed in the past few years.

29.4.2.7 HPLC-UV

HPLC-UV methods have been widely used for the chromatographic separation of palytoxin and its analogues, primarily for studies on reference palytoxin and for identification of the toxin in matrices

implicated with food poisoning incidents. Yasumoto et al. [33] analyzed and identified by HPLC reference palytoxin together with palytoxin extracted from two species of xanthid crabs, using three different combinations of analytical column and mobile phases. In all cases, retention times of crab toxins showed retention times comparable to those of reference palytoxin (Table 29.4). Similar but slightly modified methods were used by Fukui et al. [36] for the identification of toxin from the trigger fish *M. vidua* [column: TSK G3000SW (600 mm × 7.5 mm); mobile phase 0.1 M acetic acid; flow rate: 1.0 mL/min; detection: UV absorption at 263 nm] and by Alcala et al. (1988) for the identification of *D. reynaudii* toxin [column: ERC-ODS (250 mm × 6 mm); mobile phase acetonitrile : 0.05 N acetic acid (1:1); flow rate: 0.9 mL/min; detection: UV absorption at 263 nm]. A detection limit of 125 ng/injection for palytoxin was obtained by Mereish et al. [103] by use of a Bio-Sil 5 ODS column (Table 29.4) while Lau et al. [99] carried out the HPLC analysis of Caribbean palytoxin using a Novapak C18 reversed-phase column (75 mm × 3.9 mm), a gradient elution with 80% acetonitrile : water (4:1) and detection at 230 nm. Reversed-phase HPLC was also applied for the analysis of Caribbean palytoxin [column: Lichrospher 300 RP-8; mobile phase 0.1% TFA and 80% acetonitrile; linear gradient; detection: UV absorption at 230 nm] with a lower tracing quantity of 10 μg [21]. Other HPLC methods for palytoxin detection using different combinations of columns and solvent mixtures have also been reported [14,26].

After the first report of Usami et al. [23] with regard to the palytoxin-like character of the *O. siamensis* toxin (ostreocin-D) and the subsequent implication of this species in a case of clupeotoxism in which the causative agent was found to be a palytoxin analogue [26], a number of HPLC-UV methods have been developed for the extraction and quantification of palytoxin analogues from extracts of *Ostreopsis* cells. Lenoir et al. [27] analyzed the toxically active *n*-butanol soluble fraction (BSF) from *O. mascarenensis* cells derived from a natural bloom in the Southwestern Indian Ocean in comparison with reference palytoxin by HPLC-UV with diode array detection (Table 29.4) and reported the presence of two different palytoxin analogues, namely mascarenotoxin-A and -B, possessing similar retention times and identical UV spectra with reference palytoxin. In the same context, Penna et al. [29] and Riobó et al. [31] using the same HPLC-UV method (Table 29.4) have detected the presence of palytoxin-like substances presenting the same UV-spectra and retention times with reference palytoxin in cultures of *O. ovata* and *O.* cf. *siamensis* isolated from Brazil and from the western Mediterranean Sea. In these reports, however, the minimum detectable quantity for reference palytoxin was in the range of 1–2 μg/injection, while with regard to the toxic *Ostreopsis* extracts it was not always possible to detect a peak and obtain a spectrum for confirmation [31].

To the best of our knowledge till date, there are no reports for HPLC-UV methods for the quantitative determination of palytoxin and analogues in shellfish samples. Moreover, taking into consideration the proposed limit of 250 μg/kg tissue, together with the published quality parameters and especially limits of detection of the above described HPLC-UV methods (see Table 29.4), it is unlikely that they could be routinely employed for the regulatory monitoring of palytoxin and its analogues in shellfish tissues.

29.4.2.8 HPLC-FLD

It is widely recognized that HPLC-FLD methods are generally much more sensitive than HPLC-UV methods. For this reason, although palytoxin is not a naturally fluorescent substance [34], Riobó et al. [31] have established a precolumn derivatization method for the separation and quantification of palytoxin, taking advantage of the presence of one amino terminal group in the palytoxin molecule and using the derivatization reagent 6-aminoquinolyl-*N*-hyroxysuccinimidyl carbamate. The method has been successfully employed for the determination and quantification of both reference palytoxin from *P. tuberculosa* and toxins contained in the methanolic extracts of 14 different strains of cultured *Ostreopsis* cells (Figure 29.8). The cell extracts underwent SPE clean up with two retention mechanisms: anion exchange and reverse phase without silanol groups. Separation was carried out as described in Table 29.4. This method exhibited much lower detection limits for derivatized

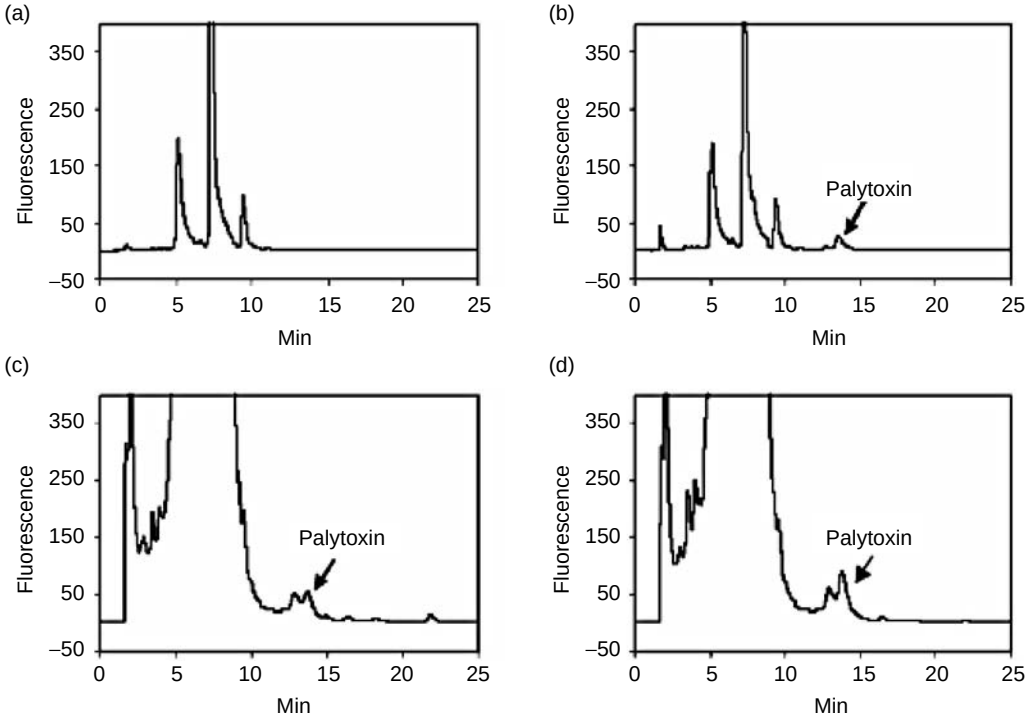

FIGURE 29.8 Representative chromatograms of derivatized blank (a) in comparison with derivatized palytoxin standard (b), derivatized MeOH:acetic acid eluate from Oasis Max cartridge of *Ostreopsis* sample (c) and the same eluate spiked with palytoxin standard. The arrow indicates the palytoxin peak. (Reprinted from Riobó, P., Paz, B., and Franco, J.M., *Analytica Chimica Acta*, 566, 217–223, 2006. © Elsevier.)

reference palytoxin (as low as 0.75 ng/injection) compared to the aforementioned HPLC-UV methods. On the other hand, the limit of quantification (2 ng), recovery (95.13% ± 7.80% S.D.), correlation with the hemolysis method ($r^2 = 0.9118$), stability of the fluorescent derivatives (reference palytoxin and spiked samples stable for up to 2 weeks at 4°C), and lack of interferences, which could affect quantification, indicated sufficient robustness of the method, which could be promising for routine use if optimized for application to shellfish extracts.

29.4.2.9 LC-MS

MS on its own has been extensively used for identification and structure elucidation of palytoxin and/or its analogues. The observed low sensitivity of HPLC-UV methods with regard to palytoxin analysis, however, has triggered interest in the use of MS in combination with LC for the determination of these substances. Till date, there are four reports of LC-MS methods (Table 29.4) targeting palytoxin and its analogues.

LC-ESI-MS methods have been employed for identification of certain palytoxin analogues by Lenoir et al. [27] and Penna et al. [29]. These researchers concluded that certain MS data (as detailed under the Mass Spectrometry section) denote the presence of palytoxin or its analogues in a matrix: (1) presence of the $m/z = 327$ fragment ion in the bivalent and trivalent positive ion MS/MS spectra, (2) presence of peaks at or near $m/z = 1340$ and $m/z = 912$ (3) multiple losses of water molecules (18 Da) from the $[M + 2H]^{2+}$ ion, and (4) molecular mass around 2600 Da.

Data on quantification of palytoxin and analogues by use of LC-ESI-MS/MS have only been provided by Ciminiello et al. [30] Four concentration levels (2.7, 0.9, 0.3, and 0.1 μg/mL palytoxin standard) were analyzed in the positive mode by selected ion monitoring (SIM) for the bi-charged

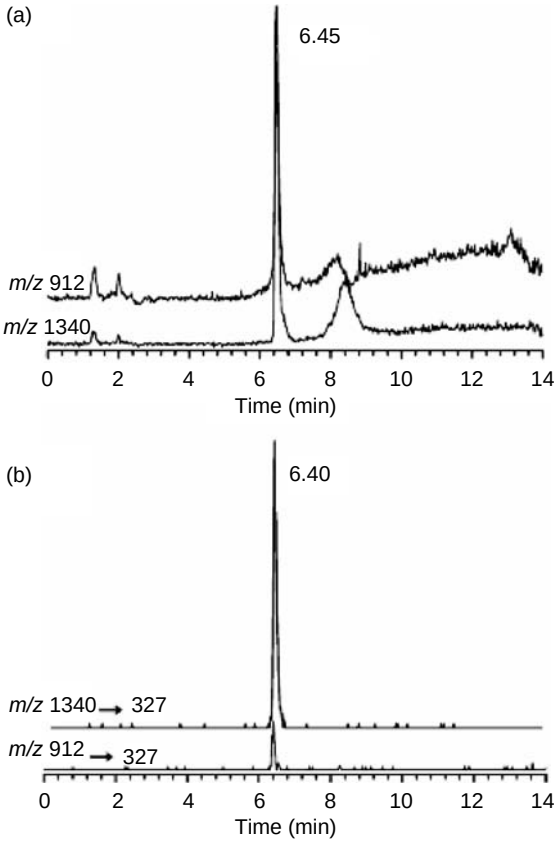

FIGURE 29.9 LC-MS analyses of a 1 μg/mL standard solution of palytoxin in positive ion mode. SIM (a) was performed for ions at *m/z* 1340 $[M + 2H]^{2+}$ and *m/z* 912 $[M + 3H + 3H_2O]^{3+}$. MRM experiment (b) was performed by selecting ion transitions consistent with the fragmentation behavior of palytoxin (*m/z* 1340→ 327 and *m/z* 912→327). (Reprinted with permission from Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Grillo, C., and Melchiorre, N., *Analytical Chemistry*, 78, 6153–6159, 2006. © American Chemical Society.)

ion *m/z* 1340 and MRM for the transition *m/z* 1340 → 327 (Figure 29.9). The minimum detection levels for matrix-free toxin on column were estimated to be 200 pg and 125 pg in SIM and MRM mode, respectively, while linearity within the tested concentration range in both cases exceeded 0.9998. When matrix effects were investigated in positive MRM mode by spiking of both sample pellet and butanol extract with the aforementioned four palytoxin levels, it was concluded that limits of detection (LOD; S/N = 3) and limits of quantification (LOQ; S/N = 10) were respectively 25 and 84 ng/mL for reference palytoxin (matrix free standard), 39 and 131 ng/mL for palytoxin-spiked butanol extract, and 38 and 127 ng/mL for spiked pellet extract. However, the slope of the curves for matrix-matched standards indicated a slight enhancement effect (6–8%) of the signal at low palytoxin concentrations (0.1–0.3 μg/mL) and a more significant suppression effect (14–20%) at palytoxin levels higher than 0.9 μg/mL. This result indicated that the matrix effect over the tested concentration range is analyte concentration-dependent, and concluded that matrix-matched standards should be used for accurate quantitation. Intraday and interday reproducibility of the method were also evaluated and the obtained values did not exceed an relative standard deviation (RSD) value of 9.6% either with pellet or butanol extracts. In the end, accuracy of the method was also tested and recoveries were estimated to be 91–98% for reference palytoxin-spiked pellet extracts and 73–82% for spiked butanol extracts with RSD values of less than 3.2% in both cases, which

indicated that the extraction efficiency for palytoxin was satisfactory. Putative palytoxin from the *Ostreopsis* cultures was also quantified and found to be present at levels 1.35 μg and 1.95 μg in the pellet and butanol extracts, respectively.

Finally, an LC-FLD-MS method has recently been reported [31]. Using this method, LOD and LOQ for reference palytoxin by quantification of the *m/z* fragment were 7.5 ng and 20 ng, respectively. Sensitivity of this method was 26-fold lower compared to the HPLC-FLD method described in the same report (Table 29.4), while correlations with hemolytic assay were also more favorable for the HPLC-FLD method (HPLC-FLD: $R^2 = 0.91$; LC-FLD-MS: $R^2 = 0.86$).

All these methods have been applied to reference palytoxin and palytoxin analogues derived from extracts of *Ostreopsis* cells, while no LC-MS methods have been published to date for the determination of palytoxin in shellfish tissues. Further research for the development of such methods is essential for future application in routine testing of shellfish tissues for palytoxin and analogues.

29.5 CONCLUSION

Palytoxin is one of the most potent marine natural products known and there is evidence that potential production sources are species of the genus *Ostreopsis*. The connection between palytoxin seafood poisoning and presence of toxic *Ostreopsis* spp. has been long suspected but has not been established with certainty until today. Further research will elucidate the potential of *Ostreopsis* derived palytoxin-analogues to enter the human food chain. Nevertheless, until this situation is clarified, vigilance is required regarding this dinoflagellate species. Toxic *Ostreopsis* spp. have been reported in the last few years to abundantly occur in temperate waters all over the world. The presence of this tropical species in temperate waters could be partly attributed to the global warming effect. Abundances as high as 400,000 cells/g (fresh weight) of macrophytes have been recorded in Greece [73], while similar or higher numbers have been reported from other Mediterranean countries. It is evident that routine monitoring of these benthic species is essential in susceptible areas. A network for the monitoring of benthic dinoflagellates has already been established in Italy, under the name BENTOX-NET [105]. The necessity for regulatory limits for palytoxin and palytoxin-analogues is already recognized in the EU and provisional limits have been proposed. The development of chemical analysis methods with sufficient sensitivity for the quantitative determination of palytoxin well below the proposed limits is therefore a priority.

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30 Diverse Chemical Structures and Bioactivities of Marine Toxin: Palytoxin and Symbiodinolide

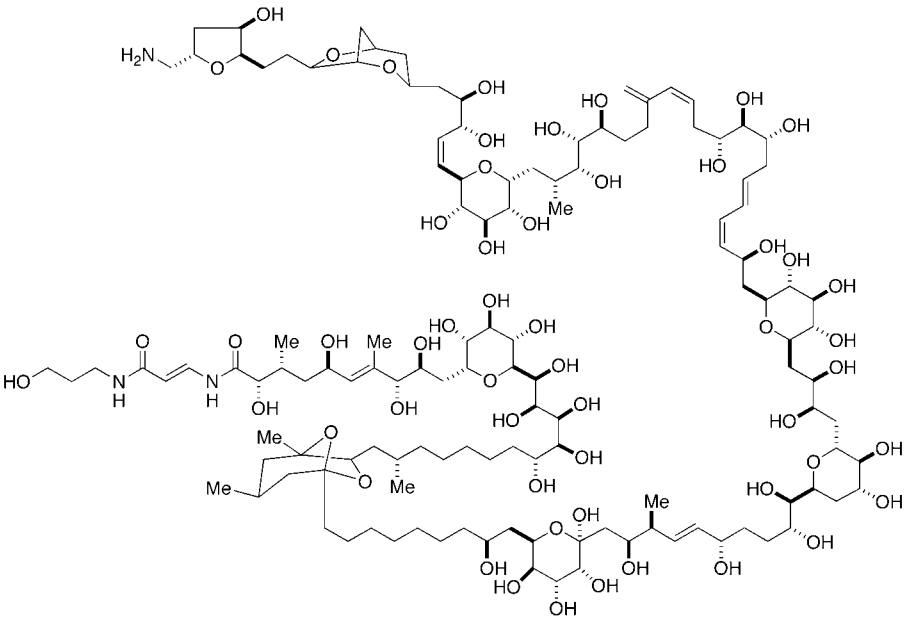
Masaki Kita and Daisuke Uemura

CONTENTS

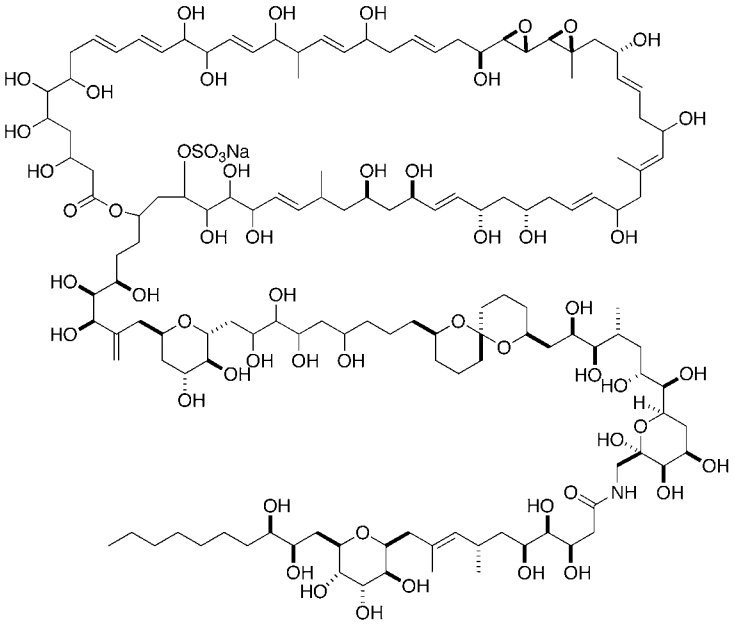
30.1 INTRODUCTION

Numerous secondary metabolites with unique chemical structures and biological activities are found in marine organisms. Their chemical structures are completely different from those of terrestrial organisms, due to their radically different habitats. Two of the most remarkable properties of these compounds are their structural and physiological diversities. In particular, huge polyol and polyether compounds composed of a long carbon backbone functionalized by oxygen atoms, such as palytoxin, halichondrin, ciguatoxin, and maitotoxin, are some of the most attractive molecules in natural products chemistry [1–5].

It has been suggested that the possible primary producers of such secondary metabolites are microalgae, bacteria, and fungi, and they are carried through symbiosis, association, a food chain, and other forms of nutrient dependency [6–9]. In fact, numerous potent bioactive compounds with highly complex structures have been isolated from various cultured marine dinoflagellates. However, the true origins or progenitors of these metabolites are not entirely clear. Determining the real producers of such bioactive metabolites would greatly contribute to knowing more seafood and freshwater poisoning. In this chapter, recent advancements of structural and biological studies on two huge polyol compounds, palytoxin (PTX) (**1**), symbiodinolide (**2**) (Figure 30.1), and some related compounds are discussed.



Palytoxin (**1**)



Symbiodinolide (**2**)

FIGURE 30.1 Structures of PTX (1) and symbiodinolide (2).

30.2 PALYTOXIN, A POLYOL COMPOUND THAT SHOWS EXTREME TOXICITY

30.2.1 Overview

PTX (**1**) is a deadly poison that is found in the coelenterate *Palythoa tuberculosa*. On the basis of local legends in Hawaii, Limu-make-o-Hana, a deadly poisonous alga, was found in the Hana District. At first, it was suspected that the poison was associated with ciguatera; however, the poison actually came not from the alga but from a species of *Palythoa toxicus* [10]. Scheuer's group reported that PTX was found in biota living in Hawaii [11]. Around the same time, Hashimoto et al. reported that a species of *Alutera scripta* living in Okinawa contained an extremely strong poison called aluterin [12]. Aluterin extracted from *A. scripta* actually originated in *P. tuberculosa*. This coelenterate spreads its polyp to form a colony 1–1.5 cm deep and produces eggs between April and June, which have the strongest toxicity. Thus, studies on the toxic constituents of *P. tuberculosa* collected on the islands of Okinawa and Ishigaki, Japan, were carried out.

In 1980, using plasma desorption mass spectrometry with californium (^{252}Cf), the exact molecular weight of PTX (**1**) was established as 2680 for the first time [13]. The planar structure of **1** was eventually clarified in 1981 [14–16], and its complete stereostructure was elucidated in 1982 [17–20]. Finally, the true stereostructure of **1** was established by an overall degradation reaction and chemical synthesis in 1982. Kishi and his coworkers completed the total synthesis of palytoxin carboxylic acid (**3**) in 1989 [21, 22], and of PTX itself in 1994 (Figure 30.2) [23,24].

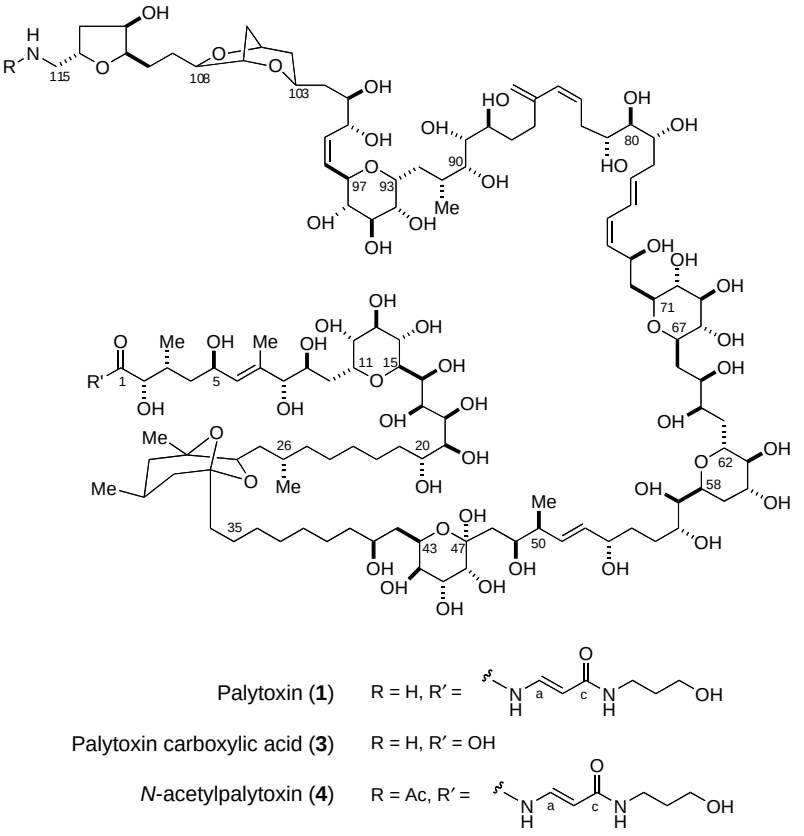


FIGURE 30.2 Structures of PTX derivatives.

PTX (**1**) is one of the largest-sized natural products that do not contain repeating units such as amino acids or monosaccharides. The molecular formula of **1** is $C_{129}H_{223}N_3O_{54}$, and the molecule contains 64 chiral centers. In 2001, using multidimensional Fourier transform techniques, all hydrogen and carbon nuclear magnetic resonance (NMR) signals of PTX (**1**) and *N*-acetylpalytoxin (NAcPTX, **4**) were successfully assigned [25].

30.2.2 Biological Activity of Palytoxin

PTX (**1**) shows extreme acute toxicity against mice (LD_{50} 0.45 μg/kg). Intoxication by PTX leads to vasoconstriction, hemorrhage, ataxia, muscle weakness, ventricular fibrillation, pulmonary hypertension, ischemia, and death. However, the most remarkable feature of PTX is to bind to the Na^+,K^+-ATPase and to convert this pump into an open channel [26–31]. Na^+,K^+-ATPase is essential in neural transmission, as it draws out three Na^+ ions from within a nerve cell and exchanges them with two K^+ ions from outside the cell. This activity is antagonized by ouabain, while PTX and ouabain bind onto different positions. Na^+,K^+-ATPase, which binds with PTX, exhibits a slight permeability to Ca^{2+}. Recently, it was shown that the effect of PTX on $[Ca^{2+}]_i$ and the downstream events induced cell death in bovine aortic endothelial cells [32].

PTX (**1**) also caused a significant reduction in the actin cytoskeleton as with other polyol and polyether toxins such as pectenotoxin-6 and maitotoxin [33]. Thus, the cytoskeleton is suggested to be an early target for the toxic effect of such toxins. Furthermore, the skin tumor promoter activity of palytoxin has gathered attention. Notably, palytoxin did not activate protein kinase C but extracellular signal-regulated kinase (ERK) through a mechanism that involves inactivation of an ERK phosphatase in keratinocytes derived from initiated mouse skin [34].

As described above, natural toxins with high specificity to their molecular targets have been contributing greatly to the study of the biology and physiology of ion channels or other bio-macromolecular targets. Especially, PTX is still a promising pharmacological reagent to investigate the mode of actions of Na^+,K^+-ATPase.

30.2.3 Molecular Shape of Palytoxin in Aqueous Solution

Conformational analyses of low molecular weight bioactive compounds have given important information on their action mechanisms. X-Ray crystallographic analysis and NMR spectroscopy have served as the most useful tools to obtain this information. However, application of such methods to palytoxin was confronted with great difficulties due to the noncrystalline nature of the compound and heavy overlaps of signals in the NMR spectra.

Recently, the molecular shapes of PTX (**1**) and NAcPTX (**4**) [35] were shown by x-ray small-angle scattering (SAXS) method [36]. SAXS data reflect the time and ensemble average of molecular solution conformations. In general, this method is only applicable to high molecular-weight bio-macromolecules. Although PTX (**1**) has an exceptionally large molecular weight for a natural product, it is actually too small to obtain a sufficient scattering intensity. Indeed, there was no precedent that used SAXS for a structural analysis of a natural product in solution. To overcome such difficulties, a synchrotron x-ray source in the radiation light facility of SPring-8 (RIKEN Harima Institute, Japan) was used, and the scattering curves for an aqueous solution of compounds **1** and **4** were obtained.

The radius of gyration (R_g) and zero-angle scattering intensity ($I(0)/C$) of PTX (**1**) and NAcPTX (**4**) were estimated on the basis of the Guinier approximation. The concentration-scaled zero-angle scattering intensity, $I(0)/C$, is proportional to molecular weight. By comparison of the $I(0)/C$ values of cytochrome C, the molecular formula of PTX and NAcPTX in solution were estimated to be 5700 and 2600, respectively. Since the molecular weight of PTX is 2680, PTX was considered to exist as a dimer, whereas NAcPTX was a monomer in solution. On the basis of the distance distribution function ($P(r)$), the maximum distance (D_{max}) values of **1** and **4** were calculated as about 35 Å and 50 Å, respectively. Furthermore, low-resolution models of these two molecules in solution were calculated

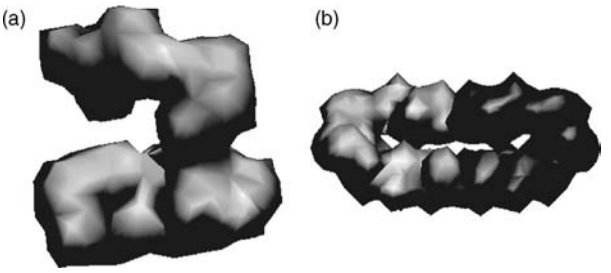


FIGURE 30.3 Low-resolution models in a solution: (a) NAcPTX (4) and (b) PTX (1).

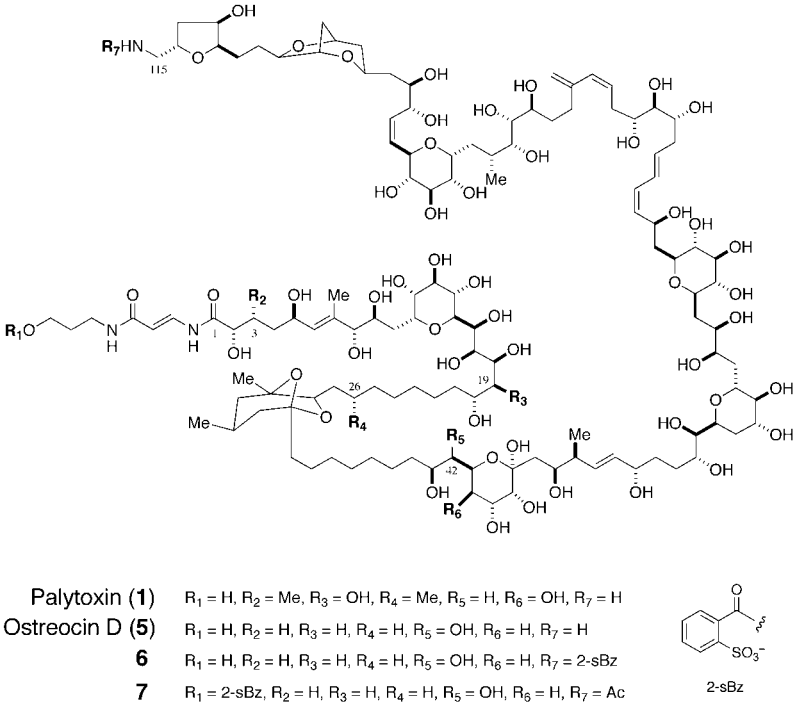


FIGURE 30.4 Structures of ostreocin-D (5) and its derivatives.

using *GASBOR* program (Figure 30.3). The representative model of NAcPTX (**4**) measured 30.6 × 23.4 × 13.0 Å with an overall horseshoe-like shape while in contrast, that of PTX (**1**) measured 52.3 × 22.0 × 15.1 Å. Each PTX unit existed in a similar shape with NAcPTX, but formed an associated dimer in aqueous solution.

It should be noted that acetylation of PTX's amino terminus decreases its toxicity to approximately 1/100 [37,38]. Thus, electrostatic interaction through the amino group would be involved in the association at the interface. These results may give quite useful perspectives for the further studies on the mode of actions of PTX.

30.2.4 Ostreocin-D, a Palytoxin Analogue from a Dinoflagellate

Although PTX (**1**) was originally found in the coelenterate *Palythoa* spp., its bioorganic origin has been questioned due to seasonal and regional variations [11,15,16]. Recently, Yasumoto and coworkers found that ostreocin D (5), a palytoxin analog from the cultured marine dinoflagellate Ostreopsis siamensis [39] (Figure 30.4). Ostreocin D was a major constituent and accounted for

70% of total toxicity. Its structure was elucidated to be 42-hydroxy-3, 26-didemethyl-19, 44-dideoxy palytoxin by detailed 2D NMR analyses of intact ostreocin D and its ozonolysis products [40].

In the structure determination study of ostreocin D (**5**), negative-ion fast-atom bombardment collision-induced dissociation tandem mass spectrometric (FAB-CID-MS/MS) methodology was quite useful [41]. The charge-remote fragmentations were facilitated by a negative charge introduced to a terminal amino group (compound **6**) or to a hydroxyl group (compound **7**) at the other terminus by a reaction with 2-sulfobenzoic acid cyclic anhydride.

The small structural changes barely affected the mice lethality of ostreocin D (**5**). The LD_{50} value of **5** was 0.75 μg/kg (i.p. injection). Ostreocin D also exhibited potent cytotoxicity against P388 cells and hemolytic potency against mouse blood cell suspension in the same order concentrations as PTX (**1**).

Furthermore, it was shown that toxins from *Ostreopsis* spp. and a parrotfish *Scarus ovifrons* showed similar delayed hemolytic activity with mouse and human erythrocytes, both of which were inhibited by an antipalytoxin (PTX) antibody and ouabain [42]. A considerable number of adherent *Ostreopsis* spp. was found in the gut contents of *S. ovifrons* during the heavy occurrence of *Ostreopsis* spp. This result indicates that the dinoflagellate *O. siamensis* is one of the biogenetic origins of PTX, and that a number of polyol compounds can also be biosynthesized by marine microorganisms as with the various causative substances of food poisoning.

30.3 SYMBIODINOLIDE, A POLYOL MACROLIDE THAT ACTIVATES Ca^{2+} CHANNEL

30.3.1 Overview

As mentioned above, marine dinoflagellates are considered rich resources of bioactive compounds, and various long-carbon-chain polyol compounds have been isolated from cultured symbiotic ones, such as amphidinols [43] and karatungiols [44] from *Amphidinium* spp., zooxanthellatoxins (ZTs) [45,46], and zooxanthellamides (ZADs) [47–49] from *Symbiodinium* spp., and durinskiols [50,51] from *Durinskia* spp. On the basis of their structural, biological, and conformational diversity and uniqueness, various potential abilities of such polyol compounds can be considered, that is, chemical communication with host animals, defense materials, or nutrient sources. However, the true physiological functions or roles of these compounds in the ecosystem or symbiotic relationship have rarely been clarified.

The symbiotic marine dinoflagellate *Symbiodinium* spp., which is a type of zooxanthellae, is found in a wide range of marine invertebrates [52–54]. Recently, a unique polyol macrolide, symbiodinolide (**2**), that activates N-type Ca^{2+} channels was isolated [55]. Here we describe the structure and biological activity of **2** and its structurally related compounds.

30.3.2 Structure of Symbiodinolide

The molecular formula of symbiodinolide (**2**) was elucidated to be $C_{137}H_{232}NNaO_{57}S$, with an overall C_{129} carbon-chain skeleton and 43 hydroxyl groups. The entire planar structure of **2** was confirmed by detailed analyses of the degradation products obtained by alkaline hydrolysis and ethenolysis using the second-generation Grubbs' catalyst. As a result, symbiodinolide was found to be a structural congener of ZTs, and had a similar 62-membered mono-sulfated macrolactone moiety, bis(epoxide) moiety, 6,6-spiroacetal and hemiacetal rings. The molecular weight of **2** was 36 mass unit (mu) smaller than that of ZT-A (**8**) [45], and 6 mu larger than that of ZT-B (**9**) [46] (Figure 30.5). Meanwhile, the terminal carbon-chain moiety (C21′–C25′) in **2** was saturated, while it is a conjugated diene moiety in ZTs. Other differences between **2** and ZTs were the presence or absence of three hydroxyl groups on C3, C76, and C100. In the case of ZT-A (**8**), the absolute stereochemistries of the six-membered ether part (C71–C75), the spiroacetal moiety (C81–C94), and the

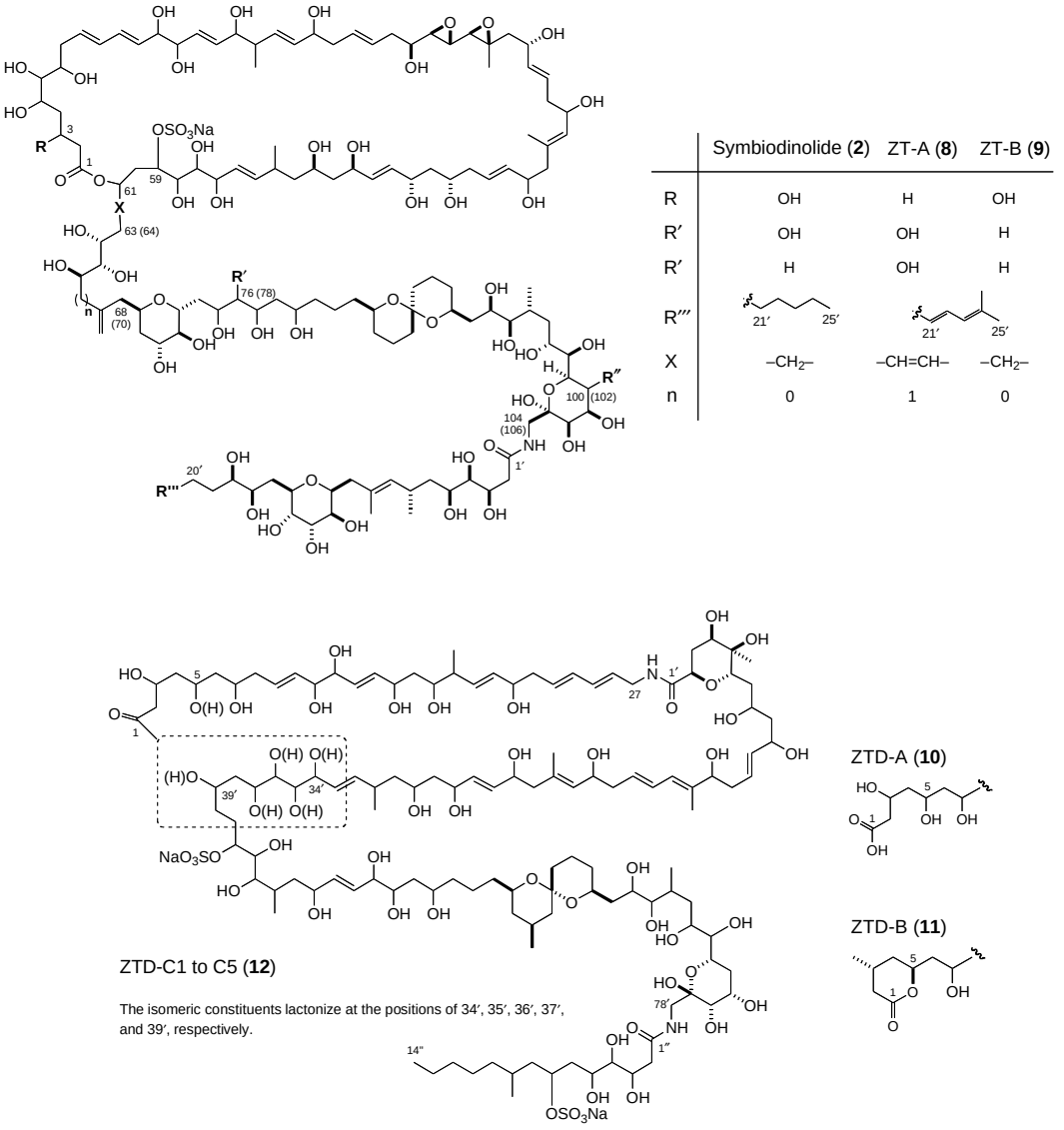


FIGURE 30.5 Structures of ZTs and ZADs.

side chain part (C3′–C7′ and C11′–C18′) were determined by chemical synthesis of its degraded fragments [56–59. Meanwhile, by detailed spectroscopic analysis, the relative configurations of C26–C32, C44–C51, and C64–C66 parts, and the absolute configurations of C69–73, C83–C103, and C3′–C18′ parts in symbiodinolide (**2**) have been confirmed.

30.3.3 Biological Activity of Symbiodinolide and Structural Diversity of Its Congeners

Symbiodinolide caused a significant increase in the intracellular free Ca^{2+} concentration at 7 nM against differentiated IMR-32 neuroblastoma cells in the presence of niphedipine (L-type Ca^{2+} channel blocker) [55]. This result revealed that symbiodinolide (**2**) possessed significant voltage-dependent N-type Ca^{2+} channel-opening activity. In contrast, **2** showed relatively weak acute

toxicity against mice (LD_{99} ~ 5 mg/kg, i.p. injection). Of the various enzymatic profiling screening assays tested, symbiodinolide (**2**) showed a significant cyclooxygenase-1 (COX-1) inhibitory effect at 2 μM (65% inhibition). Meanwhile, no potent inhibitory effects were observed against other kinds of enzymes at the same concentration, such as protein serine/threonine kinase, protein tyrosine kinase, protein tyrosine phosphatase, acetylcholinesterase, α-D-glucosidase, H^+/K^+- or Na^+/K^+-ATPase, COX-2, monoamine oxidase (MAO-A and B), nitric oxide synthase (eNOS, iNOS, nNOS), peptidases (angiotensin converting enzyme, cathepsins 1, B, D, chymotrypsin, tissue plasminogen activator, trypsin, renin, thrombin, factor Xa, and factor VIIa), or phosphodiesterase (PDE 3 to 6). Thus, it was found that the inhibitory effect of 2 toward COX-1 was quite specific.

Furthermore, to clarify the role of long-carbon-chain polyol compounds in the symbiotic relationships between microalga and their host animals, the effects of these compounds on host animals were examined. Notably, symbiodinolide (**2**) caused immediate rupture of the tissue surface of the host animal (marine acoel flatworm *Amphiscolops* spp.) at 2.5 μM [55]. Meanwhile, the dinoflagellates released from the host animals seemed to suffer no significant damage. It is largely unknown how much polyol compounds including **2** are accumulated in a flatworm. Still, our preliminary results suggest that symbiodinolide may act as a defense substance that prevents digestion of their host animal.

To date, two seco acid congeners of ZTs, ZAD-A (**10**), and ZAD-B (**11**), and 63- to 66-membered macrolides, ZAD-Cs (**12**), were found from the same dinoflagellate species [47–49]. Vasoconstrictive activity of ZTs and ZADs was reported using rat blood vessels. The EC50 values for ZT-A (8), and ZADs 10–12 were 1.2, >30 μM, >3 μM, and 0.39 μM, respectively [49, 60]. It should be noted that vasoconstrictive activity was only seen with the macrolactone congeners ZTs and ZAD-Cs, which indicated that the huge macrolactone structures were important for their activity. It has also been shown that ZT-A (8) caused aggregation in rabbit washed platelets, accompanied by an increase in the cytosolic Ca^{2+} concentration [61]. Thus, its potent voltage-dependent Ca^{2+} channel-opening activity may be a common feature of symbiodinolide and ZTs, and may be intimately involved in such constrictive activity. Further structural and biological studies of symbiodinolide are currently underway.

30.4 CONCLUSION

Along with the development of new analytical instruments and techniques over the past 40 years, a variety of seafood and freshwater toxins with unique chemical structures and biological activities have been characterized. Furthermore, chemical and biological studies on these molecules, especially huge polyol and polyether compounds, should contribute to a deeper understanding of their physiological roles in nature. In addition, intensive studies involving the comprehensive evaluation of these molecules may lead to the creation of a new field in bioscience.

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31 Palytoxins: Pharmacology and Biological Detection Methods

Carmen Vale

CONTENTS

31.1 PALYTOXIN

31.1.1 Origin and Distribution

Palytoxin (PTX) was first discovered, isolated, and purified from zoanthids belonging to the genus *Palythoa*.[1] Originally, it was only found in a single tidal pool on the Hawaiian island of Maui. This zoanthid was later identified as *Palythoa toxica*.[2] Palytoxin was also found in *Palythoa tuberculosa*[3,4] which appears to be randomly and sparingly distributed throughout the Pacific regions including Japan. The toxin was also present in *Palythoa mammilosa* and *Palythoa caribaeorum* [5] from the islands of the West Indies such as Jamaica, Puerto Rico, and the Bahamas. There is now a school of thought that suggests that the coral is simply concentrating the toxin made by dinoflagellates belonging to the genus *Ostreopsis*.[6,7] These species are widespread around the world and have

been identified in tropical and subtropical areas through the Pacific, Atlantic, and Indian Oceans.[8] In the coral reefs of the Caribbean Sea (Colombia), palytoxin has also been detected in zoanthid species of the genus *Zoanthus*, space competitors of *Palythoa* in the coral reefs.[9] A bacterial origin for palytoxin was also suggested after the finding that there are minor toxins that are also produced by *Palythoa* (homopalytoxin, bishomopalytoxin, and deoxypalytoxin), and that there is a fluorescent structural analog of palytoxin with equivalent cytotoxicity.[10–12] Despite its high lethality to terrestrial animals, the toxin has also been identified in crabs, fish, and a sea anemone.[13–15] Resistance of marine animals to the toxin may enable it to enter the food chains.

31.1.2 Pharmacology of Palytoxin

31.1.2.1 Pharmacological Targets of Palytoxin

Palytoxin is a large, water-soluble polyalcohol likely to interact with a cell-surface receptor. A broad range of studies indicates that mammalian Na^+,K^+-ATPase (or sodium pump) is a high-affinity cellular receptor for palytoxin. Initial studies had found that iodinated palytoxin binding to intact erythrocytes was fast and reversible with a Kd of 0.02 pM. Binding was facilitated by divalent cations ($Ca^{2+} > Sr^{2+} > Ba^{2+}$) and by borate. It was inhibited by K^+ (IC_{50} 2 mM), ouabain (IC_{50} 3 pM), and ouabagenin (IC_{50} 6 μM). Conversely, [^{3}H] ouabain was displaced by palytoxin (K_i 0.03 pM). Binding of ^{125}I-palytoxin and [^{3}H] ouabain was dependent on the state of the sodium pump and was decreased by ATP depletion, which decreases binding of both ligands to erythrocytes.[16] Recent experiments had led to the conclusion that the toxin binds to the enzyme and converts it into an open channel.[17–20] The channel open by palytoxin within the sodium pump transports sodium and potassium but has a low calcium permeability.[17,21] However, the effect of palytoxin on cellular permeability occurs at concentrations of the toxin that are several orders of magnitude lower than those that inhibit the sodium pump in the same cell.[12,22] Although the interaction of palytoxin with the Na^+,K^+-ATPase has been studied extensively,[17,21,23] the high diversity of preparations used to study the mechanism of action of palytoxin makes it difficult to rule out the possibility that another site may be involved, and it remains possible that palytoxin could also act through other cellular receptors.[24,25] In this sense, recent evidences indicate that the Na^+,K^+-ATPase may not be the only target of the toxin.

Research on palytoxin effects generally relates to two major areas. A broad range of studies focuses on how palytoxin regulates ion flux, which is the first effect of this toxin on the cell. Another broad class of studies concentrates on several cellular effects elicited by the toxin such as tumor promotion. The vast majority of work performed to test the mechanism of action of palytoxin has relied on the initial experiments indicating PTX binding to Na^+,K^+-ATPase;[16,26] therefore, this section will examine the main evidences supporting an action of the toxin in the sodium pump and continue with the effects of the toxin in other cellular targets.

31.1.2.2 The Na^+, K^+-ATPase as the Receptor for Palytoxin

The Na^+,K^+-ATPase or sodium pump is a transmembrane protein that, in each transport cycle, exchanges three cytoplasmatic Na^+ ions for two extracellular K^+ ions and hydrolyses one molecule of ATP. The large chemical gradients, established by the Na^+,K^+-ATPase, are essential for the maintenance of cell homeostasis. These gradients provide energy for multiple cellular functions such as membrane potential, excitability, control of cell volume, and solute homeostasis through numerous secondary active transport systems. The transport cycle of the sodium pump (known as the Post-Albers cycle) is realized by the successive binding of three intracellular Na^+ ions, their occlusion within the protein, and their release on the other side, followed by the binding, occlusion, and intracellular release of two extracellular K^+ ions.[27] The pump adopts two principal conformations, E1 with the binding sites accessible from the cytoplasm, and E2 with access from the extracellular space. Binding of a third cytoplasmic Na^+ to E1 triggers phosphorylation of the pump from ATP

and occlusion of the three Na^+ within the pump. Spontaneous relaxation to E2 opens the external gate, allowing release of the three Na^+ to the exterior and binding the two extracellular K^+ that, in turn, promotes occlusion of the K^+ ions and dephosphorylation of the pump. Subsequent binding of ATP promotes the release of K^+ into the cytoplasm.[21] The enzyme is composed of two subunits: the catalytic α subunit, which binds translocating cations and ATP, and the β subunit, which modulates cation affinity, and it is necessary for the proper folding and translocation of the sodium pump to the plasma membrane.[28] To date, four α and three β isoforms have been identified, with 85% and 45% of sequence identity, respectively. The different isoforms show a tissue-specific distribution and have a pattern of expression developmentally regulated. Biochemical evidences and transfection studies suggest that α and β isoforms can assemble in different combinations and potentially form functional pumps. The sodium pump is specifically inhibited by a series of naturally occurring steroids, such as ouabain.[29] Based on their clinical use, these substances are also referred to as cardiac glycosides or cardioactive steroids. Cardiac glycoside sensitivity is conferred by the α subunit of the Na^+,K^+-ATPase.[30] Ouabain inhibition of the Na^+,K^+-ATPase activity will alter the cation homeostasis of the cell, causing a rise in intracellular Na^+, which in turn will increase the intracellular calcium concentration. However, palytoxin uncouples the pump's gates, which normally open strictly alternately, thus allowing both gates to be open simultaneously, transforming the pump into an ion channel.[23]

31.1.3 Interaction of Palytoxin with the Mammalian Sodium Pump

Exposure of rabbit and dog muscles to an extract of partially purified PTX caused a decrease of the membrane potential and an increase in the rise time of the action potential while repolarization was shortened. These effects were reversible in a concentration-dependent manner.[31] Very low palytoxin concentrations increased the potassium permeability of erythrocytes from different species and also increased the sodium permeability of human erythrocytes; these effects of palytoxin were antagonized by ouabain.[32] The Na^+,K^+-ATPase was originally proposed as the target for palytoxin after the observation that the palytoxin effects were antagonized by cardiotonic steroids such as ouabain.[18] Besides these pharmacological studies, the first direct evidences indicating that palytoxin was acting at the Na^+,K^+-ATPase were obtained after the demonstration that ouabain prevented the palytoxin-induced K^+ efflux in liposomes containing an ouabain-sensitive form of the enzyme but not if the liposomes were filled with an ouabain-resistant enzyme.[33] A second study exploited the fact that yeast cells do not express endogenous Na^+,K^+-ATPase and, after expressing both α and β subunits of the enzyme in yeast, it was found that the toxin caused ouabain-sensitive cation fluxes. However, the toxin had no effect if the yeast expressed only the α or β subunits of the enzyme[20] or if the 44 amino acid residues of the carboxyl-terminal of the α subunit were deleted.[34] However, the catalytic activity of the sodium pump was not a requirement for the effect of palytoxin since the toxin still elicited ouabain-sensitive K^+ efflux in yeast expressing mutant sodium pumps in which the phosphate accepting residue Asp369 of the α subunit was replaced by Ala (thus eliminating the ouabain-sensitive ATPase activity), with the effect of palytoxin being inhibited by ouabain in both mutant and wild type yeast cells.[30] Palytoxin also caused K^+ efflux in yeast cells expressing a chimera in which the catalytic subunit of the sodium pump was replaced by that of the endoplasmic reticulum Ca^{2+}-ATPase.[35] Finally, palytoxin elicited single channel currents from a synthetic Na^+,K^+-ATPase obtained by incorporation of the enzyme expressing the α 3 and β 1 subunits into a planar phospholipid bilayer.[36] In spite of these experimental evidences, the role of the Na^+,K^+-ATPase in the formation of the PTX channel has not been clarified in detail. Recent elegant experiments by Artigas and Gadsby[17,21,23,37] have provided a more detailed biophysical picture on the mechanism of palytoxin action on the Na^+,K^+-ATPases. As mentioned above, the sodium pump is thought to have two gates that control the access of ions to their binding sites within the protein. These two gates normally open and close as the pump changes between its two major conformational states. To function as a pump, the inner and outer gates open and close in a sequential fashion but can never open together.

However, when palytoxin binds to the pump, the enzyme is locked into a conformation that allows simultaneous opening of both gates, thus eliciting a characteristic channel activity. Permeation studies have shown that the channels activated by palytoxin are nonselective for either Na^+ or K^+ and exhibit a slight permeability to calcium.[17] Moreover, it was recently described that the toxin affected the pump function in the state P-E2, independently of the type of phosphorylation. The palytoxin-induced modification of the protein consisted of two steps: toxin binding and a subsequent conformational change into a transmembrane ion channel. Binding of palytoxin to the pump increases the affinity of the enzyme for sodium and protons; however, even under saturating palytoxin concentrations, the ATPase activity was not completely inhibited.[19]

31.1.4 Possible Interaction of Palytoxin with Other Cellular Targets

Although the interaction of palytoxin with the Na^+,K^+-ATPases has been extensively documented, very few pharmacological targets other than the sodium pump have been evaluated to test palytoxin action. Nevertheless, different studies indicate that the sodium pump may not be the only cellular target for palytoxin. A series of alternative sites of action for the toxin were also proposed. First, the observation that, at high concentrations, the toxin induced norepinephrine release in a sodium-independent manner led to suggest that high palytoxin concentrations (1 μM) could produce a direct calcium influx.[38] At least in excitable cells, palytoxin has been shown to induce the activity of a small-conductance (9–25 pS), nonselective cationic channel that then triggers secondary activations of voltage-dependent Ca^{2+} channels and of Na^+/Ca^{2+} exchange. This results in neurotransmitter release by nerve terminals and contractions of striated and smooth muscle cells. Palytoxin-induced channels were blocked by amiloride derivatives such as 3,4 dichlorobenzamil and by high ouabain concentrations.[39] It was also described that the toxin was able to open a membrane-conductive pathway for H^+ causing proton influx and the secondary activation of the Na^+/H^+ exchanger.[40,41] Finally, palytoxin increased the cytosolic calcium concentration in chick cardiomyocytes in a manner independent of its depolarizing action or of its action on intracellular pH.[24] All this observations led Frelin and Van Renterghem to suggest that the toxin probably had more than one site of action in excitable cells and that it may act as an agonist for a family of low-conductance channels that conduct Na^+/K^+, H^+, and Ca^{2+} ions.[24]

Among the other possible cellular receptors tested, it was found that palytoxin caused K^+ efflux in yeast expressing a hybrid between the Na^+,K^+-ATPase and the H^+,K^+-ATPase, converting this enzyme into and open channel.[42] Interestingly, subsequent studies have described palytoxin-stimulated cation fluxes that were blocked by vanadate but resistant to ouabain in rat colon, suggesting that the toxin was able to convert a vanadate-sensitive H^+,K^+-ATPase into an electrogenic cation transporter and consequently, that the pore-forming action of palytoxin was not restricted to Na^+,K^+-ATPase since it was also observed with the colonic H^+,K^+-ATPase, which is related to the sodium pump.[25] The toxin was also found to interfere with the sarcolemmal calcium pump in cardiac myocytes as a secondary effect.[43] Further studies are required to determine how palytoxin interferes with each of the P-type ATPase pumps or if the effects of the toxin on these pumps are consequences of structural similarities between the enzymes.[44]

In conclusion, normally, low concentrations of palytoxin are sufficient to produce a massive increase in the permeability of cells to cations. Palytoxin stimulates sodium influx and potassium efflux, and thus produces depolarization of the membrane in several cellular systems.[12] In excitable systems, palytoxin-stimulated depolarization can modulate calcium channel activity, resulting in a rise in the intracellular calcium concentration, which can afterwards regulate calcium-dependent pathways and their related events. In muscle, depolarization causes calcium release and contraction. *In vivo*, the depolarization caused by the toxin can produce vasoconstriction, which can be lethal. In other systems, palytoxin-regulated events may not require an increase in the intracellular calcium concentration.[45] Whether the high cytotoxicity of palytoxin is merely a consequence of its disruption of the ionic environment of the cell remains to be elucidated.

31.1.5 Effects of Palytoxin on Ion Homeostasis in Primary Neuronal Cultures

As mentioned above, palytoxin produces multiple pharmacological actions at the cellular, tissue, and organism level. Alterations in the Na^+ and K^+ gradients by the toxin will initiate a complex chain of cellular events; it could alter the active transport of various ions as well as the transport processes across intracellular membranes. One important parameter that can be altered by palytoxin is the cytosolic free calcium concentration, which will produce alterations in numerous cellular processes.

We have recently started to analyze the cellular effects of palytoxin in primary cultures of murine neurons. Primary cultures of cerebellar granule cells constitute one of the most reliable, well-characterized, and highly sensitive models for the study of neuronal function and pathology as well as the effects of poisonous agents.[46,47] In this system, we found that palytoxin caused a dose-dependent membrane depolarization as evaluated with the voltage-sensitive membrane probe bis(oxonol) (Figure 31.1). Significative differences between control and palytoxin-treated cells were found at toxin concentrations of 5 nM.

Next, we evaluated the effect of the toxin on the intracellular sodium concentration. In cerebellar granule cells, loaded with the fluorescent sodium indicator Na^+ binding benzofuran isophthalate (SBFI), palytoxin at 10 nM caused an immediate rise in the cytosolic calcium concentration, which was greatly attenuated in the presence of 50 nM saxitoxin to block voltage-dependent sodium channels (Figure 31.2), thus indicating that the binding of the toxin to the sodium pump caused a depolarization followed by the secondary activation of voltage-dependent sodium channels.

Exposure of cerebellar granule cells to palytoxin caused a rapid rise in the cytosolic calcium concentration ($[Ca^{++}]_c$). In primary cultures of cerebellar granule cells, palytoxin induced a dose-dependent increase on $[Ca^{2+}]_c$. Figure 31.3 shows the effect of 1, 10, and 50 nM PTX on cytosolic calcium concentration in primary cultured neurons. The effect of palytoxin on $[Ca^{2+}]_c$ was immediate after addition of the toxin. The palytoxin-induced calcium increase showed a typical pattern characterized by an initial and fast calcium rise followed by a plateau phase.

When we analyzed the different pathways that could potentially contribute to the palytoxin-induced calcium entry in neurons, we found that the effect of palytoxin was dependent on the presence of extracellular calcium and glutamate receptors activation. Furthermore, secondary activation of voltage-dependent sodium and calcium channels and the reversal of the Na^+/Ca^{++} exchanger also contributed to the rise in calcium caused by the toxin.[48]

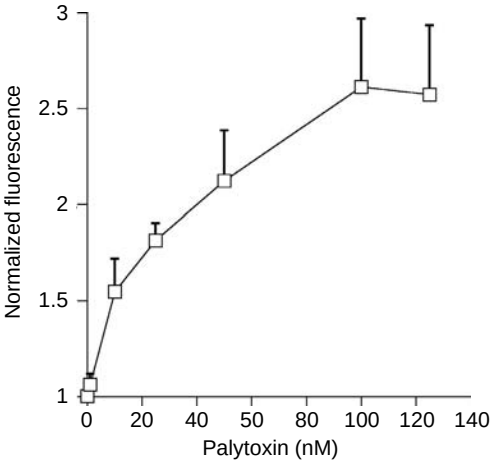

FIGURE 31.1 Palytoxin-induced depolarization of cultured cerebellar neurons. Cerebellar granule cells were loaded with the membrane probe bis(oxonol).

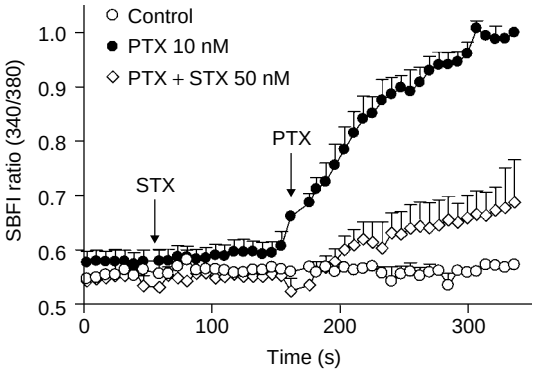

FIGURE 31.2 At 10 nM, palytoxin-induced a rapid increase in the intracellular sodium concentration in cultured cerebellar granule cells. This effect of palytoxin was sensitive to the voltage-dependent sodium channel blocker saxitoxin. Data are means ± sem of three independent experiments, each performed in duplicate. Drugs were added at the time points indicated by the arrows.

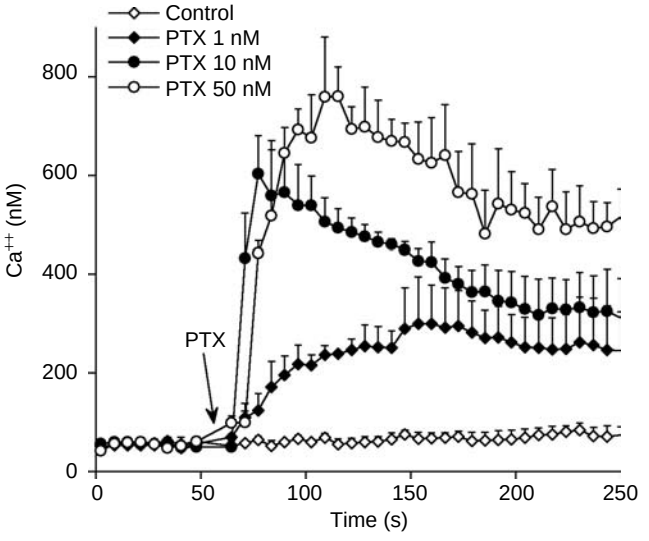

FIGURE 31.3 Effect of palytoxin on the cytosolic calcium concentration in primary cultured neurons loaded with the fluorescent calcium indicator Fura-2 AM. (Data extracted from Vale, C and Ares, I.R., in *Phycotoxins: Chemistry and Biochemistry*, Botana, L. (Ed.), Blackwell Publishing, 2007, pp. 95–118.)

In primary cultures of cerebellar neurons, palytoxin also caused a rapid intracellular acidification. Previous work had shown palytoxin-induced intracellular acidification in chick ventricular cells and osteoblasts.[40,49] The acidification induced by the toxin was reported to be dependent on the opening of proton-conductive pathways in the plasma membrane or to be a secondary effect of the interaction of the toxin with the sodium pump, respectively. In primary neuronal cultures, palytoxin caused a fast and irreversible intracellular acidification.[50,51] The decrease in intracellular pH (pH_i) caused by palytoxin was already maximal at toxin concentrations of 1 nM (Figure 31.4); at this concentration, palytoxin decreased intracellular pH by 0.6 units.

The acidification produced by palytoxin was due to an influx of extracellular calcium, since it was completely abolished in a calcium-free medium. In addition, toxin-induced intracellular acidification was completely prevented by several inhibitors of the plasma membrane calcium ATPase,

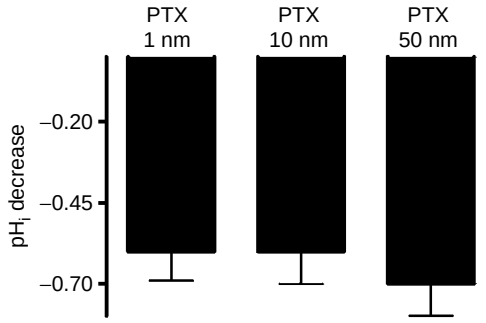

FIGURE 31.4 Intracellular acidification caused by palytoxin in primary cultures of cerebellar granule cells as assessed with the pH-sensitive fluorescent indicator BCECF-AM (2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester). (Modified from Vale, C and Ares, I.R., in *Phycotoxins:Chemistry and Biochemistry*, Botana, L. (Ed.), Blackwell Publishing, 2007, pp. 95–118.)

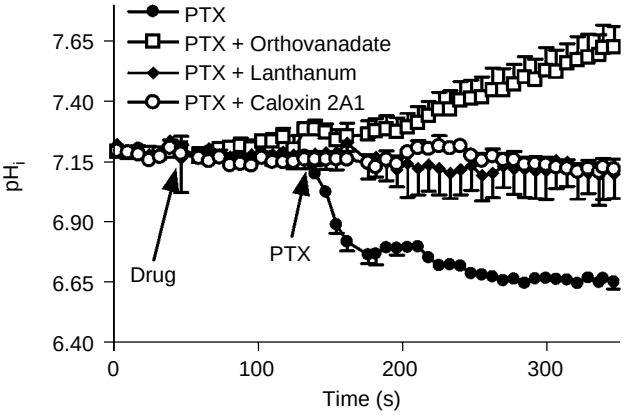

FIGURE 31.5 Effect of different blockers of the plasma membrane calcium ATPase on the intracellular acidification caused by palytoxin in primary neuronal cultures, as assessed with BCECF-AM. (Data extracted from Vale-González, C. et al., *J. Neurosci. Res.*, 85, 90, 2007.)

namely orthovanadate, lanthanum, and the caloxin 2A1 peptide (Figure 31.5). Therefore, the results obtained indicated that the plasma membrane calcium ATPase is involved in the palytoxin-induced intracellular acidification in cultured neurons. From our experiments, we concluded that the palytoxin-induced rise in the cytosolic calcium concentration will activate the calcium extrusion mechanisms through the plasma membrane calcium ATPase and, consequently, this will decrease the intracellular pH by the countertransport of protons. The contribution of the palytoxin-induced acidification to the high toxicity of this compound remains to be elucidated.

31.1.6 Palytoxin as a Skin Tumor Promoter

Palytoxin was identified as a skin tumor two decades ago. Palytoxin is a potent tumor promoter in the mouse skin carcinogenesis model.[52,53] The biochemical mechanism of action of palytoxin as a tumor promoter differs significantly from that of the prototypical phorbol ester tumor promoters. In contrast to the skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), palytoxin does not activate protein kinase C or increase ornithine decarboxilase activity in the mouse skin.[54] There are several interesting studies on the cellular mechanisms activated by palytoxin as a tumor promoter; however, the biochemical mechanisms by which palytoxin-stimulated signaling contributes to tumor promotion are currently elucidated, and it seems that they could depend on the experimental

cellular model. Some of the best studied cellular actions of palytoxin that are likely to play a role in carcinogenesis are stimulation of arachidonic acid metabolism and the production of prostaglandins, modulation of the epidermal growth factor (EGF) receptor, and modulation of mitogen-activated protein kinase (MAPK) cascades.[52]

Palytoxin shares with other tumor promoters a stimulatory action of arachidonic acid metabolism and the production of prostaglandins. Thus, picomolar concentrations of palytoxin stimulate the production of prostaglandins in a wide variety of systems.[52] Interestingly, palytoxin-stimulation of arachidonic acid metabolism[55] or prostaglandins release[56] has also been shown to be involved in the contractile action of palytoxin. The arachidonic acid released is either reincorporated into the membrane phospholipids, or it is oxidized by lipooxygenases and cyclooxygenases, producing leukotrienes, prostaglandins, and oxygen radicals. The accumulation of arachidonic acid and its metabolites results in cell membrane destruction. These changes may affect the activities and function of different ion channels, transporters, and other membrane-associated enzymes, particularly the most vulnerable, Na^+,K^+-ATPase.[57]

Another common cellular target of palytoxin and TPA is the EGF receptor. It has been described that palytoxin inhibited the binding of EGF to low- and high-affinity receptors in a manner independent of protein kinase C.[58] Moreover, picomolar concentrations of palytoxin inhibited EGF binding in the absence of cytosolic calcium increase.[45] Further studies indicated that the palytoxin effects on EGF receptor were not due to common secondary effects of sodium influx, including membrane depolarization, changes in intracellular pH, or inhibition of protein synthesis.[59]

MAPs seem to play an important role in mediating the distinct signal transduction pathways stimulated by palytoxin.[60,61] MAPKs are a family of serine/threonine kinases that coordinate the transmission of various types of signals to the nucleus.[62] Once activated, MAPKs translocate from the cytoplasm to the nucleus, phosphorylate specific transcription factors, and thereby modulate gene expression.[63] The three best-characterized members of the MAPK family are extracellular signal/regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Picomolar concentrations of palytoxin stimulated JNK in Swiss 3T3 fibroblasts,[60] and this effect was sodium dependent in COS-7 and HeLa cells.[64] In addition, palytoxin is a potent stimulator of p38 activity in a variety of cellular systems[61,65]; however, the protein kinase cascades that led to p38 activation by palytoxin differ, depending on the cell type.[52] Moreover, palytoxin stimulated ERK activity through a mechanism that involves the inactivation of an ERK phosphatase in initiated mouse skin keratinocytes.[65,66] A recent study found that palytoxin stimulated ERK activation in the BALB/c *in vitro* model through a mechanism that involved the production of prostaglandins.[67]

31.1.7 Mitogen-Activated Protein Kinases and the Effect of Palytoxin on Ion Homeostasis and Viability of Cultured Neurons

Recent work on the signaling action of the Na^+,K^+-ATPase raised interesting questions about the mechanisms by which the interaction of palytoxin with the sodium pump may regulate different signaling pathways. In addition to acting as an ion pump, one of the functions of the Na^+,K^+-ATPase is to mediate signal transduction. Inhibition of this enzyme with ouabain activates the cytoplasmic tyrosine kinase Src, transactivation of the EGF receptor, and increased production of reactive oxygen species. Activation of Src results in the formation of a structure that phosphorylates other proteins into different signaling modules. This, in turn, activates multiple protein kinase cascades including MAPs and protein kinase C (PKC) isozymes in a cell-specific manner.[68,69] Although ion flux has been implicated in palytoxin-stimulated signaling, it is also possible that palytoxin could modulate the signaling capacity of the Na^+,K^+-ATPase. For example, in HeLa cells, palytoxin could stimulate JNK and p38 activation to a greater extent than ouabain, whereas ouabain stimulated ERK activation to a greater extent than palytoxin.[61] In view of the signal-transducing function of the mammalian Na^+,K^+-ATPase and the effect of palytoxin on $[Ca^{+2}]_c$ as well as on MAPKs activation in dividing cells, we decided to explore whether protein kinases were involved in the

palytoxin-induced increase in calcium and cytotoxicity in cultured neurons. It was previously demonstrated that inhibition of the sodium pump with ouabain involved the activation of the MAPK cascade, and this effect was necessary for the ouabain-induced increase in the cytosolic calcium concentration.[70] Thus, it was logical to ask if the protein kinases that are activated by palytoxin in some cell types were involved in the palytoxin-induced neurotoxicity and increase in $[Ca^{2+}]_c$ in our in vitro model using cultured cerebellar granule neurons. The pharmacological study of the involvement of kinase pathways in the palytoxin-induced calcium increase in cultured neurons was started employing different MAPK inhibitors.

Regulation of the ERK cascade is distinguished by a characteristic core cascade of three kinases. The first kinase, Raf-1 and B-Raf, activates the second (MEK) by serine/threonine phosphorylation. Activation of extracellular signal-regulated kinase (MEK) leads to activation of ERK 1 and ERK 2 by phosphorylation of a threonine and a tyrosine residue.[71] Since Na^+,K^+-ATPase and the ERK1/2 pathway appear to be linked in some fashion in a variety of cells,[72] we first investigated the effect of inhibition of the ERK cascade on the calcium influx induced by palytoxin. To achieve this goal, we used a dual pharmacological approach by the inhibition of MEK and ERK. First, inhibition of MEK with PD 98059 reduced the calcium response evoked by the toxin, indicating that MEK are involved in the palytoxin-induced calcium influx in neurons. However, treatment of cultured cerebellar neurons with a cell-permeable ERK inhibitor completely eliminated the palytoxin-induced calcium influx. However, inhibition of JNK with SP 600125 before the addition of palytoxin slightly decreased the PTX-induced calcium-influx. Finally, inhibition of the p38 MAPK with SB 202190 also inhibited the calcium peak produced by the toxin; however, the effect of this inhibitor lasted only for about 100 s, after which the PTX-induced calcium influx reached values similar to that in the presence of the toxin alone. A summary of the effects of the different MAPK inhibitors on the palytoxin-induced calcium peak observed immediately after addition of the toxin in cerebellar neurons is presented on Figure 31.6.

Interestingly, inhibition of ERK completely eliminated the palytoxin-induced cell death in cultured neurons and MEK inhibition also partially attenuated the cytotoxic action of palytoxin; however, inhibition of JNK and p38 did not modify the toxic effect of the toxin (Figure 31.7). Altogether, these effects indicated that MAPK activation could play a pivotal role on the palytoxin effect on calcium homeostasis as well as on the cytotoxic effect of the toxin.

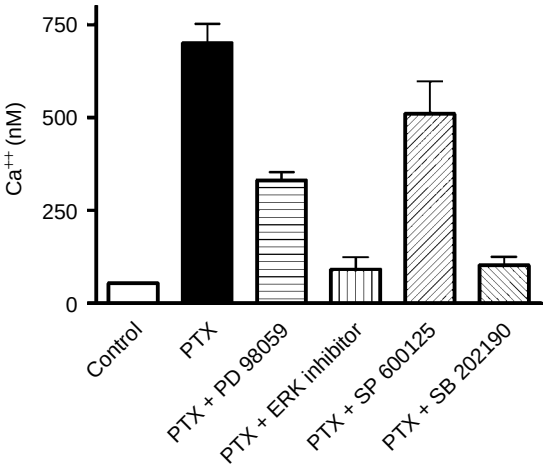

FIGURE 31.6 Effect of different MAPK inhibitors on the palytoxin-induced calcium influx in cultured neurons. Cerebellar granule cells were exposed to 10 nM palytoxin in the presence or absence of different MAPK inhibitors and the cytosolic calcium concentration was monitored with the fluorescent calcium indicator Fura-2 AM.

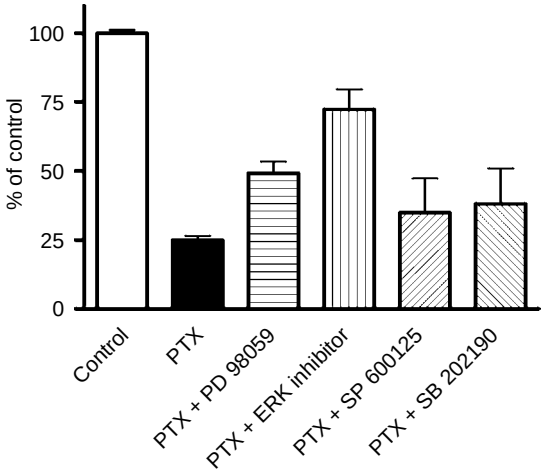

FIGURE 31.7 Effect of different MAPK inhibitors on the palytoxin-induced cytotoxicity in cultured neurons. Cerebellar granule cells were exposed to 10 nM palytoxin in the presence or absence of different MAPK inhibitors for 15 min, and cell viability was assessed with the MTT test.

31.1.8 Inhibitors of the Palytoxin Action

The study of the compounds that inhibit the action of palytoxin has focused primarily on the inhibition of the pharmacological target of the toxin, that is, the Na^+,K^+-ATPase; however, other inhibitors of several transport systems can also affect the action of palytoxin, since several transport systems are activated after the palytoxin-induced increase in the cationic permeability of the cell.

The sodium pump inhibitors are the most studied drugs that block palytoxin actions. Ouabain has been found to inhibit the action of palytoxin in most of the cellular systems studied.[12,18] Cardiac glycosides, such as ouabain, convallatoxin, cymarin, digoxin, and digitoxin, inhibited the PTX-induced K^+ release in rabbit red blood cells.[73] Their corresponding aglycones did not inhibit the K^+ release, but antagonized the inhibitory effect of the glycosides. All these cardiotonic steroids equally inhibited the activity of Na^+,K^+-ATPase prepared from hog cerebral cortex. Vanadate was also found to inhibit K^+ release and palytoxin-induced channels in red cells[22,74] and palytoxin-induced acidification in neurons.[51] However, the ubiquity of pumps and channels in the living tissues and the high diversity of preparations used to study the mechanism of action of palytoxin make it difficult to compare the effects of the several inhibitors of palytoxin action in different preparations. To date, no systematic study has been carried out to test the effects of the different inhibitors of the multiple transport systems activated, directly and indirectly, by palytoxin and its relationship to its cytotoxic effect.

31.2 OSTREOCINS

31.2.1 Origin and Distribution

Ostreocin-D is a structural analogue of palytoxin, whose mechanism of action and toxicological effects have not yet been elucidated, although seafood contamination with ostreocin-D is becoming an increasing problem in several Mediterranean countries. The dinoflagellate *Ostreopsis siamensis*, discovered originally in Thailand, was the first biogenetic origin of ostreocins.[7] Ostreocin-D was the most important compound isolated from *O. siamensis* cultures; this analogue of palytoxin exhibited a high cytotoxicity (LD_{50} 0.75 μg/kg) in mice after intraperitoneal administration. Other ostreocins, different from palytoxin, were also analyzed in the chemical analysis of these cultures. The complete structure of Ostreocin-D was recently clarified.[75,76] *O. Siamensis* is frequently present as

epiphytic on seaweeds or planktonic in some occasions in New Zealand's coastal waters. The common occurrence of low levels of unidentified lipid-soluble toxins in Rangaunu Harbour, Northland, New Zealand, since 1994, had led to restrictions on the harvesting of oyster. Studies on the marine microflora of this region between 1995 and 1997 indicated that *Ostreopsis* spp. (*O. siamensis*, *O. lenticularis*, *and O. ovata*) were the most prevalent dinoflagellate species in northern New Zealand, *O. siamensis* being the dominant species in summer.[77] Low levels of palytoxin-like material were reported in extracts of oysters from this area. The toxin present showed a higher toxicity to mice by intraperitoneal injection than by oral administration.[78]

31.2.2 Pharmacology of Ostreocin-D

Nowadays, the only available data studying the biological activity of ostreocin D has been reported in rabbit intestinal and human neuroblastoma cells.[50,79,80] The effect of ostreocin-D and palytoxin in the actin cytoskeleton was investigated by labeling filamentous actin (F-actin) with the fluorescent marker Oregon green 514 phalloidin. When the same concentrations of palytoxin and ostreocin-D were used (75 nM of each), it was found that both toxins caused a similar loss of filamentous actin after 4 h of incubation. The effect of both compounds was dependent on the presence of extracellular calcium. However, in neuroblastoma cells, ostreocin-D seemed to produce a lower membrane depolarization than did palytoxin, as assessed with the membrane potential sensitive probe bis(oxonol). In addition, the ostreocin-D-evoked depolarization seemed to be of shorter duration than the palytoxin-induced depolarization. Nevertheless, the use of the bis(oxonol) dye to asses membrane depolarization precludes any conclusion on the effects of the toxin on the real membrane properties. In the same study,[80] palytoxin and ostreocin-D, at 75 nM, caused a similar increase in the cytosolic calcium concentration in neuroblastoma cells, and this effect was dependent on the presence of calcium in the extracellular medium, indicating that both toxins elicited calcium influx in nondifferentiated human neuroblastoma cells. However, analysis of the effects of palytoxin and ostreocin-D in primary cultured neurons indicated that the potency of ostreocin-D to increase the cytosolic calcium concentration was about three times lower than the potency of palytoxin. Figures 31.8 and 31.9 illustrate the effect of palytoxin and ostreocin-D in the cytosolic calcium concentration and intracellular pH, respectively, in primary cultured neurons. Both compounds increased the cytosolic calcium concentration and acidified cultured neurons. The available data until now suggest that both palytoxin and ostreocin-D share a similar target in all the cellular

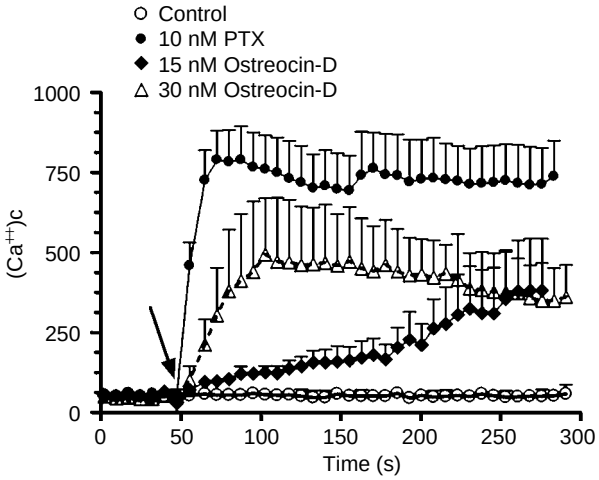

FIGURE 31.8 Effect of palytoxin and ostreocin-D in the cytosolic calcium concentration in primary cultures of cortical neurons.

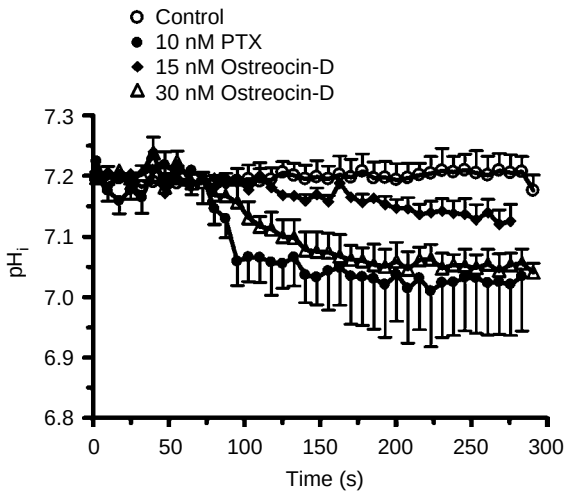

FIGURE 31.9 Effect of palytoxin and ostreocin-D in the intracellular pH concentration in primary cultures of cortical neurons.

systems evaluated; however, although both compounds displayed a similar pharmacological potency in intestinal cells and human neuroblastoma cells, ostreocin-D was about three times less potent in primary neuronal cultures.

31.3 OVATATOXIN

31.3.1 Origin and Distribution

Ostreopsis ovata is usually found in protected, inshore areas in tropical and subtropical regions of the tropical Pacific Ocean,[81] the Caribbean Sea, and the Tyrrhenian Sea,[81–83] but in recent years has been found frequently in the Mediterranean Sea.[84] During summer 2005, symptoms of rhinorrhea, cough, fever, bronchoconstriction with mild dyspnea, and wheezing were observed in people exposed to marine aerosols in Genoa, Italy.[84] Seawater samples were found to have high densities of *O. ovata* and showed the presence of "putative palytoxin" because the test could also have detected an isomer of palytoxin. Unfortunately, there is scarcity of information about the toxic compounds produced by *O. ovata*, or its potential health hazards, but they are probably related to palytoxins.

31.3.2 Pharmacology of Ovatatoxin

The only available data on the pharmacological properties of ovatatoxin refers to experiments performed using a sample purified of an *O. ovata* extract that was tested on actin cytoskeleton of isolated rabbit intestinal cells, indicating that *O. ovata* toxin interfered with the cytoskeleton as occurred with palytoxin and ostreocin-D. In addition, the purified extract had a depolarizing effect similar to palytoxin and ostreocin-D in neuroblastoma cells. Therefore, although these studies only provide very preliminary information about the toxic compound produced by *O. ovata*; they appear to point out to similar cellular targets for palytoxin, ostreocin-D, and ovatatoxin.

31.4 BIOLOGICAL DETECTION METHODS

Detection of palytoxin in biological samples can be accomplished by both analytical means and biological assays. Biological methods use the functional properties or biological activities of the toxin to ascertain its presence in a sample. However, it is often necessary to use a combination of methods

to confirm the presence of the toxin.[85] The methods listed in this section have been used successfully to detect palytoxin, and some of them are highly sensitive.

31.4.1 Mouse Bioassay

The mouse bioassay is one of the simplest ways to detect the presence of palytoxin and analogues in biological samples. In this method, the mouse lethality of a sample is evaluated after intraperitoneal injection of the sample. Characteristic symptoms in mouse after a lethal injection of palytoxin include sudden jerks, stretching of hind limbs and lower back, weakening of forelimbs, ataxia, decreased locomotion, convulsions, gasping for breath, and death. However, the results of toxin analyses in the mouse bioassay vary depending on the mouse strain, gender, and weight of the animals, and this assay is not suitable for quantitative determinations of the toxin.

31.4.2 Haemolysis Assay

Palytoxin is a potent but slow hemolysin in hog, rat, mousse, rabbit, guinea pig, and human erythrocytes.[86] The hemolytic action of palytoxin was exploited to develop an assay to detect the toxin.[87] Assays to detect the hemolytic activity of palytoxin are normally carried out following Bignami's method.[87] In the original method, whole blood from mouse was collected from the tail vein and diluted 1:9 in phosphate buffered saline (pH 7.0), and red cells were separated from plasma by centrifugation. Erythrocytes were washed once with buffer, and the cell buffer was diluted in medium containing 5% (v/v) fetal bovine serum. For the hemolysis neutralization assay, phosphate buffered saline (PBS) was supplemented with 0.1% (w/v) bovine serum albumin (BSA), 1 mM calcium chloride, and 1 mM sodium tetraborate (pH 7.2). The blood suspension was diluted 1:49 in this buffer with or without a palytoxin monoclonal antibody. Samples of the blood cell suspension with and without toxin-containing solutions are mixed and incubated at 37°C for up to 24 h. After incubation, the samples are centrifuged and the supernatants are used to measure the absorption at 540–595 nm. The time course of hemolysis has to be evaluated varying the incubation time. The amount of hemoglobin released was found to be time and concentration dependent.[86] The concentration of the toxin in the sample can be determined by incubation of red cells with palytoxin standards followed by measurement of the amount of hemoglobin released after a fixed period of time. The specific presence of palytoxin or its analogs in the sample can be demonstrated by prevention of the hemolytic activity of the toxin with ouabain[9] or with a palytoxin monoclonal antibody.[87]

31.4.3 Potassium Release

Palytoxin has been shown to induce a rapid release of K^+ from cells.[18] Palytoxin induces K^+ efflux before the onset of other effects such as hemolysis.[86] To perform this assay, determination of, the amount of K^+ released is measured using either K^+ selective electrodes, a flame photometer, or an atomic absorption photometer. The palytoxin-induced K^+ release is concentration dependent, and the method has a sensitivity of approximately 1 pM for palytoxin-induced release from rat and human erythrocytes.[18,85]

31.4.4 Cell Toxicity Assay

Several types of cells including HeLa cells[11] and fibroblast and rat 3Y1 cells have been employed to evaluate the presence of the toxin.[88] This assay is based on the morphologic changes caused by the toxin, which includes granulation of the cytoplasm and swelling of the cells accompanied by shrinkage of the nuclei during the first 10 min of exposure of the cells to 5 nM palytoxin. After these changes, cell detachment is observed followed by lysis of the cell. This assay permits the detection of palytoxin concentrations in the picomolar range.[88] The cell toxicity assay provides an alternative model to the use of animals in toxicity tests and is extremely useful for the evaluation of the toxic fractions during toxin purification.[85]

31.4.5 Monoclonal Antibody-Based Enzyme-Linked Immunoassay

The method developed by Bignami et al.[89] is based on the generation of mouse and rabbit monoclonal and polyclonal antibodies against conjugates of keyhole limpet hemocyanin and chemically defined palytoxin haptens. Palytoxin haptens were produced by derivatization of the primary amino group with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate or succinimidyl 3-(2-pyridyldithio)propionate. Selected antibodies were used to develop five palytoxin-specific enzyme-linked immunoassay formats for the quantitation of palytoxin. A sandwich enzyme-linked immunosorbent assay was capable of detecting as little as 10 pg palytoxin per test, but was subject to matrix interference. The direct competitive inhibition enzyme-linked immunoassays detected as little as 30 pg palytoxin per test, with a total assay time of only 4 h. The enzyme-linked immunoassays do not cross-react with the other marine toxins tested, but do cross-react with certain nontoxic, treated preparations of palytox in. The enzyme-linked immunoassays were used to quantitate palytoxin[89] and structural isomers of the toxin.[10]

31.4.6 Radioimmunoassay

Palytoxin, labeled with ^{125}I-Bolton-Hunter reagent on its terminal amino group, bound specifically to rabbit antipalytoxin antibody.[90] The extent of binding increased progressively with repeated immunizations. After absorption of the rabbit IgGs with a goat antirabbit IgG, binding was reduced greater than 95%. The method was very sensitive and allowed the detection of palytoxin in the picomolar range. For 50% inhibition of binding in the ^{125}I-palytoxin-antipalytoxin reaction, 0.27 pmoles of unlabeled palytoxin were required. However, this method did not allow distinguishing between biologically active and inactive palytoxins.[85]

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32 Occurrence and Toxicology of Palytoxins

Rex Munday

CONTENTS

32.1 OCCURRENCE OF PALYTOXINS

32.1.1 Primary Sources of Palytoxin Derivatives

In 1971, a toxic material, named "palytoxin," was reported to be present in a soft coral (a *Palythoa* species) in Hawaii [1]. The coral was later identified as *Palythoa toxica* [2], and chemical investigations of the toxin [3] revealed two major isomeric components, 5- or 6-membered hemiketals, of the molecular formula $C_{129}H_{223}N_3O_{54}$. The same substances were isolated from *Palythoa tuberculosa* from Japan, and it was suggested that the hemiketals exist in equilibrium, with the 6-membered isomer being favored [4,5]. Four minor palytoxins have also been isolated from *P. tuberculosa* and characterized. These are homopalytoxin, bishomopalytoxin, neopalytoxin, and deoxypalytoxin [6]. Another Japanese zoanthid, *Palythoa* aff. *margaritae*, contains a palytoxin derivative that appears to be the same as that from *P. toxica* [7]. A related material has been isolated from an unnamed species of *Palythoa* from Tahiti. This differs from the palytoxins from *P. toxica*, having the molecular formula $C_{129}H_{221}N_3O_{53}$ [3]. A major palytoxin derivative, with the same retention time on mixed-mode high performance liquid chromatography (HPLC) as the palytoxins from *P. toxica*, was isolated from the Caribbean zoanthid *Palythoa caribaeorum*. Two minor palytoxin analogues were also detected in extracts of this organism [8]. Palytoxin has also been isolated from *Palythoa mammillosa* from the Caribbean [9] and from *Palythoa vestitus* from Hawaii [10].

TABLE 32.1
Concentration of Palytoxin in *Palythoa* Species

Species	Location	Tissue	Concentration of Palytoxin (mg/kg)	Reference
P. caribaeorum	Jamaica	Whole animal	40	[13]
P. tuberculosa	Hawaii	Whole animal	2.5–5.0*	[116]
P. tuberculosa (female)	Japan	Whole animal	2.4–4.0	[12]
P. tuberculosa (female)	Japan	Eggs	1000	[12]
P. tuberculosa (male)	Japan	Testes	<0.01	[12]
P. caribaeorum	Colombia	Whole animal	0–36	[31]
P. caesia	Australia	Whole animal	0–34	[31]

* Yield of purified toxin.

Quantitative analyses of soft corals reveal major variations in palytoxin content (Table 32.1). The variation is attributable to a number of factors. While *P. toxica* contains high levels of palytoxins at all times of the year, and is therefore continually toxic [11], *P. tuberculosa* shows marked seasonal variation. The latter is highly toxic in June and July, but essentially nontoxic for the rest of the year [12]. *P. tuberculosa* colonies contain four different types of polyp—female, male, hermaphrodite, and sterile. Female polyps are highly toxic, particularly when bearing eggs, which may contain as much as 1 g/kg of palytoxin [12]. In contrast, hermaphrodites are of relatively low toxicity, while no toxicity was seen with male or sterile polyps. The seasonal variation may be explained on the basis of appearance of female colonies in March, with mature eggs being first seen in May [12]. Similar seasonal variation was observed in *P. caribaeorum* in Jamaica, but there are also large variations attributable to location. Even in June, no toxicity was observed in specimens of *P. caribaeorum* collected in Bermuda or Florida [13].

A palytoxin derivative has been isolated from the sea anemone *Radianthus macrodactylus* [14]. This substance was similar, but not identical, to palytoxins isolated from *Palythoa* species, though its structure was not determined.

Palytoxin analogues have also been isolated from dinoflagellates of the genus *Ostreopsis*, which are widely distributed in tropical and subtropical waters throughout the world. *Ostreopsis siamensis* produces a toxin named ostreocin D [15], characterized as 42-hydroxy-3,26-di-demethyl-19, 44-di-deoxy palytoxin [16,17]. This strain of *Ostreopsis* did not produce palytoxin itself. New Zealand strains of *O. siamensis* were shown to contain a palytoxin-like material, at levels between 0.1 and 0.4 pg/cell palytoxin equivalents [18,19], which was distinguishable from palytoxin and ostreocin D on liquid chromatography-mass spectrometry (LC-MS) [20]. *Ostreopsis mascarenensis* also produces compounds, named mascarenotoxins, at a level of 0.04 pg palytoxin equivalents per cell. These substances have many of the characteristic properties of palytoxin, but their molecular weights are significantly lower than those of zoanthid-derived palytoxins or ostreocin D [21]. Material with the properties of palytoxin has also been found in an unidentified Japanese *Ostreopsis* species [22], although this has not yet been fully characterized. Palytoxin-like substances have also been identified in *Ostreopsis* cf. *siamensis* and *Ostreopsis ovata* from the Mediterranean and from the coast of Brazil [23,24]. Extracts of *Ostreopsis lenticularis* from the Virgin Islands [25] and from Puerto Rico [26] were shown to contain highly toxic substances, although these have not yet been identified.

Two palytoxin analogues were reported to be produced by a red alga, *Chondria armata* [27]. These compounds were named CA-I and CA-II, but their structures do not appear to have been elucidated. The dried alga contained approximately 2.5 mg/kg of each of the palytoxin derivatives [28].

32.1.2 Secondary Sources of Palytoxin Derivatives

The palytoxin derivatives produced by the above primary sources readily enter the marine food chain, and such derivatives have been shown to be present in many marine animals. The identification of palytoxin derivatives in these organisms has been confirmed using spectrophotometry, chromatography, or effects of extracts on red blood cells *in vitro*. As discussed in Section 32.2, palytoxin causes hemolysis of red blood cells after a prolonged lag phase. The involvement of palytoxin in the hemolysis is confirmed by the use of an antibody that neutralizes the effect of palytoxin and/or by the use of ouabain, which blocks the Na,K-ATPase that is recognized as the target site of palytoxin in the erythrocyte membrane [29]. This test does not, however, distinguish between palytoxin and its analogues, and will respond to any derivative that is recognized by the antibody and/or acts through the Na,K-ATPase.

Palytoxin-like materials have been identified in polychaete worms (*Hermodice carunculata*) and starfish (*Acanthaster planci*) that feed on zoanthids, and in sponges, crustaceans, and gorgonians living in close proximity to *Palythoa* colonies [30–32].

Palytoxin derivatives have been identified in many species of fish. A toxin chromatographically indistinguishable from palytoxin, and showing the characteristic palytoxin behavior in the hemolysis test, was isolated from the sardine *Herklotsichthys quadrimaculatus* from Madagascar [33,34]. The mass spectrum was dissimilar to that of palytoxin, however, suggesting that the material was a palytoxin analogue [34]. There is evidence [34] that the toxin accumulated in the sardine following consumption of an *Ostreopsis* species, although it has recently been suggested that zooplankton are intermediate vectors in the transfer of palytoxin from dinoflagellates to this particular species of fish [35]. Similarly, palytoxin or palytoxin analogues were detected in parrotfish (*Scarus ovifrons*) in Japan [22,36], which was attributed to the consumption of *O. siamensis* by the fish [22]. Traces of a toxigenic strain of this organism were found in the gut contents of the toxic parrotfish [22]. A water-soluble toxin in parrotfish has been described [37], which appears to be distinct from palytoxin, since its molecular weight was reported to be less than 500 Da. Whether this molecular weight was erroneous, or whether parrotfish contain a low-molecular-weight toxin in addition to palytoxin, is not presently known. Palytoxin-like material has also been detected in mackerel (*Decapterus macrosoma*) from the Philippines [38], while a toxin named aluterin was isolated from the filefish, *Alutera scripta*, in Japan [39]. This substance was not characterized, but on the basis of the identification of *P. tuberculosa* in the gut contents of the fish and on the similarity of the toxic symptoms induced in mice by aluterin and by authentic palytoxin, the former substance was suggested to be a palytoxin derivative [39]. A toxic extract of the triggerfish *Melichtys vidua* from Micronesia was indistinguishable from palytoxin on the basis of chromatographic properties [40]. Material positive in the palytoxin hemolysis test was present in an *Epinephelus* spp. in Japan [41], in a puffer fish (*Tetraodon* sp.) from Bangladesh [42], and in several species of reef fish in Hawaii [43,44]. Later work [45] confirmed the presence of palytoxin in 21 out of 47 species of Hawaiian fish. Unfortunately, palytoxin levels were not quantified, but extracts of many of the palytoxin-containing fish were extremely toxic after intraperitoneal injection in mice [45]. Several species of butterfly fish (*Chaetodon* spp.), which are frequently observed feeding on *Palythoa*, contained palytoxin. In these fish, the highest levels were found in the intestines, liver, and muscle [31].

Quantitative data on palytoxin levels in fish are shown in Table 32.2. Only reports in which the presence of palytoxins was confirmed are included. Again, wide variations in tissue concentrations of palytoxin have been recorded. If, as suggested [32], palytoxin accumulates in the fish through predation, large seasonal and geographical variations are to be expected. This has not, however, been systematically investigated.

A potent toxin was reported to be present in the coral reef crab *Lophozozymus pictor* [46], although its identity was not established. All parts of the crab were toxic, and differed little in potency. In contrast, Chia et al. [47] found the toxin levels in this crab to be particularly high in the gut and hepatopancreas, while the muscle and carapace were less toxic. The toxicity of crabs kept in captivity decreased with time, with almost complete loss after 24 days [47]. The toxin was partially

TABLE 32.2
Concentration of Palytoxin in Fish

Species	Location	Tissue	Concentration of Palytoxin (mg/kg)*	Reference
Tetraodon spp.	Bangladesh	Whole animal	<0.1	[42]
M. vidua	Micronesia	Muscle	0.003	[40]
M. vidua	Micronesia	Viscera	0.3	[40]
Chaetodon spp.	Colombia and Australia	Skin	0–57	[31]
Chaetodon spp.	Colombia and Australia	Muscle	0–71	[31]
Chaetodon spp.	Colombia and Australia	Intestine	0–127	[31]
Chaetodon spp.	Colombia and Australia	Gills	0–28	[31]
Chaetodon spp.	Colombia and Australia	Liver	0–76	[31]
Chaetodon spp.	Colombia and Australia	Eggs	5.2–42	[31]
S. ovifrons	Japan	Liver	0.005	[22]
*S. ovifron*s	Japan	Muscle	0.005–0.020	[22]
S. ovifrons	Japan	Muscle	0.009	[36]
S. ovifrons	Japan	Unidentified	0.006	[36]
S. ovifrons	Japan	Viscera	0.008	[36]

* Data quoted as "mouse units (MU)" have been converted to weights, with 1 MU = 10 ng palytoxin [28,47].

purified by Teh and Gardiner [48], and palytoxin and palytoxin analogues were later identified in *L. pictor* [8,49–52], and in other Asian crabs, *Demania alcalai* [49] and *Demania reynaudii* [53]. The presence of palytoxin itself in *L. pictor* and *D. alcalai* was reported by Yasumoto et al. [49]. The toxin was found in all tissues, but was particularly high in the gills and viscera [49]. Lau et al. [50–52] later reported the presence of a fluorescent material in extracts of *L. pictor* which was of similar molecular mass to palytoxin, but which could be distinguished from the latter on the basis of chromatographic behavior and fragmentation pattern in mass spectrometry. The latter authors also showed the presence of four palytoxin analogues in *L. pictor*, one of which appeared to be unique to this source, suggesting metabolism of palytoxin after ingestion by the crab [51].

Quantitative data on palytoxin concentrations in crabs are shown in Table 32.3. Extraordinarily high levels have been recorded in these crustaceans, particularly in the gills and viscera. Again, if palytoxin accumulates in tissue through the consumption of *Palythoa* by crabs, seasonal and geographic variations are to be expected.

Palytoxin-like material has been detected in oysters, scallops, mussels, and sea urchins fed a New Zealand strain of *O. siamensis* [20,54].

32.2 TOXICITY OF PALYTOXINS TO CULTURED CELLS AND TO ISOLATED TISSUE *IN VITRO*

Palytoxin potently, yet slowly, lyses erythrocytes *in vitro*. Incubation of such cells with palytoxin results in loss of intracellular potassium, followed, after a lag period of 1–2 h, by progressive osmotic lysis and release of hemoglobin [55]. Red cells from different species show pronounced differences in response to palytoxin, with pig, rat, and mouse cells being particularly sensitive, and human cells being comparatively resistant [55]. Hemolysis is inhibited by ouabain, a specific inhibitor of Na,K-ATPase, indicating a role for the membranal sodium–potassium pump in the mechanism of the hemolysis induced by palytoxin [56,57]. Hemolysis was also inhibited by a palytoxin-neutralizing antibody [29], and delayed hemoglobin release from erythrocytes, which is inhibited by ouabain and/or antibody, is considered characteristic of palytoxin, and is widely used as a diagnostic tool

TABLE 32.3
Concentration of Palytoxin in Crabs

Species	Location	Tissue	Concentration of Palytoxin (mg/kg)*	Reference
L. pictor, male	Philippines	Carapace	3–20	[49]
L. pictor, male	Philippines	Cheliped, cuticle	2.2–4.0	[49]
L. pictor, male	Philippines	Cheliped, flesh	0.9–1.0	[49]
L. pictor, male	Philippines	Viscera	60–100	[49]
L. pictor, male	Philippines	Gills	22–24	[49]
L. pictor, female	Philippines	Carapace	1.6–14	[49]
L. pictor, female	Philippines	Cheliped, cuticle	0.4–1.7	[49]
L. pictor, male	Philippines	Cheliped, flesh	0.2–0.5	[49]
L. pictor, male	Philippines	Viscera	2–60	[49]
L. pictor, female	Philippines	Gills	1.8–30	[49]
L. pictor, female	Philippines	Eggs	7.0	[49]
D. alcalai, female	Philippines	Carapace	5.0	[49]
D. alcalai, female	Philippines	Cheliped, cuticle	1.6–4.0	[49]
D. alcalai, female	Philippines	Cheliped, flesh	0.8–2.4	[49]
D. alcalai, female	Philippines	Viscera	40–54	[49]
D. alcalai, female	Philippines	Gills	24–160	[49]
D. reynaudii	Philippines	Legs	0.8	[53]

* Data quoted as "mouse units (MU)" have been converted to weights, with 1 MU = 10 ng palytoxin [28,47].

for samples suspected to contain this toxin. By the use of appropriate standards, palytoxin can be quantitated with excellent sensitivity from the dose–response relationship between palytoxin concentration and degree of erythrocyte lysis [29].

Palytoxin is exceptionally toxic to cultured cells *in vitro*, causing cell death in many cell types at nanomolar concentrations. In HeLa cells, palytoxin causes swelling, granulation, vacuolation, and lysis [50,58], which again is associated with loss of potassium from the cells [56]. Both potassium loss and lysis are prevented by ouabain [8,56]. Similar structural changes were recorded in 3Y1 rat embryo cells [7], the A7r5 cell line derived from rat aorta [59], LLC-PK1 renal epithelial cells [60], bovine aortic endothelial cells [61], and human lymphocytes [62] exposed to palytoxin. In lymphocytes, loss of intracellular potassium and gain of intracellular sodium were recorded, and cytotoxicity was inhibited by ouabain [62]. The structural changes, ionic movements, and the effect of ouabain again indicate that cytotoxicity reflects osmotic changes associated with disruption of ion movements via the Na,K-ATPase. In rabbit enterocytes, palytoxin and ostreocin D damaged the cytoskeleton, causing loss of the microfilament network, particularly in microvilli [63]. This was associated with an influx of calcium into the cells [63], which again may be attributable to effects on the membrane ionic pump, since sodium uptake has been shown to be associated with accumulation of intracellular calcium in several cell systems [64–66], and calcium uptake was inhibited by ouabain [67]. In murine cerebellar granule cells, palytoxin, at a concentration of 10 nM, decreased cell viability by 30% within 5 min. Cytotoxicity was accompanied by increased intracellular calcium concentration and increased release of excitatory amino acids into the culture medium [68].

Cytotoxicity by palytoxins has also been demonstrated in human bronchial epithelial cells [69], 3T3 Swiss mouse cells [70], V79 Chinese hamster lung cells [71], H 2981 human lung adenocarcinoma cells [72], EL-4 murine T-lymphoma cells [72], HL-60 human promyelocytic leukemia cells [73], and by an extract of *O. siamensis* in neuroblastoma cells [19]. Palytoxin was shown to be highly toxic to P-388 lymphocytic leukemia cells [15]. Ostreocin D was significantly less toxic than palytoxin to the last-named cells [15]. Palytoxin, at very low concentration, inhibited the motility

of spermatozoa from various species [74] and caused depolarization of BE(2)-M17 human neuroblastoma cells [75].

At very low concentration, palytoxin stimulates the activation of several mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase and p38, in keratinocytes derived from initiated mouse skin [76,77]. It also stimulated an increase in mRNA for matrix metalloproteinase-13 (MMP-13) [76,77]. Kinases were similarly activated by palytoxin in a fibroblast cell line (FC_2-Rat1) [78], in HeLa cells [79], COS7 monkey kidney cells [79], and in 3T3 Swiss mouse cells [80]. MAPK activation was sodium-dependent and blocked by ouabain, suggesting that activation of these kinases is again attributable to palytoxin-induced effects on transmembrane ion transfer [78–80]. It has been suggested that ERK plays an important role in transmitting palytoxin-stimulated signals to downstream targets that are likely to affect carcinogenesis, such as c-FOS, AP-1, and MMP-13, and that such an effect could be involved in the observed tumor promotion by palytoxin *in vivo* (Section 32.3.4).

Palytoxin promotes cell transformation in Balb/c 3T3 cells initiated with 3-methylcholanthrene [81]. This effect was accompanied by profound stimulation of the synthesis of prostaglandin E_2 and prostaglandin $F_{2\alpha}$. Both prostaglandin synthesis and promotion were inhibited by the cyclooxygenase inhibitor, indomethacin [81]. The prostaglandins themselves are tumor promoters, and it was suggested that stimulation of the synthesis of these substances may be responsible for the tumor-promoting activity of palytoxin [81]. Stimulation of arachidonic acid production and prostaglandin synthesis have also been observed in other cell types *in vitro* [73,82–84]. A role for prostaglandins in palytoxin-induced bone resorption in neonatal mouse calvariae in organ culture is also indicated. Both resorption and prostaglandin synthesis were inhibited by indomethacin [85].

Palytoxin stimulates the formation of superoxide radical in neutrophils through activation of NADPH oxidase [86]. Again, it is possible that superoxide, and/or other "active oxygen" species could be involved in the tumor-promoting activity of palytoxin [86].

Incubation of rat aortic rings with palytoxin led to microvesiculation of the endothelial cell cytoplasm [87]. Dilatation of the sarcoplasmic reticulum, densification of mitochondrial cristae (possibly reflecting calcium uptake by the tissue), and disruption of myofibrils were observed in isolated rat muscle [88]. Addition of palytoxin to the perfusion medium of the perfused heart caused cardiac arrest within minutes [13], and the spontaneous beating of isolated rat auricles was rapidly inhibited [9].

32.3 TOXICITY OF PALYTOXINS TO ANIMALS

32.3.1 Acute Toxicity

32.3.1.1 Acute Toxicity by Intravenous Injection

The palytoxins are exceptionally toxic by intravenous injection, with LD_{50} values between 0.025 and 0.45 μg/kg in different species (Table 32.4). Rabbits and dogs are most vulnerable to poisoning by the palytoxins, followed by monkeys, rats, guinea pigs, and mice. At high doses, animals showed ataxia, convulsions, and dyspnea, and death occurred within minutes. Hypotension, electrocardiographic changes, cardiac arrhythmia, and cardiac arrest were observed in anesthetized dogs [9,89]. Cardiac arrest preceded respiratory arrest [9], and it has been suggested that the rapid death seen after intravenous injection of high doses of palytoxins is due to acute heart failure, reflecting severe myocardial ischemia resulting from intense coronary vasoconstriction [89,90]. This suggestion is consistent with the observation of profound vasoconstriction in the perfused heart soon after addition of palytoxin to the perfusion medium [9]. In contrast, at doses close to the LD_{50}, death occurred no sooner than 8–10 h after administration [14], and the heart continued to beat for some time after respiratory arrest [91], indicating that under these conditions, death is not attributable to heart failure.

TABLE 32.4
Acute Toxicity of Palytoxin and Extracts of Palytoxin-Containing Organisms by Intravenous Injection

Animal	Source of Palytoxin	LD_{50} (μg/kg)	Symptoms of Intoxication	Biochemical/Pathological Findings	Reference
Rabbit	*P. vestitus*	0.025 (0.024–0.026)*	Prostration, dyspnea, convulsions, death	—	[96]
Dog	*P. vestitus*	0.033 (0.026–0.041)*	Defecation, vomiting, ataxia, collapse, death	Intestinal hemorrhage	[96]
Dog	*P. mammillosa*	—	—	At low doses, reversible tachycardia, arrhythmia and hypotension. At high doses, cardiac arrest within 10 min	[9]
Dog	*P. tuberculosa*	0.05–0.06	—	At low doses, reversible hypertension and arrhythmia. At high doses, hypotension, arrhythmia and death within 5 min	[89]
Rat	*P. vestitus*	0.089 (0.080–0.098)*	Prostration, dyspnea, convulsions, death	—	[96]
Rhesus monkey	*P. vestitus*	0.078 (0.060–0.090)*	Ataxia, collapse, death	—	[96]
Guinea pig	*P. vestitus*	0.11 (0.070–0.170)*	Prostration, dyspnea, convulsions, death	—	[96]
Cat	*P. tuberculosa*	0.2	Respiratory distress, anoxia, cyanosis, death	Hypertension, then hypotension, bradycardia, arrhythmia, cardiac arrest	[104]
Mouse	*P. toxica*	0.15	Decreased locomotion, extension of forelimbs, paralysis of hind limbs, diarrhea, convulsions, dyspnea and death from respiratory failure	—	[2]
Mouse	*P. vestitus*	0.45 (0.33–0.62)*	Piloerection, prostration, dyspnea, convulsions, death	—	[96]
Mouse	*P. tuberculosa*	0.53 (0.38–0.73)*	—	—	[104]
Mouse	*R. macrodactylus*	0.74 ± 0.29**	Drowsiness, convulsions, collapse and death. At doses close to the LD_{50}, death occurred no sooner than 8–10 h after administration	—	[14]

* Figures in brackets indicate 95% confidence intervals.

** Mean ± SEM.

32.3.1.2 Acute Toxicity by Intraperitoneal Injection

Data on the acute intraperitoneal toxicity of purified palytoxin and of extracts of palytoxin-containing organisms are summarized in Table 32.5. The intraperitoneal LD_{50} of palytoxins in mice is very similar to that recorded by intravenous injection. In the rat, however, the acute toxicity by intraperitoneal injection is approximately seven times lower than the intravenous LD_{50}.

The LD_{50} values for purified palytoxin from *P. vestitus*, *P. tuberculosa*, and *P. caribaeorum* in mice are remarkably consistent, with values between 0.45 and 0.72 μg/kg. Kaul et al. [9] reported an LD_{50} of between 0.05 and 0.1 μg/kg for palytoxin from *P. mammillosa*, but it is conceivable that this reflects a calculation error of a factor of 10, rather than indicating a much higher toxicity of the palytoxin from this particular species of *Palythoa*.

The symptoms of intoxication by purified palytoxin and palytoxins derived from *Ostreopsis* and from toxic fish and crabs are also quite consistent. Ataxia and paralysis, particularly of the hind limbs, are early observations. Dyspnea, cyanosis, and exophthalmus precede death, which, at dose-levels close to the LD_{50}, may occur up to 48 h after toxin administration. Convulsions preceding death have been reported by some, but not all, observers, and diarrhea has also been described in some cases. Writhing after injection was recorded in one study [92], and in these animals peritonitis and ascites were observed. Such an effect was not, however, reported in other studies.

Histologically, edema and necrosis of intestinal villi, associated with intestinal hemorrhage, has been reported [92], and vacuolation in intestinal epithelial cells has been described [93]. Other target organs include the heart, in which single-cell necrosis was observed, and the kidney, liver, and pancreas, which showed vacuolation [93]. Necrosis of lymphocytes in lymphoid tissues was also reported [92]. Increased activity of serum creatine kinase was observed in mice given purified palytoxin or palytoxin extracted from *Ostreopsis* spp. and *S. ovifrons* [22], suggesting myotoxicity. Pretreatment of animals with ouabain did not influence the severity of the histological alterations induced by palytoxin [93]. It was shown that potassium chloride induced similar changes in the rat heart to those found with palytoxin, and it was suggested that hyperkalemia could be responsible for the cardiac damage induced by this compound [93].

32.3.1.3 Acute Toxicity by Oral Administration

Comparatively little information on the oral toxicity of palytoxins is available (Table 32.6), but it is clear that these substances are very much less toxic orally than by intravenous or intraperitoneal injection. In an early study, Vick and Wiles [90] reported an oral LD_{50} of >40 μg/kg in rats. In later work, the LD_{50} of purified palytoxin from *P. caribaeorum* was established as 510 μg/kg in mice [94]. An extract of *L. pictor* was orally toxic in mice, although no quantitative data were obtained [46]. Similarly, deaths occurred in cats fed *D. alcalai* and *L. pictor* [95]. No effects were seen with a crude extract of *O. siamensis* given orally to mice at a dose of 1 g/kg, even though the LD_{50} of this material by intraperitoneal injection was only 710 μg/kg [94].

32.3.1.4 Acute Toxicity by Other Routes of Administration

The toxicity of palytoxin via several other routes of administration has been investigated (Table 32.7). This substance is highly toxic after intramuscular or subcutaneous injection, or following intratracheal instillation [90,96]. No toxicity was recorded after intrarectal administration of palytoxin at 10 μg/kg [90]. Renal necrosis and pulmonary damage were recorded in animals given palytoxin intradermally [96], and local irritation and swelling, associated with edema and necrosis, were observed after both intradermal injection and percutaneous application. Severe irritation, involving ulceration and conjunctivitis, was induced by application of palytoxin to the eye [96].

TABLE 32.5
Acute Toxicity of Palytoxin and Extracts of Palytoxin-Containing Organisms by Intraperitoneal Injection

Animal	Source of Palytoxin	LD_{50} (μg/kg)	Symptoms of Intoxication	Biochemical/Pathological Findings	Reference
Mouse	*P. toxica*	0.4	Decreased locomotion, extension of forelimbs, paralysis of hind limbs, diarrhea, convulsions, dyspnea, and death from respiratory failure	—	[2]
Mouse	*P. tuberculosa*	0.5	—	—	[117]
Mouse	*P. caribaeorum*	0.72 (0.64–0.80)*	Abnormal gait, splaying of hind limbs, paralysis, dyspnea, death. No diarrhea	No peritonitis or ascites. No other macroscopic changes	[20]
Mouse	*P. tuberculosa*	0.60	—	—	[105]
Mouse	*P. mammillosa*	0.05–0.1	—	—	[9]
Mouse	*L. pictor*	—	Laborious movement and ataxia	Single-cell necrosis in the heart. Autophagic vacuoles in proximal convoluted tubules of the kidney. Glomeruli normal. Vacuolation and fat deposition in midzonal hepatocytes. Autophagic vacuoles in pancreatic acinar cells. Vacuolation in intestinal epithelium	[93]
Mouse	*L. pictor,* semipurified	377 (305–467)*	Death within 4 h	—	[48]
Mouse	*O. siamensis*, crude extract	710	Abnormal gait, splaying of hind limbs, paralysis, dyspnea. No convulsions. No diarrhea	—	[94]
Mouse	*O. mascarenensis,* crude extract	900	Prostration, progressive paralysis, dyspnea, convulsions and death. No diarrhea	—	[21]
Mouse	*D. alcalai, L. pictor, Z. aeneus,* crude extract	—	Motor incoordination, paralysis, dyspnea, cyanosis, exophthalmus. Death generally between 2 min and 45 min after administration	—	[95]
Mouse	*L. pictor,* crude extract	—	Hind limb paralysis, cyanosis, convulsions, respiratory collapse, exophthalmus, death within 2 h	—	[47]
Mouse	*Ostreopsis* spp., *S. ovifrons,* crude extracts, and commercial palytoxin	—	Convulsions, drowsiness, and collapse. Death within 48 h	Increased serum creatine kinase activity	[22]
Mouse	Various Hawaiian fish, crude extracts	—	Piloerection, paralysis of hind legs, respiratory distress, immobility, cyanosis, death.	—	[45]
Mouse	*M. vidua*	—	Diarrhea, cyanosis, progressive paralysis and death	—	[40]
Mouse	*D. alcalai*	—	Writhing soon after injection, diarrhea, paralysis, dyspnea. Death between 3 and 5 hr at 1.5 μg/kg. No deaths at 1.0 μg/kg at 24 h	Peritonitis, ascites, necrosis of lymphocytes in lymphoid tissues. Intestinal congestion and hemorrhage. Edema and necrosis of intestinal villi. Decreased weights of thymus, spleen and liver	[92]
Mouse	*A. scripta*	—	Loss of activity, hypersalivation, paralysis of hind limbs, dyspnea, and death.	—	[39]
Rat	*P. vestitus*	0.63 (0.44–0.91)*	—	—	[96]

* Figures in brackets indicate 95% confidence intervals.

TABLE 32.6
Acute Toxicity of Palytoxin and Extracts of Palytoxin-Containing Organisms by Oral Administration

Animal	Source of Palytoxin	LD_{50} (μg/kg)	Symptoms of Intoxication	Biochemical/ Pathological Findings	Reference
Rat	*P. vestitus*	>40	—	—	[96]
Mouse	*P. caribaeorum*	510 (311–809)*	Abnormal gait, splaying of hind limbs, paralysis, dyspnea, cyanosis, exophthalmus, death. No convulsions. No diarrhea	No macroscopic changes	[94]
Mouse	*O. sameness*, crude extract	>1,000,000	No effects recorded at this dose level	—	[94]
Mouse	*L. pictor* crude extract	—	Sedation and partial paralysis of the hind limbs after 1 h. Subsequent labored respiration, convulsions, and death. High doses killed after a few minutes. At low doses, death up to 30 h after administration	—	[46]
Cat	*D. alcalai, L. pictor*	—	Death between 27 and 47 min after administration	—	[95]

* Figures in brackets indicate 95% confidence intervals.

32.3.1.5 Acute Toxicity in Humans

Several early reports describe human poisoning by crabs of the genera *Demania* and *Lophozozymus*, although the nature of the toxin or toxins was not known at that time [97–99]. With the later discovery of palytoxin in such crabs, and in fish of the genus *Scarus*, many instances of poisoning have been attributed to this toxin. Clinical, biochemical, and pathological changes in humans following ingestion of fish and crabs that have been shown to accumulate palytoxin are summarized in Table 32.8.

Vomiting and diarrhea have been recorded in many cases of human intoxication ascribed to palytoxin, along with paresthesia of the extremities. In fatal cases, respiratory distress and cyanosis preceded death. In most of the reports listed in Table 32.8, there is evidence for muscle damage in humans, as indicated by myalgia, myoglobinuria, and elevated serum activities of muscle-derived enzymes and light myosin chain.

A recent report [100] describes rhinorrhea, cough, fever, and bronchoconstriction in individuals exposed to marine aerosols in Italy. This was attributed to an *Ostreopsis* spp., blooms of which (designated as *O. ovata*) have been recorded on the Italian coast over several years [24,101,102].

32.3.2 Toxicity to Animals after Repeated Administration

Very little information on the chronic toxicity of palytoxin is available. Ito et al. [103] gave 5, 10, 15, or 29 intraperitoneal injections of 0.25 μg/kg palytoxin (purified from *D. alcalai*) to mice, five times per week. Diarrhea and peritonitis were recorded in 60% of mice given 29 doses of the test substance, while decreased thymic weights and increased splenic weights were observed after

TABLE 32.7
Acute Toxicity of Palytoxin and Extracts of Palytoxin-Containing Organisms by Other Routes of Administration

Animal	Route	Source of Palytoxin	LD_{50} (μg/kg)	Symptoms of Intoxication	Biochemical/Pathological Findings	Reference
Rat	Intramuscular	*P. vestitus*	0.24 (0.21–0.28)*	Local irritation and swelling. Time to death longer than after intravenous injection	—	[96]
Dog	Intramuscular	*P. vestitus*	0.08 (0.05–0.14)*	Local irritation and swelling	—	[96]
Rat	Intratracheal	*P. vestitus*	0.36 (0.23–0.55)*	Respiratory distress	—	[96]
Rat	Subcutaneous	*P. vestitus*	0.40 (0.29–0.54)*	Time to death longer than after intramuscular injection	—	[96]
Mouse	Subcutaneous	*P. vestitus*	1.39 (1.07–1.80)*	—	—	[96]
Rat	Intrarectal	*P. vestitus*	>10	No effects recorded at this dose	—	[96]
Rat, rabbit, guinea pig	Intradermal	*P. vestitus*	—	Local swelling and edema	Necrosis at injection site. Renal necrosis and damage to pulmonary vessels	[96]
Rabbit	Percutaneous	*P. vestitus*	—	—	Swelling, edema and necrosis at application site	[96]
Mouse	Percutaneous	*P. tuberculosa*	Between 400 and 1700	Irritation at application site	—	[73]
Rabbit	Ocular	*P. vestitus*	—	No systemic signs of intoxication. Eye irritation, swelling, edema, ulceration and conjunctivitis	—	[96]

* Figures in brackets indicate 95% confidence intervals.

TABLE 32.8
Effects of Palytoxin-Containing Foodstuffs in Humans

Ingested Foodstuff	Symptoms of Intoxication	Biochemical/Pathological Findings	Palytoxin Identified/ Quantified?	Presence of Other Toxins Investigated	Reference
D. macrosoma	Weakness, sweating, abdominal cramps, nausea, diarrhea, circumoral paresthesia and paresthesia of the extremities, dysesthesia, muscle spasms, and tremor. Respiratory distress. Recovery	Myoglobinuria. Elevated serum levels of creatine kinase, elevated serum activities of lactate dehydrogenase and aspartate aminotransferase	Yes/No	No	[38]
S. ovifrons	Severe muscle pain, dyspnea. Two cases, one death	—	Yes/Yes	Yes (tetrodotoxin identified)	[36]
S. ovifrons	Hypothermia, hypertension. Myalgia. Abnormal electrocardiogram. Recovered	Myoglobinuria. Elevated serum activities of creatine kinase, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase. Increased serum level of light myosin chain. Serum urea and creatinine levels normal	No/No	No	[112]
S. ovifrons	Severe muscle pain, paralysis, dyspnea, cyanosis, and numbness. Nausea and vomiting were rarely recorded. In 52 cases, 4 deaths. Death time between 10 h and 5 days. Others recovered	Myoglobinuria, elevated serum activities of creatine kinase, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase	No/No	No	[37]
S. ovifrons	Muscle pain, backache, dyspnea	Rhabdomyolysis	No/No	No	[118]
S. ovifrons	—	Myoglobinuria. Elevated serum activities of creatine kinase, aspartate aminotransferase, alanine aminotransferase. Electrocardiographic changes.	No/No	No	[119]
S. ovifrons	—	Elevated serum activities of creatine kinase, aspartate aminotransferase, alanine aminotransferase	No/No	No	[120]
Epinephilus spp.	Muscle pain, low back and shoulder pain. Four cases, all recovered	Myoglobinuria, increased serum creatine kinase activity	Yes/No	No	[41]

L. pictor	Abdominal pain, dizziness, vomiting. 2 cases, both died 30–60 min after ingestion	—	No/No	No	[98]
L. pictor	Severe abdominal pain with vomiting and diarrhea. Cyanosis, shallow breathing. Died	Small intestine and proximal large intestine filled with bloodstained fluid. Mucosal lining was severely congested and showed areas of hemorrhage. Other organs normal	No/No	No	[99]
D. reynaudii	Dizziness, nausea, vomiting, diarrhea. Numbness of hands and lower extremities. Muscle cramps. Irregular heart rate, rapid and shallow breathing, cyanosis, anuria. Death after approximately 20 h	—	Yes/Yes	Yes. No evidence for the presence of saxitoxin or tetrodotoxin	[53]
Demania sp.	Severe diarrhea, nausea, and vomiting. Hypersalivation. Convulsions and respiratory arrest. Death in 7 h	Myoglobinuria, increased serum creatine kinase	No/No	No	[97]
Tetraodon sp.	55 cases. In some, nausea, vomiting, respiratory failure, muscle pain. In others, paralysis and dyspnea	Increased serum creatinine kinase in some cases	No/No	No*	[121]
H. quadrimaculatus	General malaise, uncontrollable vomiting, and diarrhea during the first 2 h. Tingling of extremities and delirium	—	Yes/Yes	No	[34]

* In later studies [42], palytoxin, together with paralytic shellfish poison, were identified in *Tetraodon* spp., although the fish analyzed were not associated with human intoxication.

10–15 doses. The latter effect was attributed to the irritant effect of injected palytoxin. The weights of these organs had returned to normal 1 month after cessation of dosing. Necrosis of lymphocytes in the cortex of the thymus was seen after 29 doses, and the number of lymphocytes in the splenic white pulp was decreased. The total dose of palytoxin administered in this experiment was up to 725 μg/kg. This is approximately 1450 times the single-dose LD_{50} of palytoxin to mice by intraperitoneal injection, suggesting that significant accumulation of palytoxin does not occur in mouse tissues when administered by this route. In contrast, 47% of mice dosed twice weekly with palytoxin by application to the skin at 20 μg/kg for 30 weeks died [73]. The total dose administered was 1200 μg/kg, which is similar to the single-dose LD_{50} by skin application [73], which could indicate a degree of accumulation of the toxin after repeated dosing. However, the skin irritation induced by palytoxins (Section 32.3.1.4) could facilitate the absorption of subsequent doses, and this could account for the relatively high toxicity observed after repeated application to skin.

32.3.3 Stability of Palytoxins and Factors Influencing Toxicity

Palytoxins are stable in neutral aqueous solutions for prolonged periods [104,105]. However, rapid decomposition occurs, with loss of toxicity, under acid or alkaline conditions [2,7,96,105,106]. Palytoxin is also detoxified by bleach [96]. Catalytic hydrogenation greatly decreases toxicity, and no effects were seen in mice after injection of perhydropalytoxin at 9.2 mg/kg [106]. Toxicity is also decreased following acetylation of the terminal amino group [106].

Animals given a sublethal dose of palytoxin intravenously or intramuscularly were partially protected against a subsequent lethal intravenous dose [90]. Hydrocortisone afforded protection when administered before the palytoxin [90], while vasodilators, injected directly into the heart, gave a degree of protection against the toxicity of intravenous palytoxin [90].

32.3.4 Tumor Promotion by Palytoxins

Palytoxin is a promoter of skin carcinogenesis in mouse skin, when 7,12-dimethylbenz[*a*] anthracene is used as the initiator [73]. The mechanism of palytoxin-induced tumor promotion has not been fully characterized. It differs from that associated with "classical" promoters, such as phorbol esters, since although palytoxin is a strong skin irritant, it does not activate protein kinase C or increase ornithine carboxylase activity in skin [73]. It does not act by growth stimulation, differential cytotoxicity, or terminal differentiation between normal versus neoplastic cells, which are proposed to be involved in the effects of other tumor promoters [69], or on gap-junctional intercellular communication, inhibition of which, through effects on cell proliferation, differentiation, and development, is also proposed as a mechanism of promotion [71]. As discussed previously (Section 32.2), effects of palytoxin on cellular signaling processes, demonstrated in cultured cells *in vitro*, could be involved in the promoting activity, possibly by a mechanism involving activation of prostaglandin synthesis.

32.3.5 Carcinogenicity and Anticancer Action

Although a tumor promoter (Section 32.3.4), palytoxin did not induce cell transformation in Balb/c 3TC cells [81], and it was not an initiator in mouse skin [73].

Palytoxin showed activity against Ehrlich ascites tumor cells *in vivo* [10,107], but although very effective *in vitro* [15], it had little effect on P-388 lymphocytic leukemia cells *in vivo* [10]. In the 1990s, because of the very high cytotoxicity of palytoxin *in vitro*, conjugates of this substance were suggested as prodrugs for cancer chemotherapy. By targeting the prodrug to a tumor, and there releasing palytoxin, selective destruction of the neoplastic cells could be achieved [72,108,109]. There appears, however, to have been no further work in this area in recent times.

32.4 EVALUATION AND RECOMMENDATIONS FOR FUTURE STUDIES

Palytoxin derivatives, produced by soft corals or dinoflagellates, readily enter the food chain, and may accumulate at very high levels in fish and crabs. While such levels of palytoxin appear to cause no harm to these creatures, the possibility of toxic effects in humans who consume them must be evaluated.

Palytoxin derivatives are exceptionally toxic to cells and tissue *in vitro*, with effects being recorded at nanomolar concentrations. Such *in vitro* studies have proved very important in identifying the effect of palytoxin on ion movements across the cell membrane involving the Na,K-ATPase, and palytoxin has proved valuable in investigating the mechanism of action of ion pumps [110,111]. In many cases, it was shown that the toxicity of palytoxin to cells was caused by osmotic lysis due to interaction with the Na,K-ATPase, as shown by the ability of ouabain to prevent cytotoxicity, but whether such effects are relevant to the toxicity of palytoxin *in vivo* is doubtful. Effects on ion transport have never been demonstrated *in vivo*, and ouabain had no effect on palytoxin toxicity *in vivo* [93].

The acute toxicity of palytoxin by injection has been very well evaluated, with good data available on the lethal doses of this substance to many species of animals by various routes of injection. There is no doubt that injected palytoxin, whether administered via the intraperitoneal, intravenous, intramuscular, or subcutaneous route, is an exceptionally toxic compound. There is, however, surprisingly little information available on the oral toxicity of palytoxin. The major focus of toxicological studies on food contaminants such as palytoxin should be risk assessment, and since such contaminants are eaten, not injected, in assessing risks to human health of food, oral data are of paramount importance. What data there are indicate that palytoxin is very much less toxic when given orally than when administered by injection. In a comparative study, more than 700 times more palytoxin was required to kill mice via gavage than by intraperitoneal injection. Similarly, a crude extract of *O. siamensis* was more than 1000 times more toxic by injection than by oral administration. The reason for the large disparity between toxicity by injection and by oral administration is presently unknown. One possibility is that palytoxin, being of such high molecular weight, is not readily absorbed from the gastrointestinal tract, and a pharmacokinetic study of this substance is of high priority.

A number of human poisoning incidents have been ascribed to palytoxin. The parrotfish, *S. ovifrons*, has been shown to contain this substance, and it is reported that between 1953 and 1999, at least 20 poisoning incidents occurred in Japan following consumption of this fish. These incidents involved 75 people, 6 of whom died [22]. Intoxication by palytoxin also occurs in other parts of the world, although Wachi et al. [43], in their paper published in 2000, stated that there had been fewer than 12 deaths due to palytoxin poisoning worldwide.

It must be pointed out, however, that the evidence that palytoxin was the agent solely responsible for the observed poisoning of humans by crabs and fish is inconclusive. While palytoxin has been positively identified in some foodstuffs that have caused poisoning in humans, care must be taken to avoid logical fallacies with regard to cause and effect. Because a substance is identified in a toxic foodstuff, it does not follow that it is that substance that is responsible for the intoxication. Marine animals may contain many toxins, so that poisoning may result from a toxin other than the one identified, or may be due to synergistic effects between the identified toxin and other toxins. Ideally, the following criteria should be satisfied before attributing toxicity to a particular food contaminant:

1. Samples of the food actually eaten by the poisoned individuals should be shown to contain that contaminant, and the levels quantified.
2. Samples of the food actually eaten by the poisoned individuals should be shown to contain only low levels of other toxic materials.
3. The symptoms of intoxication seen in humans should be the same as those observed in experimental animals exposed to that contaminant.

In none of the case studies have the above criteria been fully satisfied.

Although palytoxin was positively identified in many of the foodstuffs listed in Table 32.8, it was quantified in only three, and in these cases, the degree of quantitation was unsatisfactory. The leftovers of parrotfish from a poisoning incident were analyzed by Noguchi et al. [36]. Muscle, viscera and "the tissue suspected to be liver" were analyzed. However, it was later concluded that the last-named sample was not liver, but some other tissue. The lack of data on liver is unfortunate, since palytoxin is reported to selectively accumulate in parrotfish liver, and consumption of this is known to cause severe toxic effects in humans [37,112]. In the case of a woman who died after eating a sardine, *H. quadrimaculatus*, only the heads of the fish were available for analysis [34]. They contained only low levels of palytoxin, but data on fish heads give no information on levels in the parts of the fish that were actually eaten. In the third case, two legs of a specimen of *D. reynaudii* that had caused death in a human were assayed for palytoxin [53]. These contained 0.77 mg palytoxin/kg, but again such levels cannot be extrapolated to the parts of the crab that were eaten.

Little attention has been given to other toxins in the harmful fish and crabs. Alcala et al. [53] found no evidence for the presence of saxitoxin or tetrodotoxin in toxic specimens of *D. reynaudii*, but saxitoxin was detected in *Tetraodon* spp. [42]. Tetrodotoxin was detected in a toxic specimen of *S. ovifrons* [36].

It is interesting to note, however, that clinical and biochemical data in more than 70% of the case reports listed in Table 32.8 indicated that muscle damage had occurred in the affected individuals. There is evidence that palytoxin is myotoxic in mice, so in this respect, symptoms in humans are the same as those recorded in experimental animals. While further data are required on this point, it is possible that muscle damage could be a diagnostic symptom for palytoxin intoxication in humans.

In the acute oral toxicity study described in Reference 94, no adverse effects were observed in the mice at a dose of 320 μg/kg. By applying the standard safety factor of 100 [113], the acute reference dose of palytoxin would therefore be 3.2 μg/kg, which is equivalent to a dose of 192 μg for a 60-kg adult. If an intake of 250 g is taken as an estimate of the amount of food containing palytoxin or palytoxin derivatives consumed in a single meal, a concentration of these substances of 768 μg/kg in such food would not be expected to cause acute effects in humans. Assays of palytoxin in foodstuffs associated with poisoning incidents would be extremely valuable in testing the validity of this estimate. At present, however, analytical data on foodstuffs associated with poisoning incidents are unsatisfactory, and in future studies of outbreaks of human intoxication that may be attributable to palytoxin, it is important that analyses are conducted on the actual food that was consumed by the affected persons, and that assays not only for palytoxin but also for other potential toxicants are carried out. In view of the reports of widespread contamination of reef fish by palytoxin [45], analytical studies on fish from markets would be of great interest, in order to give information on the amounts of palytoxin that can safely be consumed by humans.

The potential importance of the reported tumor-promoting activity of palytoxin requires special consideration. Promotion was observed with palytoxin in the two-stage carcinogenesis model of murine skin cancer. It should be noted, however, that skin application is not relevant to human exposure to palytoxin, and the doses employed to demonstrate promotion in murine skin caused gross skin irritation and the death of more than 50% of the animals. Many tumor promoters cause irritation and/or show surface-active properties, and these properties are believed to be important for promotion in the mouse skin model [114]. As discussed previously, palytoxin is a powerful irritant, and it also shows surface active properties [57]. Whether irritation or surface activity are seen after oral administration of palytoxin, when it will be diluted by the contents of the stomach and intestine, is not known. Furthermore, the significance of tumor promoters in the progression of human cancers is difficult to assess, since humans are exposed to many materials that are known promoters in rodents [114,115], and some of these, such as saccharin, alcohol and oleic acid, are regularly consumed in large amounts. On the basis of presently-available information, it is considered unlikely that palytoxin will significantly increase the total bodily burden of tumor promoters. However, in order to resolve this point fully, detailed *in vivo* experiments involving oral administration of palytoxin after various known carcinogens will be required. Similarly, while palytoxin was not an initiator in the

mouse skin model, no information on the oral carcinogenicity of this material is available. Long-term feeding studies are required, but as a first stage in the evaluation of potential carcinogenicity, studies on the genotoxicity of palytoxin *in vitro* would be valuable.

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Part X

Pfiesteria Toxins

33 A Decade of Research on *Pfiesteria* Spp. and Their Toxins: Unresolved Questions and an Alternative Hypothesis

A. R. Place, K. Saito, J. R. Deeds, J. A. F. Robledo, and G. R. Vasta

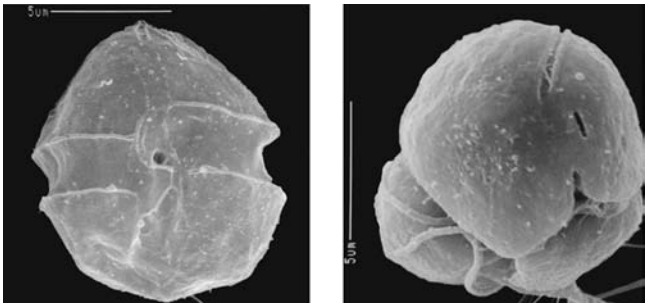
CONTENTS

33.1 SUMMARY

In the summer of 1997, Maryland was held captive by a single cell microbe called *Pfiesteria piscicida*, which was reportedly implicated in killing more than 30,000 fish and making ill more than three dozen people in Maryland's Pocomoke River. In October of that year, a workshop was organized to assess the current knowledge about *Pfiesteria*, and the critical unanswered questions and necessary research studies to address the crisis. This resulted in a white paper (Greer, J., Leffler, M., Belas, R., Kramer, J. and Place, A. (1997), *In:* Zohar, Y., Belas, R., Vasta, G., Place, A., and Kramer, J. (Organizing Committee), Molecular Technologies and *Pfiesteria* Research: A Scientific Synthesis; http://www.mdsg.umd.edu/MDSG/) that established a set of research priorities. Because of the research performed since then, cultures of *Pfiesteria* spp. are now available at the Center for Culture of Marine Phytoplankton (CCMP) to all members of the scientific community, as well as molecular probes that distinguish between *Pfiesteria* spp. and other co-occurring harmful algal blooms (HABS). Further, critical knowledge has been generated that has contributed to a rigorous interpretation of the above mentioned initial environmental and laboratory observations. We now know that physical contact is necessary for *P. piscicida* to kill fish, and that live fish and/or their products induce excystment and proliferation of *P. piscicida.* Moreover, the standard tank fish bioassay contains a microbial assemblage that makes it impossible to discern the cause of fish death, even when inoculated with unialgal clones of *Pfiesteria*. Cultures of *Pfiesteria* contain a bacterial assemblage that is unique, stable, and needed for optimal growth. Members of the assemblage may also be responsible for the reported toxicity. No evidence for a diffusable toxin being responsible for fish death in nature can be rigorously attributed to *Pfiesteria* spp. In contrast, a co-occurring dinoflagellate, *Karlodinium veneficum*, that was present in the Pokomoke in 1997, produces water soluble toxins that kill fish through gill disruption. The toxins have been isolated not only from laboratory cultures but also from water samples obtained at the site of fish kills, and their structure has been determined. *K. veneficum* produces a suite of toxins (karlotoxins, KmTxs), which have not only been shown to possess ichthyotoxicity but are also hemolytic, cytotoxic, and antifungal in nature.

33.2 INTRODUCTION

An apparent increase in the frequency over the past two decades of ocean disease events, usually affecting only marine life, has contributed to rising awareness of health issues in the marine/estuarine environment and the need for rigorous scientific approaches to address them [1]. During the summer of 1997, mass fish mortalities observed in the Chesapeake Bay and its tributaries were the subject of intense and widespread media coverage. These events were unusual, because human health effects among individuals exposed to the presumably contaminated waters were reported, leading to descriptions of a new toxic exposure syndrome characterized by unique skin lesions, respiratory problems, and neurological complications primarily including short-term memory loss [2,3]. The symptoms were reported severe enough to require immediate medical treatment, and in several cases full recovery required more than 6 months. This led to closures of public waterways for both commercial and recreational use, and resulted in substantial economic losses in the local seafood and tourism industries (http://www.dnr.md.us/Bay/cblife/dino/pfiesteria/Lilltest.html). Although the observed fish kills and reported human health problems in the Chesapeake Bay estuary greatly resembled those described for the Pamlico Sound of North Carolina that were attributed to the heterotrophic dinoflagellate *P. piscicida* ([4,5]; http://www.epa.gov/OWOW/estuaries/pfiesteria/, http://www.pfiesteria.seagrant.org/) after 10 years, the cause(s) of these events are yet to be conclusively determined. *P. piscicida* was originally isolated from an outbreak at an aquaculture facility by Burkholder et al. in 1992 and has been described as the causative agent of massive fish kills along the Atlantic Coast of the United States, including the Pamlico Sound of North Carolina [5,6] and rivers that drain from the agriculturally rich region of the Eastern Shore of Maryland into the Chesapeake Bay [7].



Pfiesteria piscicida *Karlodinium veneficum*

FIGURE 33.1 Scanning electron micrographs of *Pfiesteria piscicida and Karlodinium veneficum* isolated from the 1997 Pocomoke fishkill in Maryland. The characteristic apical pore in *K. veneficum* is readily apparent as is the thecate structure for *Pfiesteria piscicida*. (Images provided by the Florida Fish and Wildlife Conservation Commission.)

Actually a fish kill event a year earlier occurred in Maryland at HyRock Fish Farm, Princess Anne, Maryland [8]. On July 30, 1996 a large mortality of approximately 15,000, 1–1.25 lb. (2.20–2.75 kg) reciprocal cross hybrid striped bass (*Morone saxatilis* male × *Morone chrysops* female) occurred following copper sulfate treatment (<2 mg/L) to arrest a dense dinoflagellate bloom. The bloom had been developing for over 1 week before the events of July 30, but no mitigating actions were taken due to the misdiagnosis of the deep mahogany water coloration as tannins. Limited fish mortalities had occurred in the days preceding July 30. To avoid low dissolved oxygen problems, paddlewheel aeration was initiated maintaining dissolved oxygen levels >5 ppm. Water quality conditions immediately before the main kill were within normal midsummer ranges for HyRock Fish Farm. Foaming and a "petroleum-like" odor were present in the days preceding the large fish kill. Treatment using potassium permanganate (<4 mg/L), a strong oxidizing agent, of a neighboring pond with similar mixed dinoflagellate populations, appeared to arrest the bloom without fish mortality.

The bloom was subsequently determined to be dominated by a 10–15 μm, nonthecate, mixotrophic dinoflagellate *K. veneficum* (see Figure 33.1), formerly *Gyrodinium galatheanum* [9], (about 6×10^4 cells/mL), with <1000 cells/mL of an unidentified dinoflagellate (*Gymnodinium* spp.) and several additional <10 μm unidentified species (Wayne Coats, Smithsonian Environmental Research Center, Edgewater MD, personal communication). Some of the <10 μm dinoflagellates present resembled life history stages of *P. piscicida*, as described in Burkholder et al. [4] and Steidinger et al [6]. Samples were forwarded to the laboratory of Dr. Karen Steidinger (Florida Marine Research Institute, Florida Fish and Wildlife Conservation Commission, St. Petersburg, FL) who confirmed through scanning electron microscopy (SEM) and light microscopy that *P. piscicida* was present in the sample (about 300 cells/mL). Pathological examination of both live and preserved specimens at the time of the kill suggested that suffocation due to gill inflammation was the cause of mortality, although brain tissue also showed some abnormalities (Eric May, University of Maryland Eastern Shore, Salisbury MD, personal communication). In subsequent years, two additional fish mortality events occurred with blooms of this organism—8000 adults on August 13, 1997, and 5000 fingerlings on May 15, 1999. In both circumstances, mortality was stopped following a <4 mg/L potassium permanganate treatment, with fish returning to feed in 3–5 days. In an attempt to minimize the continued introduction of bloom-forming dinoflagellate species, common in the Manokin River, an ozone generation system was added inline with the main water supply in the summer of 1998 and no further fish mortalities were recorded. This was the first reported co-occurrence of *K. veneficum* and *Pfiesteria* spp. and as we will show later *K. veneficum* produces a polyketide toxin that forms pores in membranes with an appropriate sterol and is highly ichlthyotoxic. Strong oxidizing agents destroy the toxin [8].

At the time, the association of *P. piscicida* with the observed fish kills and reported human health problems relied on the morphological identification of *P. piscicida* dinospores in water samples collected from sites where fish mortality had occurred [10,11], or close to areas where unusual dermal, respiratory, and neurological symptoms were reported by individuals upon exposure to environmental water [2,3,12–14]. By analogy to other dinoflagellates, it was proposed that *P. piscicida* produces one or more toxins that affect fish and other organisms, causing skin lesions, loss of neural function, and eventually death [4,6,15–17]. However, rigorous experimental data in support of the aforementioned claims has been lacking, and the existence of the amoeba stage and *Pfiesteria* toxin(s) have been recently questioned in detailed studies [18–21]. The situation was complicated further by the description of its unusually complex life cycle having 24 stages of variable toxicity, and the presence of nontoxic or temporarily nontoxic strains that would revert to toxicity under certain environmental cues (http://www.whoi.edu/redtide/pfiesteria/glossary.html) [22–24]. Despite significant efforts by several laboratories directed toward the isolation and identification of the proposed toxin(s), limited success has been reported in the peer-reviewed literature only recently [25]. Further, the identification of *P. piscicida* as the causative agent of fish kills and human health problems has been questioned by several laboratories [18,20,26]. Although the presence of *P. piscicida* was confirmed from a fish kill at an aquaculture facility in Maryland in 1996, and another unidentified *Pfiesteria* spp. was isolated from a tropical fish aquarium in Florida; a number of isolates from Neuse River in North Carolina and the 1997 Pokomoke River outbreak in Maryland, which were associated with *Pfiesteria*-like fish kills and lesions, were later identified as a different species. The widespread confusion on sole attribution to *Pfiesteria* species and their toxins of the above-mentioned fish lesions and deaths, and deleterious effects on human health along the mid-Atlantic estuaries [3,4,6,10,11,13–16,27,28] has been the result of both the difficulties inherent in the field identification of these organisms, and the species complexity, heterogeneity, and dynamics of the local algal blooms. These problems have also revealed the pressing need for rigorous experimental approaches based on the use of clonal dinoflagellate cultures, purified toxins, and reliable bioassays. These would enable the establishment of causal links on which to base mechanistic investigations and potential production of the proposed bioactive/toxic agent(s) necessary for its characterization, and assessment of this species' potential toxigenicity. This chapter reviews the current status of knowledge and recent progress on several aspects of *Pfiesteria* spp., focusing on questions regarding its biology and toxicity in the context of mixed algal blooms in which the morphologically similar species *Karlodinium* spp., of well described toxicity is also present. This chapter also proposes that much of the fish kill activity attributed to *Pfiesteria* spp. is due to *K. veneficum*, a far more common and abundant harmful algae that *Pfiesteria* spp. co-occurs with.

33.3 THE *PFIESTERIA* COMPLEX

33.3.1 Morphological and Genetic Information, and Taxonomic Placement

P. piscicida, initially described as "the phantom dinoflagellate" [4], is now considered to be one of the multiple species of a *Pfiesteria* complex. These dinoflagellates differ from most known toxin-producing algae in that they are not pigmented and thus, giving no visual evidence of their blooms, even when *Pfiesteria* spp. may be present at higher cell densities than most other algal species [29–31]. The *Pfiesteria* complex constitutes a relatively recent discovery and, consequently, there is a good deal of uncertainty about its biology, its impacts on fish, shellfish, human health, seafood safety, and water quality. Although, there has been remarkable progress on several aspects of its biology, there is still uncertainty about almost every aspect of its natural history. Further, some findings on *Pfiesteria* remain highly controversial, particularly those indicators of potential toxin activity by *Pfiesteria* spp., including the deep sores in fish and fish kills, and the impacts on human health [18,20,32].

Ultrastructural analyses by scanning electron microscopy (SEM) are still the primary basis for species identification of dinoflagellates [33,34], which includes roughly 2000 known species. Classification of both fossil and modern dinoflagellates is based primarily upon the thecal or armored plates of the vegetative cells or cysts. These cellulose structures are composed of two valves in prorocentroids, while peridinioids thecae have multiple plates and gymnodinioids are athecate or lightly armored [35]. The main class of dinoflagellates, Dinophyceae, are further classified by flagellar position. Prorocentroids are desmokonts (apical flagella), and peridinioids and gymodinioids are dinokonts (flagella by sulcus) [36,37]. *Pfiesteria* spp. share basic morphological features with most Dinoflagellata, *Pfiesteria* spp. were formerly identified primarily by the shape and number of the plates in the theca. The morphological identification requires stripping away the outer membrane to reveal the shape and number of these plates under SEM [38]. *Pfiesteria* and *Pfiesteria*-like dinospores are heterotrophic, typically with a dinokaryon nucleus in the hypotheca and a food vacuole in the epitheca (Figure 33.2a). The longitudinal flagellum has two parallel parts and the peduncle, a feeding organelle emerges from under the flexible right sulcal plate. The sulcus is not straight but offset. The genus *Pfiesteria* is characterized as having a plate formula/tabulation of apical pore complex (pore plate, closing plate, and X plate), 4′, 1a, 5″, 6c, 4s, 5‴, 0p, 2‴ (Figure 33.2a,b,c). It has a triangular 1a (first intercalary plate) (Figures 33.2b and 33.3b) on the left shoulder, which is one of the characteristics of the genus. *Pfiesteria shumwayae*, has a larger, almost rectangular 1a plate and a plate formula distinct from that of *Pfiesteria* (six precingular plates instead of five). This different plate tabulation has raised questions about the affinities with *Pfiesteria* spp. and whether *P. piscicida* or *P. shumwayae* should remain in the same genus [38].

It has become increasingly apparent that identification and classification of microorganisms can be enhanced by the vast genetic and structural information available in DNA, RNA, and protein sequences. Criteria based on sequences of ribosomal RNA (rRNA) genes have been used to

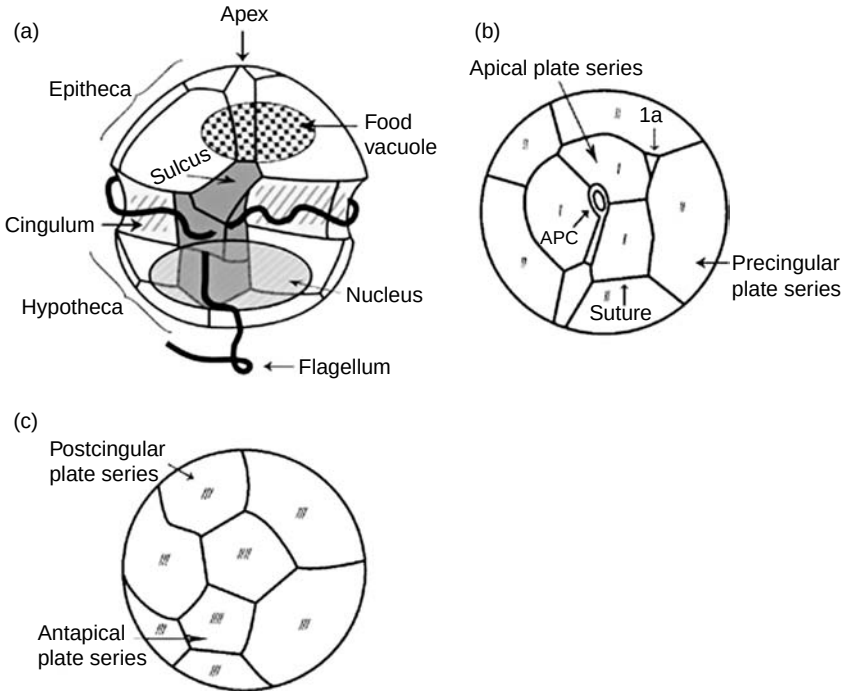


FIGURE 33.2 (a) Ventral view of motile cell with food vacuole in epitheca and nucleus in hypotheca. (b) Apical view with horizontal series of plates, that is, apical plates, intercalaries, and precingulars. (c) Antapical view with postcingular and antapical plate series. (Steidinger, K. et al., *Environ. Health Perspect*, 661, 2001.)

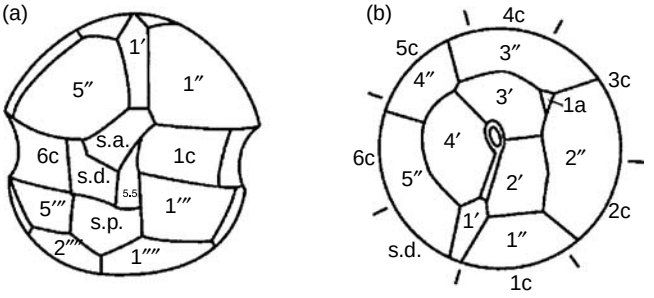


FIGURE 33.3 Schematic illustrations of *Pfiesteria* and *Pfiesteria*-like dinoflagellate. These four panels show how the epithecal plates fit together and how they are labeled. (a) Ventral view of *P. piscicida*. (b) Apical view of *P. piscicida*. (Steidinger, K. et al., *Environ. Health Perspect*, 661, 2001.)

taxonomically define a number of bacterial, protozoan, and higher eukaryotic species [39–47]. Within the Alveolata, which includes ciliates, apicomplexans, perkinsozoans, and dinoflagellates, current taxonomy relies on both morphological characters and phylogenetic analysis. Similarly, although earlier species descriptions were mostly based on morphology, classification of dinoflagellates is often revised following new morphological findings and mostly DNA sequence information. *P. piscicida* was first reported/described in 1988, but its taxonomic placement was not reported until 1996 [6]. Similarly, *P. shumwayae* was detected in 1995 and classified in 2001 [48]. The above-mentioned massive fish kills during the summer of 1997 led to increased scientific and governmental interest on the identification of the causative agent(s). This has resulted in a large number of nucleotide sequences becoming available, thereby providing a larger database suitable for a comprehensive phylogenetic analysis. A search for Dinophyceae nucleotide sequences in GenBank revealed 72,623 sequences (release December 4, 2006) with 4391 ribosomal RNA gene including 191 *Pfiesteria* spp., 120 *P. piscicida*, 42 *P. shumwayae*, and 29 *Pfiesteria* spp. or *Pfiesteria*-like sequences.

In dinoflagellates, analysis of large-subunit (LSU) sequences has been used to revise the phylogeny [49]. These data confirmed the division of three existing groupings of peridinioids, prorocentroids, and gymnodinioids; however, *Prorocentrum lima* was positioned more closely to peridinioids and may be considered an ancestral species. LSU analysis also provided strong evidence that *Alexandrium minutum* and *A. lustianicum* were actually synonymous species as they had identical sequences. However, the rate of sequence change in the LSU is significantly faster than the small subunit (SSU), and thus may not be as useful as the SSU in discriminating the deepest branches of phylogenetic trees [43]. For different taxa, the rates of evolution of ribosomal genes can vary greatly, presenting difficulties for accurate phylogenetic placement. An additional problem presented by protozoa and dinoflagellates is the presence of extrachromosomal DNA in the form of plastids, chloroplasts, kleptochloroplasts, or microbial symbionts, which may be preferentially amplified by polymerase chain reaction (PCR) and are sometimes difficult to discriminate from nuclear coding regions. Frequently, conserved genes of multiphasic organisms will be comprised of gene families whose differential expression is life stage dependent. The evolution of the different members of these gene families may vary and present different profiles upon phylogenetic analysis. Therefore, for accurate classification and understanding of the evolutionary origins of related species, it is essential to consider several genes.

On the basis of the morphological analysis, *P. piscicida* was initially placed into the order Dinamoebales, within the class Dinophyceae. However, phylogenetic analysis based on the SSU of ribosomal DNA sequence placed *P. piscicida*, *P. shumwayae*, and *Cryptoperidiniopsis* spp. into the family Pfiesteriaceae, order Peridiniales, class Dinophyceae [38]. Later in 2005, based on plate tabulation differences between *P. piscicida* and *P. shumwayae* and a phylogenetic analysis of ribosomal DNA sequence data [50] *P. shumwayae* was reclassified and assigned to a new genus, *Pseudopfiesteria* gen. nov. This proposal for reassignment was further supported by additional plate

TABLE 33.1
Comparison of Morphological Differences between *Pseudopfiesteria shumwayae* and *Pfiesteria piscicida*

	Pseudopfiesteria shumwayae	*Pfiesteria piscicida*
Epitheca	Po, cp, X, 4, 1a, 6"	Po, cp, X, 4', 1a, 5"
Typical size range	10–25 μm	7–14 μm
Mean plate length ± SD	1.7 ± 0.3 μm, *n*=29	1.5 ± 0.2 μm, n=22
1a plate	4-sided (rectangular)	3-sided (triangular)
1a placement	Touches 2", 3", 2', 3'	Touches 2", 3", 3'

Source: Litaker et al. *J. Phycol.* 41, 643, 2005.

tabulation differences between the two species. *Pseudopfiesteria shumwayae* has a rectangular or diamond-shaped intercalary plate and six precingular plates, whereas *P. piscicida* has a triangular intercalary and five precingular plates (Figures 33.2 and 33.3, Table 33.1) [38]. Accordingly, *P. shumwayae* was placed into the genus *Pseudopfiesteria* (Po, cp, X, 4′, 1a, 6″, 6c, PC, 5+s, 5‴, 0p, 2⁗) and emend the genus *Pfiesteria* to include only species with a Kofoidian plate tabulation of Po, cp, X, 4′, 1a, 5″, 6c, PC, 5+s, 5‴, 0p, 2⁗ by Litaker et al. (2005) [50]. Therefore, *P. shumwayae* has been reclassified into the family Congruentidiaceae, order Gonyaulacales, class Dinophyceae [50]. However, the phylogenetic position of *P. piscicida*, *P. shumwayae*, and *Pfiesteria*-like species within same or separate genus is still under debate. Six out of nine earlier studies—although the earliest two studies did not include *P. shumwayae* [51,52]—using various phylogenetic methods (maximum likelihood, parsimony, distance, minimum-evolution tree, Bayesian, maximum parsimony, and neighbor-joining analyses) provided weak to moderate support for *P. piscicida* and *P. shumwayae* as sister taxa with similar consistent topology [53–58]. Nonetheless, the order of divergence among the major dinoflagellates groupings has been poorly resolved owing to short internal branch lenghts and, consequently, weak bootstrap support. Although the molecular phylogenetic analyses have become important tools for classification of genospecies, in taxa such as the dinoflagellates where the sequence database is still relatively small, traditional taxonomy of morphospecies is critical.

33.3.2 Life Cycle and Exogenous Factors That Induce Proliferation and Encystment

The complex life cycle earlier proposed for *P. piscicida* included polymorphic and multiphasic forms, further contributing to difficulties in identification, population assessment, and evaluation of environmental factors relevant to this potential fish and human health hazard. Its life cycle was described as comprising at least 24 stages with bi- and triflagellated forms of gymnodinioid or peridinioid types, as well as amoeboid and cyst stages of multiple forms present in both benthic and planktonic stages in the estuarine environment (Figure 33.4) [17, 23]. While the species lacks chloroplasts, kleptochloroplasts, or photosynthetic prey in food vacuoles are common as a result of phagocytic ingestion. It has been observed to actively feed on fish tissue by means of a peduncle typical of heterotrophic dinoflagellate species. Thus, metabolism is either heterotrophic or mixotrophic. The toxicity of *Pfiesteria* species would depend on the stage of their life cycle, of which 20 stages are harmless, but four are toxic. Cysts are dormant, whereas flagellate and amoeboid *Pfiestieria* can be toxic. However, studies published in 2002 challenged the methods, findings, and objectivity of former reports [19]. For example, studies conducted by Dr. Wayne Litaker and Dr. Patrick Gillevet, demonstrated than no amoeboid stage(s) is found in clonal *Pfiesteria* culture [19], and the commonly depicted *Pfiesteria* amoeboid stage is very likely a species of *Korotnevella* [21], respectively.

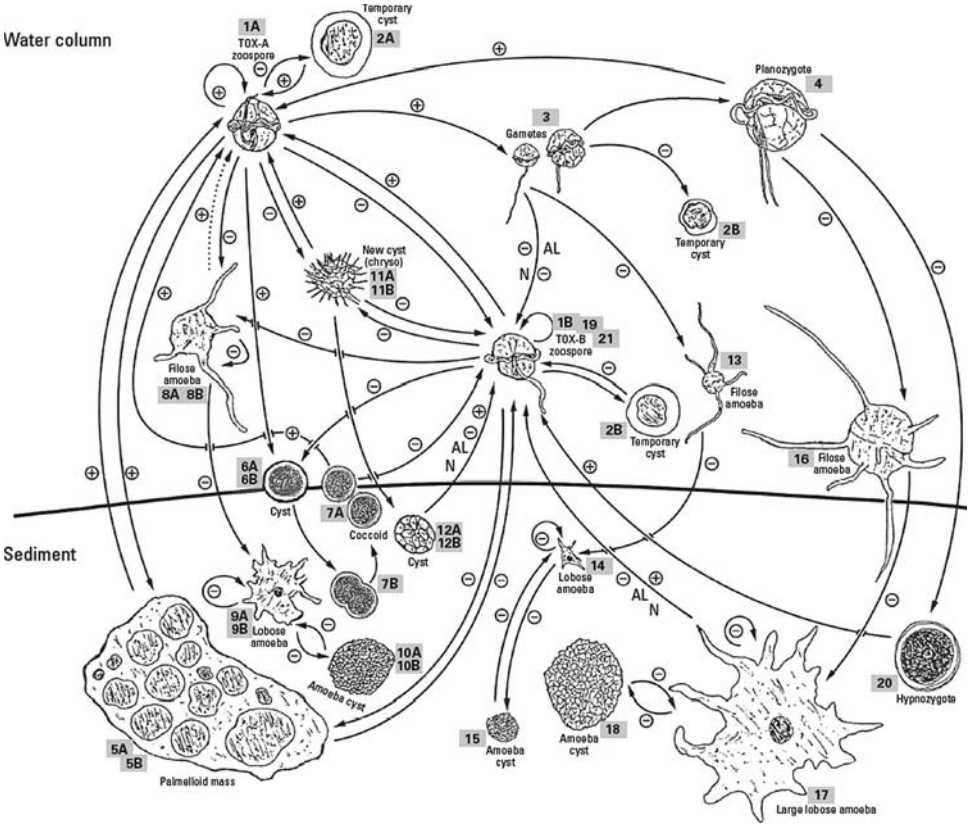


FIGURE 33.4 Schematic of the complex life cycle of *Pfiesteria* spp. as presently understood, showing stages and pathways that have been verified (solid lines) for toxic strains of *P. shumwayae*, and shared in common by *P. piscicida*. It should be noted that *P. piscicida* has been under study for a longer period, and several additional stages and pathways have been verified for that species. The pathways indicate the presence (+) versus the absence (-) of live finfish; AL = presence of cryptomonads and certain other algal prey; N = nutrient enrichment as organic and/or inorganic N and P; S = environmental stressor such as sudden shift in temperature or salinity, physical disturbance, or prey depletion. Dashed lines = hypothesized pathways. Stages have been conservatively numbered to facilitate description. Also note that varied approaches have been used for numbering stages. For example, complex life cycles of other dinoflagellates have sometimes been numbered as morphologically identical stages at each pathway or sequence of occurrence which, if applied in this schematic, would result in at least nine additional stages. TOX-B zoospores (haploid; ploidy confirmed as in Burkholder) are the temporarily nontoxic functional type in the absence of live fish prey (referred to as nontoxic zoospores in the life cycle schematic previously published for toxic strains of *P. piscicida*), although they can carry residual toxicity. TOX-B zoospores become TOX-A zoospores and produce toxin when sufficient live fish are added. As TOX-A and TOX-B zoospores are actually the same cells in the presence versus the absence of live fish. To stress that point we have designated morphologically identical stages as TOX-A versus TOX-B zoospores, or derived from those zoospores, with the same number followed by "A" or "B" (stages #1, 2, 5–12). TOX-B zoospores produced from diploid amoebae (stage #17) or hypnozygotes (stage #20) (rather than from stages directly derived from TOX-A or TOX-B zoospores) have been numbered as additional stages (stages #19, #21) in recognition of their distinct origin. TOX-A zoospores (stage #1A) can transform to filose (stage #8A) and lobose (stage #9A) amoebae (maximum cell dimension 15–60 μm). TOX-B zoospores (stage #1B) can transform to filose (stage #8B) and, less commonly, to lobose state #9B amoebae of similar size as those transformed from TOX-A zoospores. Planozygotes (stage #4) can transform to larger filose (stage #16) and lobose (stage #17) amoebae (maximum cell dimension 40–120 μm). Small filose (stage #13) and lobose (stage #14) amoebae (length 5–10 μm) can also be produced by gametes. Cysts include stages with

(Figure caption continued on opposite page)

Litaker et al. (2002) [19] reported that *P. piscicida* has a haplontic life cycle typical of free-living marine dinoflagellate using nuclear staining techniques combined with traditional light microscopy, high-resolution video microscopy, electron microscopy, and *in situ* hybridization with a suite of fluorescently labeled peptide nucleic acid (PNA) probes. Using clonal isolates of *P. piscicida* grown on algae, the complete life cycle would include temporary cysts, resting cysts, and hypnocysts (Figure 33.5). Cell division would occur in division cysts rather than in motile cells and sexual reproduction would be by fusion of homothallic gametes that form a two-celled hypnozygote. Although these organisms are still considered as potentially toxic, the recent studies suggest a simpler life cycle, more representative of dinoflagellates, than earlier proposed. Indeed, the *P. piscicida* sexual life cycle findings published in Litaker et al. (2002) [19] were partially supported by Parrow and Burkholder (2004) [59]. The *P. piscicida* planozygotes reported, however, had larger size (25–60 μm) [6], distinctive shape [19], or formed morphologically distinctive hypnozygotes from which four flagellated offspring cells emerge [19].

In addition, interstrain variability of toxin production of *Pfiesteria*-complex has been proposed as these organisms often temporarily lose toxicity in the absence of fish. The first report at the "*Pfiesteria* Interagency Coordination Workshop Group (PICWG)," Woods Hole Oceanographic Institute (WHOI) 2000 (http://www.whoi.edu/redtide/pfiesteria/glossary.html) described that *Pfiesteria* spp. can be subdivided into TOX-A (actively toxic and killing fish), TOX-B (temporary nontoxic and potential to be toxic) and NON-IND (noninducible to be toxic) based on toxic activity, as other natural populations of toxic microorganism such as cyanobacteria [60], prymnesiophytes [61], and *Alexandrium* spp. [62]. Temporary nontoxic TOX-B cells are not engaged in toxin production because of the absence of sufficient stimulation from live fish; therefore, TOX-B strain culture is produced by removing TOX-A populations from access to additional live fish and growing on algal prey. However, TOX-B can retrieve residual toxicity by exposing to live fish [22,24]. In contrast, NON-IND clones consistently fail to show its toxicity, especially after extended time in culture (in PICWG documents, 2000). The classification of *Pfiesteria* spp. functional toxic types has been controversial because the toxic strains were not made openly available to the scientific community, and studies by others were simply refuted for not using them [63,64].

Estuarine fish kills attributed to *Pfiesteria* spp. generally occurred during the summer, in shallow, poorly oxygenated, eutrophic water (~≤3–4 m, 2–35 ppt salinitty and 15–33°C) [17]. *P. piscicida* has been propagated in the laboratory by maintaining environmental samples under appropriate conditions [4]. Early laboratory studies using clonal cultures established that *P. piscicida* is active at a range of temperatures (9–33°C), although proliferates better at 18°C or above [15]. Although it grows optimally in seawater within a range of salinities (2–20 ppt), it can multiply in fresh water containing high concentration of calcium. The observation of *Pfiesteria*-associated fish kills occurred in estuaries with a high nutrient load suggested that the outbreaks were somehow related to nutrient-enriched environments. *In vitro* experiments demonstrated that *Pfiesteria* spp. proliferate faster on both algal prey with nitrogen- or phosphate- enriched medium (500 μg NO_3^- or PO_4^- /L) [23]; however, *P. shumwayae* prefers high levels of nitrogen while *P. piscicida* apparently favors a high-phosphorus environment [65]. However, both these conditions also enhance the growth of the cryptomonad prey so the effect maybe indirect. Nonetheless, the specific links

(a) roughened or reticulate covering (from amoeboid stages; haploid except when derived from diploid amoebae; stages #10A, B, #15, #18; diameter 4–30 μm); (b) scaled covering ± bracts; from TOX-A and TOX-B zoospores; these chrysophycean-like cysts (stages #11A,B) are 4–25 μm (rarely 30 μm) in diameter and can lose their bracts (stages #12A,B) and scales over time so that they have a smooth covering. Also note that transitional forms to these cysts are not shown but occur in *P. shumwayae* as in *P. piscicida*; and (c) hyaline covering, with darkened contents; and hypnozygote (diploid cyst at lower right). Zoospores and gametes also form temporary cysts with thick mucus covering, which may settle out of the water column (arrows not shown). (Burkholder, J.M. et al., *Environ. Health Perspect*, 109, 667, 2001.)

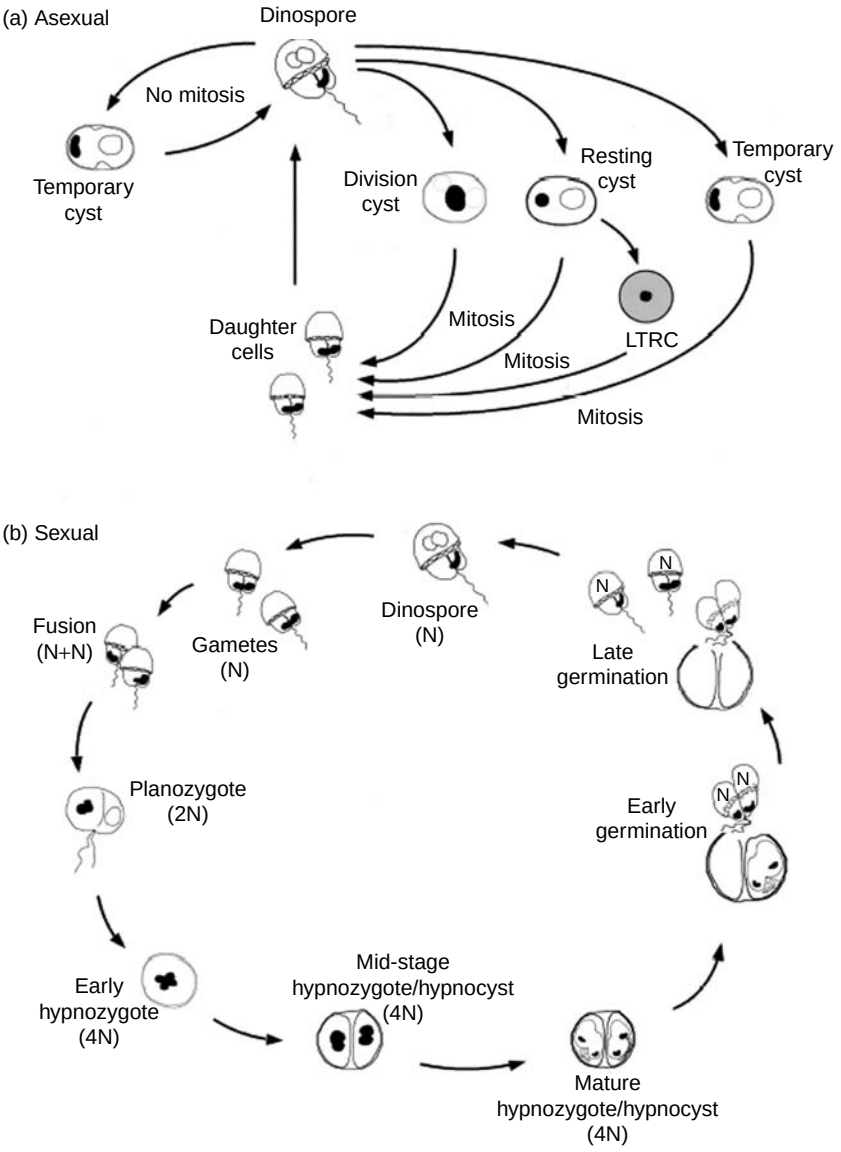


FIGURE 33.5 (a) Schematic diagram showing paths of asexual reproduction and temporary cyst formation of *P. piscicida*. (b) Diagram of the sexual life cycle stages. (Litaker, R.W. et al., *J. Phycol.* 38, 442, 2002.)

between environmental nutrients and *Pfiesteria* spp. proliferation during dinoflagellate blooms has not been fully resolved.

Clonal *P. piscicida* maintained with algal prey in f/2 medium [66] has a doubling time of 12 h during the exponential growth phase with 14 h light/10 h dark cycle. It reaches stationary phase within the range of $1–5\times10^5$ cells/mL and 5–25 μm cell size distribution. When cultures are incubated in the dark, *P. piscicida* dinospore proliferation rate exhibited lower than when maintained with light [67,68]. *Pfiesteria* spp. are heterotrophic dinoflagellates that do not have chloroplasts and cannot photosynthesize. Instead, the possibility of *P. piscicida* conducting kleptoplastidy, acquiring functional chloroplasts from algal prey, has been suggested based on earlier reports that retention of prey chloroplasts in *Pfiesteria* food vacuoles may benefit its growth [69]. The photosynthetic

inhibitors (methyl viologen (500 μM), DCMU (10 μM) and antimycin A (20 μM)) decreased the growth of *P. piscicida* on algae with light. However, light stimulated *P. piscicida* growth was diminished coinciding with rapid disappearance of prey-derived pigments (RUBISCO monitored by immunodetection, and actin and chloroplastic GAPDH determined by RT-PCR) from *P. piscicida* culture when the prey *Rhodomonas* spp. was depleted by grazing, and then *P. piscicida* became vulnerable to cannibalism with light but stimulated to encystment in darkness when prey algae is absent [67,68]. Because these dinoflagellates graze bacteria, algae, and live and dead animal tissues, for uptake of nutrients, it is very difficult to demonstrate the nutrient acquisition and growth enhancement by kleptoplastidy. The mechanism for gaining nutrients in culture is highly complex, both direct and indirect enrichment through photosynthesis by prey algae or *Pfiesteira* kleptoplastic activity, which can be affected by light conditions enhancing their proliferation rate.

On the basis of the observation that encystment of *Gonyaulax polyedra* is initiated by modifying the photoperiod [70], stationary phase *P. piscicida* dinospores were maintained at 4°C in the dark to induce encystation, a process that could be reverted by maintaining the encysted culture at 23°C with a standard light cycle [68]. Although faster encystment in the dark has been described [71], the detailed effects of shorter light periods on *P. piscicida* encystment remain unknown. Two types of cysts, both of round shape, have been described from clonal *P. piscicida* cultures with light (12 h light/12 h dark) and in darkness [71,72] with differences in wall thickness: "Cyst A" with a thick cell wall, and "Cyst B" with a thin wall [17]. The greater *P. piscicida* "Cyst A" production (A: B = 10:1) was obtained by incubation at 4°C in the dark [69] suggesting that the lower temperature and complete darkness might lead *P. piscicida* to selectively form "Cyst A" as the resting cysts.

A wide range of fish species were reported to be susceptible to *P. piscicida* toxin (Burkholder et al., 1992). Mortalities were reported to occur within 48 h and were preceded by sloughing of epidermal tissues resulting in open lesions [7]. Toxicity correlated with increased cell numbers and the presence of motile zoospores (>500/mL) and the sexual planozygote stage (>50/mL). Removal of fish or fish products interrupted the sexual stage, and zoospores reverted to benthic amoeboid stages, eventually leading to formation of cysts. However, *P. piscicida* can be cultured in a nontoxic form indefinitely by maintaining adequate numbers of live algal (*Crypotomonads*) as food/prey [17] although the recently described toxin from *P. piscicida* was isolated from a strain grown (CCMP 1921) only on cryptomonads [25, 73].

Pfiesteria is strongly attracted toward fish and fish-derived materials such as mucus [17,74]. However, *Pfiesteria* did not show any specificity and has been reported to kill equally numerous fish and shellfish species including native estuarine and marine, and exotic species in their natural habitat and in laboratory bioassay [17]. Growth and toxicity were induced in culture by the presence of live fish, fish feces, fish serum, or filtered (0.2 μm) water from fish cultures. Alternatively, toxic flagellated forms could be maintained under phosphorus-enriched conditions (>100 mg/mL of PO_4) without live fish [17]. When live fish were exposed to environmental sediments, emergence and proliferation of *P. piscicida* and *Pfiesteria* spp. were stimulated. Emergence of *P. piscicida* was observed about 10 days earlier than other *Pfiesteria*-like dinoflagellates. It has also been shown that exposure of starving (no algal prey) *P. piscicida* to fish-derived factors contributed to its survival and proliferation. Furthermore, the *P. piscicida* dinospore doubling time on algal prey was twice faster in the presence of fish-derived factor(s) during exponential phase, although the depletion rates of prey algae did not show difference between the presence and absence of fish factors. Interestingly, this effect was lost when the aquarium water was filtered (0.2 μm and/or 0.4 μm) and boiled (100°C), suggesting that the factor(s) affecting *P. piscicida* growth were most likely microorganism(s) and/or protein(s) [75]. The yet unidentified factor(s) derived from live fish that actively stimulates *Pfiesteria* spp. could be used as a supplement(s) to maintain toxicity of clonal *Pfiesteria* culture rather than exposing to live fish [23,24,73]. Clearly, more research is needed to rigorously identify the fish component(s) that triggers *Pfiesteria* proliferation.

Pfiesteria species can also be grown axenically on fish cell lines [76] and grown to high densities apparently in the absence of bacteria and algal prey. This is not unique to *Pfiesteria* spp. species

since another heterotrophic dinoflagellate (*Crypthecondinium* spp.) can also be maintained on fish cells [77]. It would be of interest to see if these cultures also produce *Pfiesteria* toxic activity.

33.3.3 Proposed Environmental Impact(s) of *Pfiesteria* Spp. Blooms

Fish lesions and kills: In North Carolina, fish kills caused by *P. piscicida* occurred inclusively from May to December during 1991, an unusual warm and dry year, which had conditions similar to those when *Pfiesteria*-like outbreaks were recorded in Maryland during the summer of 1997. Among the major fish kills (>1000 fish deaths [78]) between 1991 and 2000 in North Carolina, 53 events were attributed to toxic *Pfiesteria* spp. as the primary cause, and 79 were recognized as caused by anoxic stress or other causes [79,80]. Given the co-occurrence of *Pfiesteria* spp. and *K. veneficum* in North Carolina (see later) this conclusion needs to be readdressed. Early studies suggested that the characteristic "punched out" skin lesions in fish were attributable to exposure to the toxic forms of *P. piscicida* [4,81]. However, the association of *Pfiesteria* spp. with fish lesions remains highly controversial [20,82,83]. Current data indicate that most ulcers in menhaden (*Brevoortia tyrannus*), the fish most commonly affected in the Chesapeake Bay, are due to a highly invasive fungal species, *Aphanomyces invadeans* [20]. Although this work was criticized, the explanation based on a presumptive *Pfiesteria* hydrophilic toxin (tentatively named PfTx) has not yet been confirmed either [18,73,84–86]. Given the uncertainty about the causal relationship between *Pfiesteria* spp. and fish lesions, the absence of a rigorous assay for the specific detection of the toxin in environmental samples represents a critical problem [87,88].

Human health impacts: Exposure to *Pfiesteria* blooms or laboratory cultures was reported as resulting in deficits in human learning and memory [16]. Upon accidental exposure to *Pfiesteria* cultures and their aerosols, laboratory staff sustained respiratory, visual, and neurological problems. The most striking symptom was severe cognitive impairment involving learning disabilities and Alzeimer's-like short-term memory dysfunction, which required days to months for recovery to within-normal range function. Other symptoms reported were asthma-like respiratory problems, epidermal lesions that did not respond to antibiotics, severe headaches that did not respond to normal treatment, vision problems, joint and muscle pain, vomiting and stomach cramping [16]. Long-term effects, lasting for years and linked to the same exposures, have included autoimmune and central nervous system dysfunction and suppression. Significant reductions of cognitive function and chronic learning disabilities in rats were recorded upon injection of 0.2 μm filtered or unfiltered *Pfiesteria* culture [13,86,89], and were proposed to be the result of a toxin produced by the dinoflagellate. In the absence of a purified *Pfiesteria* toxin standard, verification of links between chronic and low-level environmental exposure and health risks (as seen among occupationally exposed commercial fishermen) has not been possible, particularly in light of studies suggesting that *Pfiesteria* strains are widespread in the estuarine environment in the United States mid-Atlantic region. Thus, the strategy that most researchers adopted for field studies to detect human health effects derived from recurring occupational exposure to an environment in which the presence of the candidate agent was monitored with an assay sensitive and specific for the "toxic" strains. The assay used, however, was not quantitative and not validated in specificity, thus generating additional uncertainties. It was also proposed that the neuropsychological deficits experimented by individuals exposed to the Pocomoke River water in the summer of 1997 were reversible [2]. On the basis of the recent findings from laboratory-exposed individuals [90], experimentally exposed animals [13,86,89], and the *in vitro* response of specific neuroreceptors [91], it is plausible that under very unique, isolated instances, and in association with specific, unusually toxic strains or other toxic dinoflagellate species, *Pfiesteria* spp. can cause human health effects. This possibility is being discussed later in this review. Nevertheless, recent studies in Maryland, Virginia, and North Carolina provided reassurance that in the absence of a bloom in which a particularly toxic strain is specifically identified, the routine occupational exposure to estuarine waters in which *Pfiesteria* is known to be present does not represent a significant human health risk [26,92,93].

33.4 ENVIRONMENTAL DETECTION OF *PFIESTERIA* SPP.

33.4.1 Isolation and Culture

The initial discovery and subsequent detection of *P. piscicida* relied on microscopic and culturing techniques [15]. Later on, the identification required the resolution of the thecal plate structures by SEM (see above) [6,38,48]. The classic technique for isolation and cloning free-living unicellular microorganisms including dinoflagellates has been widely used for *Pfiesteria* spp. in various research laboratories. For heterotrophic dinoflagellates, it is a combination of single cell isolation by limited serial dilution or micropipetting and culturing the separated single cell in medium with algal prey [66,94]. Further, it has been established that some heterotrophic dinoflagellate species do not graze well under axenic conditions, because they may require the presence of symbionts [95–97]; therefore, the *Pfiesteria* culture media lack antibiotics.

In 2001, the so-called "gold standard" method for isolation and cloning of "toxic" *Pfiesteria*-complex was established [98]. The three-step process starts with observation of the field samples under the light microscope, followed with seeding of the possible *Pfiesteria* containing water sample and sediment into a bioassay tank containing live fish. If fish deaths are detected within 3 weeks, the dinoflagellates are isolated for examination of the thecal plate structure using SEM. Because *Pfiesteria* cells have to be maintained in the presence of live fish to retain its toxicity, the culture on algal prey should be reexposed to fish in bioassay aquarium after every 4–6 weeks of algae feeding. One alternative to manual cloning is to use sorting systems (The AutoClone, Beckman Coulter, Inc., Fullerton, California); those systems allow precise and rapid sorting of single cell into multiwell microculture plates. This procedure, described for obtaining "axenic clonal" cultures of toxic *Pfiesteria* spp., does not produce "sterile clonal" isolates since *Pfiesteria* still carries bacteria after the treatment [96,97,99]. In addition, in order to maintain *Pfiesteria* cultures, prey must be added, thus, "clonal cultures" for *Pfiesteria* spp. are formally defined as being derived from isolated *Pfiesteria* cells with symbiont bacteria, together with added prey (in PICWG documents, 2000). Nevertheless, to identify *Pfiesteria* in environmental samples and to obtain its clonal culture using either classic or "gold standard" methods are labor intensive and require a high degree of expertise and a relatively long period of time.

In the United States, the Provasoli-Guillard National CCMP, which is an integral part of the Bigelow Laboratory for Ocean Sciences, curates the national culture collection of marine phytoplankton [100]. The CCMP has 2242 strains from around the world, mostly marine phytoplankton, but also benthic, macrophytic, freshwater, and heterotrophic organisms. Regarding *Pfiesteria* spp., the collection includes 18 clonal isolates distributed in 13 *P. piscicida* isolates and 5 *P. shumwayae* isolates; in addition, there are 50 of *Pfiesteria*-like dinoflagellates. All heterotrophic dinoflagellates in the CCMP collection are maintained in standard marine phytoplankton culture media with algae prey [66]. The collection's web page (http://ccmp.bigelow.org/) offers the search for strains using taxonomic, geographic, and other parameters. Strain records provide information on collection and isolation, including a map of collection site, medium recipes, and growth conditions for culturing, photographs, GenBank accession, and the taxonomic database links (as on December, 2006 search).

33.4.2 Molecular Approaches

The use of nucleic acid probes to detect the presence of specific target sequences in complex sample mixtures is an established and rapidly expanding technique. Species-specific DNA probes have been developed from ribosomal sequences that provide extremely accurate, and rapid diagnostic tools for the evaluation of clinical and environmental samples. Especially in combination with the PCR, these probes are invaluable tools for the rapid detection and identification of an increasing number of etiological agents both in routine and research applications. PCR offers sensitivity and specificity beyond standard microscopy and immunological methodologies, which can be more time-consuming and subject to problems of misinterpretation and cross-reactions with related species. The sensitivity

of PCR for detection of trace quantities of foreign DNAs in heterogeneous samples has made this technology an ideal choice for identifying infectious agents and has been used with great success to screen protozoan pathogens in aquaculture [41]. The ability of a PCR assay targeting DNA to distinguish among genetically related species and subspecies has been documented in other marine organisms. Similar studies with the pink barnacle [101], red snapper [102], blue mussel [103], and calanoid copepods [104] all provide evidence for genetic drift among closely related populations based on small differences in nucleotide sequence of the genome.

Within the dinoflagellates, the first molecular probes were developed for *Alexandrium* spp. [105–108] and for *G. polyedra* [109]. These probes targeted rRNA sequences, but their application has not been widespread. For the vast majority of dinoflagellate species, molecular probes are not available. A number of alternative strategies are available for the development of diagnostic probes and include the use of specific nucleotide sequences from rRNA, mitochondrial DNA (mtDNA) [110], or other conserved sequences such as those from structural genes like actin. In addition, other noncoding regions such as repetitive DNA sequences, microsatellite DNA [111], or even random sequences have been used for species-specific discrimination [112,113]. Probe development generally involves the use of universal primers to amplify specific DNA from isolated organisms. The sequences from these PCR products can then be used to design species-specific primers that will detect a particular organism in mixed populations from environmental samples.

Since their discovery, *Pfiesteria* spp. have been a concern because of the proposed association with massive fish kills and human health problems. Consequently, much of the research was directed toward development of sensitive and species-specific environmental detection methods for *Pfiesteria* spp. to prevent potential health risks associated with exposure. Because morphology of *Pfiesteria* spp. resembles other estuarine dinoflagellate species, particularly *Karlodinium* spp., the microscopy-based methods used of field identification were not reliable. Conversely, the detection of toxicity to fish using the abovementioned "gold standard" bioassay for *Pfiesteria* spp., is labor intensive and requires experienced personnel with specialized equipment and a complex experimental setup. Therefore, molecular approaches were introduced for *Pfiesteria* detection. The qualitative or semiquantitative PCR-based methods targeting rDNA genes reduced the analytical time to 1–2 days, depending on the DNA extraction method used before PCR amplification [52,114,115]. These were soon followed by the development of RT-PCR, which further reduced the sample analysis time [116]. When commercial DNA extraction kits are combined, the diagnostic time for water samples could be as little as 2–3 h from the sample arrival at the laboratory.

Progress in the development of molecular detection methods for *Pfiesteria* spp. was rapid. Various methods using different technique(s) and target gene(s) such as regular PCR [52,114,115, 117,118], heteroduplex mobility assays [52], fluorescent in situ hybridization, RT-PCR [116], denaturing gradient gel electrophoresis (DGGE) [119], and PCR-fluorescent fragment detection [119] were developed. Targets for amplification genes were most likely SSU rDNA gene but also the nontranscribed spacer (NTS) [115] and the internal transcribed spacer [117] of the rDNA genes, and mitochondrial cytochrome *b* [118] genes. In addition, using RT-PCR for amplifying the cytochrome c oxidase subunit 1 and cytochrome b transcripts [72] can enable not only identification of *Pfiesteria* but also evaluation of the viability of environmental cysts. However, molecular assays aimed at evaluating toxicity of *Pfiesteria* spp. are not yet available.

The molecular detection of dinoflagellates from environmental samples can be complicated by both the presence of coextracted polymerase inhibitor(s) in the sample DNA and false positives derived from unspecific amplification. Therefore, our approach was to develop a competitive PCR targeting the NTS rDNA, a spacer known to be less conserved than the other regions of the rRNA genes, and very useful for developing specific PCR-based assays [115,120]. In this method, an internal standard, a plasmid containing the same priming sites of the target but generating an amplicon of size different from target, is added to the PCR reaction mixture to identify potential false negative results. Moreover, because both target gene and plasmid are amplified with the diagnostic primers, this methods enables quantification of the number of targets/cells in the original sample from

the relative amount of the coamplified products, target, and competitor, based on over the range of coinoculated competitor plasmid. This assay has been very successfully applied to field samples and only requires basic research laboratory equipment [115].

The development of molecular detection assays for *Pfiesteria* spp. enabled high-throughput sample analysis, thereby facilitating extensive environmental monitoring in the United States and throughout the world. Thus, although some of the most notable blooms have occurred in the East coast of the United States, between Delaware and North Carolina, *Pfiesteria* spp. has been found worldwide. Blooms have been reported in estuaries and costal rivers and sounds as well. They are not generally found in either open water or inland rivers/lakes. The use of molecular monitoring has significantly contributed to assessing rigorously the distribution range of *Pfiesteria* spp. confirming their presence between northward to Rhode Island and New York, and southward to Texas and Mississippi on US east coast region [121–123], and Norway [53] and New Zealand [124].

Many dinoflagellate species, including *Pfiesteria* spp., form resting cysts as part of their life cycle. *Pfiesteria* spp. have two cyst types commonly observed in laboratory culture [17, 24, 71]. For several species, dinoflagellate blooms have been linked specifically to the germination of resting cysts [125–128], suggesting that "seed banks" of dinoflagellate cysts play an important role in bloom initiation [129]. Therefore, the detection of dinoflagellate cysts in environmental sediment to map the location of natural "seed bank" of toxic species is critical for understanding future harmful blooms. As pointed out earlier, molecular methods offer an efficient alternative to microscopic identification of dinoflagellate cysts in environmental sediment samples. However, molecular analysis of sediments samples has problems inherent to the relatively low cell density in the sediment sample, and the viability of the cysts detected. One approach is to induce the excystment before PCR amplification as an indirect measurement of the cyst viability [75]. Another alternative has been to enumerate cysts by targeting specific messenger RNA transcripts by RT-PCR, allowing only viable cysts of *P. piscicida* to be detected [72].

33.4.3 Alternative Detection Methods under Development

As alternatives to the molecular approaches discussed above, several detection methods are currently under development. These include an electrochemical-based assay [130] and antibody probes for cell-surface epitopes [107]. The use of lectins (carbohydrate-binding protein) to distinguish *P. piscicida* and *P. shumwayae* from other *Pfiesteria*-like dinoflagellates in environmental samples is another alternative method suitable for field monitoring. This method relies on ability of lectins to bind specific sugar-chain structures on cell surface. The "lectin western blot" applied to cultured dinospores, including *P. piscicida*, *P. shumwayae*, *Prorocentrum minimun*, *Pr. micans*, *K. veneficum*, *Amphidinium carterae*, *Karenia mikimotoi*, and *Rhodomonas* spp., suggested that lectins from *Galanthus nivalis* (GNA), *Sambucus nigra* (SNA), and *Arachis hypogaea* (peanut, PNA), may be useful for species discrimination. Lectin immunofluorescence analysis on paraformaldehyde fixed dinospores with the β-galactose-binding lectin PNA revealed a distinct staining spotted pattern on *P. shumwayae*, not observed with *P. piscicida* [68]. The application of lectin(s) as a tool for live whole cell identification and selection is promising, though it is not yet fully developed.

Actin and tubulin are abundant cytoskeletal proteins in all eukaryotic cells and in eubacteria. The cell cortex formed by network of actin filaments and various actin-binding proteins interacts with various myosins to control cell-surface movements, such as the formation of long, thin microspikes or sheet-like lamellipodia, or they can pull the plasma membrane inward to divide the cell in two [131,132]. In dinoflagellates, actin probably plays a role in intracellular transport, cell organization, and cell division in the same manner as in other unicellular eukaryotes, which was confirmed by whole cell histochemistry analysis using rhodamine-phalloidin. Moreover, especially with its nonphotosynthetic species, such as *Pfiesteria* spp., a peduncle for attachment, and osomotrophic and phagotrophic processes is a most likely form of extended plasma membrane supported by a flat web of actin filaments. On the other hand, the dynospore flagella are constituted by microtubules,

which are linear polymers of tubulin. Therefore, the assessment of relative actin and tubulin expression was proposed as a useful approach for the detection of *Pfiesteria* cysts and dinospores. Indeed, tubulin expression is minimal in cysts but abundant in dinospores, as measured by western blot and histochemical analysis using anti-ß tubulin universal antibody [68].

33.5 BIOASSAYS FOR TOXICITY ASSESSMENT OF *PFIESTERIA* SPP.

Optimization of the initial fish bioassay designed to analyze the potential association of fish kills with toxin production by *Pfiesteria* spp. led to the so-called "standard fish bioassay" described above [4,15,133] as well as a variety of bioassay formats in aquaria, culture flasks, and plates that used a variety of finfish and shellfish species. To address the need for more sensitive and rigorous biotoxicity assays and mechanistic aspects of the proposed toxic activity *in vitro* bioassays using genetically engineered cell lines were established. Finally, a murine model was adapted to assess the possible links of *Pfiesteria* toxic exposure to the neurological and behavioral impairments reported. Further, due to concerns about potential exposure to aerosols containing *Pfiesteria* toxin(s), believed to cause serious human health problems [2,16,134], the use of biosafety level 3 facilities and the design of the pertinent protocols for assessing *Pfiesteria* toxicity were strongly recommended [22,96,133,135].

33.5.1 *In Vivo* Toxicity Assays

33.5.1.1 Biotoxicity Assay Formats Using Fish and Shellfish

Aquarium assay format: This assay format has been widely used in *Pfiesteria* studies to assess toxicity of *Pfiesteria* spp. or its derived products by exposing fish to either *Pfiesteria* spp. containing environmental sediments, water samples, or clonal *Pfiesteria* cultures. Several formats and variants of the fish bioassay have been reported that differed in the use of various experimental fish and shellfish species (tilapia, hybrid striped bass, southern flounder, eel, menhaden, sheepshead minnow, blue crab, bay scallop, and eastern oyster), and assay conditions, including salinity, the size, age, and density of fish, aquarium size, and time course [4,15,17].

Because no *P. piscicida* toxin had been rigorously identified, purified, or characterized, Marshall et al. [22] characterized the "gold standard" fish bioassay as the only reliable technique available for assessing the *Pfiesteria* toxin production. However, this study also reported for the first time a brief characterization of the microbial community present in the bioassay setup, which included fish pathogens such as *Vibrio* spp. and *Aeromonas* spp. Thus, although during the following year several publications documented the standardized fish bioassay procedure thoroughly [23,24,81,98], the presence of a complex and dynamic associated microbial community in the bioassay setting hindered the unambiguous establishment of cause–effect relationships linking *P. piscicida* and fish death. We examined this aspect of the aquarium format bioassay using molecular methods to analyze the microbial communities associated to aquaria containing environmental water and sediment, and established not only that fish deaths occurred as described in studies reported earlier [23,24,81,98], but also the presence of diverse and dynamic microbial assemblage that included *Pfiesteria* spp. [136]. Fingerprinting analysis revealed the presence of potential fish pathogens, thus preventing the rigorous identification of *Pfiesteria* spp. as the only cause of fish death. Cell-free water from the bioassay aquaria failed to show ichthyocidal activity in flask and plate bioassay formats with adult and larvae fish (see below), respectively, and, moribund and dead fish from assay aquarium did not have the characteristic "pushed out" skin lesions that are known as the signature indicator for *Pfiesteria* fish kills by histopathological examination [4,15,16]. Interestingly, fish larvae introduced into the aquaria in "cage," containers sealed with membranes with different pore sizes, died at higher rates when the mesh size was larger than *Pfiesteria* spp. dinospores. Those results suggested that direct contact with organism(s) and/or molecule(s) larger than 10 μm could be responsible for the fish death. However, a 10% increase in fish death relative to the controls was

observed in cages fitted with a membrane through which only soluble factors could diffuse, suggesting that a soluble factor(s) present in the bioassay may have a limited, yet significant, contribution to the overall fish mortality observed. However, no evidence was obtained that would support attributing its source to *Pfiesteria* spp., or to any particular component of the bioassay microbial flora. Thus, although extensively reported in the literature, the aquarium format bioassay may be not suitable for rigorous assessment of toxic production and ichthyocidal activity of *Pfiesteria* spp. [136].

Flask assay format: The recommended use of biosafety level 3 protocols and facilities, together with the need for including enough replicates in the experiments in this limited space setting, led to the development and optimization of a scaled-down bioassay format in which fish are exposed to clonal dinoflagellate strains or environmental consortia (e.g., environmental water or sediment samples) in 750 mL culture flasks [137]. This bioassay format provides higher throughputs, and generally a shorter experimental time course than the aquarium format assay. Environmental variables including water quality (pH, dissolved oxygen, ammonia, nitrate and nitrite concentrations), temperature, and salinity were monitored, and the effect of the presence of fish on the proliferation and compositions of protist (dinoflagellate, protozoa, diatom) and bacterial populations and the time of fish death could be assessed in detail. The results indicated that the flask assay enables the assessment of acute effects of ichthyocidal dinoflagellates during the first 10 days of the experimental course. Fish deaths during the subsequent 10–20 days may be attributed to the proliferation of ichthyocidal dinoflagellates, pathogenic bacteria, and deteriorating water quality, whereas those beyond a period of approximately 3 weeks can be, most certainly, attributed to deteriorated water quality. Thus it was concluded that the flask format assay should prove useful in monitoring blooms for the presence of *P. piscicida* and other dinoflagellate species potentially harmful to fish, when implemented with cautious interpretation [137]. It should be noted that *K. veneficum* did not proliferate under this flask format assay as it did not under the tank format assay, and hence the formats are highly selective in their species proliferation.

Plate assay format: The need for more reproducible, sensitive, rapid, and high throughput biotoxicity assays for *Pfiesteria* toxicity led to the miniaturization of the above formats by using finfish or shellfish juveniles or larvae, or small invertebrates in multiwell plates. For this assay format, usually live dinospores at predetermined cell densities, culture supernatants, or fractionated dinospore extracts are placed in multiwell plates in direct contact with the test fish or invertebrates, and their behavior or time to death monitored throughout the experimental time course. This method allows conducting biotoxicity experiments with the required multiple replicates. Because of small assay volume, however, except for establishing the starting water quality conditions, no-water monitoring is usually conducted, and the analysis is completed within 24–48 h. Larval or juvenile sheepshead minnow (*Cyprinodon variegatus*), a resilient fish species easy to acclimate to a wide range of salinity, has been frequently used for biotoxicity assay [24,85]. Brine shrimp (*Artemia* spp.) [85], larval eastern oysters (*Crassostrea virginica*), and bay scallops (*Argopecten irradians*) have also been used in an attempt to shorten assay times, for some species, to ≤30–60 min [138]. We used a multiwell plate assay format to examine the toxicity of water from aquaria in which fish had been exposed to clonal *P. piscicida, K. veneficum*, or sediments containing *P. piscicida* cysts, and to directly expose fish larvae to dinoflagellate dinospores. For *Pfiesteria* spp. the former had no effect on the target fish, whereas the latter indicated that the effects on behavior or death observed with clonal *P. piscicida* culture were directly proportional to dinospore cell density to which the fish were exposed, but the cell-free supernatants had no effect. Under the microscope, it became clear from the dinospores clustered around the mouth, eyes, and operculi that fish health may be affected by their direct interaction with skin, gill epithelia, or mucous surfaces, rather than by the effect of *Pfiesteria* soluble components [136]. Similar results were reported by Berry et al. (2002) using *P. shumwayae* that concluded that *Pfiesteria* is able to kill fish by physical contact rather than releasing by a toxin into the water [18]. An alternative method to test for the release of soluble toxins by *Pfiesteria* is to place dinospores and fish in the same assay container, separated with permeable membrane inserts to prevent physical contact of dinospores with fish, which allows fish to be exposed to only *Pfiesteria*

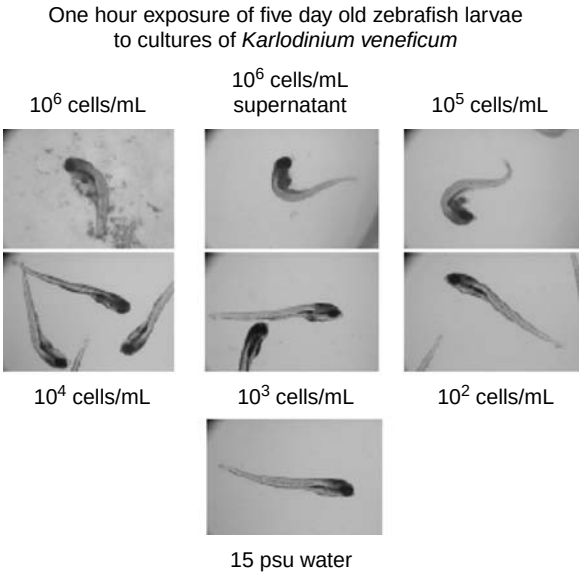


FIGURE 33.6 *In vitro* exposure of zebrafish larvae (5 day old) to cultures of *K. veneficum* (CCMP 1974). The 1×10^6 cell/mL was obtained by centrifuging the 1×10^5 cell/mL at $500 \times g$ for 10 min. The zebrafish larvae were dead (no heart beat) in the exposures from the 1×10^4 cell/mL to the 1×10^6 cell/mL including the supernatant from this culture within 1 h.

exotoxins [20]. In contrast, exposure to cultures (and supernatants) of *K. veneficum* at cell densities above 1.0×10^3 cells/mL killed all larvae within 1 h (see Figure 33.6) [139,140].

33.5.1.2 Biotoxicity Assay Formats Using Mammalian Models

To examine the possible cause–effect relationship between human exposure to *Pfiesteria*-derived materials and the reported neurobehavioral impairments, a rat model was developed [89]. Using a radial-arm maze, learning and memory was examined in rats exposed to *Pfiesteria* extracts. Although it was proposed that individuals were affected by *Pfiesteria* toxin(s) most likely by breathing aerosol in the estuarine environment, possible ingesting of water, or by transdermal absorption by direct contact with contaminated water, the actual route(s) of exposure remained uncertain. Therefore, the subcutaneous injection of *Pfiesteria* cells or toxic bioassay aquarium water to experimental animals was chosen to ensure the delivery of a reliable toxin dose. Abnormal behaviors caused by injection of the abovementioned *Pfiesteria*-derived materials were reported to be relatively specific to the acquisition phase in the training procedure. When rats were pretrained, *Pfiesteria* treatment did not affect performance. Nevertheless, factor(s) affecting rat performance in the radial-arm maze remain unknown, because the purified *Pfiesteria* toxin has not been available.

33.5.2 *In Vitro* Toxicity Assay Formats

Cell-based *in vitro* assays do not require prior knowledge of the initial cellular target for the toxin, and therefore, they are the method of choice for screening samples of unknown toxicity such as *Pfiesteria* spp. extracts or culture supernatants. The GH_4C_1 rat pituitary cell line used for those assays has been applied to characterize signaling pathways for a variety of first messengers [141]. The assay detects signals from living cells, and it is dependent on their degree of activation. This method can therefore be used to measure cytotoxicity, proliferation, or activation. The algae-derived toxins, including water samples possibly containing *Pfiesteria* toxin(s), cause an increase in ionic conductances and an elevation of cytosolic free calcium in GH_4C_1 cells [142,143]. For *Pfiesteria*

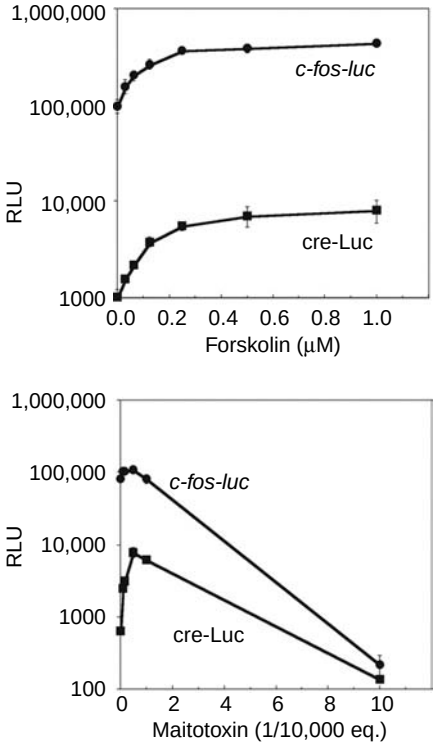


FIGURE 33.7 Concentration dependence of luminescence to forskolin and maitotoxin of *c-fos-luc* and pCRE- Luc stable transfectant cell lines. The maitotoxin was a semipurified fraction obtained from the Marine Biotoxins Program (Charleston, South Carolina). The curves for the two different response elements were nearly identical except for a higher basal expression of luciferase with the *c-fos-luc* cell lines. RLU, relative luminescence units.

toxin, cytotoxicity was measured in a microtiter plate using the mitochondrial indicator 3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide (MTT) [144]. GH_4C_1 cells (30,000 cells per well) in 100 μL of appropriate media and 2 μL of methanol extracted test samples are incubated for 24 h, and then 15 μL of MTT (5 mg/mL phosphate-buffered saline) are added for determination of cell survival. Mitochondrial dehydrogenases in live cells convert the MTT to an insoluble formazan crystal, which is solubilized by the addition of 1% sodium dodecyl sulfate in 0.1 N HCl, resulting in a purple color that indicates toxic activity of the samples. The cytotoxicity assay is regularly used for first screening of *Pfiesteria* toxic activity.

The luciferase reporter gene assay [145,146] was also used to confirm the results of first assay for *Pfiesteria* biological activity. This modified cell-based assay changes the end point from the mitochondrial indicator for toxicity (MTT-dye based assay) to specific gene reporters [145]. This assay uses a cell line that stably expresses reporter gene constructs, which contains the entire c-*fos* regulatory region gene ligated to the coding region of reporter molecule such as firefly luciferase. The c-*fos* gene, which immediately responds to algae-derived toxins in neuronal cells expressing c-*fos* [147,148] and in whole animal models [149], has been used as a biomarker. The c-*fos* promoter has multiple interdependent transcription control elements [150] and is thus prone to interference from other potential signaling molecules such as serum-derived factors. By using only the cyclic AMP responsive element one can target responses to those molecules that either activate adenylate cyclase, promote increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$), or cause increased phosphorylation of the cAMP response element binding protein (see Figure 33.7). The GH_4C_1 cell line expresses the purinergic P2X7 receptor and is thus susceptible to adenosine

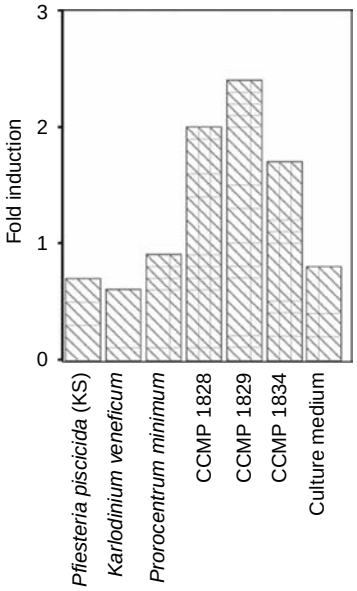


FIGURE 33.8 Activation of pCRE-Luc cell lines to culture media from recent isolates of *Pfiesteria piscicida*. The cell densities in all exposures were 1×10^3 cell/mL.

triphosphate (ATP)-induced cell death. The c-fos-luc and pCRE-Luc transfectant cell lines respond to specific algal toxins (e.g., maitotoxin) by producing luciferase in a concentration-dependent manner (Figure 33.7). The serum responsiveness has been eliminated with the pCRE-Luc cell lines (place unpublished). Attached GH_4C_1 c-*fos-luc* and pCRE-Luc transfected cells (30,000 cells per well) are treated with samples for 4 h at 37°C. After removal of experimental media from the well, cells are lysed with 20 μL of cell lysis buffer, and then the luminescence generated from each well with added luciferase and ATP in the luminometer is measured. The sensitivity of the reporter gene assay could not be precisely determined because purified *P. piscicida* toxin standards were not available, and the assay was calibrated based on *P. piscicida* cell number. Dose–response curves yielded a bell-shaped response, with incremental increases in luciferase activity, until inhibition of luciferase activity presumably due to cytotoxicity co-occurs (Figure 33.7). Cell-based biotoxicity assays are usually carried out parallel with the fish bioassay standard method described above to confirm that the *Pfiesteria* cytotoxic activity is associated with its ichthyocidal properties. *Pr. minimum* and *K. veneficum*, the two dinoflagellates isolated from the Chesapeake Bay, failed to activate luciferase expression (Figure 33.8). However, recent isolates obtained from CCMP containing *P. piscicida* induced nearly a twofold activation of luciferase expression. The reporter gene assay provides a rapid and sensitive screening for biotoxic activity, and has been used to monitor toxic cultures and environmental samples, and potentially guide *Pfiesteria* toxin purification. However, it has primarily been the cytotoxicity assay of GH_4C_1 cells that has been used in for guided purification of *Pfiesteria* spp. toxins [25].

33.6 ONGOING INITIATIVES FOR IDENTIFICATION AND CHARACTERIZATION OF TOXINS FROM *PFIESTERIA* SPP.

Perhaps, the most critical aspect of the *Pfiesteria* controversy is the source and structure of the toxin. Part of the problem is that it was initially claimed that *Pfiesteria* must feed on fish to produce the toxin [17]; yet recent data clearly shows the activity is present in algae-grown strains [25]. Second, the toxic activity was originally described as hydrophobic (lipophilic) and of high molecular weight (>10,000 Da) [151]. As noted before, the fish bioassay tanks contain numerous organisms, some

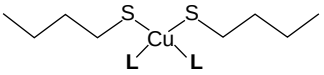


FIGURE 33.9 Putative *Pfiesteria* toxin molecular framework. The approximate mass of this framework is 311 amu with a range up to greater than 700 amu. The L stands for ligands of diverse nature.

of which are identified pathogens, which makes attribution of a toxic compound from this complex matrix impossible [136]. A putative phalate ester toxin was isolated from these cells [85], but turned out to be a plasticizer contaminant originating from the instant ocean media used to grow the *Pfiesteria* cells [85]. The next partial structure announced for the *Pfiesteria* toxin was at the tenth annual HAB and are summarized in an article in *Science* to be "…a glycoside, a molecule that's half sugar, half some other chemical group that has not been identified" [87,152]. The final structure of this toxin has never been released.

At about the same time, others groups began investigating the toxicity of *Pfiesteria* using alternative approaches. One group examined organic extracts of lyophilized *Pfiesteria* cells, all of which failed to kill larval fish [18]. Since most known algal toxins have a polyketide structure, other researchers used molecular primers to demonstrate that at least *P. shumwayae* most likely lacked the polyketide synthases needed to synthesize this class of toxins [18].

Most recently the *Pfiesteria* toxin was announced to be a ligated copper compound with numerous congeners [25] (see Figure 33.9). It was hypothesized that the "rapid, free-radical-mediated toxicity of *Pfiesteria* toxins may occur via production of a redox-cycling metal center and free radical(s) that can lead to specific reactions with 'pro-toxins' which, in turn, can produce more active toxic species." Under this scenario sunlight and metal exposure are the two primary environmental factors that would combine to initiate *P. piscicida* toxicity during toxic algal blooms. "Light exposure could initiate redox-cycling of the metal ion(s) resulting in radical formation and release of the toxin species." Unfortunately, no direct evidence that free radical formation actually takes place in estuarine water was presented and given the high organic load in these waters it seems unlikely that the reaction distance would be very great. More importantly, no dose–response data were presented to confirm the concentration required to kill fish nor was it shown that this putative material would activate the *c-fos-luc* assay described earlier. Without these data it is not possible to rigorously establish the environmental relevance of the toxin. Toxicity was inferred primarily from the work presented by Burkholder et al. (2005) [73].

There are several inconsistent observations in Burkholder et al. (2005) paper [73]. The first issue is the toxin cell quota of *Pfiesteria* toxin. *Pfiesteria* spp. have approximately 100 pg total carbon per cell. Yet the reported toxin concentrations for several of the "toxic" *P. shumwayae* cultures was on the order of 43–251 pg/cell or 0.5–2.5 X the total C of a *Pfiesteria* cell. Given that this activity is claimed to be an exotoxin, such high toxin cell quotas is unprecedented. This would provide nearly 200 mg of toxin from a 90 L culture of 10,000 cells/mL. Next, the toxicity of the extracts was reported as time of death of a fish exposed to 50 μL of *Pfiesteria* toxin [73]. This raises the question as to why when the toxin had been quantified, the volume of toxin added was reported instead of the actual concentration of toxin as would normally be done in a dose–response assay. Also, the activity of the *Pfiesteria* toxin as measured by cytotoxity to cultured tissue cultures was nonexistent in cells grown on algae, but high in cells grown on fish (Figure 3 of Burkholder et al. (2005) [73]) yet the chromatogram shown in Figure 33.4 of the original online version of Burkholder et al. (2005) [73] showed that the noninducible strain CCMP 1832 grown on algae had the same peak as the toxic 2089 strain grown on fish. Moeller et al. (2007) [25] purified the toxin from cultures grown on algae.

Standard fish bioassays were used to assess the killing ability of *Pfiesteria,* which allowed direct contact between fish and *Pfiesteria,* making it impossible to determine what portion of the observed fish mortalities were due to micropredation versus and the actual production a toxin. As discussed above, over 90% of the observed mortality is likely due to micropredation [20]. Another aspect of the study was the use of plating techniques and DGGE gel techniques to assess the bacterial similarity

or difference between the control and experimental treatments where fish mortality was occurring. The goal was to demonstrate that no one bacteria was unique to the cultures where *P. piscicida* or *P. shumwayae* were killing fish. Unfortunately the plating techniques used are only capable of supporting the growth of less than 2% of estuarine bacteria. Since 98% or more of the bacteria will not show up using this assay, any similarities or differences noted between treatments are meaningless. The premise behind the DGGE experiment is that the absence of a unique randomly amplified 16S band in the killing cultures would indicate that bacteria are not involved in the production of the toxin. Unfortunately, DGGE tends to picks up only the more abundant bacteria. Many species present in significant densities are not picked up by this method. The lack of a uniform band cannot therefore be used to exclude bacteria as a potential source of the toxin as implied in the paper. This leaves the potential bacterial source of the toxin in limbo. Although given that *Pfiesteria* species can be grown axenically on fish cells, it is critical to demonstrate that the toxin can be isolated from these cultures. The study also shows cytotoxicity data from two cultures of *P. shumwayae*. One culture showed no activity at all and the other marginal activity likely within the noise level of the assay (Figure 33.3 of Burkholder et al. (2005) [73]). No 16S ribosomal DGGE data are presented to confirm that these cultures are actually axenic (bacteria free) despite the fact that the techniques were used to assay bacterial abundance in other samples. It is on the bases of these data that it was concluded that bacteria-free *Pfiesteria* cultures produced toxins, thereby eliminating bacteria as the source of the toxin.

Another inference in the paper is data showing that *Pfiesteria* cell densities at a fish kill can be high (>1000 cell/mL). The samples analyzed in Burkholder et al. 2005 [73] were collected in 1998 and 2000 and analyzed years later. These samples stored in Lugols will have gradually deteriorated over time implying that the cell concentration were even higher than reported. Interestingly these are the only quantitative PCR data published from fish kill sites and are several orders of magnitude higher than previously reported by other investigators. Moreover, the 2000 sample obtained from a fish kill recorded in Arnell Creek, Delaware, had already been shown to contain large numbers of *K. veneficum* by PCR.

These data indicate that much research remains to be done in order confirm the structure and source of "*Pfiesteria*" toxin, despite the fact that identical ^{13}C MNR spectra were shown from standard fish bioassay tanks and in the toxin description paper [25,73]. Clearly a bioactive compound can be extracted from these cultures given that some unknown concentration was able to cause epithelial ulcerations (Figure 5 of Burkholder et al. (2005) [73]). The most important remaining question, however, is whether this bioactive compound is environmentally relevant and unique to *Pfiesteria*. The answer is likely to be no for the following reasons: (a) cell densities in nature are generally low (<2 cell/mL) [153] meaning that that there are too few cells generally present to cause a problem even if the toxin proved highly potent, (b) the histology of lesioned fish from fish kill sites does not mimic that seen in the laboratory and is inconsistent with exposure to significant amounts of *Pfiesteria* toxin in the environment and (c) the toxin is very unstable, which severely limits the period of effective exposure. Not until quantitative dose versus mortality curves are established for the *Pfiesteria* toxin and the toxin is found to be present in the environment at quantities indicated by the dose mortality curve to be of concern can a *Pfiesteria* toxin be considered of consequence ecologically or toxicologically. Moreover, until a fish kill can be found in which *K. veneficum* is not observed and only *Pfiesteria* spp. are found can the "cell from hell" be implicated. *K. veneficum* fish kills, however, have met all of the above criteria.

33.7 ALTERNATIVE INTERPRETATION(S) OF THE AVAILABLE EVIDENCE

33.7.1 Co-occurrence of *Pfiesteria* Spp. and *Karlodinium veneficum*

With the successful development of PCR probes for *Pfiesteria* spp. and *K. veneficum*, routine screening of environmental water samples for these species has been ongoing for almost 10 years.

TABLE 33.2
Relative Abundance of *Karlodinium veneficum* and *Pfiesteria* spp. in Water Samples Screened with Species Specific Molecular Probes

	Percent of Samples Taken Positive with PCR Probe (2003)			
	Delaware[a] (Water Column)	Maryland[b] (Water Column)	South Carolina[c] (Sediment)	North Carolina[d] (Water Column)
Karlodinium veneficum	45.8%	39.6%	17.9%	55%
Pfiesteria piscicida	6.3%	1.07%	57.1%	31%
Pseudopfiesteria shumwayae	4.2%	0.0%	3.6%	0.0%

[a]Edythe Humphries DNREC; [b]Peter Tango, MDNR; [c]Alan Lewitus, SCNDR; [d]Wayne Litaker; NOAA

Table 33.2 summarizes the results for just the year 2003. As clear from this one year of sampling, *K. veneficum* is far more abundant and prevalent than *Pfiesteria* spp. in mid-Atlantic estuaries. A recent reexamination of 7000 phytoplankton samples taken by Maryland DNR from 1981 to 2001 found *K. veneficum* to be present in 18% of the samples [154]. Moreover, since 1998, *K. veneficum* has been documented extensively in the estuaries of North Carolina [155,156] while before 1998 it frequently was misidentified as *Pfiesteria*.

Moreover, when examined for co-occurrence, *K. veneficum* and *P. piscicida* co-occur more frequently than expected. A contingency analysis of 878 samples (ending February 2004) in which molecular detection for *Pfiesteria* spp. and *K. veneficum* in Maryland samples was performed found that *P. piscicida* co-occurred with *K. veneficum* more frequently than expected ($p = .009$, Fisher's Exact test). We believe that this co-occurrence is driven by a preference for a common prey item, crytophytes.

33.7.2 Natural History of *Karlodinium* Spp.

This small gymnodinioid dinoflagellate has been associated with toxic activity ever since its discovery in the 1950s [157] and has seen its name changed multiple times over the last 50 years. Until recently, the genus *Gymnodinium* F. Stein comprised a diverse assemblage of naked (unarmored) dinoflagellates. A recent comparative study using morphological features, particularly the outline of the apical groove, in addition to the composition of photosynthetic pigments and nuclear-encoded LSU rDNA sequences, clearly showed that *Gymnodinium* was polyphyletic [9] and two fucoxanthin-containing genera, *Karenia* Gert Hansen et Moestrup (with three species), and *Karlodinium* J. Larsen (also with three species) were proposed, and the description of *Gymnodinium* was emended [9]. Since then, the known diversity of *Karenia* has escalated to 10 species while the number of described *Karlodinium* species has grown to 4. A third fucoxanthin-containing genus, *Takayama* de Salas, Bolch, Botes et Hallegraeff, has been erected and shown to be related to *Karenia* and *Karlodinium* [158]. This lineage shares a single synapomorphic character relating to the chloroplasts (symbiont)—fucoxanthin and its derivatives as the major accessory pigments. One of the morphological characters separating *Karlodinium* and *Karenia* is the presence of a ventral pore and a unique type of amphiesma with plugs in *Karlodinium*:

Synopsis. *K. veneficum* (Ballantine) J. Larsen
Basionym: *G. veneficum* Ballantine [157]

Taxonomic synonyms: *K. micrum* (Leadbeater et Dodge) J. Larsen in Daugbjerg et al. (2000) [9], *G. galatheanum* Braarud sensu [159], and their nomenclatural synonyms: *Gymnodinium micrum* (Leadbeater et Dodge) Loeblich III, *Gyrodinium galatheanum* (Braarud) Taylor, *W. micra* Leadbeater et Dodge (1966) [160]. Daugbjerg et al. (2000) [9] discussed including *Gyrodinium*

corsicum Paulmier, Berland, Billard et Nezan in *Karlodinium* [9], but information on the amphiesma of this species was not available at that time. Also Hulburt described *Gyrodinium estuariale* from Great Pond and Salt Pond, Woods Hole, Massachusetts. to be very simlar to *Gymnodinium veneficum* (Ballantine).

Type locality: Hamoaze, over Rubble Bank, off King William Point, South Yard, Devonport, England.

Distribution: *K. veneficum* has been reported from the North Sea; British Isles; Oslo Fjord, Norway; Whangakoko, South Island, New Zealand; Swan River, Perth, Western Australia; St. Johns River, Florida, USA; Neuse River, North Carolina; Maryland: Princess Anne Co., Manokin River, Hyrock fish farm, Chesapeake Bay; South Atlantic Ocean: Walvis Bay, South Africa, and the eastern coast of Kamchatka, Avachinskay Gube Inlet, Russia. Owing to the similarity between the different species of *Karlodinium*, the reports need to be confirmed using molecular data.

33.7.3 Bioassays for Assessment of Toxicity of *Karlodinium* Spp.

The toxic activity is easily released from *K. veneficum* cells either by filtration and/or by centrifugation [139] (see Figure 33.6). However, under normal conditions greater than 90% of the toxin is cell bound [161]. A hemolytic assay based on the lysis of fish erythrocytes is routinely utilized to screen for karlotoxin in *Karlodinium* species [139]. Cultures and culture fractions that are positive in the hemolytic assay are tested further using assays for ichthyotoxicity and cytotoxicity as described above for *Pfiesteria* toxins. Erythrocyte suspensions are prepared as described in Edvardsen et al. (1990) [162]. Karlotoxins are extremely "sticky," binding to plastic, glass, and teflon in aqueous solutions. Elution with methanol can usually recover the bound toxin. Karlotoxins are also readily degraded by natural bacterial assemblages. An active glass fiber (GF/F) filtrate kept at room temperature for 24 h will have only 10% of the original activity remaining.

33.7.4 Identification and Characterization of Toxins from *Karlodinium* Spp.

In an attempt to determine the cause of repeated fish kills in an estuarine aquaculture facility in Maryland, we have shown that *K. veneficum* produces a unique suite of compounds with hemolytic, cytotoxic, and ichthyotoxic properties, which we refer to as karlotoxins (KmTx [139]). Thus far, we have been able to detect these compounds in clonal isolates collected from estuarine waters from the U.S. states of Maryland, North and South Carolina, Georgia, Florida [163], as well as isolates from New Zealand, Norway, and the English Channel. In addition, we were able to isolate these same toxins directly from water samples collected during fish kills in a South Carolina brackish water pond [140] as well as a fish kill in a tributary of the Chesapeake Bay. In both cases, high densities of *K. veneficum* were found. The principal toxin isolated from both cultured cells and directly from water samples collected during the fish kill in South Carolina (KmTx2), was similar but not identical to the main toxin isolated from cultures and the fish kill in Maryland (KmTx1) (Figure 33.10). Hence, two different toxin types occur in *Karlodinium* spp. from the U.S. Atlantic coast [163]. Toxins observed in Chesapeake Bay cultures and field samples differ from those found in strains south of the Chesapeake based on chromatographic profiles and UV absorption spectra [163]. All *Karlodinium* strains from Chesapeake Bay produce KmTx1 (λ_{max} 225 nm) while all *Karlodinium* strains south of the Chesapeake Bay produce KmTx2 (λ_{max} 235 nm). A genetic marker difference correlates with these toxin phenotypes [164]. Moreover, the level of toxin per cell equivalent at the fish kills is nearly tenfold higher than that observed in cultures.

Mass spectrometry has found a molecular mass of about 1322 Da for KmTx1 and about 1344 Da for KmTx2. Each of these species also has a +16 Da congener that copurifies on a C8 reverse phase column (Figure 33.10). Separation on an amine column under normal phase conditions separates these two congeners. We do not know at this time if both compounds are equally toxic or if the combination is synergistically toxic. Additional toxic compounds (e.g., KmTx4 and KmTx5, etc.), less active on a per weight basis than KmTx 1 and 2, are still in the process of being

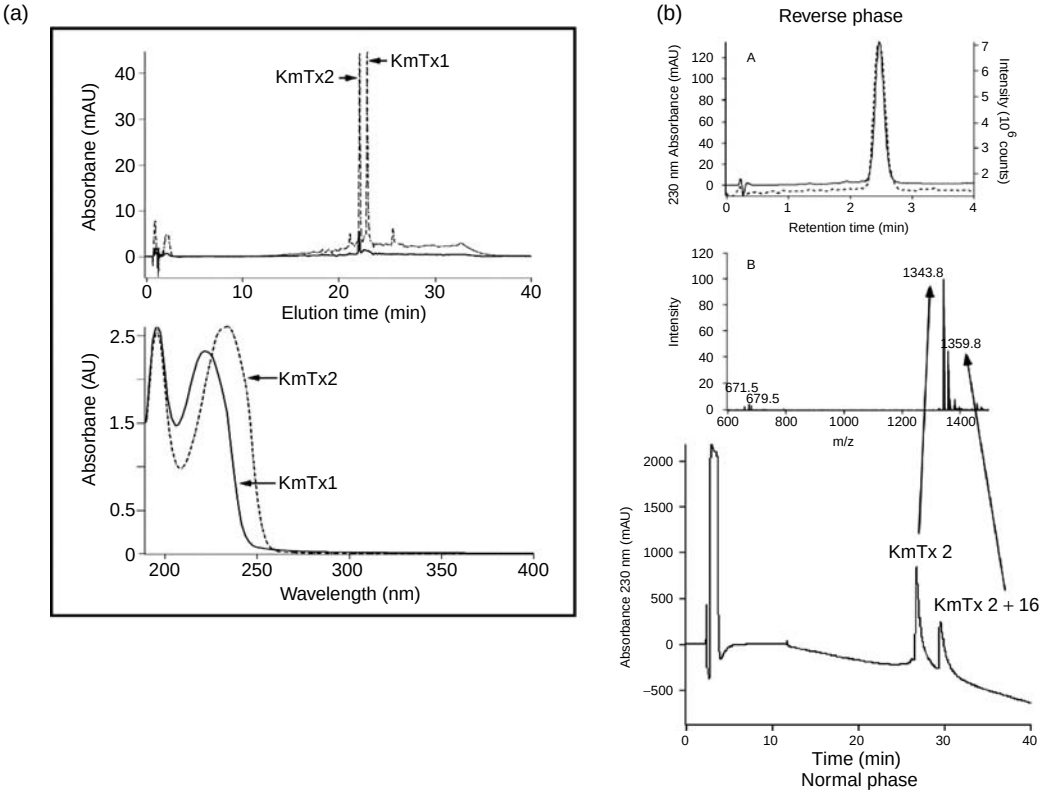


FIGURE 33.10 (a) Co-injection of purified karlotoxins (KmTx1 and KmTx2) on a reverse phase C18 column. (b) UV spectra of KmTx1 and KmTx2. Each karlotoxin is purified with a +16 congener which can be isolated on a normal phase (NH_2) column.

purified and characterized. On the basis of assays for hemolytic activity and ichthyotoxicity, KmTx1 appears to be more potent than KmTx2.

To assess the effects of karlotoxins on eukaryotic microorganisms, we conducted growth inhibition and cytotoxicity assays, respectively, on model fungal and dinoflagellate species. *K. veneficum* is immune to its own toxin showing no growth inhibition up to 1 µg/mL added toxin. We chose *Aspergillus niger* as a representative filamentous fungus and *Candida albicans* as a representative yeast species. Minimal inhibitory concentrations (96 h MIC) of 8 and 16 µg/mL KmTx2 were found for *As. niger* and *C. albicans*, respectively. Amphotericin B was used as a positive control in the fungal growth inhibition assays. For amphotericin B, 96 h MIC values of 0.5 µg/mL and 0.125 µg/mL were found for *As. niger* and *C. albicans*, respectively. Interestingly, in hemolytic assays, KmTx2 and amphotericin B showed the opposite order of potency, with hemolytic LC_{50} values of 0.37 µg/mL (range: 0.190–0.605) and 3.8 µg/mL (range: 2.07–7.86), respectively.

In the 1970s, the well known "barrel-stave model" was proposed for the amphotericin B ion channel in which about eight pairs of amphotericin B/sterol form the channel in a lipid bilayer. Its selective toxicity against microbes could be attributed to a higher affinity for ergosterol, the principle sterol in fungi, over cholesterol comprising mammalian plasma membranes. Amphotericin B is reported to have higher affinity for ergosterol than for cholesterol, which is the entire basis for its use as a treatment for human systemic fungal infections. We incubated fish erythrocytes with either cholesterol or ergosterol (0.001–10 mM) then tested for hemolysis by KmTx2. Both cholesterol and ergosterol began to inhibit KmTx2-induced hemolysis in the 10–100 nM range. For ergosterol, inhibition plateaued at about 1 mM, whereas inhibition increased with cholesterol concentration until complete inhibition was attained at about 10 mM [165].

In comparison, incubation with the common membrane lipid phosphatidylcholine [0.001–10 mM; both synthetic (16:0) and natural (from brain) dipalmitoyl] had no effect on hemolytic activity (data not shown). As described above, depending on the species tested, KmTx2 as an antifungal agent was less effective than amphotericin B by 10- to 100-fold. As a hemolysin, however, KmTx2 was more effective than amphotericin B by 10-fold. Taken together, these studies suggest that KmTx2 is more active against cholesterol-containing membranes than ergosterol-containing membranes. Thus, it was natural to ask what factors underlie this apparent immunity of *K. veneficum* to karlotoxin. For several cytolytic pore-forming compounds, membrane sterols play a critical role in toxicity. For example, the polyene antibiotic, amphotericin B, can bind to membranes devoid of sterols, but sterols in the target membrane are reported to be required for permeabilization leading to cell lysis. Similarly, incubation with either cholesterol or ergosterol inhibited the activity of prymnesins, potent hemolytic, and ichthyotoxic polyketides produced by the haptophyte *Prymnesium parvum*. Leblond and Chapman (2002) [166] recently found that the sterol profile of *K. veneficum* is dominated by (24S)-4α-methyl-5α-ergosta-8(14),22-dien-3β-ol (gymnodinosterol). This profile is almost unique—being shared only by the closely related dinoflagellates *Karenia brevis* and *K. mikimotoi*. We first confirmed that our *K. veneficum* cultures indeed have gymnodinosterol as their major sterol. We then asked whether this unusual sterol plays a role in protecting *K. veneficum* from the membrane-disrupting effects of its own toxin. Earlier, we showed that co-incubation with cholesterol or ergosterol inhibited KmTx2 hemolytic activity. In contrast, co-incubation with gymnodinosterol had no effect on KmTx2-induced hemolysis. We infer from these results that cholesterol and ergosterol can complex with KmTx2 and sequester the toxin away from erythrocyte membranes, and thus afford protection against hemolysis. Furthermore, simple phosopholipids and gymnodinosterol cannot complex KmTx2 and, therefore, offer no protection. These observations suggest that by utilizing a sterol that is structurally different from sterols found in the membranes of heterologous species, *K. veneficum* renders itself impervious to KmTx2. Further studies have shown that any organism that has des-methyl sterols will be sensitive to karlotoxin while those with predominantly 4-α-methyl sterols are insensitive [161,165,167]. *Pfiesteria* spp. are insensitive to karlotoxin [165].

The purified toxins reproducibly kill fish in a dose-dependent manner in laboratory assays targeting the gills [165]. The pathology observed in the laboratory-treated fish gills is similar to that observed in gills from fish kills in nature. This activity is similar to prymnesin, isolated from *Prymnesium parvum* [168,169] and to amphidinols, a family of hemolytic toxins isolated from the dinoflagellate genus *Amphidinium* [170]. The primary human impact of *Karlodinium* blooms is fish kill events, but it seems likely that KmTxs, like prymnesin may function as antigrazing or allelopathic compounds [161,171] as well as prey immobilization agents for mixotrophy.

33.7.4.1 Structural Characterization of *K. veneficum* Toxins

The structure of KmTx2 from *K. veneficum* (CCMP 2064) has recently been determined using high-resolution NMR on ^{13}C enriched material [172] and shown to have an amazing structural similarity to amphidinol (Figure 33.11). The molecular formula $C_{67}H_{121}ClO_{24}$ was deduced from detailed analysis of NMR data and the high-resolution ESI-TOFMS molecular ion series 1367.7844 $[M+Na]^+$ (calcd. 1367.7829, error −1.1 ppm), 695.3883 $[M+2Na]^{++}$ (calcd. 695.3866, error −2.4 ppm), in addition to the comparison with simulated isotope pattern data for this formula. The KmTx1 toxins do not appear to have a terminal chlorine. The absolute configuration of the 27 chiral carbons are currently being determined as are the structures for KmTx1 toxins. Molecular simulations are being conducted to determine the nature of the sterol specificity. An LC-MS method has been developed to detect these toxins in nature [164].

33.7.4.2 Search for Genes Involved in Putative Biosynthetic Pathways

Given the polyketide nature of karlotoxin and its amazing similarity to amphidinol, current efforts are underway to isolate components of the synthetic machinery from both genuses. This may be

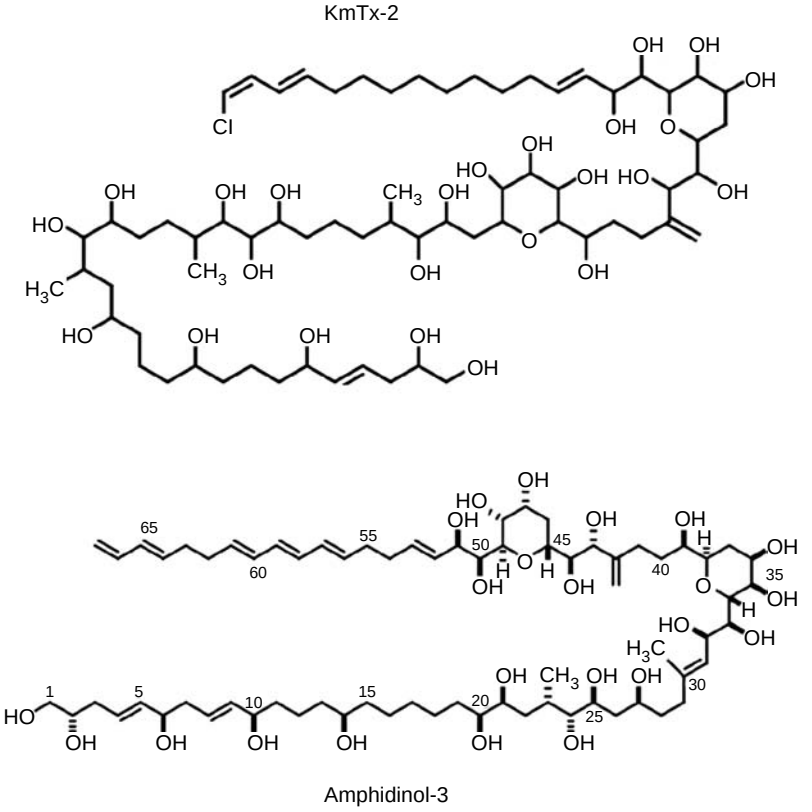


FIGURE 33.11 Structure of karlotoxin-2 (KmTx2) [172] compared to absolute configuration of amphidinol-3. Karlotoxin has 27 chiral carbons, 2 more than amphidinol-3.

extremely difficult given the recent findings of Kubota et al. (2004) [173], which indicates the genes for amphidinolide synthesis in *Amphidinium* spp. are not arranged in arrays like other polyketide synthases. A large-scale expressed sequence tag library is currently being developed for both *Amphidinium* and *Karlodinium* spp.

33.8 AN INTEGRATED HYPOTHESIS FOR THE TOXICITY OF THE *PFIESTERIA/KARLODINIUM* MIXED BLOOMS

On the basis of almost 10 years of published and unpublished work we envision the following scenario for the 1997 Pocomoke event. *K. veneficum* (which was present before the event) bloomed in response to an increased abundance of its cryptophyte prey. *Pfiesteria* spp. in the water column would also have increased in abundance because of this increase in prey abundance. Approaching nearly 10,000 cells/mL (the lethal level for toxicity), the *K. veneficum* bloom was grazed on by menhaden in the area. As the *K. veneficum* cells passed over the gill rakers karlotoxin was released and caused cell death of chloride cells and supportive cells in the menhaden gills. This damage caused osmoregulatory imbalance and inability to efficently absorb oxygen in the menhaden. The *K. veneficum* bloom crashed, lowered dissolved oxygen levels, and further stressed the menhaden, which began to die. The dead and dying menhaden induced excystment and proliferation of *Pfiesteria* spp., which then fed on the epithelia cells of the dying gill-damaged menhaden. When sampled at the time of the fish kill *Pfiesteria* spp. cell densities would have been greatly elevated. When this water sample was then taken through the fish standard bioassay the role of *K. veneficum* would not be evident because of the species bias of the assay.

For the last 2 years (2005, 2006) late summer fish kills associated with *K. veneficum* blooms on the Corsica River, an eastern-shore subestuary of the mesohaline portion of the Chesapeake Bay, have been recorded. In September 2005 approximately 50,000 dead fish were collected on the Corsica River associated with a *K. veneficum* bloom (upwards of 56,000 cells/mL and 229–1809 ng KmTx1/mL) (http://mddnr.chesapeakebay.net/hab/news_100505.cfm). The 2006 Corsica River fish kill (approximately 2000 fish, mostly white perch) contained *K. veneficum* cell densities from 8×10^3 to 100×10^3 cells/mL, and karlotoxin levels and 640–841 ng KmTx/mL, both of which are conditions expected to result in fish mortality [165]. The Corsica River is recognized by the environmental protection agency (EPA) as an "impaired" water body and Maryland state officials announced in September 2005 the Corsica River pilot project with the long-term goal of having the Corsica River removed from the EPAs "impaired" list. Water quality parameters including Chl *a* fluorescence (Maryland Department of Natural Resources' "Eyes on the Bay" program and nutrients (http://hpl.umces.edu/aimes/sitelocations) are continuously monitored in the Corsica River, and in the case of the 2005 *K. veneficum* event show a very high input of NH_4 preceding the bloom and fish kill. No *Pfiesteria* spp. were detected at either kill.

Similarly, the years 2005 and 2006 also saw significant, toxic blooms of *Karlodinium* in the Inner Harbor of Baltimore (Patapsco River, Maryland). The temporal duration of blooms observed in the Inner Harbor and elsewhere is typically on the order of days to 2 weeks. Karlotoxins KmTx1-1 and 1–3 were detected associated with this bloom, showing an average cell quota of 5.6 pg KmTx 1/cell (SD = 9.21) and a KmTx 1-1:1–3 ratio of 0.59 (SD = 0.100). Peak *K. veneficum* cell density was associated with reduced salinity and temperature, indicative of a local runoff event that *K. veneficum* was able to exploit for growth. *Karlodinium veneficum* has formed persistent blooms associated with fish kills in the Swan River Estuary upstream of Perth, Australia (S 31 57', E 115 52') during the austral autumn for the last 4 years. These blooms have occurred in the upper, highly eutrophic reaches of the Swan estuary during a time of year characterized by exceedingly dry conditions, high temperature and salinity (>25 ppt), generally low (but highly variable dependent on rainfall) surface inorganic N, high (>1 mM) surface inorganic P concentrations, and generally low bottom water oxygen levels [174]). Autumn is also the time of year when cryptophyte blooms tend to occur in the Swan River Estuary [175]. Before 2000, blooms of *K. veneficum* were not observed in this system although now they have become a recurring event (W. Hosja, pers. comm.).

33.9 CONCLUSIONS

The events of 1997 had a major impact on research funding for HABS and spurred the adoption of molecular techniques for detection and monitoring of HAB species. *Pfiesteria* spp. can now be detected and identified in natural water and sediment samples and also at fish kill events. The current data from these studies indicates a low abundance in nature for these species and no indication of involvement in any fish kill event since 1998. While a toxic free radical generating activity has been described, the uniqueness to *Pfiesteria* spp. for this activity and the *in situ* detection of the activity have not been established. In contrast, *K. veneficum* has a worldwide distribution, and has been implicated in numerous fish kill events around the world since 1950, a toxin has been isolated, its structure determined, and rigorously quantified at specific fish kill events. Therefore, we present a scenario for the co-occurrence of *K. veneficum* and *Pfiesteria* spp., which explains the fish kill events of 1997, and subsequent similar events attributed to *Pfiesteria* spp. The cause(s) for the proposed human health detrimental effects of these events are still unclear.

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Part XI

Azaspiracids

34 Pharmacology and Epidemiological Impact of Azaspiracids

Gavin Ryan, Kevin Cunningham, and Michael P. Ryan

CONTENTS

34.1 PHARMACOLOGY AND EPIDEMIOLOGICAL IMPACT OF AZASPIRACIDS

Azaspiracids (AZAs) are marine biotoxins that were first observed in mussels (*Mytilus edulis*) in 1995. The discovery of this toxin occurred when AZA contaminated shellfish of Irish origin were consumed in the Netherlands. The symptoms observed were nausea, vomiting, severe diarrhea, and stomach cramps.[1] Satake et al.[2] later identified that AZA was the causative agent. Limited epidemiological data based on a small number of recorded azaspiracid poisonings (AZPs) is currently available. The limited nature of this data has made the determination of relevant safety levels difficult.[3] In the absence of sufficient epidemiological data it has become necessary to examine the effects in *in vivo* and *in vitro* models. These models clarify the mechanism of action of the multiple AZA analogues.

34.2 EPIDEMIOLOGICAL AND GEOGRAPHICAL DATA

The epidemiological data for AZAs to date is limited. Several issues have contributed to this—difficulty in acquiring suspect samples postconsumption, limited reporting of these cases, and few countries have identification methods other than the mouse bioassay, which is not capable of accurately testing for AZAs specifically. Owing to these difficulties, the possibility that AZA is more widespread than reported so far cannot be ruled out. AZP has been reported in Ireland, the Netherlands, United Kingdom, Italy, France, Norway, and Spain. The majority of these incidents have been traced back to Irish sources, all of which were before 2001, when more sophisticated monitoring

TABLE 34.1
Azaspiracid Occurrences to Date

Occurrence	Area of Origin	Date	Species Involved	Estimated AZA (μg/g)	AZP Illnesses
Netherlands	Killary Harbor, Ireland	November 1995	Mussels (*Mytilus edulis*)	1.14[a]	8
Ireland	Arranmore Island, Ireland	September/October 1997	Mussels (*Mytilus edulis*)	0.865[b]	20–24
Italy	Clew Bay, Ireland	September 1998	Mussels (*Mytilus edulis*)	1.0	10
France	Bantry Bay, Ireland	September 1998	Scallops (*Pecten maximus*)	1.1–1.5	20–30
United Kingdom	Caster, England	August/September 1998	Mussels (*Mytilus edulis*)	0.13	Unknown
Norway	Sognefjord, Norway	August/September 1998	Mussels (*Mytilus edulis*)	0.883	Unknown
United Kingdom	Bantry Bay, Ireland	August 2000	Mussels (*Mytilus edulis*)	0.85	4
Spain	Galicia, Spain	2001	Mussels (*Mytilus galloprovincialis*)	0.24	Unknown
France	Brittany, France	2001	Scallops (*Pecten maximus*)	0.32	Unknown
Norway	Norway	November 2005	Crabs (*Cancer pagurus)*	0.177–0.399	Unknown

[a] Samples harvested from same waters 5 months later.
[b] Samples harvested from same waters 1–2 months later.

methods were implemented and the European Union maximum limit of 0.16 μg/g edible shellfish was introduced. However, AZA contamination has since been confirmed throughout Western Europe, including England, Spain, France, and Norway. Table 34.1 summarizes the occurrences of AZP since 1996.

34.2.1 Ireland

In November 1995, at least eight people in the Netherlands became ill after eating mussels (*M. edulis*) cultivated at Killary Harbor, Ireland.[1,4] The symptoms included nausea, vomiting, severe diarrhea, and stomach cramps. A toxin then called Killary Toxin-3 or KT3 was detected. Satake et al.[2] elucidated the structure of KT3 and called the toxin AZA. Mussels collected at Killary Harbor in April 1996 (5 months after the incident) were found to contain 1.14 μg AZA/g of meat, 0.23 μg AZA-2/g of meat, and 0.06 μg AZA-3/g of meat (total AZAs 1.4 μg/g of meat).

Since 1996, mussels produced in Ireland have resulted in a number of AZP incidents. In 1997, cases of contamination recurred in the Arranmore Island region of Donegal, Northwest Ireland. Mussels harvested in Ireland have caused human intoxication in other European countries. Mussels originating from Arranmore Island caused AZP in an estimated 20–24 people in Arranmore, Ireland in September/October 1997. The symptoms included nausea, vomiting, and diarrhea. AZA-1, AZA-2, and AZA-3 were identified in samples. After the initial intoxication in Arranmore Island and Killary Harbor, the toxin persisted for a further 7–8 months. Mussels traced to Clewe Bay, Ireland, caused AZP in ten people in Ravenna, Italy in September 1998. About the same time, AZP occurred in France in 20–30 people, which was traced to scallops (*Pecten maximus*) from Bantry Bay, Ireland. The last recorded incident of AZP traced to Irish shellfish occurred in the United Kingdom in

August 2000. Frozen mussels were responsible for AZP in up to 16 people, which originated from Bantry Bay, Ireland. Again, the symptoms included nausea, diarrhea, abdominal pain, and cramps.

34.2.2 United Kingdom

The first identification of AZAs from mussels harvested outside Ireland occurred in the United Kingdom. AZAs were identified in mussels (*M. edulis*) harvested from Craster, England in August 1998.[5] Using liquid chromatography-mass spectrometric (LC-MS) methods an overall AZA concentration of 0.13 μg/g was detected, the majority of which was AZA-1 along with AZA-2 and AZA-3.

34.2.3 Spain and France

Magdalena et al.[6] have reported that AZAs (0.24 μg/g) were identified in mussels (*Mytilus galloprovincialis*) from Galicia, Spain. Separation of the toxins, AZA (AZA-1) and analogues, AZA-2 and AZA-3, was achieved using isocratic reverse phase liquid chromatography coupled, through an electrospray ionization source, to an ion-trap mass spectrometer. Toxin profiles were similar to those found in the equivalent shellfish in Ireland in which AZA-1 was the predominant toxin.[6] Azaspiracids (0.32 μg/g) were simultaneously identified in scallops (*P. maximus*), from Brittany, France. Similar to mussels harvested in Spain, toxin profiles resembled those found in mussels in Ireland.[6]

34.2.4 Norway

The first identification of AZAs in Norway was in mussels (*M. edulis*) harvested in Sognefjord, southwest Norway, in August–September, 1998.[5] In the fall of 2002 and the winter 2002/2003, the first closures of mussel farming areas in southern Norway occurred owing to the presence of AZAs above the regulatory limit in blue mussels (*M. edulis*). The AZAs reoccurred in mussels in the fall of 2003 and the winter of 2003/2004.[7] Identification of AZAs was initiated by a positive response in the mouse bioassay for lipophilic toxins in the absence of other lipophilic toxins by LC-MS. The symptoms in the mice were consistent with those described for the AZAs, and their identity was confirmed with LC-MS. Additional LC-MS/MS analysis of a sample from one location (Fløde vigen) was performed for a full profile of AZAs present, confirming AZA-1 to AZA-6 present with indications of AZA-7 to AZA-10. AZA contamination was also reported in crabs (*Cancer pagurus*) in November 2005. The mouse bioassay was subsequently found to be positive for AZAs. Although contaminated shellfish were withdrawn from the market, some contaminated shellfish were already on the market and presumed to be consumed, but no AZP was reported.[8]

Because of the limited reports of AZP in humans, in order to attempt to elucidate the pharmacology and toxicology of AZAs, it is necessary to draw information from *in vivo* animal studies and *in vitro* studies.

34.3 PHARMACOLOGICAL EFFECTS OF AZASPIRACID ANALOGUES IN *IN VIVO* MODELS

To further investigate the pharmacological and toxicological effects of AZA, Ito et al.[9–11] carried out several *in vivo* studies in mice. These studies observed the toxicological effects of AZA-1 in multiple tissues, including the small intestine, liver, and T and B lymphocytes in the spleen and thymus. Unfortunately, the limited availability of pure AZA toxin has limited the statistical value of the *in vivo* studies. No study has yet been carried out to establish a no observed adverse effect level (NOAEL) in these animal studies. Nevertheless, these *in vivo* animal studies have provided valuable data and have clearly confirmed that the gastrointestinal tract is a major target for AZA toxicity.

34.4 PHARMACOLOGICAL EFFECTS OF AZASPIRACID ANALOGUES ON ENZYMES AND OTHER BIOCHEMICAL PARAMETERS

To date there have been a relatively small number of studies investigating the mechanism of action of AZA in the *in vitro* systems. Early studies found that unlike the diarrhetic shellfish poisoning (DSP) toxins, AZA did not inhibit protein phosphatase 2A.[12] Further studies on targets of AZA-1 at the cellular level where those of Roman et al.[13] They reported on potential cellular targets of AZA-1 in excitable neuroblastoma cells. The systems studied were membrane potential, F-actin levels and mitochondrial membrane potential. AZA-1 did not modify mitochondrial activity but decreased F-actin concentration. These results indicated that the cytoskeleton seems to be an important cellular target for AZA-1 and that it did not induce apoptosis. In addition to this, AZA-1 did not alter membrane potential, suggesting that it does not produce neurotoxic effects. In human lymphocytes, adenosine cAMP, cytosolic calcium, and cytosolic pH (pHi) levels were also studied. AZA-1 increased cytosolic calcium and cAMP levels and did not affect pHi. The cytosolic calcium increase seemed to be dependent on both the release of calcium from intracellular Ca^{2+} pools and the influx from extracellular media through Ni^{2+}-blockable channels. AZA-1 induced Ca^{2+} increase was negatively modulated by agents that regulate protein kinase C (PKC) activation, protein phosphatases 1 and 2A (PP1 and PP2A) inhibition, and cAMP increase. The effect of AZA-1 on cAMP was not extracellular Ca^{2+} dependent and insensitive to okadaic acid (OA).[13]

Further studies by Roman et al.[14] examined the effect that AZA-2 and AZA-3 had on intracellular cAMP, cytosolic Ca^{2+}, and cytosolic pH (pHi) levels in freshly isolated human lymphocytes. This data indicated that AZA-2 and AZA-3 could increase cytosolic cAMP levels. AZA-2 increased intracellular Ca^{2+} by release from internal stores and Ca^{2+} influx from extracellular medium when cultured initially in Ca^{2+}-free medium. AZA-2 induced Ca^{2+} influx was mediated through Ni^{2+} and SKF96365 blockable channels and was additive with thapsigargin-induced Ca^{2+} influx [thapsigargin (Tg) is a drug that passively releases Ca^{2+} from internal stores and consequently, induces Ca^{2+}-influx through store-operated Ca^{2+} channels]. AZA-3 did not, however, empty intracellular Ca^{2+} stores but did increase cytosolic Ca^{2+} levels. The Ca^{2+} influx was not additive with Tg-induced Ca^{2+} influx. Adenylyl cyclase modulation inhibited AZA-2 and AZA-3-evoked Ca^{2+} increase and AZA-3 induced pHi rise.

In a similar model, AZA-4 was reported not to modify cytosolic calcium in resting human lymphocytes. However, AZA-4 inhibited the increase in cytosolic calcium levels induced by Tg in a dose-dependent manner but did not affect the Ca^{2+}-release from internal stores induced by this drug.[15] These effects were reversible and not regulated by cAMP pathway. AZA-4 appeared to be a novel inhibitor of plasma membrane Ca^{2+} channels, affecting store-operated channels, showing an effect different from other AZA analogues.[15] It was the author's opinion that the inability of AZA-4 and AZA-5 to increase Ca^{2+} influx lowered their relative toxicity compared to AZA-1 and AZA-3.

Alfonso and colleagues further assessed the role of different AZA analogues on intracellular pH and the influence of Ca^{2+} on regulating this in human lymphocytes. These included the naturally occurring analogues AZA-1 to AZA-5 and the synthetic analogues AZA-6 to AZA-9. It was observed that AZA-1 to AZA-5 were able to modulate cytosolic Ca^{2+} levels although not all could affect intracellular pH. The different synthetic analogues also showed differing effects. These results highlighted a structure–activity relationship between the different AZA analogues on intracellular pH and Ca^{2+} levels.[16] The structure–activity relationship of the different domains of AZA-1 on cytotoxicity on primary neuronal cultures reported that the complete chemical structure of AZA-1 was necessary to produce neurotoxic effects.[17] The different mechanistic behavior between the different analogues in the human lymphocyte model indicates a need to correlate structural activity with toxicity.

Kulagina et al. examined the pharmacological effect of AZA-1 on action potential generation and voltage-gated currents in cultured spinal cord neuronal networks from primary cultures.[18] Extracellular recordings of action potentials or spikes from cultured networks of spinal cord neurons derived from E13 mice were made using a neuronal network-based biosensor. This biosensor incorporated spinal cord networks grown on 64 channel microelectrode arrays. The mean spike rate for a network was computed as a function of time and used as an assay for the efficacy of AZA-1 to alter the behavior of the neurons in the network. AZA-1 decreased the mean spike rate of the spinal cord neurons with an IC_{50} of approximately 2.1 nM. A partial recovery of activity occurred after removal of AZA-1. The neuronal networks showed an enhanced irreversible inhibition of mean spike rate to AZA-1 after preexposure to the $GABA_A$ receptor antagonist bicuculline, suggesting that the $GABA_A$ system may play a modulatory role in this model. Whole-cell patch clamp recordings demonstrated that AZA-1 had no effect on the voltage-gated Na^+, K^+, and Ca^{2+} currents found in spinal cord neurons. This data suggested that AZA-1 may be affecting synaptic transmission in the neuronal networks through a mechanism that does not involve voltage-gated channels.[18]

During the examination of cytotoxicity of AZA-1 on mammalian cell lines, it was reported that AZA-1 was capable of rearranging cellular F-actin in nonadherent cells, that is, Jurkat cells (lymphocyte T cells). This was apparent with the concurrent loss of pseudopodia and cytoplasmic extensions that function in mobility and chemotaxis, before cytotoxicity.[19] Additional evidence for the ability of AZA-1 to induce cytoskeletal changes were reported in adherent cells, that is, neuroblastoma and Caco-2 cells. AZA-1 was able to induce the rearrangement of actin filament bundles and loss of focal adhesion points in these cells. These effects did not affect the quantity of polymerized actin nor alter the microtubular cytoskeleton but did alter cell shape and morphology. Detachment and growth inhibition after exposure to AZA-1 appeared to be related to reorganization of the actin cytoskeleton.[20] AZA-1 has been reported to alter the cellular pool of the adhesion molecule E-cadherin, impairing cell–cell adhesion.[21] This was observed to be due to a dose- and time-dependent accumulation of an E-cadherin fragment after exposure to AZA-1, independent of F-actin depolymerization. It was also noted that AZA-1 had no effect on N-cadherin, suggesting a cadherin specific effect. Altogether, these reports indicate that AZA-1 is capable of inducing alterations in the cytoskeleton of both adherent and nonadherent cell types.

34.5 *IN VITRO* MODEL TO MIMIC EFFECTS SEEN IN HUMANS

With gastrointestinal disturbances as one of the major adverse effects of AZA-1, the effect of AZA-1 on gastrointestinal permeability was examined in an *in vitro* model in our laboratory. The human colonic cell line Caco-2 was used to assess the impact of AZA-1 on intestinal barrier function. Barrier function was measured by transepithelial electrical resistance (TEER). TEER works by measuring the rate of flux of ions across the paracellular pathway as an electrical resistance. Disruption of the paracellular barrier is a contributing factor to increased fluid secretion in diarrhea. AZA-1 was found to decrease TEER in a dose-dependent manner over time. A significant decrease was observed with 5 nM at 24 h (Table 34.2). This decrease in TEER corresponded with an increase in paracellular permeability. On the basis of the ability of AZA-1 to disrupt the paracellular barrier, further studies examining the tight junctions were assessed. Tight junctions are responsible for regulating paracellular barrier function. Several different tight junction proteins were examined, namely, the claudin family (claudins 1, 2, and 4), ZO-1, and occludin. Alteration in the levels and localization of these proteins was observed and correlated with the increased paracellular permeability.[22]

These studies may provide a mechanism for the toxicity demonstrated in the epidemiological studies to date. This *in vitro* model has the potential to be developed as a functional assay for detection of AZA and other marine toxins. The assay may well be more suitable than the mouse assay. The assay has high sensitivity with very significant effects being demonstrated at 5 nM AZA.

TABLE 34.2
Effect of AZA-1 on the Transepithelial Electrical Resistance across Caco-2 Cell Monolayers

	ΔTEER (Ω·cm²)		
[AZA-1] nM	**24 h**	**48 h**	**72 h**
Control	0	0	0
0.1	−5.7 ± 2.1	−0.6 ± 4.4	−2.5 ± 1.4
1	−19.2 ± 4.3	−10.5 ± 7.5	−13.9 ± 3.3
2.5	−21.3 ± 6.8	−55.5 ± 33.9	−104.4 ± 53.6
5	−502.5 ± 26.8*	−686.2 ± 12.3*	−673.0 ± 5.0*
7.5	−551.3 ± 31.9*	−708.0 ± 13.6*	−712.7 ± 3.7*
10	−590.1 ± 18.0*	−712.1 ± 11.9*	−717.9 ± 4.3*
100	−604.6 ± 16.8*	−712.3 ± 12.7*	−719.4 ± 5.2*

* Indicates statistically different to control, $p < .01$. Each value represents the mean ± SEM of three experiments.

34.6 RISK ASSESSMENTS OF AZASPIRACID

A number of risk assessments of AZAs have been carried out. The first risk assessment was carried out by the Food Safety Authority of Ireland (FSAI) in 2001. All of the risk assessments to date have had to rely on the limited epidemiological evidence. As indicated earlier in this chapter, the animal *in vivo* studies have been limited by lack of availability of AZA toxin to carry out statistical studies aimed at establishing a NOAEL. Recently the FSAI has reviewed the AZA risk assessment in the light of experience gained since 2001 and new findings on the basis of developments of chemical analysis. Evidence from routine monitoring on profiles of analogues, studies on heat stability of AZAs as well as the distribution of AZA throughout different mussel tissues allowed the revision of the initial intake for people involved in one of the first poisoning incidents in Ireland. These data, in combination with the experience of the risk management board during the period 2001–2005, suggested the establishment of a safe level shellfish of 0.16 mg/kg AZA in shellfish flesh. This risk assessment was adopted by the FSAI in August 2006 and has been made available to international bodies including the European Food safety Authority and *Codex Alimentarius.*

It is anticipated that as more AZA toxin becomes available further studies aimed at elucidating the precise actions of this toxin will provide more scientific basis for future interpretations of the risks involved in incidents of AZA intoxication.

34.7 ACKNOWLEDGMENTS

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35 Azaspiracids: Chemistry, Bioconversion, and Determination

Kevin J. James, Daniel O'Driscoll, Javier Garcia Fernández, and Ambrose Furey

CONTENTS

35.1 INTRODUCTION

Azaspiracid poisoning (AZP) is the most recently discovered of the toxic syndromes from shellfish consumption. The symptoms of acute AZP intoxication closely resembles those associated with diarrhetic shellfish poisoning (DSP), and include diarrhea, vomiting, and headache and the first confirmed incident was in 1995 (Netherlands), following the consumption of mussels (*Mytilus edulis*) that were cultivated in Ireland (Satake et al., 1998). Since these symptoms are similar to bacterial enterotoxin poisoning, and analytical methods for the detection of these toxins have only been developed in recent years, it is probable that there have been many unreported cases of this poisoning. The first azaspiracid to be identified was AZA1 (Figure 35.1, $R^1 = R^3 = R^4 = H$; $R^2 = CH_3$), which was isolated from mussels from Killary, Ireland, that had been implicated in human illness in consumers. It was found that this toxin possesses a polyether backbone, in common with many dinoflagellate toxins, but it also has unique structural features (Satake et al., 1998). Following a second human intoxication caused by the consumption of Irish mussels, two analogues of AZA1, AZA2, and AZA3, were isolated and identified as methyl and demethyl analogues, respectively (Ofuji et al., 1999a). AZA6 is an isomer of AZA1, but it has not yet been isolated in sufficient quantities for structural confirmation using nuclear magnetic resonance (NMR) (Furey et al., 2002). Seven hydroxylated analogues of azaspiracids, all of which are probably bioconversion products of AZA1, AZA2, AZA3, and AZA6, have been identified in shellfish and of these, AZA4 and AZA5 have been structurally confirmed using NMR (Table 35.1) (James et al., 2003a).

Following the development of liquid chromatography-multiple tandem mass spectrometry ($LC\text{-}MS^n$) methods for azaspiracids (discussed later), which display both high sensitivity and selectivity, a wide geographical distribution of azaspiracids in Western Europe was established (James et al., 2001a). In 2000, azaspiracids were first identified in mussels harvested from Sognefjord in southwest Norway and along the eastern coast of England. $LC\text{-}MS^3$ analysis revealed a similar toxin

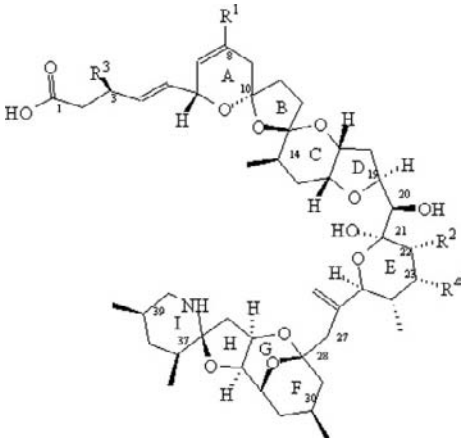

FIGURE 35.1 Structures of azaspiracids (see Table 35.1 for the identity of the substituents R^1–R^4.

TABLE 35.1
Azaspiracid Structures (See Figure 35.1) and Precursor and Product Ion Masses in Positive Mode

Toxin	R^1	R^2	R^3	R^4	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-H_2O-C_9H_{10}O_2R^1R^3]^+$
AZA1	H	CH_3	H	H	842	824	672
AZA2	CH_3	CH_3	H	H	856	838	672
AZA3	H	H	H	H	828	810	658
AZA4	H	H	OH	H	844	826	658
AZA5	H	H	H	OH	844	826	674
AZA6	CH_3	H	H	H	842	824	658
AZA7	H	CH_3	OH	H	858	840	672
AZA8	H	CH_3	H	OH	858	840	688
AZA9	CH_3	H	OH	H	858	840	658
AZA10	CH_3	H	H	OH	858	840	674
AZA11	CH_3	CH_3	OH	H	872	854	672

profile to that found in Ireland (James et al., 2002b). Thus, AZA1 was the predominant toxin with the remaining comprising lower levels of AZA2 and AZA3. In 2002, mussels cultivated in Denmark were implicated in over 400 human poisonings in Belgium, and both DSP toxins-low levels of azaspir and acids (0.06 μg/g) were identified in these shellfish (De Schrijver et al., 2002). Azaspiracids were also identified in scallops (*Pecten maximus*) from Northern France and in mussels (*Mytilus galloprovincialis*) from Galicia, Northwest Spain (Braña Magdalena et al., 2003a).

LC-MS/MS was also used to identify the microalgae, *Protoperidinium crassipes*, as the organism that produces of azaspiracids that accumulate in filter-feeding bivalves—a surprising finding as this ubiquitous genus was previously considered to be toxicologically benign (James et al., 2003b).

Toxicological studies using mice have demonstrated that azaspiracids can cause widespread organ damage, including necrotic atrophy of lamina propria of the villi, and repeated doses induced lung tumors. These effects, together with the low rate of recovery from azaspiracid intoxication, indicate that these toxins are potentially more dangerous to human health than previously known classes of polyether marine toxins (Ito et al., 2000, 2002). The intracellular pH levels in human lymphocytes is modified by azaspiracids and AZA4 appears to be a novel inhibitor of plasma

membrane Ca^{2+} channels, affecting enter by store-operated channels in calcium signalling, an effect that is different from other AZA analogues (Alfonso et al., 2005, 2006). It has been demonstrated that AZA1 is a potent teratogen to finfish (Colman et al., 2005). The European Union regulatory limit for azaspiracids has been established as 0.16 μg/g total tissues, a similar level to that used for DSP toxins in shellfish (EU Commission, 2002).

35.2 STRUCTURES AND CHEMISTRY OF AZASPIRACIDS

One and two-dimensional NMR and MS techniques were used to elucidate the structure of the toxin. As with several other classes of marine toxins, the azaspiracids are structurally characterized by a series of fused polyether rings. Fast atom bombardment (FAB) MS/MS experiments, in positive and negative modes, also provided valuable structural data. The positive fragmentation spectra were dominated by water loss fragment ions $[M+H\text{-}nH_2O]^+$. The negatively charged ion $[M–H]^-$ underwent charge-remote fragmentation resulting in sequential cleavage of polyether rings that provided spectra that were consistent with the proposed structure for AZA1 (Satake et al., 1998). The unique structural features of azaspiracids included a trispiro ring assembly, an azaspiro ring fused with a 2,9-dioxabicyclo[3.3.1]nonane, and a terminal carboxylic acid group.

However, the first proposed structure was shown to be stereochemically inaccurate following the synthetic studies of Nicolaou et al. The revised structure (Figure 35.1, $R^1 = R^3 = R^4 = H$; $R^2 = CH_3$), was confirmed by synthesis and direct comparison with the toxin that was isolated from shellfish (Nicolaou et al., 2003a,b). Two azaspiracid analogues, AZA2 and AZA3, were isolated from mussels (Ofuji et al., 1999a) and these are usually less abundant than AZA1 (James et al., 2002a; Furey et al., 2003). In positive ionisation mode, the mass spectrum of AZA2 gave a molecule-related ion, $[M+H]^+$, at *m/z* 856, 14 Da greater than AZA1 (*m/z* 842), indicating an additional methyl substituent. The location of this methyl group was established using NMR as being on C_8. The corresponding MS of AZA3 gave a molecule-related ion, $[M+H]^+$, at *m/z* 828, which was 14 amu smaller than for AZA1, indicating a demethyl analogue. NMR revealed that the signal for the 22-methyl was absent (Ofuji et al., 1999a) and total synthesis has also been achieved for AZA2 and AZA3 (Nicoloau et al., 2006). Usually, AZA1 is the predominant toxin (50%–80%) in both shellfish and phytoplankton with the remaining comprising AZA2 (10%–30%) and AZA3 (5%–20%) (James et al., 2001, 2002a, 2003b).

35.2.1 Mass Fragmentation Pathways of Azaspiracids

Studies of the mass fragmentation pathways are useful for establishing characteristic cleavages in MS that may prove valuable for the identification of new analogues, metabolites, or degradation products. The investigation of new azaspiracid analogues demands the high sensitivity that is offered by MS. Quadrupole ion-trap (QIT) MS can provide MS^n data which reveals the sequential processes of mass fragmentation. Hybrid quadrupole time-of-flight (QqTOF) MS provides high mass accuracy data for analytes and product ions produced by collision induced dissociation (CID) and this complements the QIT MS data by confirmation of the proposed formulae.

In positive mode, azaspiracids undergo CID charge remote fragmentation toward the positive charged nitrogen on ring G. When fragmentation was studied using deuterated AZA1, it was observed that the first water loss was 20 Da, due to D_2O, which demonstrates that the diol moiety at C_{20}–C_{21} is the location for this process. The next stage in fragmentation is a retro-Diels-Alder reaction involving the ring-A, a very important processes for the discrimination of azaspiracids (Figure 35.2). Subsequent fragmentation occurs at the C-ring, C_{19}–C_{20}, E-ring and C_{27}–C_{28} as shown in the annotated QIT MS^3 spectrum of AZA1 (Figure 35.3) that contains the identification of the major product ions and processes.

The above studies were exploited to assign an isomer of AZA1, named AZA6, as 8-methyl-22-demethylazaspiracid (Figure 35.1, $R^1 = CH_3$; $R^2 = R^3 = R^4 = H$) (Furey et al., 2002). Both AZA1 and AZA6 have a molecule-related ion, $[M+H]^+$, at *m/z* 842 and the water-loss ion $[M+H\text{-}H_2O]^+$,

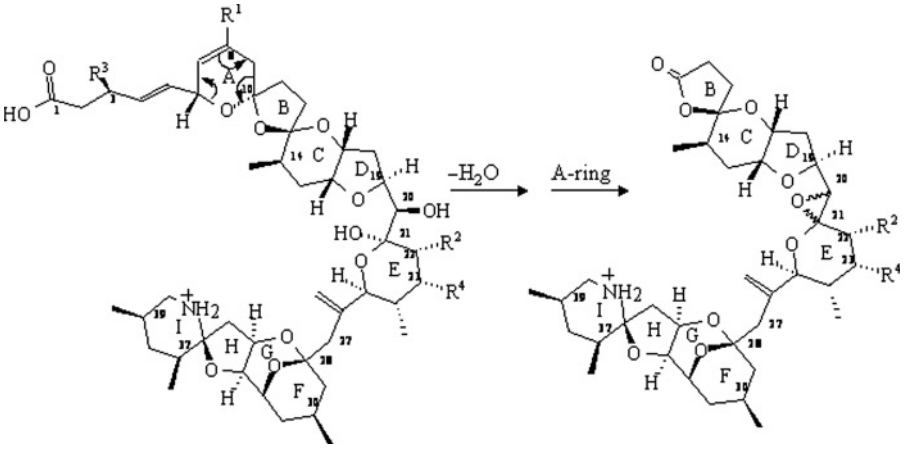

FIGURE 35.2 The first two stages of the CID fragmentation of azaspiracids and the mechanism of A-ring cleavage.

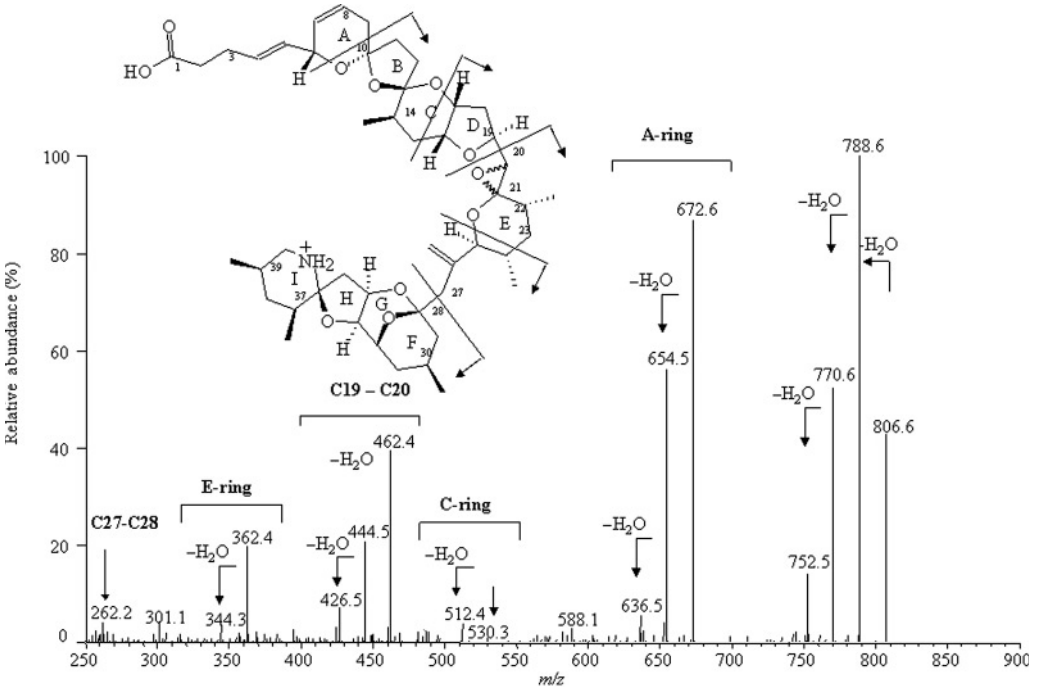

FIGURE 35.3 The MS^3 spectrum of AZA1 produced using QIT MS. The sequential fragmentation of AZA1 produces product ions by processes shown by the annotations.

at *m/z* 824. However, the main difference in the mass spectra between these two products is due to the A-ring fragmentation. Thus, AZA1 displays an ion at *m/z* 672 and for AZA6 the corresponding ion is at *m/z* 658 (Table 35.1) The AZA6 product ion is 14 amu smaller than the corresponding ion from AZA1 and the remaining spectral data for AZA6 and AZA3 are virtually identical, indicating a similar C_{10}–C_{46} region. NMR will be required to confirm that the assignment of the methyl group of AZA6 at C_8 but this may prove difficult owing to the low natural abundance of this toxin.

Two other low abundant azaspiracids in mussels, AZA4 and AZA5, were identified as the 3- and 23-hydroxy analogues of AZA3, respectively (Ofuji et al., 2001). Since these toxins were

not detected in *Protoperidinium* spp., the progenitors of azaspiracids (James et al., 2003b), it is probable that they are produced by bioconversion in shellfish. The structures of the other azaspiracids are proposed on the basis of multiple tandem MS data and by analogy to the confirmed toxin structures.

35.3 AZASPIRACID DETECTION AND DETERMINATION METHODS

35.3.1 Bioassays

Early attempts at AZP monitoring relied exclusively on nonselective mouse or rat bioassays, which had previously been developed to monitor DSP toxicity in shellfish (Yasumoto et al., 1984). However, the implementation of these bioassays repeatedly failed to prevent acute human intoxications. Polyether toxins belonging to the DSP group tend to accumulate in the digestive glands (hepatopancreas) of scallops (Murata et al., 1982). The exclusive use of the hepatopancreas of shellfish for DSP toxin detection using animal bioassays was consequently adopted in most European countries but this was implemented apparently without consideration that toxin distribution patterns may vary with toxin class and shellfish species. It has been shown that azaspiracids can migrate to other shellfish tissues (James et al., 2002b) and the failure to assay total tissue has led to false negatives and subsequent incidents of intoxications from the consumption of shellfish (James et al., 2001b). Not only does the mouse bioassay suffer from poor reproducibility but the lack of specificity is such that false positives result from the presence of free fatty acids in the extracts (Kogawa et al., 1989). The diminished confidence in the use of mouse bioassays for the regulatory control of shellfish toxins has led to a drive to establish LC-MS methods to quantify individual toxins. There have been attempts to establish screening tests for azaspiracids on the basis of determination of cell viability following a 24-h exposure to a shellfish extract (Flanagan et al., 2001). However, detection limits were not reported and these tests have not been validated using standard toxin.

35.3.2 Liquid Chromatography-Mass Spectrometry

Liquid chromatography for detection using a single quadrupole mass spectrometer (LC-MS) has been widely used for the analysis of polyether toxins in shellfish. However, when using LC-MS for the analysis of a complex matrix, such as shellfish tissue, interferences are to be expected, especially ion suppression (Suzuki and Yasumoto, 2000). These interferences are usually less evident in the analysis of toxins in marine phytoplankton (Dahlmann et al., 2003) but most LC-MS methods have not employed the minimum of three selected ions per analyte that is required for reliable identification. One way of reducing interferences and improving the reliability and robustness of a method for the determination of marine toxins in shellfish is to use a cleanup step prior to analysis. Solid phase extraction (SPE) has been successfully employed as a cleanup step for the determination of AZA1–AZA3 using LC-MS (Ofuji et al., 1999b). A comprehensive study of the clean up and toxin recoveries using various types of SPE phases identified the best octadecyl (C_{18}) and diol SPE for azaspiracid analysis (Moroney et al., 2002). Although SPE is not essential with all multiple tandem MS methods, these sample preparation steps will be essential for the development of routine analytical methods for the determination of azaspiracids in seafood that do not have comparable specificity or sensitivity to MS.

Liquid chromatography, coupled with mass spectrometry and tandem mass spectrometry (LC-MS/MS), is a universal technique for determining toxins in food. The first multiple reaction monitoring (MRM) method for polyether marine toxins was used for the determination of DSP toxins and this technique has been successfully applied to the simultaneous determination of various groups of polyether toxins in shellfish (Draisci et al., 1998, 1999). MRM involves the selection of a precursor ion and its product ion from CID fragmentations and it is highly selective. For azaspiracids, the first validated LC-MS/MS method was developed for the determination of AZA1

in mussels using a triple quadrupole instrument (Draisci et al., 2000). Volmer et al. expanded this method for the determination of other azaspiracids but method validation was not possible as they were unable to purify any azaspiracid toxin from shellfish extracts for use as a standard and used partially purified azaspiracid mixtures to examine instrument parameters (Volmer et al., 2002; Blay et al., 2003).

LC-MS^3 methods, using a QIT instrument, have been validated for the determination of AZA1–AZA3 and also applied to the discovery and determination of all of the other azaspiracids (Furey et al., 2002; Lehane et al., 2002; James et al., 2003a). The full separation of eleven azaspiracids using C_{18} reversed phase chromatography has been achieved (James et al., 2003a; Lehane et al., 2004). QIT MS does not offer the equivalent high sensitivity that can be achieved using MRM with a triple quadrupole MS instrument. However, not only does LC-MS^3 provide greater sensitivity than LC-MS/MS using QIT MS for the determination of azaspiracids, but full spectral data are also obtained without diminution of sensitivity—a feature unique to ion-traps and particularly valuable in this field of research for analyte confirmation when analytical standards are rarely available (Lehane et al., 2002). The sensitivity enhancement in MS^3 is a consequence of the trapping process that eliminates most interferants that leads to a dramatic decrease in background noise (Kamel et al., 2002).

At the MS^3 stage, a significant fragmentation is the loss of the A-ring in azaspiracids and this results in the loss of the C_1–C_9 portion that contains the R^1 and R^3 substituents, leaving a residual ion, $[M+H\text{-}H_2O\text{-}C_9H_{10}O_2R^1R^3]^+$, which retains R^2 and R^4 (Figure 35.2). Since AZA1–AZA3 have different masses, it is possible to determine these toxins without chromatographic resolution. However, although the isomers, AZA4 and AZA5 (Figure 35.2, Table 35.1), have the same mass these toxins can be conveniently discriminated without the need for chromatographic resolution by the selection of characteristic product ions formed by A-ring fragmentation using LC-MS^3 since the selected ions and spectra for AZA4 (*m/z* 658) and AZA5 (*m/z* 674) are different. Figure 35.4 shows the LC-MS^2 chromatograms and spectra (AZA4, Figure 35.4b; AZA5, Figure 35. 4c) obtained for the analysis of these toxins in mussels.

35.3.3 Dynamics and Bioconversion of Azaspiracids

The rates of intoxication and detoxification of shellfish by polyether toxins is somewhat unpredicatable and hence the need for efficient and regular monitoring of all shellfish cultivation areas to ensure public health. The azaspiracid contamination of mussels (*M. edulis*) in a shellfish farm in County Cork, Ireland, during September 1999–August 2000, is shown in Figure 35.5. Mussels were sampled 36 times during this year and data showed that periods of high intoxication occurred throughout year and was not confined to the summer months.

The dynamics of azaspiracids in shellfish are different from other polyether marine toxins and toxicity is not always confined to the digestive glands (hepatopancreas). The transportation of toxins to other mussel tissue compartments is unpredictable but has been observed during a prolonged period of high levels of azaspiracid intoxication (Braña Magdalena et al., 2003b). This is of concern as the regulatory mouse bioassay involved testing only the hepatopancreas extract. Azaspiracid can exist as a zwitterion in a pseudomacrocyclic ring system due to the proximity of the positive charge on nitrogen and the negative charge on the terminal carboxyl moiety and thus can impart detergent properties to this molecule. The apparent ease with which azaspiracids can move through phases of different polarities probably plays a significant role in the increased penetration of these toxins in shellfish and mammalian tissues.

35.3.4 Bioconversion of Azaspiracids

The discovery of the phytoplankton source of azaspiracids was achieved using LC-MS^n determination on monocultures (200 cells) of *Protoperidinium* spp., manually collected from mixed populations of phytoplankton (Figure 35.6). The toxin profile observed contained a somewhat

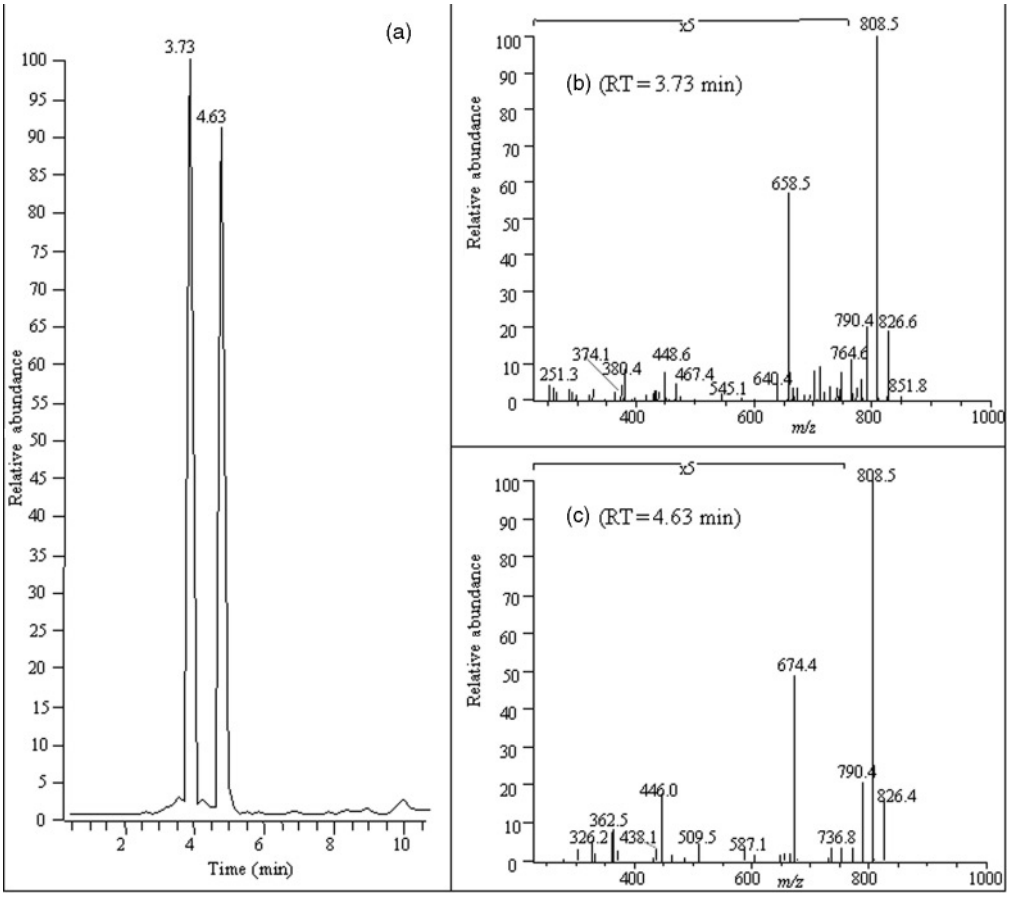

FIGURE 35.4 The LC-MS^2 chromatogram produced by QIT MS of AZA4 and AZA5 in a mussel extract, together with the accompanying spectra corresponding to each chromatographic peak. (From Lehane, M., Braña-Magdalena, A., Moroney, C., Furey, A., and James, K. J., *J. Chromatogr.* **950**, 139–147, 2002. With permission. Copyright (2002) Elsevier).

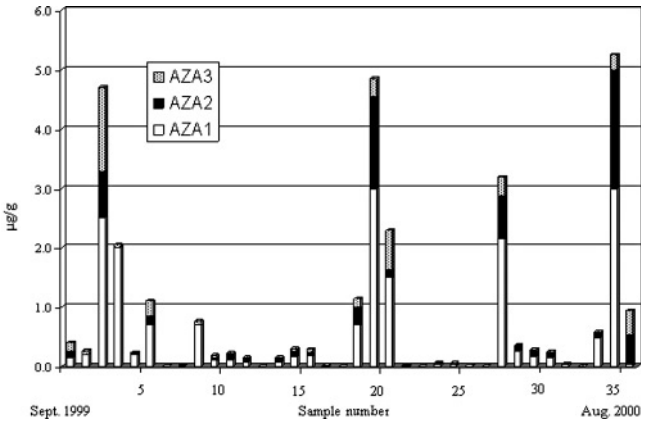

FIGURE 35.5 The azaspiracid toxin profiles in mussels cultivated in County Cork, Ireland, which were sampled 36 times during a yearlong study.

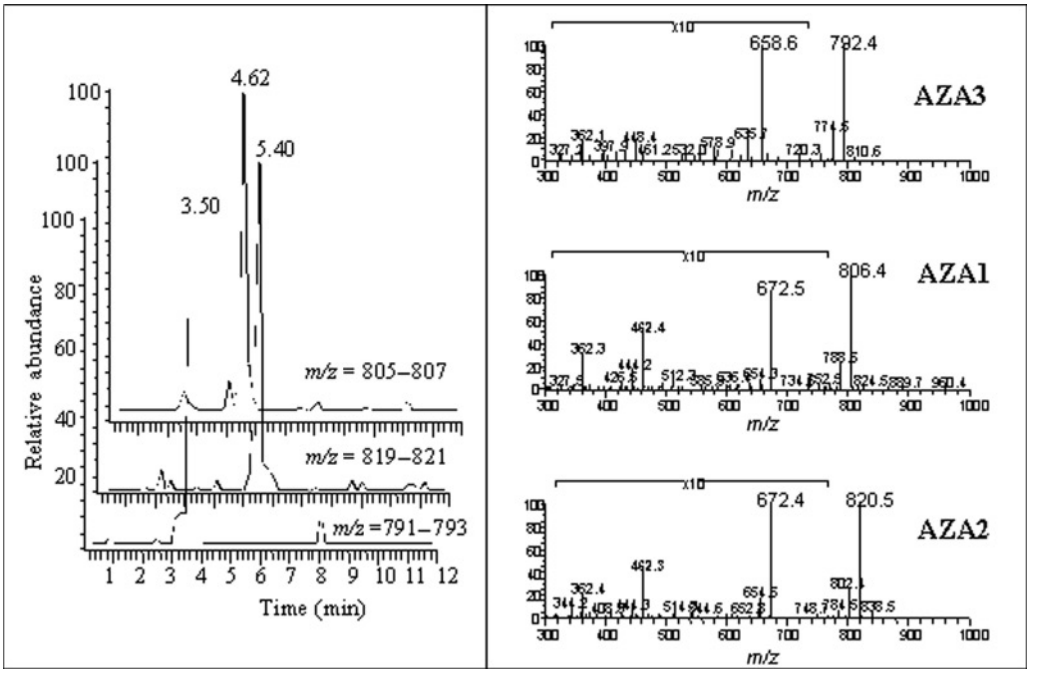

FIGURE 35.6 Chromatograms obtained from the LC-MS^3 analysis of an extract of *P. crassipes* cells (equivalent to 200 cells). The average content was 1.8 fmol total AZA toxins per cell with a composition of AZA1 (82%), AZA2 (10%), and AZA3 (8%). (The peak height for the main component in each chromatogram was scaled to 100%.). Mass spectra correspond to the chromatographic peaks at 4.62 (AZA1), 5.40 (AZA2), and 3.50 (AZA3) min, respectively. (From James, K. J., Moroney, C., Roden, C., Satake, M., Yasumoto, T., Lehane, M., and Furey, A., *Toxicon* **41**, 145–151, 2003b. With permission. Copyright (2003) Elsevier).

higher proportion of AZA1 than is commonly found in mussels (AZA1, 82%; AZA2, 10%; and AZA3, 8%). However, the hydroxylated analogues of AZA3, AZA4, and AZA5, were not detected in phytoplankton thus indicating that the latter are probably bioconversion products in shellfish. It seemed logical that this bioconversion should not be confined to AZA3 and that there should be other hydroxylated analogues of azaspiracids in mussels. Using a similar approach to that described to discriminate the isomers, AZA4 and AZA5, LC-MS^3 was used to identify AZA7–AZA10 as the hydroxyl analogues of AZA1 and AZA6 (Figure 35.1, Table 35.1) (James et al., 2003b).

The four isomers, AZA7–AZA10, could be discriminated by LC-MS^3 analysis even though they have identical molecule-related ions, $[M+H]^+$ at *m/z* 858, and product ions due to the water-loss, $[M+H\text{-}H_2O]^+$ at *m/z* 840. Figure 35.7 shows the MS^3 spectra obtained for AZA7–AZA10, with the distinguishing ion from A-ring fragmentation circled. The A-ring fragmentation results in the loss of the C_1–C_9 portion that contains the R^1 and R^3 substituents, leaving a residual ion, $[M+H\text{-}H_2O\text{-}C_9H_{10}O_2R^1R^3]^+$, that retains R^2 and R4. The ion sequences for an initial water-loss, followed by the A-ring fragmentation, are: AZA7 (*m/z* = 858.5 → 840.5 → 672.4); AZA8 (*m/z* = 858.5 → 840.5 → 688.4); AZA9 (*m/z* = 858.5 → 840.5 → 658.4); AZA10 (*m/z* = 858.5 → 840.5 → 674.4). This was exploited in another LC-MS^3 method that targeted the product ions from A-ring fragmentation. It was therefore possible to determine AZA1–AZA10, without complete chromatographic separation, by exploiting the ability of multiple tandem MS to resolve isomers by prescribed mass selection (Lehane et al., 2002, 2004).

An analogue of AZA3 with carboxylic acid moieties has also been suggested, on the basis of MS data but a structure was not proposed (Brombacher et al., 2002). Other AZA bioconversion products will probably emerge during larger scale isolation studies.

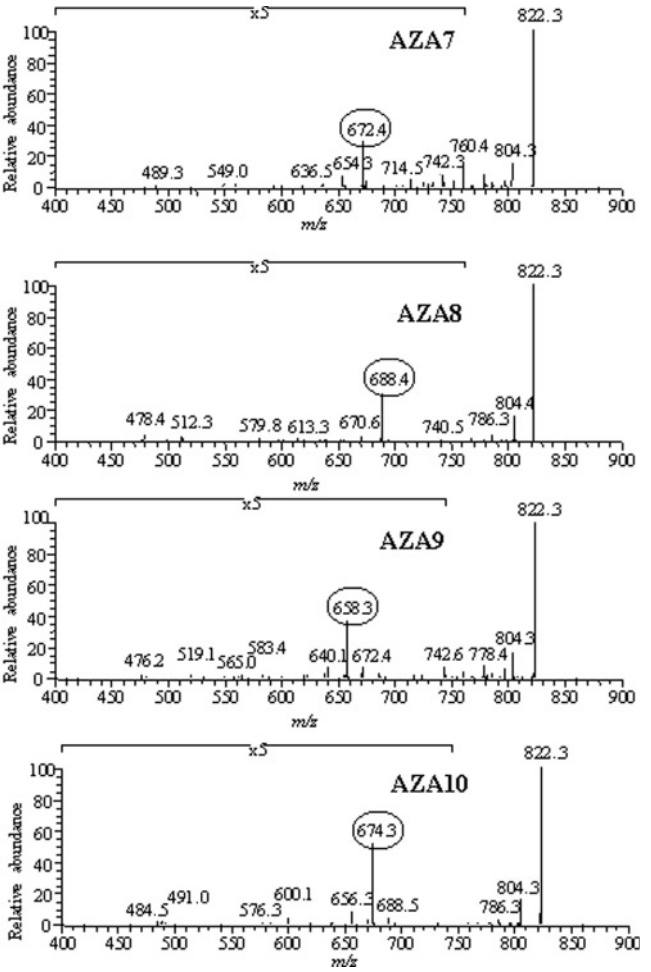

FIGURE 35.7 Analysis of a mussel extract using QIT MS. LC-MS^3 of AZA7–AZA10 using the selected ions, $m/z = 858.5 \rightarrow 840.5 \rightarrow 822.5$. The four spectra show different product ions (circled) due to A-ring fragmentation. (From James, K. J., Diaz Sierra, M., Lehane, M., Braña Magdalena, A., and Furey, A., *Toxicon* **41**, 277–283, 2003a. With permission. Copyright (2003) Elsevier).

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36 Toxicology of Azaspiracid-1: Acute and Chronic Poisoning, Tumorigenicity, and Chemical Structure Relationship to Toxicity in a Mouse Model

Emiko Ito

CONTENTS

36.1 INTRODUCTION

Azaspiracid poisoning (AZP) was reported first in the Netherlands in 1995. The poisoning was by imported mussels from Ireland, which caused symptoms including nausea, vomiting, diarrhea, and stomach cramps in humans. An acute bioassay using mice showed some neurological symptoms (McMahon and Silke, 1996, 1998). In the case of humans, the lowest observable adverse effect level (LOAEL) was 23–86 μg for acute gastrointestinal effects; based on this the FAO/IOC/WHO consultation established a provisional acute reference dose of 0.04 μg /kg b.w. in 2005. Azaspiracid-1 (AZA-1) is the main component of the AZAs but the potencies in mice through intraperitoneal injection were AZA 2 >3 >1 >4 >5 (Ofuji et al., 1999, 2001), and those of several other members (AZA-6–11) were estimated (James et al., 2003).

To clarify the pathological changes and to evaluate the risks posed by AZA, this work studied the morphological changes in mice following acute and chronic per oral (p.o.) exposure to azaspiracid-1.

By using synthetic azaspiracid-1, similarities in toxicity with natural AZA-1 and correlation to the chemical structure were studied pathologically.

36.2 MATERIALS AND METHOD

In the present experiments, azaspiracid-1 (AZA) was used for pathological studies. AZA was extracted from blue mussels, *Mytilus edulis*, collected in Ireland in 1996 and 1997 (Satake

et al., 1998a,b). A stock solution of AZA was prepared by dissolving 1.0 mg of the toxin in 2 mL of aqueous 50% ethanol. A total of 39 male ICR (4-week-old) mice were used for the acute poisoning experiment and 25 mice were used for a recovery experiment. In addition, 126 mice were used for chronic experiments (+control $n = 71$). The AZA was dissolved in a 0.2 mL portion of saline and administered orally by the gastric tube (Table 36.1).

36.3 RESULTS AND DISCUSSION

36.3.1 Acute Poisoning in Mice

36.3.1.1 Lethality and Changes

Mice did not show any symptoms after administration for at least several hours, and thereafter they gradually weakened—even at 900 μg/kg within 4 h there was no change. The lethal toxicity of AZA-1 to mice by oral administration was about 250–500 μg/kg (b.w.). Mice showed individual differences in sensitivity to the toxin, especially aging-induced death, even at as low a dose as 250 μg/kg (Ito et al., 2002) (Table 36.2).

Pathologically, multiple organ damages were observed mainly in three organs (1) Liver: fatty change and single-cell necrosis, (2) Small intestine: erosion at villi, (3) Lymphatic organs: lymphocyte necrosis in the thymus and spleen. In severe cases there were inflammation and edema in the lung and stomach (Ito et al., 2000).

36.3.1.1.1 Liver changes

As shown in Table 36.3, the liver swelled and increased in weight, which showed fatty degeneration after 16–24 h, as the organ became yellowish, microscopically, causing very fine fatty droplets to

TABLE 36.1
Experiment Procedure for Pathological Observation

Oral Giving	Observation	Organ Fixing	Specimen	Staining
AZA-1 ⇒ mouse	Light microscope	10% formalin	Paraffin section	H&E (general)
			Paraffin section	PAS (polysaccharide)
			Frozen section	Sudan III (lipid)
	Scan microscope	(1) 4% paraformaldehyde (2) 1% OsO_4 (3) 1% tannic acid (4) 1% OsO_4	Critical point dry	Coated with platinum–palladium

TABLE 36.2
Lethality of AZA-1 with p.o. Administration in Mice

Dose (μg/kg)	Mice Age	Survival	Survival Rate (%)
600	5-week	3/6	50
500	5-week	3/3	100
450	4-week	5/16	31
350	6-week	3/3	100
300	4-week	2/2	100
300	6-week	0/3	0
250	5-month	0/2	0

TABLE 36.3
Weight of Organs and Decreased Number of Nongranulocytes after 24 h in the Spleen with Azaspiracid-1 p.o. at 500 μg/kg (n = 3)

Group	Body Weight (g)	Liver Weight (g)	Spleen Weight (g)	Nongranulocytes
Control	29.9 ± 0.5	1.96 ± 0.1	0.114 ± 0.01	24 ± 3.2 ($\times 10^6$)
Azaspiracid-1	28.5 ± 1.3	2.71 ± 0.1	0.106 ± 0.01	16 ± 2.8 ($\times 10^6$)

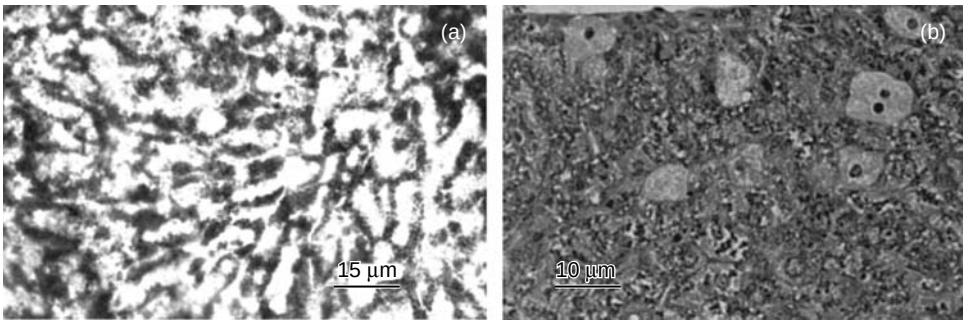

FIGURE 36.1 Liver changes by AZA-1. Fat droplets (black) distributed in the entire liver and located at periphery of the hepatocytes (a). The architecture of the liver (H&E) was not clear by the background of fatty change, but had swollen cells with degenerated nucleus that were seen sporadically (b).

distribute homogeneously in the liver lobule (Figure. 36.1a). By Hematoxylin and Eosin staining (H&E), enlarged, clear, and degenerating nucleus-containing cells were seen distributed sporadically (Figure 36 1b).

36.3.1.1.2 Small intestinal changes

The small intestinal villi eroded epithelial cells with higher doses at 700–900 μg/kg in several hours (Figure 36. 2a-i and ii), but these changes were not distinguished from sublethal doses of okadaic acid (OA) or dyniphysistoxin-1 or -3 (150 μg/kg<) (Ito and Satake, 2002). Characteristic changes were confirmed at lower doses (Figure 36.2b i–iii).

36.3.1.2 Recovery Terms from the Serious Condition

The surviving 10 mice (Table 36.2: 5–450, 3–350, and 2–300 μg/kg) did not show changes in condition after 24 h; therefore, the second oral administration (250 or 300 μg/kg) was given depending on their age to induce severe condition. After this, the mice continued to be weak for 3 days, and on day 4 they conquered the illness. Every organ of the mice was observed for 3 months (Figure 36.3).

Lymphoid tissues recovered first within 10 days, next the liver within 15 days, and the lung after 8 weeks. Complete recovery in the gastrointestinal mucosa was not evident until 3 months (Ito et al., 2002) because these organs caught bacterial infection in addition to the injuries and light erosions that were seen during the experimental period.

Thus, the recovery was expected even after such a serious condition as that caused by AZA-1; in contrast, with OA the mice usually showed nothing or died, without any induced serious condition for days, and recovery was impossible (Table 36.4).

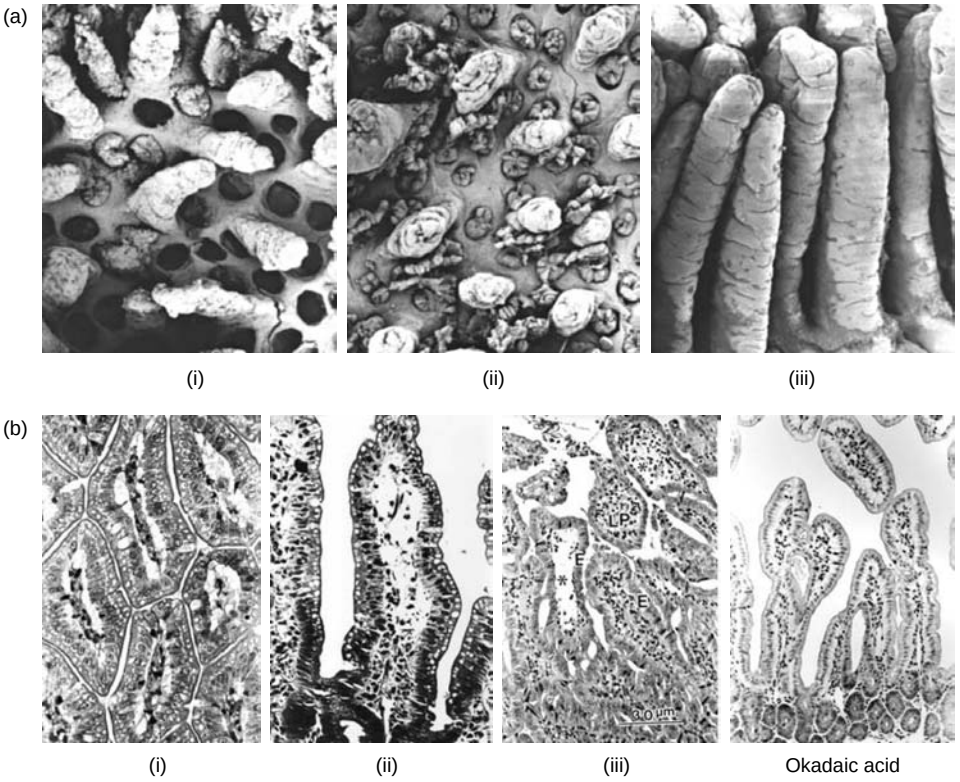

FIGURE 36.2 (a) Small intestinal changes caused by higher doses. With 900 μg/kg after 4 h (i), epithelial cells of villi shedded together with crypt cells; with 700 μg/kg after 8 h (ii), epithelial cells shedded resulting in exposure of shortened lamina propria and crypt cells. Normal villi (iii) have the heights and are covered with epithelial cells. (b) Characteristic changes at small intestine by AZA-1 (300 μg/kg) and the process (i–iii): atrophy at lamina propria and vacuoles in epithelial cells at 4 h (I), necrosis at lamina propria after 8 h (ii), empty at lamina propria still necrotic at16 h, but epithelial cells were recovering (fused each other) (iii). Okadaic acid p.o. at 300 μg/kg-16 h, recovery stage of villi had balanced at epithelial cells and lamina propria.

Organ	0 day~	10 days	15 days	20 days	8 weeks	3 months
Lymphocytes						
Liver						
Lung						
Gastro-intestinal						

FIGURE 36.3 Recovery observation in each organ from serious condition.

TABLE 36.4
Comparison of Okadaic Acid in Small Intestine and the Possibility of Survival with Serious Condition

Toxin (μg/kg)	Eroding Time	Fluid	Characteristic	24 h	Recovery from Serious Condition (μg/kg)
AZA-1 (300)	4 h –?	?	Lamina necrosis	Eroding	Possible (ca. 300 × 2)
OA (75–150)	15–60 min	+	Edema at villi	Finished	Difficult (500–600)

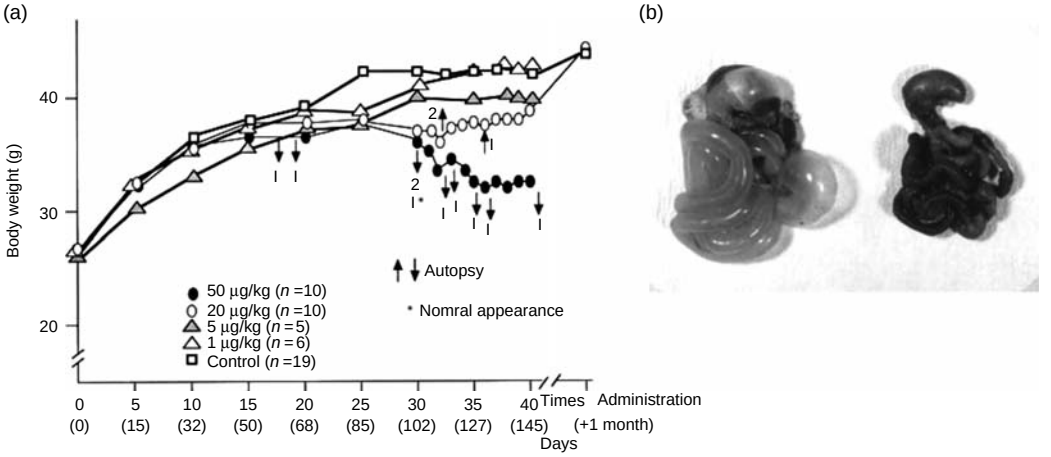

FIGURE 36.4 Mice body weight change and lethal cases at repeated administration of AZA-1 (a), weakened cases showed a lot of gas accumulation in the gastrointestinal organs (b), nontreated organs from control (right).

36.3.2 Subacute Poisoning in Mice

Mice were given a lethal dosage of AZA-1 twice a week: 1/10 lethal level: 50 μg/kg (n = 10), 20 μg/kg (n = 10); 1/100 lethal level: 5 μg/kg (n = 5), 1 μg/kg (n = 6), and nontreated and uncontrolled (n = 19) (Figure 36.4a).

At the highest dose level of 50 μg/kg twice a week, all mice died within 40 treatments (100% in 5 months). Even with 20 μg/kg twice a week, mortality appeared 30% (3/10) after 5 months. However, no effects were observed at the lower level of 5 and 1 μg/kg. The second experiment was conducted using a narrower dose range of 5–20 μg/kg. In the second study that was repeated, 45% of the (9/20) mice died at 20 μg/kg after 15 administrations (Figure 36.6, line 5), and therefore mortality was higher than in the first experiment. These weakened mice commonly showed prominent decrease of body weight before death and weakness progressed gradually for several days. Mice in terminal stage were declared dead and sacrificed under anesthesia. They had a lot of gas accumulation in the digestive organs (stomach, small and large intestines, Figure 36.4b), where villi were short and sparse. In addition to the insufficient recovery, repeated administration induced a cumulative effect on the villi and induced anorexia-like low body condition and peristaltic deficiency resulting in gas accumulation. Other chronic changes were observed in the stomach: hyperplasias (6/10) in mice treated with 20 μg/kg in the first group (Figure 36.5a), and lymphatic nodule in the lung (Figure 36.5 c,d and lines 5–6 in Figure 36.6).

All ten mice could survive for 1 year at decreased doses (20→15→10 μg/kg) depending on their body weight conditions after 33 dosages (Figure 36.6, lines 9). Repeated dosages of 20 μg/kg (<1/10 of the 100% acute lethal dose) became a lethal dose at relatively fewer exposures (Figure 36.6 lines 2–5).

36.3.3 Tumor Appearance in Mice

Four lung tumors appeared in a chronic study (Figure 36.6, lines 1 and 2, Figure 36.7 Nos 1–4); to confirm the tumorigenicity, additional experiments were conducted. The second study was with dosage at 50 μg/kg and 20 μg/kg used more animals (Figure 36.6, lines 5–8).

In an 8 months observation carried out according to the first experiment schedule, no tumor was observed in the lymphatic nodules in the lung (10/25: Figure 36.6, ✦ in Figure 36.5c,d); however, study of this group was unsuccessful.

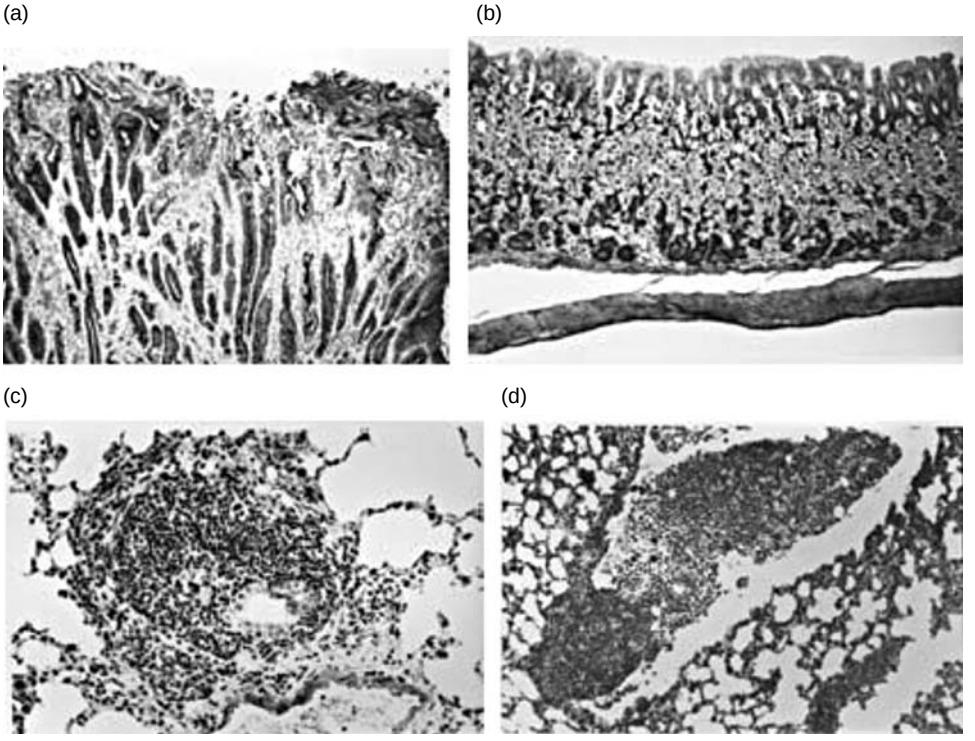

FIGURE 36.5 Chronic changes. The stomach hyperplasia (a), normal (b). lung, lymphatic nodules (c, d).

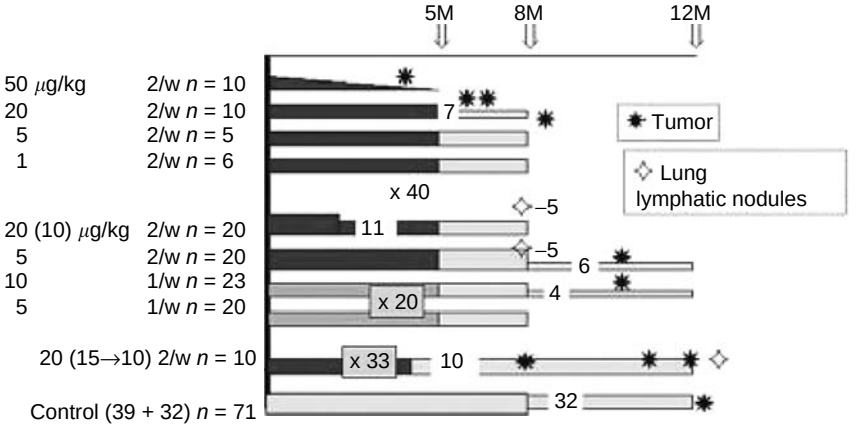

FIGURE 36.6 Outline of chronic administration of AZA-1 and appearance of tumors (✶) and polylymphatic nodules (✦) in the lung.

The surviving ten mice (Figure 36.6, lines 6 and 7) and the newly added ten mice (dose at 20→15→10 μg/kg depending on body condition) were observed for 1 year (Figure 36.6, line 9).

Indeed, in the 1-year study, five more tumors were observed. As a result, a total of 9 tumors out of 126 treated mice had appeared (✶ in Figure 36.6). The dosage of 20 (or 15) μg/kg proved effective for tumor formation. Among the nine tumors observed, seven were lung tumors and two were malignant lymphomas (Figure 36.7). Only 20 mice could be observed after 12 months, whereas the others were either sacrificed after 8 months or weakened during dosage period. More tumors could

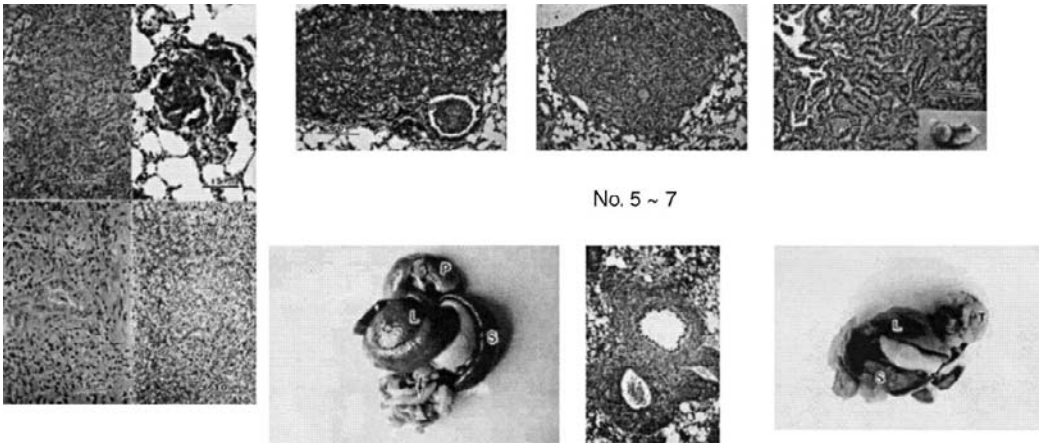

FIGURE 36.7 Tumors. Nos 1–4 lung tumors (1st chronic experiment). Lung tumors from the 2nd chronic experiment (No. 5: originated from bronchial epithelium, No. 6: adenocarcinoma from bronchial glandular epithelium, No, 7: from alveolar epithelium). Malignant lymphomas (Nos 8, 9) with lymphocytes infiltrated systemically.

TABLE 36.5
Spontaneous Tumor of ICR Mice at 2 Years, and AZA-Induced Tumors at 1 Year

Organ	AZA-1 , 1-year, $n < 126$[a]	Control, 1-year, $n = 71$[b]	Reference, 2-year, $n = 2037–2575$
Lung (%)	>5.5	1.4	21.1
Liver	0.79	11.2	17.2
Stomach	0	0	0.15
Small intestine	0	0	0.24
Large intestine	0	0	0.12
Kidney	0	0	0.42
Thymus	>0.79	0	0.34
Whole body	>1.58	0	7.5

[a] 106 mice finished until 8 months, residual $n = 20$ observed at 1 year.
[b] 39 until 8 months, 32 until 1 year.

have been observed if all mice had survived up to 1 year. Since 10 out of the 27 mice that were sacrificed at 8 months had multiple lymphatic nodules in the lungs (✦ in Figures 36.6 and 36.5 c,d), some of them might correlate with malignant lymphoma.

The ratio of tumor-bearing mice was >7% (9/126), while control mice showed only one lung tumor. Lasting till the end was 7%, but 30% in the last 1-year group. Within our experiment, tumor ratio was clearly larger than control (1.4%, 1/71), but, ICR mice showed a relatively high ratio of spontaneous tumors at 2 years (Table 36.5) (Giknis, 2000); therefore, nine tumors might be spontaneous.

As a result, azaspiracid can be either tumorigenic by itself or a promoter to the early appearance. Future work should consider a larger number of mice, not only for better statistical assessment of the observed effects but also to show whether the observed lymphomas will lead to cancer after the exposure is ceased.

36.3.4 Correlation between Toxicity and Chemical Structure

Azaspiracid-1 synthesis was successfully accomplished by Nicolaou et al. (2003a,b, 2004a,b); toxicity identification and correlation to the chemical structure in mice were studied through an acute experiment after 24 h. Among these compounds, upper two compounds of the correct structure of AZA-1 and the isomer showed toxicity at the p.o. dose up to 3 mg/kg; components were also tested intraperitoneally (i.p.) (Figure 36.8).

As shown in Figure 36.9a, with synthetic AZA-1, the liver showed changes similar to those seen with the natural compound, seen in Figure 36.1 (a and b), namely, fatty degeneration and sporadic necrotized hepatocytes. The small intestine erosion and characteristic changes of late recovery at lamina propria were confirmed (Figure 36.9a–i and ii).

With use of the isomer, the liver showed fatty degeneration, but necrotized hepatocytes were seen at the central area (Figure 36.9b-liver), and the injury to the small intestine was unclear. (Figure 36.9b).

Both syn-AZA-1 and the isomer showed degeneration of lymphocytes in the thymus and spleen; these pathological changes are summarized in Table 36.6.

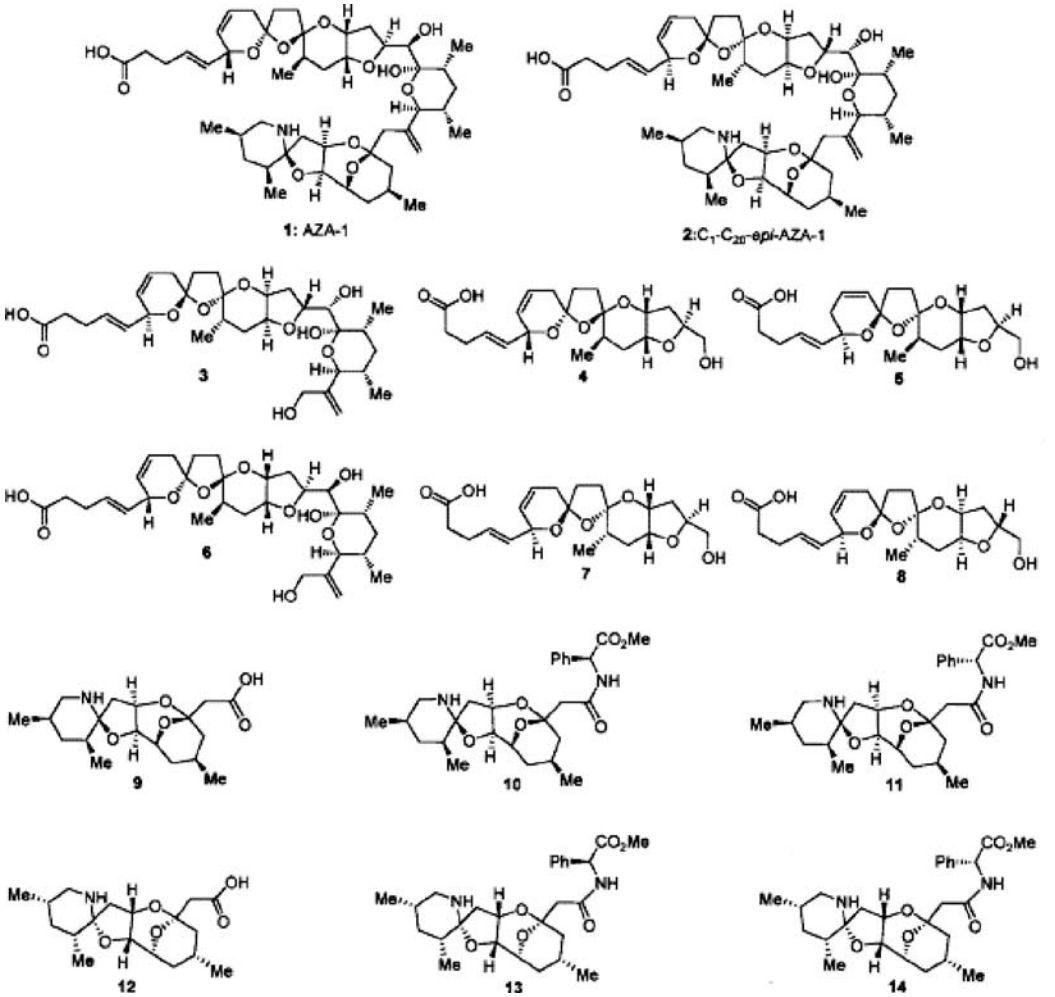

FIGURE 36.8 Molecular structures of synthetic azaspiracid-1 (1), the isomer C_1C_{20}-*epi*-AZA-1 (2), and truncated analogs (3–14).

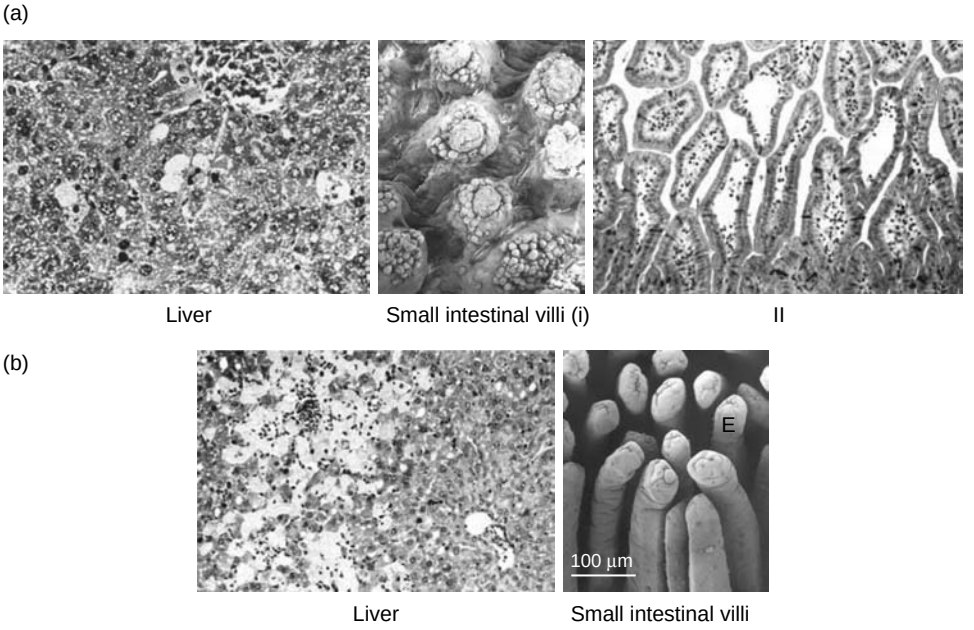

FIGURE 36.9 (a) Synthetic AZA-1 (24 h) injured the liver and the small intestine. Erosion still be seen after 24 h (i), and characteristic change of late recovery at empty lamina propria can be seen in (ii). (b) Isomer injured the liver but ballooning hepatocytes appeared at central area, and did not injure small intestinal villi.

TABLE 36.6
Effective Dose and Pathological Changes of Synthetic AZA-1 and Natural Product

AZA-1	Effective Dose (μg/kg)	Liver	Lymphocytes	Stomach	Small Intestine
Natural	450–500 (lethal dose)	Fatty change and sporadic ballooning	Many picnosis	Light erosion	Erosion and empty lamina
Synthetic	≥700–900	Fatty change and sporadic ballooning	Many picnosis	Light erosion	Erosion and empty lamina
Isomer	≥3000	Fatty change and ballooning (central area)	Many picnosis		

Synthetic AZA-1 showed similar characteristic changes as the natural product; however, its effective dose levels (≥700–900 μg/kg) were lower than those of the natural one (lethal level: 450–500 μg/kg).

The reason for this was not clear, but one reason could be the individual differences in impurity or sensitivity of each mouse. The isomer also showed toxicity, but with higher doses (≥3 mg/kg) had no common activity on the gastrointestinal organs.

In conclusion, characteristic pathological changes were induced only by all the components in correct position (Ito et al., 2006).

We have only studied about AZA-1 in mice; and azaspiracid poisoning is complex poisoning with other AZAs, and there must be a correlation with shellfish component as well. At present since other AZAs are not available for study, synthetic AZAs are expected to be used for elucidation in this field in the future.

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Part XII

Cyanobacterial Toxins

37 Cyanobacterial Toxins in Aquaculture

Paul T. Smith

CONTENTS

37.1 INTRODUCTION

Aquaculture has a very long tradition and began as an activity of subsistence for farmers in Asia. In fact, the first known book on fish culture was written more than 2000 years ago by Fan Li on the results of his work with breeding and raising carp in Wushi, Kiangsu Province, eastern China (Fan L, 473 BC; pp. 3–4, Tapiador et al., 1977). Traditional methods have been handed down through the generations and typically a variety of species of Chinese carps (Figure 37.1) are grown in earthen ponds that have been fertilized to enhance the growth of microalgae, natural foods, and cyanobacteria. In addition, since ancient times, ornamental fish have been cultured in ponds near temples and castles and this tradition continues even today (Figure 37.2).

FIGURE 37.1 Common carp, a member of the cyprinids, is grown in earthen ponds for food security in developing countries. More than 18 million tons of cyprinids, valued at more than US$16 billion are harvested annually worldwide (FAO, 2006).

FIGURE 37.2 Ornamental carp have been cultured for centuries in fish ponds at palaces and temples in China and other Asian countries.

Recent technological advances have facilitated the development of many diverse forms of aquaculture with at least 442 fish and shellfish species worldwide (FAO, 2006; p. 10). Aquaculture production has grown at an average rate of 8.8% p.a. since the 1950s and development continues to expand in almost all regions of the world (FAO, 2006; p. 5). In 2004, world aquaculture production was 59.4 million tons and was valued at US $70.3 billion (FAO, 2006). China, with its abundant lakes, reservoirs, ponds, and rivers as well as suitable fish species, is the leading nation in both marine and freshwater aquaculture. Recent figures show that China accounts for 69.6% of worldwide production (41.3 million tons in 2004) and the rest of Asia-Pacific region accounts for a further 21.9%.

Aquaculture is recognized as the only means of meeting the future demand for seafood as well as being an important source of employment, income, and food security in developing countries (FAO, 2006). Of the remaining wild fish stocks, less than 25% are moderately exploited, while the rest are either fully exploited (52%), over-exploited (17%), or depleted (8%). The inland communities of developing countries have staple diets of rice, wheat, cassava, maize, and potato, which are high in carbohydrates but lacking in protein, essential nutrients, and fatty acids. For example, a survey of fish farmers in inland communities of Papua New Guinea revealed that for new fish farmers who had not harvested, the median period between meals that contained some form of meat was 14 days ($n = 156$). Whereas for experienced fish farmers who had harvested at least once, the median period was significantly lower at 7 days ($n = 106$) (Smith et al., 2007). This type of finding provides a very strong argument for the benefits of fish farming to smallholder farmers in developing countries and it supports the health message coming from nutritionists for everyone to regularly consume fish (Elvevoll and James, 2000; Halwart, 2006).

The application of organic manures and inorganic fertilizers is fundamental to the development of pond ecology and development of natural foods for the culture of fish and crustaceans (Massaut, 1999).The main fertilizers used in most forms of pond aquaculture in China and other Asian countries are animal manures (Shingang, 1989; Chapter 3). Manures are broken down by pond microorganisms and the nutrients that are released are absorbed by cyanobacteria and microalgae. In addition, the decomposing bacteria are nutritious to zooplankton, resulting in blooms of zooplankton and phytoplankton approximately 7–10 days after application of manures. In particular, organic fertilizers are used in the preparation of nursery pond so that the peaks in blooms of phytoplankton and zooplankton coincide with the feeding requirements of fish fry (Shingang, 1989). The main disadvantage of organic manure is their tendency to reduce the level of dissolved oxygen; hence, manures are generally used in small quantities every 7–10 days. Inorganic fertilizers are also used because they are fast acting, convenient, concentrated, and do not reduce the dissolved

oxygen level. However, fertilization with phosphorus-based fertilizers inevitably leads to strong cyanobacterial blooms.

The implications for aquaculture of cyanobacterial blooms have been considered previously and the general conclusion was that there are relatively few cases of cyanotoxic effects of cyanobacteria in aquaculture (Sevrin-Reyssac and Pletikovic, 1990; Paerl and Tucker, 1995; Smith, 2001). It is high time the situation is reexamined, given the continued worldwide expansion of the aquaculture industry, improvements in detection methods, as well as the increased awareness of the risks that cyanotoxins pose to both human health and ecological systems.

Cyanobacteria are known to produce a huge diversity of cyanotoxins and secondary metabolites and these compounds and methods for detection have been reviewed elsewhere (Carmichael, 1988, 1994; Moore, 1996; Haider et al., 2003; Shimizu, 2003; Dorigo et al., 2005; Msagati et al., 2006). The occurrence of toxic cyanobacterial blooms in freshwater and brackish water environments has increased noticeably in frequency and severity in recent years (Codd et al., 2005a) The alteration of water quality by human activity appears to be a contributing factor, principally through eutrophication of water bodies. This is a global problem, so efforts are being made to document the occurrence and effects of cyanobacterial blooms (Codd et al., 2005a).

This chapter reviews and discusses the occurrence of cyanobacterial blooms in aquaculture, the types of cyanotoxins that have been identified and the impacts of cyanotoxins on aquaculture. Also, reports of assimilation and depuration of cyanotoxins by various species in aquaculture will be examined and the risks posed to human health by cyanobacterial toxins in aquaculture will be considered.

37.2 OCCURRENCE OF CYANOBACTERIAL BLOOMS IN AQUACULTURE

The input of feed and fertilizers are conducive to the growth of cyanobacteria, and cyanobacterial blooms can dominate freshwater fishponds and marine shrimp ponds (Figure 37.3) for a substantial period of the growth cycle (see Tables 3 and 4, McIntosh, 2006). For instance, cyanobacteria are the

FIGURE 37.3 A farmer checks the consumption of pellet feed by shrimp (*Penaeus monodon*) from a feed tray at a shrimp farm in Hat Yai, Thailand. A Secchi visibility depth of approximately 30–50 cm is generally maintained by the addition of fertilizers and pellet feeds. Ponds are stirred by electrical or diesel powered paddle wheels in order to keep dissolve oxygen levels above *ca*. 3 mg/L. Planktonic and benthic cyanobacterial blooms are a constant threat to pond management and productivity in shrimp farming.

most dominant component of phytoplankton biomass in channel catfish ponds in the United States (Paerl and Tucker, 1995). Factors that contribute to the development of cyanobacterial blooms in catfish ponds are regular addition of supplementary feed, strong sunlight, and warm temperatures (Tucker and Lloyd, 1984; Tucker and Boyd, 1986). In addition, benthic cyanobacterial mats have been shown to be a highly digestible fish feed for tilapia (*Oreochromis niloticus*) and silver carp (*Hypophthalmichthys molitrix*) (Ekpo and Bender, 1989).

Traditionally, in the Philippines and other Asian countries, brackish water aquaculture of milkfish (Figure 37.4) (*Chanos chanos*) and penaeid species of marine shrimp relies on the growth of benthic cyanobacterial mats (*lablab*), which contain a complex community of filamentous and unicellular cyanobacteria as well as diatoms (Figure 37.5). Farmers traditionally encourage the

FIGURE 37.4 View of ponds used to grow milkfish (*Chanos chanos*) in South Sulawesi, Indonesia. Families traditionally live in huts by the side of ponds and encourage benthic cyanobacterial blooms (called *lablab*) with organic fertilizers to provide natural food for fish. Production is critical to food security for these farmers and worldwide milkfish production is approximately 1 million tons worth US$1 billion.

FIGURE 37.5 Microscopic view of a benthic bloom from a marine shrimp farm near Hiroshima, Seto Inland Sea, Japan. Benthic blooms are commonly called *lablab* and this bloom consists of cyanobacteria (*Oscillatoria* spp. and *Spirullina* spp.) and diatoms (400× magnification).

development of *lablab* in earthen ponds by the addition of organic fertilizers to shallow water at the start of the culture cycle. Milkfish feeding on *lablab* have been reported to have a higher growth rate than fish feeding on mats of filamentous green algae (Lückstädt et al., 2005). Analysis of the chemical composition of *lablab* reveals that it has adequate levels of essential amino acids, high levels of carotenoids and minerals, but the levels of fatty acids are less than that required for growth of milkfish (Lückstädt et al., 2005). Traditionally, *lablab* is used in the early stages of the growth cycle, and is followed by a planktonic microalgal bloom as the depth of the pond water is deliberately increased.

Cyanobacterial mats grow naturally on silage, such as grass and organic agricultural wastes, producing a biomass increased by photosynthesis. Feeding studies revealed that when tilapia were fed cyanobacterial mats on silage, they grew faster than when fed commercial fish feed. This finding suggests that cost savings could be significant, particularly for subsistence farmers in developing countries (Phillips et al., 1994). Also, cyanobacterial mats and algal scrubbers have been used to treat effluent from aquaculture to sequester nutrients and produce a useful, protein-rich product (Bender and Phillips, 2004). Large-scale culture of *Spirulina* in seawater "micro-oceans" has been suggested as an industry of the future (Fox, 1999). It could be a means of producing protein and alleviating malnutrition in developing countries, as well as reducing the greenhouse effect by removing carbon dioxide from the atmosphere.

In summary, each form of aquaculture has an inherent risk of developing cyanobacterial blooms based on the method of production, environment, and organic inputs. Table 37.1 quantifies the production characteristics of the various forms of modern aquaculture and the risk of cyanobacterial blooms. Carp and catfish culture have a high risk of cyanobacterial blooms while tilapia, milkfish, and shrimp farming carry a medium to high risk. In general, the forms of aquaculture with the higher risks of cyanobacterial blooms are those that are based on feeding animals in earthen ponds in either freshwater or brackish water. Notwithstanding this, in a simple form of aquaculture, fish are stocked in reservoirs or water bodies, allowed to grow by eating natural feeds, and later harvested. This form of aquaculture (culture-based fisheries) can also be at medium to high risk from cyanobacterial blooms, depending on the nutrient load in the water body.

In spite of the deliberate culture of *lablab* and other forms of cyanobacteria as natural food source for fish in traditional aquaculture (Figure 37.6); the development of benthic cyanobacterial blooms is often discouraged in many modern forms of aquaculture for a variety of reasons.

First, on sunny days benthic cyanobacterial mats rise to the surface and are blown to the edge of the ponds where they decompose. This generally causes an increase in the levels of ammonia, a decrease in the dissolved oxygen, and outbreaks of disease. Hence cyanobacterial blooms can cause husbandry problems and fish kills. Cyanobacteria are also associated with reduced productivity in penaeid shrimp farms (Yusoff et al., 2001).

Second, off-flavors can be acquired by some fish when grown in earthen ponds and this presents marketing problems. Off-flavors are mainly caused by the rapid absorption of compounds produced by cyanobacteria. The most common off-flavors in aquaculture are two metabolites of *Oscillatoria* spp. and *Anabaena* spp.—geosmin and 2-methylisoborneol. The sensory properties and kinetics of uptake and depuration of these chemicals in fish have been reviewed elsewhere (Howgate, 2004). The off-flavors produced by cyanobacteria have no adverse affect on the health of the fish or the consumer; however, they do have a substantial impact on marketability. The elimination of these compounds is much slower than the rate of uptake, and the depuration rate is reduced as water temperature decreases.

The filamentous cyanobacterium, *Oscillatoria perornata* is common to catfish farms in west Mississippi, USA (Schrader and Harries, 2001), and it produces 2-methylisoborneol, which is rapidly absorbed across the gills and accumulates in the adipose tissue. The fish cannot be harvested when levels exceed quality standards. This results in an average yield reduction of 15% in the U.S. catfish farming (Tucker, 2005). Depuration or purging of fish with clean water can take several days to reduce off-flavors and this results in further financial losses to the industry (Tucker, 2000).

TABLE 37.1
Summary of Worldwide Data on Types of Aquaculture and the Relative Risk of Cyanobacterial Blooms

Aquaculture Families and Grouping	Main Regions for Production[a]	Environment[b]	Method of Production	World Production (Million tons)[c]	Value of Production (Billion $US)[c]	Relative Risk of Cyanobacterial Blooms
Kelp (Laminariaceae) and other aquatic plants	China, Asia-Pacific	Marine	Rope or pole	13.9	9.7	Low
Bivalves and other molluscs[d]	Western Europe, Asia-Pacific	Marine and estuarine	Suspended rope, raft or rack	13.2	14.2	Low to medium
Shrimp and other crustaceans	China, Asia-Pacific, Latin America	Estuarine	Earthen pond	3.7	20.4	Medium to high
Cyprinidae carps	China, Asia-Pacific	Freshwater	Earthen pond	18.2	16.3	High
Salmonids, mainly Atlantic salmon and rainbow trout	Western Europe, Latin America, North America	Marine and freshwater	Fish cage, raceway, tank and pond	1.8	6.1	Low
Clariids including channel catfish	North America, Asia-Pacific, China	Freshwater	Earthen pond	1.0	1.0	High
Oreochromis niloticus and other tilapias	North Africa, China, Asia-Pacific	Freshwater and estuarine	Earthen pond	1.2	1.0	High
Milkfish (*Chanos chanos*)	China, Asia-Pacific	Estuarine	Earthen pond	1.4	1.2	Medium to high
Ornamental and aquarium fish and other aquatic animals[e]	worldwide	Mainly freshwater	Aquarium tanks and small ponds	Not applicable	0.9	Low
Cultured Pearl farming[f]	Asia-Pacific region	Marine and freshwater	Suspended rope	0.0012	0.46	Low

[a] Because of China's dominance in aquaculture production, China is separated from the rest of Asia and the Pacific region.

[b] World aquaculture production in 2004 from marine environments was 30.2 million tons (50.9% of total), from freshwater environments was 25.8 million tons (43.4% of total) and from brackish water environments was 3.4 million tons (5.7% of total) (FAO, 2006; p. 10).

[c] Data from FAO, 2006 (pages 6–12).

[d] Production is dominated by oysters (41%), clams (34%) and mussels (14%) and scallops (11%) (Lovatelli, 2006).

[e] Oliver, 2001.

[f] Anon, 2005.

FIGURE 37.6 A fish farmer in Western Highlands Province in PNG is seen feeding his common carp with vegetable scraps. Mats of cyanobacteria are floating near his hand.

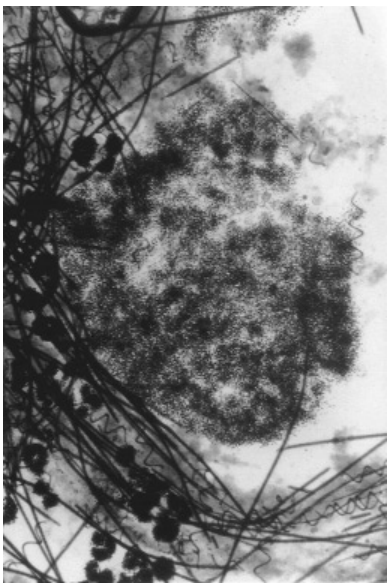

FIGURE 37.7 Microscopic view of a planktonic bloom consisting of *Microcystis* spp., *Oscillatoria* spp., and *Anabaena* spp. collected from a fish pond on the Clarence River, New South Wales, Australia. The ponds were used for culturing silver perch (*Bidyanus bidyanus*) (40× magnification).

Off-flavors have also been reported in silver perch (*Bidyanus bidyanus*) from Australian fish farms (Rowland et al., 1995). Microscopic examination of the planktonic blooms reveals a wide variety of cyanobacterial species (Figure 37.7) and mouse bioassays show that they can be toxic (unpublished). In rainbow trout farms in the United Kingdom, the cause of off-flavors was unknown until a study attributed it to blooms of *Oscillatoria* spp. in the rivers that feed the trout farms (Robertson et al., 2006). Levels of geosmin in fish tissue were typically in the range of 1.0–3.0 μg/kg, while

rivers supplying the farms had levels of geosmin of up to 25 ng/L during summer. Temperature and phosphorus concentration were found to be positively correlated with levels of geosmin in intake water from the rivers.

Selective algicides have been used to minimize cyanobacterial blooms and reduce off-flavors. For example, weekly doses of copper sulfate have been successfully used (Hanson and Schrader, 2006). In addition, treatment of ponds with diuron at 0.01 mg/L on a weekly basis has been suggested as an effective means for reducing cyanobacterial blooms and controlling 2-methylisoborneol (Zimba et al., 2002). More recently, challenges tests with a range of compounds have indicated that 9,10-anthraqinone, a naturally occurring chemical in rye grass (*Lolium perenne*), may be selectively toxic to *Os. perornata* (Schrader, 2006).

The third major area of concern for aquaculture is the possibility of cyanotoxicity, and this will be the focus for the remainder of this chapter.

37.3 CYANOTOXINS IN AQUACULTURE

While fish kills have occurred during cyanobacterial blooms, the direct link between mortalities in fishponds and cyanotoxins has been elusive, because fish kills can often be explained by low levels of dissolved oxygen or increases in ammonia concentration when the bloom crashes (Seymour, 1980; Sevrin-Reyssac and Pletikosic, 1990). For example, recently in a Japanese shrimp farm, a bloom of *Chroococcus turgidus* was associated with mass mortalities of farm-raised *Penaeus japonicus*, but the direct cause was the increase in pH of pond water (Momoyama, 2004).

Further, although cyanobacteria are ubiquitous and can produce a range of toxins, there is a relatively low level of reporting of scientific investigations of cyanobacteria in aquaculture. For example, at the 2006 annual conference of the World Aquaculture Society, only two out of 1068 presentations were relevant to the topic of cyanotoxins in aquaculture. One paper described a plan to evaluate the safety of eating crayfish caught in lakes in Tuscany, Italy by fishermen when blooms of *Microcystis* are common (Tricarico et al., 2006). The other paper compared the advantages of light microscopy and MALDI-TOF MS in the search for a means of identifying toxic blooms of *Microcystis* (Sejinohova et al., 2006). In spite of this apparent lack of scientific activity, a risk survey of the main aquaculture industries in the United States found that the catfish industry rated toxic algae as the third most significant risk, behind diseases and bird predation (Sempier et al., 2007).

On closer examination, microcystins have been reported to cause hepatotoxic effects in farmed catfish (Zimba et al., 2001) and farmed salmon (Andersen et al., 1993). Microcystins also produce pathological effects in tilapia (Preito et al., 2006), silver carp (Li et al., 2007), common carp (Fischer and Dietrich, 2000), and loach (Liu et al., 2002). The neurotoxin anatoxin-a, is produced by several genera of cyanobacteria and it has been reported to cause mortalities in juvenile common carp under experimental conditions (Oswald et al., 2006). Exposure to 0.768 μg/g dry weight of freeze-dried *Anabaena* spp. caused mortalities with 28 h.

The occurrences of cyanotoxins in aquaculture can be usefully considered on a regional basis because of the difference in the methods of aquaculture between regions. Also, some studies from wild fisheries are relevant to aquaculture, and are included where appropriate.

In Asian countries, fish are often released into freshwater reservoirs, ponds, and lakes or held in cages. These water bodies are being increasingly impacted by inputs from cities, industries, agriculture, and fish farming. Figure 37.8 shows an example of an extensive fish kill as observed in Spring of 2004 in a lake in Wuhan. The fish mortalities occurred during a planktonic cyanobacterial bloom and such blooms and associated fish kills have become a frequent occurrence in freshwater lakes in China in recent times. Those fish kills are associated with blooms of *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nostoc*, *Planktothrix*, *Oscillatoria*, and *Synechococcus* (Kaya, 2005). The blooms generally occur in spring, summer, and autumn and the cyanotoxins that were detected are anatoxin-a, saxitoxins, cylindrospermopsin, microcystins, and thionsulolipid. Also, paralytic shellfish poisons (PSPs) have been confirmed by mouse bioassay following analysis of

FIGURE 37.8 Dead grass carp and other carp species floating in a bloom of *Microcystis* spp. in a water reservoir in Wuhan, China.

strains of *Aphanizomenon flos-aquae* collected from Lake Dianchi in Yunnan Province in southwestern China (Liu et al., 2006). Lake Dianchi is the sixth largest lake in China and has been an important source of drinking water and fish farming. The findings indicate that hazards from PSP toxins in freshwater lakes and reservoirs in modern China may be more serious than previously believed (Liu et al., 2006).

Mortalities of fish have been reported for cyanobacterial blooms in earthen fish ponds and experiments have shown that microcystins were involved, indicating that cyanotoxins may enter the food chain through fish farming (Kaya, 2005). In addition, in a fishpond in Wuhan, China, the cyanobacterium, *Raphidiopsis curvata* was found to produce the hepatotoxins, cylindrospermopsin at 0.56 μg/g and deoxycylindrospermopsin at 1.3 mg/g (Carmichael et al., 2001).

In Europe, there have been a number of reports on mortalities with wild fish and shellfish. Ireland has experienced fish kills associated with microcystins (Codd et al., 2005b). Summer fish kills in Kucukcekmece Lagoon in Istanbul, Turkey, coincide with thick blooms of *Microcystis* and increased concentrations of microcystins (Albay et al., 2005). Aquatic fauna from Greek freshwaters have been screened for microcystins and the concentrations ranged from 0.020 μg/g to 1.5 μg/g dry weight for eight species of fish (Gkelis et al., 2006). For mussel (*Anodonta* spp.) and water snail (*Viviparus contectus*), the microcystin concentration ranged from 1.650 μg/g to 3.495 μg/g dry weight (Eriksson et al., 1989).

In Lake Ammersee in southern Germany, increasing oligotrophy and regular blooms of *Planktothrix* spp. have been associated with the reduced body weight and lower abundance of wild whitefish, *Coregonus lavaretus* (Ernst et al., 2007). The fish were found to consume the cyanobacteria, and microcystins were detected in gut and liver tissue. Microcystin-RR is known to inhibit glycogen metabolism and extracts made from *Planktothrix* spp. were found to be cytotoxic to whitefish eggs and larvae. Exposure caused malformations in fish typical of cyanotoxin exposure (Ernst et al., 2001).

Juvenile roach, *Rutilus rutilus*, is able to maintain liver glycogen and muscle protein concentrations when fed with the cyanobacterium, *Aphanizomenon*. However, when the roach was fed on *Microcystis*, glycogen levels were reduced (Kamjunke et al., 2002). Incomplete digestion of *Microcystis* was the main reason provided for the negative growth rate of roach when fed on this cyanobacterium.

Under experimental conditions, high doses of hepatotoxins from cyanobacteria have been shown to cause liver damage in some fish. Microcystin-LR has been reported to cause liver damage in Atlantic salmon (Andersen et al., 1993), rainbow trout (Tencalla et al., 1994; Fischer et al., 2000), and common carp (Fischer and Dietrich, 2000), while nodularin produces similar effects in

Baltic sea trout (Kankaanpää et al., 2002). Gavage feeding of common carp (*Cyprinus carpio*) with a single sublethal dose of toxic *Microcystis aeruginosa* (containing microcystin-LR of 400 μg/kg) caused rapid pathological changes. Observations included damage to the hepatopancreas characterized by dissociation of hepatocytes and early onset of apoptotic cell death, while damage to the renal proximal tubules included vacuolation of individual tubular epithelial cells, apoptosis, and cell shedding (Fischer and Dietrich, 2000).

When sea trout (*Salmo trutta m. trutta* L.) were exposed to a single dose of food containing nodularin from *Nodualria spumigena*, nodularin-like compounds accumulated in the liver (1.2 μg/g by day 8) and muscle (up to 0.125 μg/g). The nodularin produced severe but reversible damage to the liver and was detoxified rapidly (Kankaanpää et al., 2002).

In Brazil, the eutrophication of water bodies has been associated with increasing occurrence of blooms of toxic *Microcystin aeruginosa* in freshwater lakes, lagoons, and reservoirs (Huszar et al., 1998). In addition, since 1956, species of tilapia have been introduced to these water bodies to enhance fish stocks. Studies reveal that in laboratory experiments and field studies, microcystins accumulate in the liver and muscle of tilapia to levels of 2.8 μg/g and 0.8 μg/g, respectively (Magalhães et al., 2001; Soares et al., 2004). Microcystins have also been shown to accumulate in *Or. niloticus* in a fish farm in Egypt (Mohamed et al., 2003).

Mortalities of channel catfish, *Ictalurus punctatus*, have been attributed to ingestion of toxic blooms of *Microcystis aeruginosa* (Zimba et al., 2001). The mortalities followed sudden temperature drops of 5°C and necropsy of fish revealed congested liver and spleen tissue, which indicated microcystin toxicosis. Other channel catfish farms in southeastern United States using brackish well water have experienced fish kills that have been reported to be associated with thick blooms of a halophytic cyanobacteria, *Anacystis marina* rather than *Microcystis* (Snyder et al., 2002).

In Australia, cyanobacterial blooms have become a serious problem in recent years (Burch and House, 2005) and toxic blooms of cyanobacteria have resulted in accumulation of a variety of toxins in shellfish and fish. Edible mussels accumulated nodularin during a bloom of *Nodularia spumigena* (Falconer and Choice, 1992) and under experimental conditions (Strogyloudi et al., 2006). Freshwater mussels accumulated high levels of PSPs when fed toxic *Anabaena circinalis* (Negri and Jones, 1995). The marine cyanobacterium, *Trichodesmium erythraeum*, has caused substantial mortalities of farmed pearl oysters, *Pinctada maxima*, in Western Australia (Negri et al., 2003). Redclaw crayfish from an earthen aquaculture pond, which had a bloom of *Cylindrospermopsis*, accumulated 4.3 μg/g of cylindrospermopsin in the hepatopancretic tissue and 0.9 μg/g in freeze-dried muscle tissues (Saker and Eaglesham, 1999).

On occasions, cyanotoxins have been reported to cause mortalities of farm-raised penaeid shrimp. Blooms of *Spirulina subsalsa* were associated with an outbreak of hemocytic enteritis and mortalities of *Pe. styliostris* in United States of America (Lightner, 1978). Blooms of *Shizothrix calcicola* have also been reported to cause hemocytic enteritis (Lightner, 1996). A neurotoxic effect associated with a bloom of *O. corakiana* was considered to be the cause of mortalities of *Pe. monodon* and *Pe. japonicus* in Australian shrimp farms (Smith, 1996). Since then, nodularin was detected in blooms of *N. spumigena* in Australian shrimp farms that coincided with shrimp mortalities (Figure 37.9). However, challenge tests by intramuscular injection indicated that *Pe. monodon* can rapidly eliminate nodularin (Smith and Kankaanpää, 2001; Kankaanpää et al., 2005; Pflugmacher et al., 2005). Also, mouse bioassays and chemical analysis of supernatants and pellets of three other blooms from shrimp (*Pe. Monodon*) farms in Australia revealed a range of hepatotoxins, including microcystin-LR, microcystin-RR, microcystin YR, microcystin LA, and other unidentified forms of microcystin (Smith, 2003).

In United States toxic blooms of *Microcystis aeruginosa* and *Anabaena* spp. were associated with mortalities in shrimp ponds at a hatchery producing *Litopenaeus vannamei* (Zimba et al., 2006). The concentration of free microcystin-LR was 55 μg/g in dead shrimp (19 g) and 45 μg/L in water samples from the affected ponds. In comparison, the concentration of microcystin-LR was not detected in shrimp or water from ponds without mortalities.

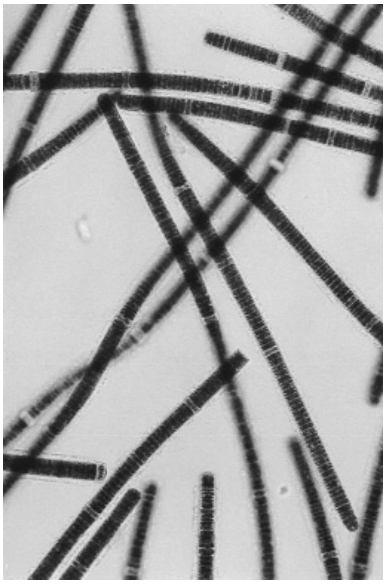

FIGURE 37.9 Microscopic view of *Nodularia spumigena* which occurs on occasions as a toxic planktonic bloom during summer months in earthen ponds at shrimp farms (*Penaeus monodon*) on Palmers Island, northern New South Wales, Australia (trichome width of 12 μm, 400× magnification).

37.4 ASSIMILATION AND DEPURATION OF CYANOTOXINS BY SPECIES IN AQUACULTURE

Many aquatic organisms have an ability to assimilate, detoxify, and depurate cyanotoxins. The process of detoxification of microcystins in fish is related to a conjugation reaction of microcystins to glutathione through the activity of glutathione S-transferase (Pflugmacher et al., 1998). The excretion of microcystins in fish and other aquatic species is mainly in bile and via feces as microcystin bound to glutathione or as a final product of the detoxification process (Sahin et al., 1996; Mohamed and Hussein, 2006).

When zebra mussels (*Dreissena polymorpha*) were provided with a diet of *Microcystis aeruginosa* as a single food source, the free unbound microcystin-LR attained a maximum level of 10.8 μg/g dry weight within 1 week. Conjugates of microcysin that result from detoxification (Pflugmacher et al., 1998) were very low, at ca. 1% of free microcystin-LR; however, covalently bound microcystins reached 62% of free microcystin levels (Pires et al., 2004). Zebra mussels appear to be able to reject selectively the more toxic strains of *Microcystis aeruginosa* under experimental conditions (Juhel et al., 2006). Molluscs appear to be efficient at assimilating microcystins and during depuration, the toxin levels rapidly decreased in 6–13 days to very low or negligible levels in various species of mussels and clams (Vasconcelos, 1995; Prepas et al., 1997; Pires et al., 2004).

When mussels (*Mytilus edulis*) were exposed to toxic *Nodularia* cells under laboratory conditions, the amount of nodularin in mussel tissue increased linearly with toxin concentration and the digestive gland had the highest concentration of nodularin (Strogyloudi et al., 2006). Depuration was relatively rapid, with a 75% reduction in nodularin levels in 72 h. Bioaccumulation of nodularin-like compounds also occurred in a study with toxic *N. spumigena* and the marine clam *Macoma balthicafo* (Lehtonen et al., 2003). Other related screening studies have demonstrated accumulation of nodularin and induction of oxidative stress during blooms of *N. spumigena* in the Baltic Sea with mussels (*My. edulis*), clam (*Ma. balthicafo*), flounder (*Platichthys flesus*), Atlantic cod (*Gadus morhua*), herring (*Clupea harengus*), and brown alga (*Fucus vesticulosus*) (Sipiä et al., 2001a, b, 2002, 2006; Pflugmacher et al., 2007). The concentrations of nodularin in brown alga was

0.014 μg/g fresh weight (Pflugmacher et al., 2007), herring was 0.010 μg/g dry weight (Sipiä et al., 2006), three-spined stickleback was 0.0028–0.70 μg/g dry weight (Sipiä et al., 2006), and blue mussel was up to a maximum of 2.0 μg/g fresh weight (Sipiä et al., 2001b).

A study of physiological effects in the green-lipped mussel (*Perna viridis*) to exposure to blooms of *Nodularis spumigena* reveals a complicated picture (Davies et al., 2005). As the bloom density increased and post-bloom lysis occurred, the activity of antioxidative, detoxification enzymes changed in opposite directions, and different organs of the mussel appear to come under toxic stress.

A study of seasonal dynamics in microcystin concentrations in aquatic organisms in Lake Suwa, Japan revealed that the freshwater snail, *Sinotaia histrica*, was able to accumulate and efficiently depurate microcystins (Xie et al., 2007). The digestive tract had the highest microcystin concentration (mean 9.03 μg/g dry weight), followed by the gonad (mean 6.90 μg/g dry weight) and the hepatopancreas (mean 5.38 μg/g dry weight). Of the three fish that were also studied, *Carassius auratus* had the highest concentration of 0.079 μg/g body-weight. In another study of bioaccumulation and detoxification by *Lymnaea stagnalis* of mycrocystin through ingestion of toxic cyanobacteria, *Planktothrix agardhii* (Lance et al., 2006) the water snail showed a preference for the cynaobacteria and accumulated concentrations of microcystins of up to 80.4 μg/g dry weight. After removal of *Plan. agardhii*, detoxification was rapid, with 64% of the toxins lost from snail tissue within 7 days, but was at 3.5 μg/g dry weight after 21 days.

The transfer of and depuration of microcystins within a model food web of cyanobacteria, zooplankton, and sunfish (*Lepomis gibbosus*) has been investigated. The fish initially accumulated microcystins in liver and muscle tissue to levels of 0.011 μg/g wet weight on day 4 (Smith and Haney, 2006). However, thereafter, the concentration of microcystins decreased and by day 9, it was 0.003 μg/g wet weight, which indicated that a detoxification and excretion pathway was induced.

37.5 POSITIVE AND NEGATIVE ASSOCIATIONS BETWEEN AQUACULTURE AND TOXIC CYANOBACTERIA

In China and other Asian nations, a culture-based fishery in water reservoirs provides protein to rural communities (Li, 1988). Water reservoirs are managed by the local communities who determine which fish are stocked, the stocking density, and timing of recapture. Culture-based fisheries use fewer resources and have less negative environmental impacts than many other forms of aquaculture (Li, 1988; De Silva, 2003; Wijenayake et al., 2005). When small seasonal reservoirs are used, such as in Sri Lanka, the recapture rate can be very high and the average fish yield per unit area is 892 kg/ha, which is comparable to semi-intensive aquaculture in earthen ponds (De Silva, 2003; Wijenayake et al., 2005).

Filter-feeding freshwater fish, most notably silver carp (*H. molitrix*) and bighead carp (*Aristichthys nobilis*), are routinely stocked in water reservoirs in China and other Asian countries because of the increase fish yield that is obtained from the reservoir and the ability of the fish to reduce noxious cyanobacterial blooms (Smith, 1985; Xie and Liu, 2001). In an experiment to demonstrate biomanipulation of cyanobacterial blooms by fish, pens were stocked with silver carp and bighead carp at a low density of 40 g/m^3 (Ke et al., 2007). *Mycrocystis* was the major phytoplankton in summer months and on average constituted 43% of the phytoplankton in the gut of silver carp. Filtration rates of phytoplankton by silver carp were 0.22–1.53 L/g/h and these rates were substantially higher than for bighead carp (Ke et al., 2007). Inside the pens, the biomass of *Microcystis* and concentration of microcystins were both lower than in the surrounding water, though variability was too high to be conclusive. In another study, silver carp was found to be efficient at controlling *Microcystis* blooms, reducing microcystin levels and increasing water clarity inside enclosures in Lake Shichahai in Beijing, China (Zhang et al., 2006).

In a similar study, silver carp was reported to be capable of achieving good growth rates when stocked in a huge cage or pen (1.088 km^2) in Lake Taihu, China and grown *in situ* with toxic *Microcystis* blooms (Chen et al., 2006). Examination of the gut contents of the fish revealed that

FIGURE 37.10 Culture of tilapia, *Oreochromis nioliticus*, and carp species in cages in Yonki Reservoir, a freshwater lake in Eastern Highlands Province, Papua New Guinea. Cage culture of fish has become very common in many Asian countries and is a cause of eutrophication and cyanobacterial blooms.

silver carp ingests *Microcystis* (up to 84% of gut contents) and the concentration of microcystins in intestines, liver, and muscle of silver carp were 24.3, 0.957, and 0.197 μg/g dry weight, respectively. The results suggest that silver carp ingests more microcystins than mussels and other fish but accumulates less microcystins. Nevertheless, silver carp should not be consumed during dense *Microcystis* blooms (Chen et al., 2006). In a related study, bighead carp (*Ar. nobilis*) was found to be resistant to microcystins and appeared to be a suitable fish for controlling cyanotoxin contamination in eutrophic waters (Chen et al., 2007).

Nevertheless, fish cages (Figure 37.10) in freshwater lakes and water reservoirs cause eutrophication whenever supplementary feeds are applied. Also, higher levels of nutrients have been detected near shrimp hatcheries and elevated levels of cyanobacteria occur in waters adjacent to the hatcheries (Sarojini and Subbarangaiah, 2001). The discharge of waters from shrimp ponds with high densities of cyanbacteria and elevated levels of nutrients has the potential to cause an impact on receiving waters.

Discharges from a trout farm beside the Lizhma River, Russia have been reported to impact on the structure and distribution pattern of phytoplankton and zooplankton. The numbers of cyanobacteria as well as the chlorophyll concentration were found to increase significantly in the region of discharge from the fish farm (Sterligova et al., 2001).

In recognition of environmental problems by effluent from fish farms, freshwater mussel, *Elliptio complanata*, has been used to treat pond water by removing cyanobacterial blooms of *Microcystis* from aquaculture systems (Stuart et al., 2001). Similar studies have been carried out to treat effluent from shrimp farms and other aquaculture operations.

The water quality parameters for streams above and below channel catfish farms in Alabama, USA, have few differences (Boyd et al., 2000). The environmental impacts of channel catfish farms are reduced through minimal discharge of pond water and using accumulated sediment to repair pond walls rather than discarding it outside of the ponds.

37.6 RISKS FROM AQUACULTURE TO FOOD SOURCES AND HUMAN HEALTH

Impacts on the health of consumers through exposure to cyanotoxins from aquacultural products have not been demonstrated. However, Table 37.2 summarizes reports, mainly from wild fisheries,

TABLE 37.2
The Concentration of Cyanotoxins in Fisheries and Aquaculture Species

Species	Comment	Cyanotoxin	Concentration (μg/g Dry or Wet Weight)	Reference
Algae				
Brown alga (*F. vesticulosus*)	Wild plant	Nodularin	0.014 WW	Pflugmacher et al., 2007
Molluscs				
Water snail (*Anodonta* spp.)	Whole body of wild animal	Microcystins	1.650–3.495 WW	Gkelis et al., 2006
Freshwater snail (*S. histrica*)	Wild animal	Microcystins	9.03 DW	Xie et al., 2007
Freshwater snail (*L. stagnalis*)	Wild animal	Microcystins	80.4 DW	Lance et al., 2006
Zebra mussel (*D. polymorpha*)	Whole body of wild animal	Microcystin-LR	10.8 DW	Pflugmacher et al., 1998
Blue mussel (*M. edulis*)	Wild animal	Nodularin	2.0 WW	Sipiä et al., 2001b
Crustaceans				
Redclaw (*C. quadricarinatus*)	Hepatopancreas and muscle of farm animal	Cylindrospermopsin	4.3 and 0.9 DW	Saker and Eaglesham, 1999
Shrimp (*L. vannamei*)	Whole body of farmed animal	Microcystin-LR	55 DW	Zimba et al., 2006
Fish				
Eight species of freshwater fish	Whole body of wild animal	Microcystins	0.020–1.5 DW	Gkelis et al., 2006
Sea trout (*S. trutta*)	Liver and muscle for wild animal	Nodularin	1.2 and 0.125 WW	Kankaanpää et al., 2002
Tilapia spp.	Liver and muscle of wild animal	Microcystin	2.8 and 0.8 WW	Magalhaes et al., 2001
Herring (*C. harengus*)	Wild animal	Nodularin	0.010 DW	Sipiä et al., 2006
Three-spined stickleback	Wild animal	Nodularin	0.0028–0.07 DW	Sipiä et al., 2006
C. auratus	Wild fish	Microcystins	0.079 DW	Xie et al., 2007
Silver carp (*H. molitrixon*)	Intestine, liver, muscle of farm fish	Microcystins	24.3, 0.957, 0.197 DW	Chen et al., 2006

that indicate that this route is potentially of concern and the risks need to be continually evaluated. Experienced researchers have suggested that the most likely route for human exposure to cyanotoxins is through drinking contaminated water (oral route) or recreational use of lakes and rivers (dermal route) (Falconer, 1996). Dietary supplements made from cyanobacteria could also pose a health risk because these products could be accidentally contaminated with microcystins or other cyanotoxins (Carmichael and Falconer, 1993; Carmichael, 1994; Hider et al., 2003; Bruno et al., 2006).

There are reports of uncomfortably high levels of cyanotoxins in some seafood, but this mainly relates to wild aquatic animals or laboratory trials. For example, a bloom of *N. spumigena* in the Gippsland lakes area of southern Victoria, Australia, in 2001 posed a threat to the health of consumers. Hepatotoxin concentrations in wild mussels and marine prawn tissue were at or above acceptable levels and wild harvest of seafood was restricted (Van Buynder et al., 2001). For microcystins

and nodularins the healthy, alert levels (in μg/g) were 0.25 for fish, 1.1 for prawns, and 1.5 for mussels (Van Buynder et al., 2001).

In fish caught in Sepetiba Bay, Brazil organic pollution has resulted in regular blooms of the cyanobacteria, *Synechocystis aquatilis f. aquatilis,* that produces microcystins (Magalhães et al., 2003). Microcystins were detected in all of the wild fish and crustacean tissue that were sampled, with a maximum level of 103.3 μg/kg (tolerable daily intake, TDI; of 0.52 μg/kg/day) and 19% of animals were above the limit recommended for human consumption. World Health Organization (WHO) recommended that the maximum TDI for consumption of foods containing cyanobacterial toxins and microcystins is 0.04 μg/kg/day (Chorus and Bartram, 1999).

In laboratory experiments with tilapia (*Or. niloticus*), fish accumulate microcystins in muscle tissue to a level of 0.1 μg/g (Zhao et al., 2006). Should the fish be consumed the ingestion would be up to 0.075 μg/kg body weight/day, which exceeds the TDI for humans. The findings indicate that when tilapia is feeding on toxic cynaobacteria, it is not suitable for human consumption (Soares et al., 2004; Zhao et al., 2006).

Finally, workers at aquaculture operations where toxic cyanobacterial blooms do occur need to be vigilant and take actions to reduce risks (Smith, 2000). They need to reduce contact with potentially toxic scums and minimize the dermal route of cyanotoxin exposure. In addition, aerosols whipped up by mechanical aerators could greatly increase the risk of inhaling cyanotoxic foam.

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Part XIIA

Neurotoxins

38 Cyanobacterial Neurotoxins, Anatoxin-A and Analogues: Detection and Analysis

Kevin J. James, Justine Dauphard, Janet Crowley, and Ambrose Furey

CONTENTS

38.1 CYANOBACTERIAL NEUROTOXINS

Neurotoxins are produced by various species of cyanobacteria, including *Anabaena*, *Planktothrix* (formerly *Oscillatoria*), and *Aphanizomenon* spp. In mouse bioassays, symptoms of cyanobacterial neurotoxin poisoning include tremors, convulsions, muscle fasciculations, and death due to respiratory arrest occurs within 30 min. There are no apparent signs of organs damage visible by autopsy (Sivonen et al., 2000). Three classes of neurotoxins have been identified in cyanobacteria: (i) anatoxin-a (AN) and analogues, (ii) anatoxin-a(s), and (iii) saxitoxin and analogues. The saxitoxin class were first identified in the marine environment and are responsible for the toxic syndrome called paralytic shellfish poisoning (PSP). Several previous reviews have dealt with the potential environmental and health hazards associated with toxic cyanobacteria (Harada, 1999; Sivonen et al., 2000; Briand et al., 2003). Before the identification of AN as the causative toxin in cyanobacterial-induced animal fatalities, it was known as a very fast death factor (VFDF) (Gorham, 1972).

In 1992, a methylene analogue of AN, homoanatoxin-a (HMAN) (Figure 38.1; R=C_2H_5), was isolated from *Planktothrix* (*Oscillatoria) formosa* in Norway (Skulberg et al., 1992). AN and HMAN act by enhancing the release of acetylcholine (ACh) from peripheral cholinergic nerves (Lilleheil et al, 1997). HMAN is much less common than AN but has been found in Ireland and Japan (Furey et al., 2003a), (Namikoshi et al., 2003). Anatoxin-a(s) is structurally unrelated to AN but was also

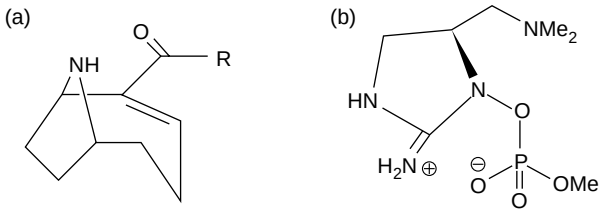

FIGURE 38.1 Structures of (a) Anatoxin-a (R=CH_3), Homoanatoxin-a (R=C_2H_5); (b) Anatoxin-a(s).

found in some *Anabaena* spp. and differs toxicologically from AN by being a potent inhibitor of acetylcholinesterase (Hyde and Carmicheal, 1991).

The present authors have recently published two reviews, one on the synthetic approaches to anatoxins (Armesto et al., 2006) and another on the discovery, distribution and toxicology of these neurotoxins (James et al., 2006). The main focus of this review is therefore confined to the methods used for the detection and analysis of anatoxins from cyanobacteria.

38.2 ANATOXIN-A(S)

38.2.1 Introduction

A study of the neurotoxins in strains of *Anabaena flos-aquae* from North American lakes revealed a new toxin that had similar poisoning symptoms as AN. However, other symptoms included severe salivation and lachrymation in mice, rats, and chickens, and in rats there was also chromodacyrorrhea (bloody tears). This new toxin was named anatoxin-a(s), abbreviated herein as AN-a(s), due to the observed salivation. It was structurally elucidated as a unique *N*-hydroxyguanidine methyl phosphate ester (Figure 38.1) (Matsunaga et al., 1989). Unfortunately, the similarity in the names, anatoxin-a and anatoxin-a(s) has led to some confusion in the literature. AN-a(s) is not only chemically unrelated to AN but it also has a very different physiological mode of action. AN-a(s) is an acetylcholinesterase inhibitor (Mahmood and Carmichael, 1987). In general, the action of AN-a(s) is similar to the synthetic organophosphate insecticides that also act by inhibiting acetylcholinesterase (Hyde and Carmichael, 1991).

AN-a(s) has been reported to be responsible for the fatal intoxications of ducks and swine (Cook et al., 1989). AN-a(s) was also implicated in the deaths of nine dogs at Richmond Lake, SD, USA in 1985 (Mahmood et al., 1988). One of the first reports of this toxin outside North America was when it was identified in the stomach contents of poisoned birds from a lake in Denmark (Henriksen et al., 1997; Onodera et al., 1997). There have been other suspected occurrences of AN-a(s), including in Brazil (Molica et al., 2005), based on observed anticholinesterase activity but the lack of a commercially available standard and the toxin's lability render absolute confirmation difficult. AN-a(s) readily loses the methyl phosphate moiety on storage to produce a nontoxic product.

38.2.2 Bioassays and Biosensors

Most of the published studies of AN-a(s) in cyanobacteria have involved live animal bioassays; a study using mice showed that this toxin has an LD_{50} of approximately 50 μg/kg (i.p.) (Mahmood and Carmichael, 1986). The same authors also observed the acetyl cholinesterase inhibition that is characteristic of AN-a(s). The inhibitory effect of this toxin remains the most effective means of detection and has been exploited in an assay using electric eel acetylcholinesterase (Mahmood et al., 1988) and in a number of subsequent bioassays. Although the inhibitory effect of AN-a(s) is similar to synthetic organophosphate insecticides, it was shown that AN-a(s) as an active site-directed

inhibitor of acetylcholinesterase and was resistant to oxime reactivation owing to the structure of its enzyme adduct (Hyde and Carmichael, 1991). Differences in activity have been exploited in the development of a biosensors for AN-a(s).

A biosensor based on the electrochemical detection of the activity of electric eel acetylcholinesterase was developed. Disposable amperometric sensors were produced that gave a detection limit of 1 μg AN-a(s)/L. Oxime reactivation was also used to discriminate between the toxin and potential insecticides present in water samples (Villatte et al., 2002).

Another biosensor was developed for AN-a(s) by Devic et al. (2002) that addressed the lack of sensitivity and specificity of detection of some bioassays. An acetylcholinesterase was engineered to increase its sensitivity and a combination of mutants was used to obtain increased analyte specificity. The sensitivity of detection achieved was reported to be below 1 nM/L. By using a four-mutant set of acetylcholinesterase variants, two of which are sensitive to AN-a(s) and two are sensitive to the insecticides, an improvement in the specificity of the biosensor for the cyanobacterial neurotoxin was achieved (Devic et al., 2002).

38.3 ANATOXIN-A AND ANALOGUES

38.3.1 Introduction

Anatoxin-a (AN) has not been implicated in human toxicosis but there have been many confirmed incidents where this toxin led to rapid animal fatalities following the consumption of lakewaters containing cyanobacteria. AN was isolated and determined to be a bicyclic secondary amine, 2-acetyl-9-azabicyclo[4.2.1]non-2-ene (Figure 38.1; R=CH_3), incorporating an α,β-unsaturated ketone moiety and was named because of its occurrence in *Anabaena* spp. (Devlin et al., 1977). Although the first confirmed detection of AN poisoning was recorded in Canada (Gorham, 1972) the majority of reported occurrences and toxic incidents have occurred in Europe. The first case of AN in benthic cyanobacteria was also reported in Scotland. The neurotoxic bloom consisted mainly of *Planktothrix* spp. and was associated with canine fatalities in 1990 and 1991. AN was identified in the stomach contents of the 1991 fatality (Edwards et al., 1992).

In Finland, Sivonen et al. (1990) performed the first survey of cyanobacterial blooms in 1985–1987 and found that 13 out of 30 bloom samples contained AN. A number of cattle poisonings associated with *Anabanea* blooms occurred (Sivonen et al., 1990). The first identification of HMAN was isolated from a strain of *Planktothrix formosa* (Skulberg et al., 1992). AN was also implicated as the causative agent in incidents of fatal canine neurotoxicosis in Ireland (James et al., 1997) and France (Gugger et al., 2005). AN and HMAN were found in *Anabaena* and benthic *Planktothrix* spp. (Furey et al., 2003a). AN and HMAN have also been reported in Japan and were isolated from *Raphidiopsis mediterranea* (Namikoshi et al., 2003).

AN and HMAN readily degrade in natural samples to their dihydro- and epoxy-analogues (Figure 38.2) and it is important to protect samples from light and to acidify them to limit the rate of degradation before analysis. Only AN is commercially available and therefore most published analytical methods are limited to the determination of this toxin alone.

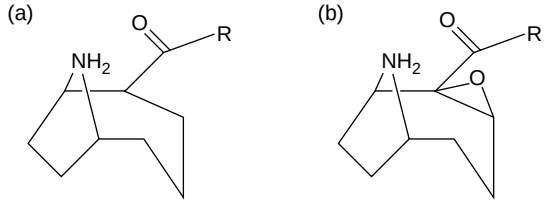

FIGURE 38.2 Structures of (a) dihydroanatoxin-a (R=CH_3), dihydrohomoanatoxin-a (R=C_2H_5); (b) epoxyanatoxin-a (R=CH_3), epoxyhomoanatoxin-a (R=C_2H_5).

38.3.2 Bioassays

Bioassays have been important in assessing the toxicity of cyanobacterial toxins. Usually the pure toxin or an extract of the toxin from a bloom is injected intraperitoneally (i.p.) to mice (Al-Layl et al., 1988; Baker and Humpage, 1994), or rats (Astrachan et al., 1980), (Adeyemo and Siren, 1992). The symptoms of AN poisoning are rapid and include muscle fasciculation, convulsions, exaggerated abdominal breathing, and death within minutes (5–10 min) due to respiratory failure. The signs of toxicity can be observed for 24 h and then mice can be subjected to postmortem autopsy to observe any abnormalities in organs (Watanabe et al., 2003). Relying only on observation of clinical symptoms, the mouse or rat bioassay suffers from poor sensitivity and is inadequate for monitoring sublethal levels of AN (Stevens and Krieger, 1988). Furthermore, it is also a time consuming, cumbersome, as well as ethically questionable monitoring technique. Al- Layl et al. (1988) also showed that the bioassay fails to reveal whether toxic alkaloids and peptides are produced by the same natural bloom since death owing to the alkaloids is really rapid (Al-Layl et al., 1988). The effects of subacute doses of AN were studied by administering the toxin orally and i.p. to rats. No changes were observed in body weight or food consumption and the study concluded that a subacute dose of AN does not cause any apparent toxic effects (Astrachan and Archer, 1981).

Lahti et al. (1995) assessed three bioassays to determine which were the ones allowing a rapid detection of cyanobacterial hepatoxins and neurotoxins (Lahti et al., 1995). *Artemia salina*, luminescent bacteria, and *Pseudomonasputida* were evaluated and compared. The study showed that only the *A. salina* test detected the toxicity of microcystins, nodularin, and AN. Moreover, it also showed that hepatotoxins cause larvae death whereas AN only affects the ability of the larvae to move forward. Therefore, this assay can be used as an indicator but obviously not for quantification.

38.3.3 Thin Layer Chromatography

Thin layer chromatography (TLC) is a simple and inexpensive technique that has been used in earlier studies to screen, isolate, and purify AN in bloom material (Devlin et al., 1977; Harada et al., 1989). It allows a fast screening of multiple samples. Devlin et al. (1977) used silica TLC for the final purification of AN from *A. flos-aquae* strains. However, TLC is only a semiquantitative analytical technique and all the positive sample need to be reanalyzed by another chromatographic method to achieve full quantitative data. Al-Layl et al. (1988) used high performance TLC (HPTLC) for both purification and identification of AN, also from *A. flos-aquae* strains (Al-Layl et al., 1988). Sivonen et al. applied TLC to identify AN obtained from lyophilized algal bloom material (Sivonen et al., 1989). TLC has also been used to purify AN as a reference standard to screen 23 clone cultures of *A. flos-aquae* (Kangatharalingam and Priscu, 1993). The diazonium reagent, Fast Black K salt, was used as a derivatizing reagent for the detection of AN using TLC (Ojanpera et al., 1995). This method was based on an *in situ* color reaction of algal extracts containing AN and was reported to have a limit of detection of 10 μg AN/g in lyophilized algal material.

38.3.4 Gas Chromatography–Electron Capture Detection

Gas chromatography with electron capture detection (GC-ECD) is a very sensitive analytical method for analytes containing halogen substituents. Since anatoxins do not contain halogens, it is advantageous to derivatize these toxins with a reagent containing multiple halogen atoms. One such method was developed for monitoring low levels of AN, the comparison of toxic and nontoxic strains and one that was compatible with a variety of possible matrices (Stevens and Krieger, 1988). This method involved derivatizing AN, and an internal standard, *sec*-butyl nipecotate, using trichloroacetic anhydride before analysis using GC-ECD. This was found to be over three orders of magnitude more sensitive (5 ng on-column) than the previously developed methods, including mouse bioassay and liquid chromatography–ultraviolet detection (LC-UV). However, a somewhat complicated cleanup procedure was required involving an initial C_{18} solid-phase extraction (SPE)

step before derivatization and a second C_{18} SPE step to remove impurities for analysis by GC-ECD. Haugen et al. developed another GC-ECD method involving a single SPE step that involved derivatization with heptafluorobutyric acid anhydride (Haugen et al., 1994). This method was used to analyze both AN and HMAN and gave a limit of detection of 3 ng/L. A more sensitive method with a limit of detection of 2.5 pg on-column was developed, which involves the derivatization of AN and the internal standard, 3-π-perazinobenzotrifluoride (PBTF), to form *N*-pentafluorobenzyl-anatoxin-a and *N*-pentafluorobenzyl-3-piperazinobenzotrifluoride, respectively, using pentafluorobenzylbromide. This method was used to detect AN in laboratory cultures and later, to monitor the toxin in 80 German lakes and reservoirs (Bumke-Vogt et al., 1999).

38.3.5 Gas Chromatography-Mass Spectrometry

Derivatization of AN has been widely used for GC-MS analysis because it improves the sensitivity of the detection. This step also increases the volatility of the analytes, thus reducing thermal degradation and the increase in the overall molecular weight of the compound of interest often results in a more elaborate fragmentation pattern. Most of the early GC-MS methods followed Devlin et al. (1977), which determined AN as its *N*-acetyl derivative, (Devlin et al., 1977). Several methods for the derivatization of anatoxins before GC-MS have been developed. Himberg et al. developed a modified method for the determination of AN in both water and algal material. The freeze-dried algae was extracted by liquid–liquid extraction and the residue derivatized after evaporation of the solvents. The derivatization involved acetic acid and acetic anhydride and the reaction takes several hours (the mixture is left overnight to react). This reaction is followed by a SPE clean up and GC-MS analysis. Water samples were also subjected to liquid–liquid extractions and derivatization. However, in this case the SPE clean up was not necessary because of the relatively simple matrix. This method gave a detection limit of 5 μg AN/L (algae) and 1 μg AN/L (water) (Himberg, 1989). Zotou et al. (1993) used GC-MS to determine AN as its *N*-butyl derivative. Although this derivatization aids confirmation of AN in cyanobacteria, it involves a long reaction time (3 h) and requires a narrow range of quantity of AN (2.5–10 μg) to be present in the sample. These authors also developed an LC-UV method for both AN and HMAN (Zotou et al., 1993). Differences in AN and HMAN production in strains of *Planktothrix* (*Oscillatoria)* were studied using GC-MS (Araoz et al., 2005).

Pentafluorobenzyl bromide has been used as a derivatizing reagent before GC-MS to confirm the identification of AN in algae from a lake in Italy (Bruno et al., 1994). This halogenated reagent is also applicable to the determination of AN in water using GC-ECD (Bumke-Vogt et al., 1999). Bruno et al. used different ions including the derivative *N*-pentafluorobenzyl-anatoxin-a (*m/z* 345), the product ion, *m/z* 302, the ions for the pentafluorobenzyl (*m/z* 181), and the anatoxin fragments (*m/z* 164). Ross et al. studied various ionization methods for the determination of *tert*-butyldimethylsilyl-anatoxin-a and concluded that desorption chemical ionization/mass spectrometry (DCI/MS) gave the lowest detection limits; for pure AN it was 10 pg, and 100 pg of the toxin can be analyzed directly from urine using DCI/MS/MS. The *t*-butyldimethylsilyl derivative was chosen for its favorable GC characteristics (longer retention time and narrow peak width) and gave a unique and abundant high mass ion (Ross et al., 1989).

A solid phase microextraction GC-MS (SPME–GC-MS) method has been developed for the direct analysis of AN in aqueous samples and cyanobacteria extracts. This method involved sorbing analytes onto a polyamine fiber that was immersed in the aqueous sample followed by insertion of the fiber into the heated injection port of the GC where the analytes are volatilized (Figure 38.3). A long SPME sampling time (30 min) per sample was required as this increased the quantity of analytes extracted, and a limit of detection (LOD) of 11 ng/L was reported (Ghassempour et al., 2005). Another SPME method utilized before derivatization of AN by the addition of hexylchloroformate to the alkaline sample (pH = 9.0). The derivatized AN was extracted an SPME procedure using a PDMS fiber in 20 min sampling period. GC-MS was used to identify and quantify the analyte in selected ion monitoring (SIM) mode using norcocaine as an internal standard. The calibration

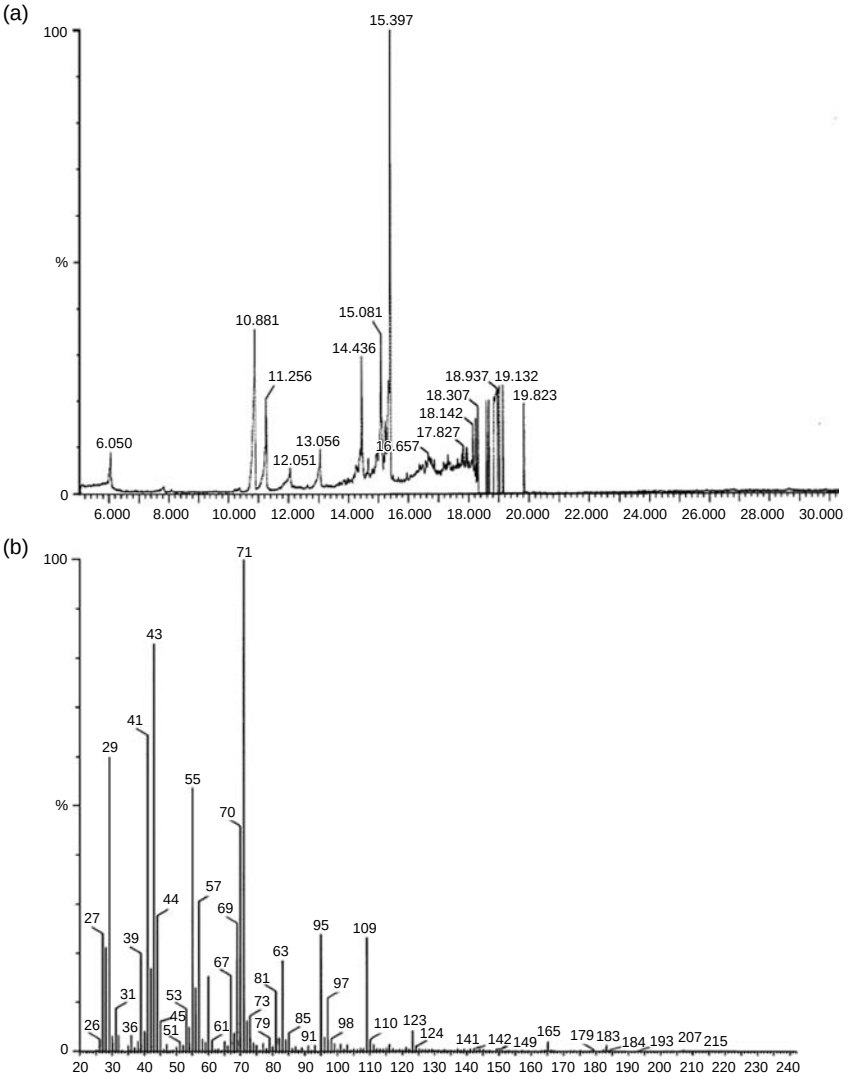

FIGURE 38.3 Total ion chromatogram (TIC) and MS spectrum from the analysis of a cyanobacteria sample (*Nostoc carneum*) using SPME–GC–MS. The peak in 11.26 min is related to anatoxin-a (Reprinted from Ghassempour, A., Najafi, N. M., Mehdinia, A., Davarani, S. S. H., Fallahi, M., and Nakhshab, M, *J. Chromatogr.* A 1078, 120–127, 2005. With permission. Copyright (2005) Elsevier.)

curve showed linearity in the range of 2.5–200 ng/mL and the LOD was 2 ng/mL that was sufficient to allow this method to be used to monitor AN for water quality control purposes (Rodriguez et al., 2006).

Freeze-dried algae spiked with AN was analyzed directly by GC-MS without prior derivatization (Dagnino and Schripsema, 2005). An acceptable linear correlation between the peak area and the amount injected was obtained with a reported detection limit of 0.5 ng AN/injection.

38.3.6 Liquid Chromatography-Ultraviolet Detection

Liquid chromatography with ultraviolet detection (LC-UV) is often used for the analysis of freshwaters suspected to contain anatoxins. Indeed, both AN and HMAN have a strong absorbance at 227

nm (log ε = 4.10) owing to an α,β-unsaturated ketone moiety (Devlin et al., 1977; Astrachan and Archer, 1981; Harada et al., 1989). On the other hand, the dihydro- and epoxy-degradation products of AN and HMAN do not possess this unsaturated ketone, making them undetectable by LC-UV. This presents a significant problem when investigating suspected anatoxin poisoning as previously discussed (see Section 38.3.1) since both AN and HMAN have half-lives of less than 1 day in natural water bodies. Harada et al. used reversed-phase LC-UV for the analysis of AN extracted from *A. flos-aquae* strains; they obtained an excellent linearity in their calibration curve in the range, 20–100 ng (on-column) (Harada et al., 1989). Wong and Hindin studied the applicability of LC-UV for the isolation, identification, and quantification of AN. Both normal phase and reversed phase chromatography were examined, but both phases showed poor chromatograms with broad peaks with extensive band tailing (Wong and Hindin, 1982).

When several dogs died after drinking water from Loch Insh in Scotland, the dog's stomach contents needed to be analyzed to determine if their deaths were due to neurotoxins. The previous methods did not allow dealing with complex matrices such as stomach contents. The LC-UV method that was developed using a C18 column with a gradient mobile phase consisting of a phosphate buffer (pH = 2.5) and acetonitrile (Edwards et al., 1992). Later, a styrene-divinylbenzene SPE cartridge to concentrate AN from spiked water samples before LC-UV determination using a gradient of acetonitrile/water, both containing 0.1% TFA, was used to determine AN in water samples (Powell, 1997). Zotou et al. used an isocratic ion-pair reversed-phase LC with an eluent containing acetonitrile/phosphate buffer (pH = 3) together with the ion-pair reagent, sodium dodecyl sulfate. This method was used to separate AN, HMAN, and the internal standard atropine sulphate (Zotou et al., 1993).

LC-UV was used to study the biodegradation and adsorption of AN and hepatotoxins on lake sediments and a detection limit of 10 ng/mL was achieved (Rapala et al., 1994). LC-UV method was one of three methods used for the first identification of HMAN in Ireland (Furey et al., 2003a). Figure 38.4 is a chromatogram from the LC-UV analysis of the cyanobacterium, *R. mediterranea* Skuja, isolated from a Japanese lake, and this was used to show the simultaneous production of AN, HMAN and a new nontoxic compound, 4-hydrohomoanatoxin-a (Namikoshi et al., 2003).

In addition to a failure to detect dihydro- and epoxy-anatoxins, the sample matrix may cause serious interferences in LC-UV. Samples with many contaminants require cleanup steps and it can

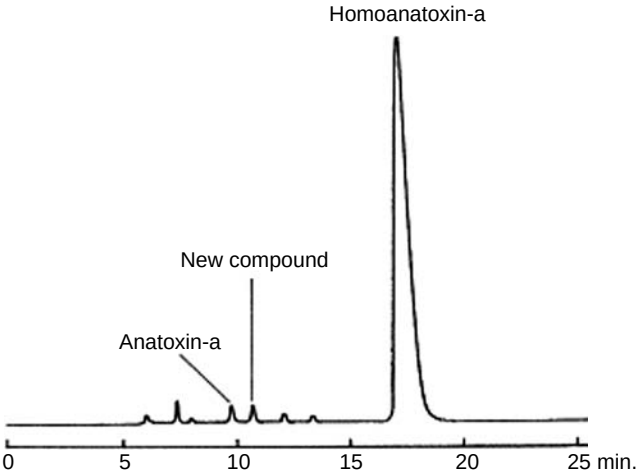

FIGURE 38.4 LC–UV chromatogram of an extract from the cyanobacterium, *Raphidiopsis mediterranea*, showing the simultaneous production of AN, HMAN, and a new nontoxic compound, 4-hydrohomoanatoxin-a (Reprinted from Namikoshi, M., Murakami, T., Watanabe, M. F., Oda, T., Yamada, J., Tsujimura, S., Nagai, H., and Oishi, S, *Toxicon* 42, 533–538, 2003. With permission. Copyright (2003) Elsevier.)

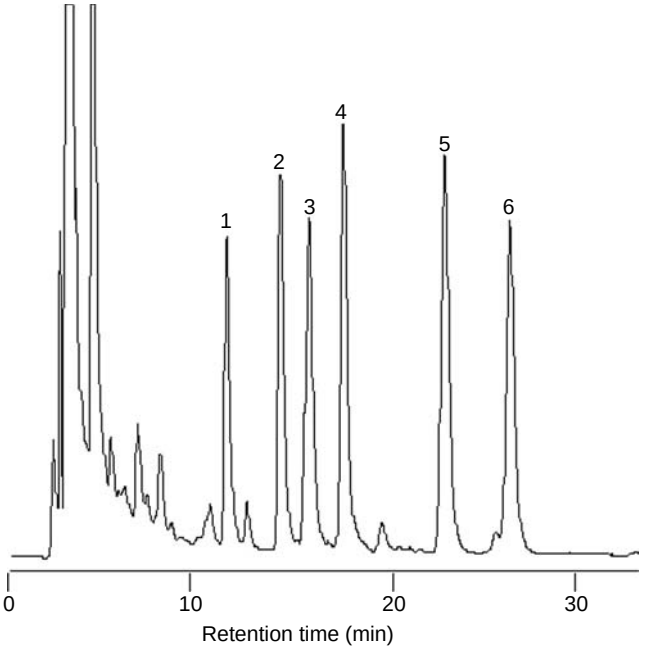

FIGURE 38.5 LC–FLD chromatogram showing the separation of six anatoxins that were derivatized using NBD-F. Chromatographic peak identification, 1. Epoxy-AN, 2. AN, 3. Dihydro-AN, 4. Epoxy-HMAN, 5. HMAN, 6. Dihydro-HMAN (Adapted from James, K. J., Furey, A., Sherlock, I. R., Stack, M. A., Twohig, M., Caudwell, F. B., and Skulberg, O. M, *J. Chromatogr.* 798, 147–157, 1998. With permission. Copyright (1998) Elsevier.)

become difficult to accurately quantify the amount of AN in some samples using LC-UV (Harada et al., 1993).

38.3.7 Liquid Chromatography-Fluorescence Detection

A highly sensitive method was developed using liquid chromatography with fluorescence detection LC-FLD method for the determination of AN, HMAN, as well as their epoxy- and dihydro-degradation products—the first time that all six anatoxins could be determined simultaneously (Figure 38.5). Water samples or algae extracts were first subjected to a cleanup step using a weak cation exchange SPE cartridge. This was followed by derivatization of the compounds using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). This particular derivatizing agent is used because it allows a rapid derivatization reaction (10 min) under mild conditions owing to its high reactivity with amines. NBD-F does not exhibit a strong fluorescence but the six anatoxins all gave highly fluorescent products. This high fluorescence allows the mixture to be directly analyzed by LC-FLD without performing any other cleanup steps to remove excess unreacted NBD-F reagent. This method has a detection limit of 0.01 ng/mL and proved useful for the analysis of water samples and for the environmental toxicological analysis of cyanobacteria (James et al., 1997, 1998).

A modification of the above LC-FLD method included SPME using fibers coated with Carbowax-temperated resin. In this method, the NBD-F reagent solution was placed onto the fibre coating before insertion of the fibre in the sample solution. A limit of detection of 20 ng AN/mL was obtained and none of the other five anatoxins were studied. This method cannot be used to monitor potable waters as the required detection limit <1 ng/mL. Another modification to the NBD-F method was the use of o-phthalaldialdehyde (OPA) and mercaptoethanol to cleanup samples. They were added to the extracts from algae before derivatization with NBD-F (Rawn et al., 2005).

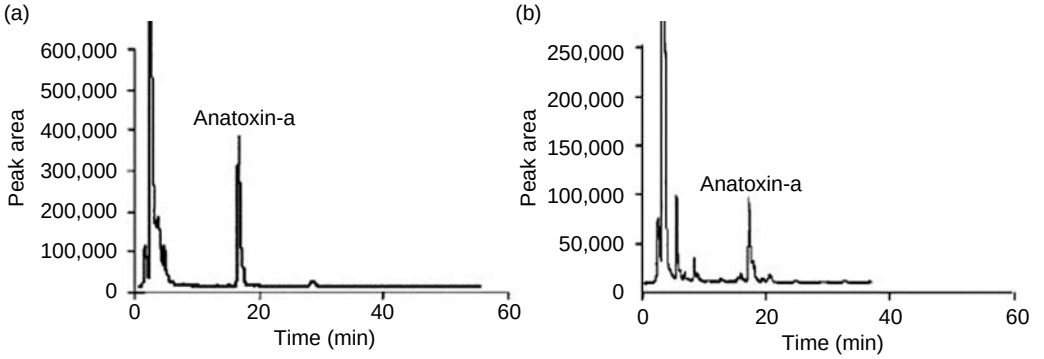

FIGURE 38.6 Examples of the application of the SPME method using PDMS/DVB fibre with on-fiber derivatization for the LC–FLD analysis of anatoxin-a, (a) a river water sample spiked with 1000 ng of anatoxin-a; (b) a cyanobacteria sample naturally contaminated with anatoxin-a (Reprinted from Rellan, S., Osswald, J., Vasconcelos, V., and Gago-Martinez, A, *J. Chromatogr.* A 1156, 134–140, 2007. With permission. Copyright (2007) Elsevier).

Primary amine contaminants in samples were removed and improved chromatograms were obtained when extracts were cleaned up in this manner. Rellan et al. analyzed freshwater samples, cyanobacterial cultures, finfish, and mussel from Portugal. A comparative study of SPE–LC-FLD and SPME–LC-FLD with derivatization of AN using NBD-F was carried out. Using SPE, analysis of AN was achieved with a high degree of sensitivity (ng/L range) with appropriate recoveries for all studied matrices. However, a high background noise was obtained for both mussels and fish matrices. Figure 38.6 shows the chromatograms for the determination of AN that were obtained from spiked water samples and a naturally contaminated cyanobacteria sample. Using SPME–LC-FLD, LODs were not as good as with SPE because of the lower sample volumes used in SPME (Rellan et al., 2007).

38.3.8 Liquid Chromatography-Mass Spectrometry

One of the early LC-MS methods for anatoxins utilized thermospray (TSP) as the ionization source and this method was used for the first identification of anatoxins in Japan (Harada et al., 1993). This method was used to determine both AN and epoxyanatoxin-a using acetyltropine as an internal standard to overcome reproducibility problems with TSP LC-MS. A detection limit of 0.5 ng was reported. The more popular electrospray ionization (ESI) source was first used for the LC-MS determination of AN in algae by Poon et al. AN in methanol, containing 1% acetic acid, gave a strong protonated molecule-related ion, $[M+H]^+$ at *m/z* 166 and these authors reported ESI-MS to be a simple and reliable method for analyzing thermally labile molecules (Poon et al., 1993).

To improve the sensitivity of LC-ESI–MS for the determination of AN in freshwaters, Takino et al. studied two aspects; (a) LC-MS using volatile ion-pairing reagents and (b) LC-MS with online precolumn derivatization. Pentafluoropropionic acid was found to be the best of four ion-pairing reagents studied for delivering appropriate chromatographic retention. This method gave an LOD of 3.3 μg AN/L but signal suppression was observed. Derivatization of AN using fluorenyl methylchloroformate (FMOC) produced an LOD 2.1 ng AN/L using SIM and was considered sufficiently sensitive for the determination of trace AN in environmental waters (Takino et al., 1999). In 2000, Hormazábal et al. used LC-MS for the determination of AN and four microcystins in both fish muscle and water samples and obtained detection limits of 0.4 μg AN/L in water (Hormazábal et al., 2000). An LC-ESI–MS method for the simultaneous determination of 11 marine and cyanobacterial toxins was reported with the MS in positive multiple ion detection (MID) mode and an LOD of 0.5 ng (on-column) for AN was reported (Dahlmann et al., 2003).

However, it will be shown later that single stage MS can be unreliable for the analysis of AN as it cannot discriminate this toxin from the isobaric compound, phenylalanine (Phe), that occurs commonly in environmental water bodies.

38.3.9 Liquid Chromatography-Multiple Tandem Mass Spectrometry

Draisci et al. developed a liquid chromatography–multiple tandem mass spectrometry (LC-MS/MS) method for the determination of neurotoxins in blue-green algae food supplements (Draisci et al., 2001). The neurotoxins included AN, HMAN, and their degradation products. After SPE clean up, the toxins were ionized in an ionspray interface, operated in the positive ion mode. The protonated molecules $[M+H]^+$ were used as precursor ions for collision-induced dissociation (CID) and diagnostic product ions were identified by selected reaction monitoring (SRM) analysis. This analysis relied on mass discrimination, as there was very little chromatographic resolution of these anatoxins (Draisci et al., 2001). LC-MS/MS has also been used for the simultaneous determination of hepatotoxins and AN in water samples (Pietsch et al., 2001). Ion-pair supported enrichment with SPE and reversed-phase LC-MS/MS) gave limits of quantification in water samples of approximately 50 ng AN/L l for microcystins and AN. The transition used for this study was 166/149 that corresponds to a loss of 17, that is, $[AN\text{-}NH_3]^+$. Unfortunately, this MS/MS transition can be inadequate since an isobaric compound with $[M + H]^+ = 166$ that undergoes a loss of water can give a similar signal with a low-resolution MS instrument (Furey et al., 2005). A method for the simultaneous analysis of PSP toxins, AN, and cylindrospermopsins was proposed that utilized hydrophilic interaction chromatography (HILIC) (Dell'Aversano et al., 2004). Unfortunately, one of the proposed transitions for AN was 166/149, which is open to interferences with Phe.

LC-MS^n, using a quadrupole ion-trap (QIT) MS instrument, was used to analyze AN and HMAN by Furey et al. MS^5 fragmentation of HMAN provided valuable data that permitted the construction of an MS fragmentation pathway. Similarly, LODs, 0.3 ng AN (on-column), equivalent to 0.6 ng/L for a 10 mL lakewater sample, were obtained for both LC-MS^2 and LC-MS^3 (Furey et al., 2003b). Not only is this an important feature of QIT MS instruments but unlike triple quadrupole MS instruments, spectra for each analyte can also be obtained without any diminution of sensitivity. Figure 38.7 shows the chromatograms and corresponding spectra obtained from the analysis of anatoxins in cyanobacteria (*Planktothrix* spp.) from Caragh Lake, County Kerry, Ireland, using LC-MS^2, where a series of incidents of fatal canine neurotxoicosis occurred. This is particularly important for analyte identification in the study of anatoxins since only AN is available commercially (James et al., 2005). These fragmentation studies were extended to include nanoelectrospray hybrid quadrupole time-of-flight mass spectrometry (QqTOF–MS) for AN, HMAN, and their dihydro and epoxy analogues. The high mass accuracy data from QqTOF–MS and QIT MS^n were used to construct fragmentation pathways for six anatoxins. Comparisons between the spectra of compounds that differ in side-chain length (the AN and HMAN series) were used to identify ions that are characteristic of the homologues. Significant differences between the fragmentation pathways of anatoxins were observed. Figure 38.8 shows the fragmentation pathways for dihydro-AN and dihydro-HMAN, which shows a loss of NH_3 at the MS^3 stage. However, in the corresponding fragmentation pathways for epoxy-anatoxins, the major products retained nitrogen (James et al., 2005).

Not only are forensic investigations of suspected AN poisonings frequently hampered in its detection due to its rapid decay but the widespread presence of the amino acid, Phe, also presents major analytical problems (Gugger et al., 2005). Since these compounds are isobaric (both molecular weights = 165) and elute similarly in liquid chromatography, misidentification of AN is possible. Approaches to prevent the misidentification of AN that have been explored in these studies included (1) fluorimetric LC following derivatization, (2) methylation using diazomethane before LC-MS determination leaves AN unaffected, (3) multiple tandem MS using a QIT (LC-MS^3) since the fragmentation pathways of Phe and AN are different, and (4) hybrid QqTOF as the high mass accuracy obtained readily distinguished between AN (165.1154) and Phe (165.0790) (Furey et al., 2005).

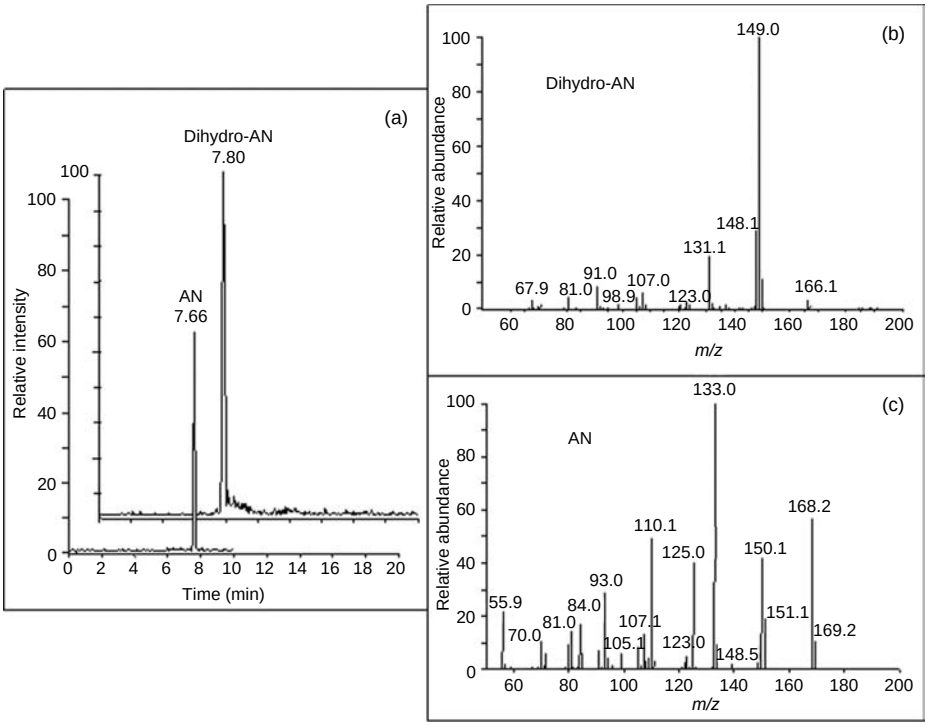

FIGURE 38.7 Determination of anatoxins in cyanobacteria (*Planktothrix* spp.) from Caragh Lake, County Kerry, Ireland, using LC–MS^2. (a) Chromatograms of anatoxin-a (7.66 min) and dihydroanatoxin-a (7.80 min). The QIT MS^2 spectra of (b) dihydroanatoxin-a and (c) anatoxin-a, corresponding to these chromatographic peaks (Reprinted from James, K. J., Crowley, J., Hamilton, B., Lehane, M., Skulberg, O., and Furey, A., *Rapid Commun. Mass Spectrom.* 19, 1167–1175, 2005. With permission. Copyright (2005) Wiley & Son, Ltd.)

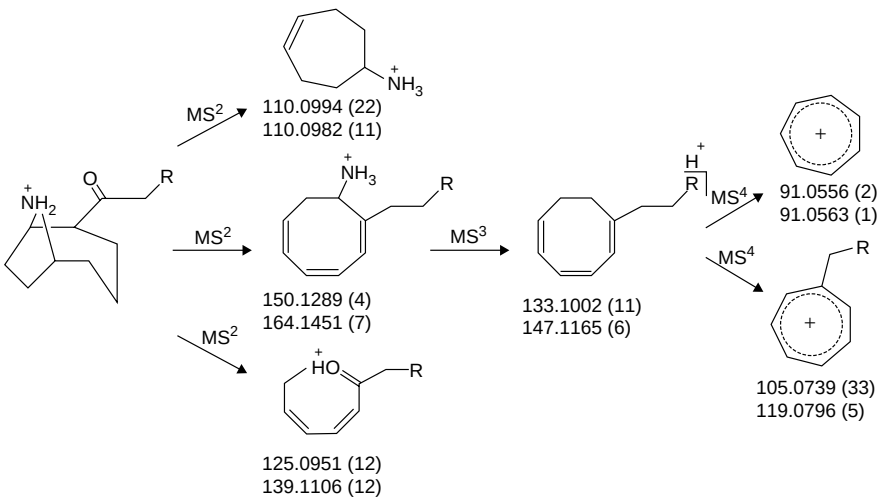

FIGURE 38.8 Proposed CID fragmentation pathways showing the typical losses and structures of product ions from protonated dihydroanatoxins. The observed masses corresponding to the product ions in QqTOF MS of dihydro-AN are shown above the corresponding dihydro-HMAN data, with error values (ppm) in parenthesis (Reprinted from James, K. J., Crowley, J., Hamilton, B., Lehane, M., Skulberg, O., and Furey, A., *Rapid Commun. Mass Spectrom.* 19, 1167–1175, 2005. With permission. Copyright (2005) Wiley & Son, Ltd.)

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Part XIIB

Hepatotoxins

39 Cyanobacterial Hepatotoxins: Chemistry, Biosynthesis, and Occurrence

Martin Welker

CONTENTS

39.1 INTRODUCTION

The earliest scientific report on toxic compounds produced by cyanobacteria was published in the late nineteenth century,[1] reporting on the death of sheep after drinking water from Lake Alexandrina, South Australia. At the time of the incident a heavy bloom of *Nodularia spumigena* was present in the lake forming scums "thick and pasty as porridge." By feeding bloom material to a sheep that died within hours, the poisoning by toxins produced by the cyanobacterium was evidenced. A postmortem examination showed symptoms that are now known to be typical for cyanobacterial poisoning.[2] A number of animal mortalities have been reported since, including flamingos, dogs, cattle, and rhinoceros.[3–7]

The potential toxicity of cyanobacteria became an issue of concern to public health authorities after several water-related illnesses that could not be explained by other compounds or pathogens. Several epidemic outbreaks of cyanobacteria-related gastroenteritis requiring clinical treatments have been reported: in Brazil an outbreak was observed in communities that received cyanobacteria infested water from Itaparica Dam[8]; in Armidale (Australia) where raw drinking water came from Malpas Dam infested with *Microcystis aeruginosa*[9]; on Palm Island (Australia) where all affected people received water from Solomon Dam at that time infested with a bloom of *Cylindrospermopsis raciborskii*.[10] The most severe outbreak with more than 70 deaths is reported from Caruaru (Brazil) where haemodialysis patients received improperly treated water from a reservoir carrying a *Microcystis* bloom at that time.[11,12] These and other incidences indicated first, that cyanobacteria

can be a severe health risk and second, that cyanobacterial toxicity occurs worldwide and independent of climatic zones and geographic location.

Parallel to medical and veterinary researchers, biochemists and ecologists were interested in the effects of cyanobacterial toxins on aquatic communities and toxin production by individual strains. The earliest reports on toxic isolates (i.e., cyanobacterial strains grown under laboratory conditions) came from the late 1950s[13] together with first characterizations of toxic compounds, then termed "fast death factor."[14] At the same time eutrophication of many inland waters became a major issue in water quality management, partly, due to the increased frequency and intensity of cyanobacterial blooms.[15] The obvious resistance of cyanobacterial blooms to grazing by zooplankton initiated studies on the toxicity of cyanobacteria to grazers like *Daphnia*. In experiments, toxicity of bloom forming cyanobacterial taxa like *Microcystis* or *Anabaena* to zooplankton could be shown[16,17] and finally related—though not exclusively—to microcystins.[18,19]

Advances in analytical techniques allowed the identification of the toxins (see below) in the 1980s and subsequent routine analyses of water samples showed that cyanobacterial hepatotoxins are likely to be detected in any water body carrying a cyanobacterial bloom.

Although cyanobacterial hepatotoxins have been studied intensively under many aspects in the last three decades, the role of the compounds in the cyanobacteria's physiology and ecology is still not evident.[20,21]

The question whether cyanobacterial hepatotoxins constitute a human health risk is still debated and is not easy to answer since in comparison to other waterborne diseases, e.g., cholera epidemics, the number and severity of cyanotoxin related illnesses appears less dramatic. On the other hand, microcystins and cylindrospermopsins are potent toxins with LD_{50}s similar to or even lower than that of some of the most notorious natural toxins, like α-amanitin (*Amanita phalloides*), strychnine (*Strychnos nux-vomica*), or aconitine (*Aconitum* sp.). Compared to these natural toxins, exposure to cyanobacterial toxins is much harder to avoid and the cyanotoxin-related epidemics indicate that potentially a large number of people can be affected when, for example, drinking water is contaminated. Among chemicals to which humans are exposed through water, cyanobacterial toxins probably occur most frequently in a global perspective.

Therefore, the understanding of the biology and ecology of toxigenic cyanobacteria is crucial for risk assessment and management and the protection of public health.

39.2 STRUCTURE AND CHEMISTRY OF CYANOBACTERIAL HEPATOTOXINS

39.2.1 Microcystins and Nodularins

In the early 1980s several groups working on cyanobacterial toxins proposed a peptidic nature for the most common and most toxic compound. Finally, Botes et al.[22,23] succeeded in isolating the toxin from a *Microcystis* strains and fully elucidating the structure, then named cyanoginosin (or cyanoviridin). The peptidic nature could thus be confirmed and soon structural variants were isolated. At this early stage it was already known that microcystins—as they were eventually named[24]—occur in many structural variants (congeners).

Figure 39.1a shows the generic structure of microcystins (MC), with the two most variable positions marked by X and Z. The numbering of the individual positions was done before the elucidation of the biosynthetical pathway and is arbitrary (see below). MCs are cyclic heptapeptides with several structural peculiarities. The moiety characteristic of microcystins and nodularins is the Adda, abbreviated for 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid, a β-amino acid that is not known from other natural products. Further peculiarities of the molecule are D-amino acids. These stereoisomers of commonly occurring L-amino acids are not found in ribosomally synthesized proteins and peptides and are therefore called "nonproteinogenic" amino acids. *N*-methyl-dehydroalanine (Mdha) in position 7 also is a nonproteinogenic amino acid as it is the case with D-erythro-β-methyl-iso-aspartate (D-MeAsp).

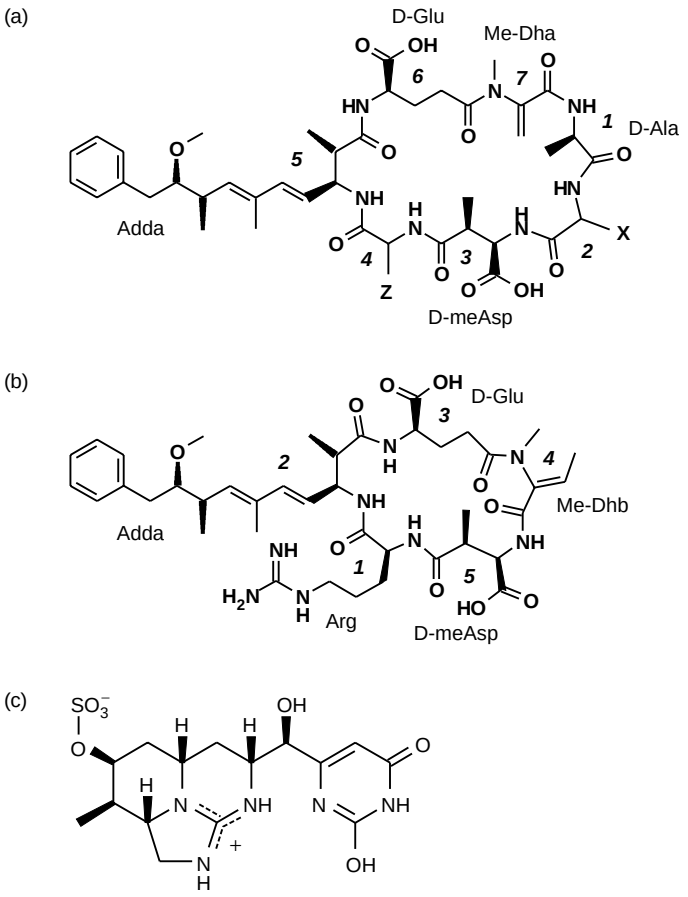

FIGURE 39.1 Structures of microcystins (a), nodularin (b), and cylindrospermopsin (c). For abbreviations of amino acids see text.

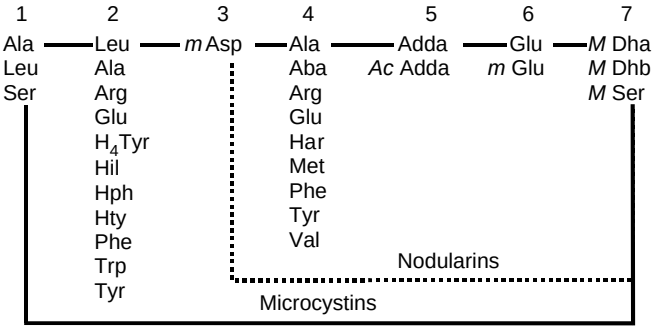

FIGURE 39.2 Schematic structure of microcystins and nodularins. For each position in the peptide ring (see Figure 39.1), all amino acid moieties are listed that have been found in that particular position. Prefixes indicate *N*-methylation (*M*), *O*- or *C*-methylation (*m*), acetylation (*Ac*). For abbreviations of amino acids see text.

Structural variants—of which some 80 have been reported[25]—differ primarily in the amino acids in positions 2 and 4. These amino acids were used for a naming system by applying the standard one-letter code for proteinogenic amino acids as suffix. MC-LR, for example, has a leucine and an arginine moiety in positions 2 and 4, respectively. In Figure 39.2 all structural variations

are summarized in a schematic structure. The first line represents microcystin-LA and below each position, all moieties found in particular positions so far are listed. In positions 2 and 4 these are mainly proteinogenic amino acids but also homovariants like homo-tyrosine (Hty) and homo-isoleucine (Hil). In position 1 leucine and serine in D-configuration have been found besides D-alanine but in the majority of congeners the latter one is present. In position 3 the β-methyl-group can be absent and respective congeners receive the suffix [Asp^3]. The *N*-methylation in position 7 can also be absent or the dehydroalanine can be replaced by Dhb that stands for dehydrobutyrine, also known as aminobutenoic acid. At the glutamate in position 6 an *O*-methylation has been reported and at the Adda moiety the *O*-methyl-group can be absent or replaced by an acetate residue. More congeners will be likely discovered in the ongoing research: a permutation based on the reported moieties leads to several thousand possible variants. Most likely, not all of these theoretical congeners will be synthesized *in vivo*, but recent studies showed that new congeners are still to be found.[26,27]

When the structures of MC and nodularins (NOD, Figure 39.1b) are compared, striking similarities are immediately evident.[28] NOD is composed entirely of amino acids that can also be found in microcystins like the Adda moiety, a D-glutamate linked to the following amino acids by a δ-peptide bond, and MeAsp. But the NOD molecule is composed of five amino acids and the two amino acids lacking compared to MC are D-alanine and the variable one in position 2. This suggested that nodularins could be regarded as incomplete microcystins and by studying the biosynthetical pathway and corresponding genes this could be confirmed (see below). Structural variability is less pronounced in NODs compared to MCs and mainly given by the lack or addition of methyl groups at the Adda, Mdhb, mAsp, and D-Glu moieties.[29] A compound, motuporin, similar to NOD except for a valine in position 1, has been isolated from a marine sponge.[30]

The molecular weight of MCs ranges between 900 and 1100 Da for individual congeners and for NODs between 760 and 840 Da.

Similar to molecular weights, other chemical properties also span a wide range. Although all MCs are soluble in water, hydrophobicity varies considerably, which is primarily important for the uptake of the toxins by organisms and cells. Hydrophobicity depends on the variable amino acids and the methylation at positions 3 and 7 in microcystins.

Both, MCs and NODs, are chemically very stable, considering their peptidic nature. Spontaneous hydrolysis apparently occurs only at negligible rates. Boiling of MC at neutral pH does not lead to considerable decay for weeks and even at pH 1 and 40°C the half-life time is some three weeks.[32] Further, MC was found to be resistant to enzymatic cleavage by common proteases like trypsins.

Since microcystins and nodularins do not absorb UV-light in the spectrum of natural sunlight ($\lambda > 290$ nm) no photolysis of the pure compounds occurs. Indirect photodegradation occurs in the presence of photosynthetical pigments[33] or humic substances[34] under natural conditions. The most important degradation pathway of microcystins (and likely of nodularins) in natural waters is bacterial degradation. Biodegradation has been observed with a number of bacterial consortia[36,37] and several strains capable to degrade microcystins have been isolated.[38,39] For most bacterial consortia, a lag-phase of some few days to several weeks has been observed before the degradation commenced and was then completed rapidly.[35] Once a bacterial consortium is preconditioned, biodegradation apparently is efficient and can be used in biological treatment processes like slow sand filtration.[40] Degradation of NOD is achieved in a similar way by individual bacterial strains.[41]

MC and NOD inhibit mammalian protein phosphatases 1 and 2a, important enzymes with regulatory functions in cell biology.[42] The target organ is the liver due to the active transport of the toxins into hepatocytes by bile acid carriers.[43,44] The LD_{50} for oral administration of MC-LR is 5000 μg/(kg body weight) in mice.[45] On the basis of toxicological studies[46,45] a tolerable daily intake (TDI) for microcystin-LR of 0.04 μg/(kg body weight ∗ day) has been proposed by the World Health Organization (WHO). From the TDI a provisional guideline value of maximally 1 μg/L in drinking water was derived.[47]

39.2.2 Cylindrospermopsins

In 1979, an outbreak of hepatoenteritis on Palm Island (Australia) was reported that was linked to compounds released from cyanobacteria in a drinking water reservoir after algicide treatment.[10] In due course, strains of the dominant cyanobacterium *Cylindrospermopsi raciborskii* were isolated and found to produce a compound that was hepatotoxic to mice.[48] The toxic compound was isolated from this species and named cylindrospermopsin (CYN).[49] The structure (Figure 39.1c) consists of a tricyclic guanidino group linked to an uracil moiety by a hydroxylated bridging carbon. Owing to the sulfate and guanidino groups the molecule is a zwitterion around neutral pH and thus highly soluble in water. Two further structural variants have been described, deoxycylindrospermopsin[50] and 7-epi-cylindrospermopsin,[51] both varying in the hydroxy-group of the bridging carbon.

To date only little information is available on the stability and degradation of cylindrospermopsin. UV-light in the solar spectrum did not degrade the toxin as it was found for (short-term) boiling, while photoinduced degradation in algal extracts was achieved rapidly.[52] Chemical degradation by chlorination was found to be effective.[53] No data have been published yet on bacterial degradation.

The toxicity of CYN is primarily based on their inhibition of protein synthesis.[54] The target organ in mammals is the liver and to a lesser degree the kidneys.[55,56] A guideline value of maximally 1 μg/L in drinking water is debated.

39.3 BIOSYNTHESIS OF CYANOBACTERIAL HEPATOTOXINS

39.3.1 Biosynthesis of Microcystins and Nodularins

Early on in cyanotoxin research, the structural peculiarities of microcystins and nodularins led to the hypothesis that microcystins are synthesized by large multienzyme complexes, so-called nonribosomal peptide synthetases (NRPS). This biosynthetical pathway is common in bacteria and fungi where it is responsible for the synthesis of a wide array of natural products, many of which show bioactivity like gramicidin or tyrocidine.[57,58] Candidate gene sequences were indeed found in strains producing microcystins[59] and a knockout mutant could be generated that no longer produced microcystins after the insertion of a resistance cassette in a NRPS gene.[60] The complete gene cluster (*mcy*) has now been sequenced from several strains belonging to distant taxa in the cyanobacterial phylogenetic tree: *Microcystis*,[61,62] *Planktothrix*,[63] and *Anabaena*[64] (Figure 39.3). Although the sequence similarity of particular genes is very high in *Planktothrix* and *Microcystis*, the arrangement of the genes in the *mcy*-gene cluster is different. In *Microcystis*, the transcription starts bidirectionally from a central

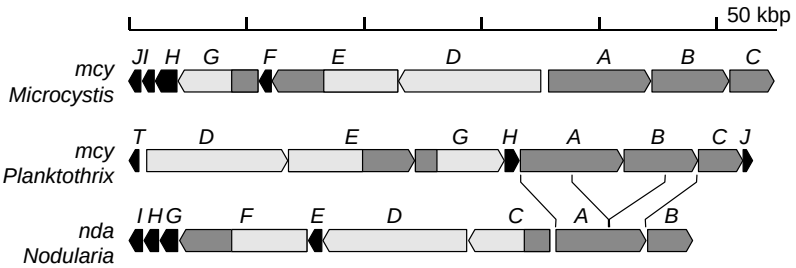

FIGURE 39.3 Schematic illustration of the microcystin and nodularin gene synthetase clusters. Sequence data from Tillett et al. 2000[62] (*mcy*, *Microcystis*), Christiansen et al. 2003[63] (*mcy*, *Planktothrix*), and Moffitt and Neilan 2004[71] (*nda*, *Nodularia*). Light grey: polyketide synthase genes; dark grey: nonribosomal peptide synthetase genes; black: other genes. For *ndaA* lines indicate homologue regions found in *mcyAB*.

promoter between *mcyA* and *mcyD* as two polycistronic operons, *mcyABC* and *mcyDEFGHIJ*[65] whereas in *Planktothrix* all genes are coded on the same strand and may be transcribed as a single operon.[63] The *mcy*-gene cluster of *Anabaena* has a gene arrangement similar to that of *Planktothrix* (not shown).

The larger genes in the *mcy*-cluster are either NRPS-genes (*mcyA*, *B*, and *C*), polyketide synthase (PKS) genes (*mcyD*), or hybrid PKS/NRPS genes (*mcyE* and *G*). *mcyA* and *mcyB* both code for two NRPS modules consisting of a condensation, an adenylation, and a thiolation domain. Further, in *mcyA* a *N*-methyltransferase and an epimerization domain is encoded, corresponding to the methylation of Mdha and the D-configuration of alanine in position 1.

Synthesis starts with the Adda moiety for which the starting unit is a phenylacetate.[66] Further synthesis involves four subsequent PKS elongations with acetate units[67] involving reductase, dehydrogenase, and C-methyl-transferase reactions. To the Adda a glutamate unit is then linked by an NRPS module. The genes of corresponding PKS/NRPS domains are found in *mcyG, D*, and *E*. Further biosynthesis is exclusively by NRPS and the five modules coded in the genes correspond to the five amino acids incorporated. In position 7 a serine is incorporated that is putatively reduced by a dehydrogenase (McyI). Further tailoring enzymes are McyJ that is responsible for the *O*-methyltransfer to Adda[63] and McyF that functions as racemase.[68] In *Planktothrix*, *mcyI* and *mcyF* are lacking and it is not known whether respective enzymatic steps are performed by enzymes coded outside of the gene cluster.

Variability of the amino acid sequence of McyB apparently is determining the substrate specificity of the adenylation domain and thus the structural variants that are synthesized by particular strains.[69]

Interestingly, in close vicinity to NRPS and PKS genes for the microcystin synthetases a gene is found coding for an enzyme with high similarity to ABC-transporters in all three (four when including *Nodularia*) genera for which the gene cluster sequence is available. Although MCs occur intracellular to a high percentage in all studies published so far, this transporter is crucial for microcystin biosynthesis and when the corresponding gene (*mcyH*) is disrupted, microcystin production seizes.[70]

Biosynthesis of NOD apparently is very similar to that of MC considering the high structural similarity.[67] Indeed, in the *nda* gene cluster a number of genes are found homologous to *mcy* genes, for example, *ndaF* and *mcyE* or *ndaC* and *mcyG*, but the arrangement of the genes is different compared to the *mcy*-clusters in *Microcystis* and *Planktothrix*.[71] A new gene is formed by fusion of the 5′-end of *mcyA* and the 3′-end of *mcyB*, forming *ndaA* coding for two NRPS modules—two less than in *mcyA* and *mcyB*—well in agreement with the peptides' structure.

39.3.2 Biosynthesis of Cylindrospermopsins

Feeding studies revealed the origin of most of the carbon and nitrogen atoms in CYN.[72] Guanidinoacetic acid serves as starter unit and the further biosynthesis involves the repeated incorporation of acetic acid units and a glycine. This indicated a hybrid NRPS/PKS pathway as it has been reported for other cyanobacterial bioactive metabolites.[21] A number of genes for putative CYN synthesis enzymes have been identified, including those with PKS and NRPS homologies[73,74] and an amidino transferase.[75] Attempts to generate a knockout mutant—the ultimate proof of the gene clusters' function—have failed until now.

39.4 CYANOBACTERIAL TAXA PRODUCING HEPATOTOXINS

39.4.1 Microcystin and Nodularin-Producing Taxa

An intriguing issue in the study of toxic cyanobacteria is the taxonomic distribution of the ability to produce particular toxic compounds.[76] MC-producing strains can be found in all major taxonomic groups of cyanobacteria, among chroococcales, oscillatoriales, nostocales, and stigonematales (the data available for pleurocapsales are very scarce). Within these orders or sections[77] genera are

known to produce MCs whereas closely related genera so far have not yet been reported to be able to do so. In nostocales (nonbranching, filamentous, heterocyst-forming taxa), for example, *Nostoc* and *Anabaena* strains have been reported to produce MCs[78,79] while this was never reported for *Aphanizomenon*, a genus frequently forming blooms in eutrophied lakes. A similar distribution of the capability to produce MCs is found in individual genera or species where producing and nonproducing strains have been identified, for example, among *Microcystis*, *Planktothrix* (formerly *Oscillatoria*), *Anabaena*, and *Nostoc*.[26,27,80–82] Toxin-producing strains include symbiotic (*Nostoc* isolated from a lichen[26]), terrestrial (*Hapalosiphon* spp.[83]), and halophil strains.[84] MCs can be found in strains and samples from tropical, temperate, and polar habitats.[85,86] Thus, the ability of microcystin production is neither confined to particular types of habitat nor to climatic regions, although most toxigenic strains reported so far were isolated from pelagic freshwater samples. This finding is, however, surely biased by the public interest that is focused rather on drinking water reservoirs than on microbial communities on rock overhangs, where cyanobacteria may also thrive and potentially produce toxins.

In natural plankton communities, MC-producing and nonproducing strains or clones have been reported to coexist. In the *Planktothrix agardhii* isolates, for which analytical results are shown in Figure 39.4, MC was detected only in those strains that were positive in a *mcyA*-specific polymerase chain reaction (PCR) experiment, that is, nonproducing strains lacked the *mcy*-gene cluster. Producing strains differed markedly in the MC content and the number of structural variants produced: from 0.3 DW to 3 mg/g DW and from 2 to 11 structural variants.[27]

Before molecular tools have been widely available, strains had to be isolated and cultivated to determine toxin production.[87] More recent studies employing either molecular genetics tools such as *mcy*-specific PCR,[88–90] mass spectrometry,[91,92] or immunosorbent assays[93] on single filaments or colonies indicate that a coexistence of multiple clones, a number of which produce MCs, is typical for most water bodies infested with cyanobacteria rather than an exception.[94]

The production of MCs by individual strains and the influence of growth conditions has been studied for a good number of strains and factors like light intensity, concentrations of macronutrients (P,N), and concentrations of trace metals in batch or in chemostat cultures.[79,95–101] A detailed

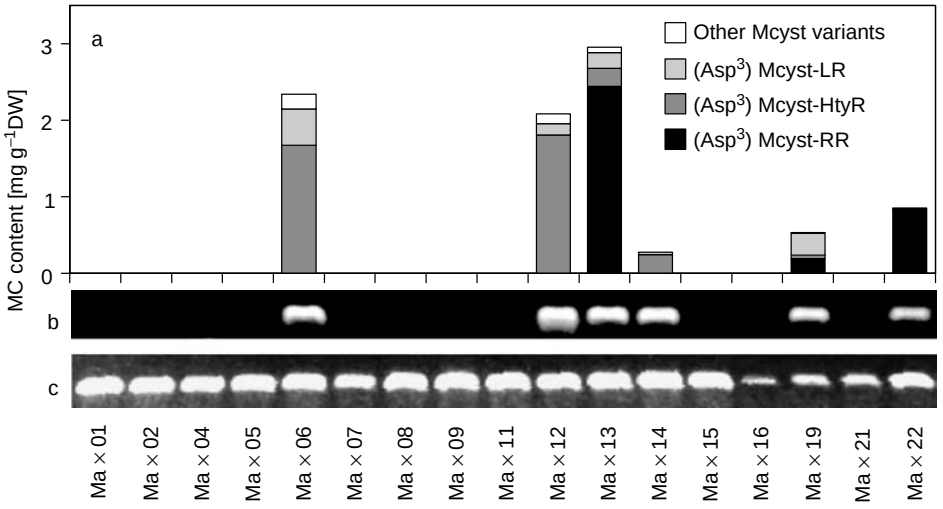

FIGURE 39.4 Microcystin content of *Planktothrix* strains isolated from a single sample taken in Maxsee, Germany.[27] Up to 11 structural variants could be identified in individual strains (a). Partial photograph of an agarose gel showing bands (297 bp) of DNA amplified from genomic DNA with primers specific for the microcystin synthetase gene *mcyA* (b). Partial photograph showing bands (349 bp) of DNA amplified with primers specific for the phycocyanin operon gene of *Planktothrix* spp. (c). (From Welker et al., *Arch. Microbiol.*, 182, 288, 2004.) Permission granted by Springer-Verlag, Heidelberg.)

compilation of factors and effects can be found in Sivonen and Jones.[25] In summary, all experimental approaches showed that MC content in an individual strain can vary for a factor of three to eight at maximum, and thus the MC cell quota of an individual strain is fairly stable, considering that the treatments in culture experiments spanned for wide ranges. Orr and Jones[102] showed that the MC net production rate is closely related to the cell division rate and consequently, the cell quota (MC per cell) are stable. Part of the differences between studies can be attributed to the different reference parameters to which MC content is related. MC content has been reported in relation to biovolume, dry weight (DW), cell number, protein or chlorophyll, all of which are themselves functionally dependent on growth conditions.

Recent studies applying continuous cultures indicate that the MC cell quota vary for a factor of two to three[96–98,100] as a function of growth rates. The direction of the functional response, however, seems to be strain specific and highest MC contents can be reached at high,[96] low,[100] or intermediate growth rates.[98] Most culture studies performed so far were focused on inorganic growth factors like nutrients or light that represent only a part of the factors that determine cyanobacterial growth in natural waters. A few studies dealing with the effect of biotic factors on MC production have been conducted[103,104] indicating, that the presence of planktivorous fish, for example, could influence the MC content of *Microcystis* strains significantly[105] although likely not in a range of orders of magnitude.

In contrast, the differences in MC contents between individual isolates spans a range much wider than reported for any individual strain cultivated under different conditions, from the detection limit in the low μg/(g DW) range to around one percent of DW, that is, potentially for more than two orders of magnitude.[27,82,87] This indicates that the MC content of an individual clone is genetically determined within a rather narrow range but with possible values spanning a wide range. Until now, no strain has been reported to stop microcystin production by physiological regulation. Mutations, however, can naturally lead to microcystin-free strains as has been reported for *Microcystis*[106] and *Planktothrix*.[107] The regulation of MC production and thus cellular content in strains with functional gene clusters remains nearly completely unknown. Light quantity and quality has been shown to influence transcriptional regulation of the *mcy*-genes[108] resulting in alternate transcripts, depending light intensity.[65] As for the downstream processes, no information is available yet on the translational regulation leading to the formation of the multienzyme complexes and the regulation of the activity of the NRPS-enzymes themselves.

In contrast to the wide taxonomic distribution of MCs, NODs are apparently confined to strains of the genus *Nodularia*, and primarily of *Nodularia spumigena*.[109] No other genera have been reported so far producing NOD. The NOD production seems to be regulated in a similar way than MC production in other strains, that is, NOD is produced constitutively in a strain specific narrow range of cell quota.[110,111] The dominance of toxin producing *Nodularia* in the Baltic Sea led to the hypothesis of an allelopathic effect of NOD[112] but recent studies could not support this.[113] Besides toxin producing isolates nontoxic ones can be found in the same genetic lineages, indicating a loss of the ability to produce NOD.[114]

39.4.2 Cylindrospermopsin-Producing Taxa

Similar to MC, CYN is produced by a number of different taxa. Most of them however belong to the order nostocales and the only known exception is the only planktonic species of the order stigonematales, *Umezakia natans*.[115]

The first species from which CYN was isolated, *C. raciborskii*, is considered as a tropical species expanding its distribution to temperate zones, likely driven by climate warming.[116,117] Isolates of this species from Europe, however, did not produce detectable amounts of CYN[118] but other, unidentified toxic compounds.[119] Further species to produce CYN have been recently reported as *Aphanizomenon ovalisporum*,[120] *Anabaena bergii*,[73] *Anabaena lapponica*,[121] and *Aphanizomenon flos-aqua*,[122] the latter one being very common and forming dense water blooms in temperate eutrophied freshwaters.[123]

Among the species for which CYN producing isolates have been reported, isolates that do not produce CYN generally lack the genes thought to be involved in the toxin production.[74,122] Interestingly, Brazilian isolates not producing CYN were found to produce saxitoxin, a potent alkaloid neurotoxin.[124] Only in a few laboratory studies strain specific CYN production was investigated. Saker and Griffiths[125] reported a reduction of CYN content with increasing temperature and an apparent inhibition of toxin production at 35°C. Further, they observed fluctuations in the ratio of extracellular CYN to total CYN in two strains. Bácsi et al.[126] reported a reduction of toxin content in *Aph. ovalisporum* resulting from phosphate and sulfate starvation.

The toxin contents reported were in a range similar to that found for MCs from a maximum of about 1% w/w (10 mg CYN per g DW) to levels just above detection limit around 1 μg/g DW.

39.5 OCCURRENCE OF CYANOBACTERIAL HEPATOTOXINS IN SURFACE WATERS

39.5.1 Factors Influencing Toxin Concentrations

In the last two decades the health risk of cyanobacterial toxins has been widely recognized and has triggered many national and international monitoring programs. Nowadays, several countries have developed guideline values for MC in drinking and bathing waters[127] following the guideline values proposed by WHO.[47,128] Results of extensive screening programs showed that MCs and also CYN are present in lakes, rivers, reservoirs, and ponds in various latitudes and altitudes all over the world. Thus, potential health risk of human exposure to toxic cyanobacteria is considered a global problem.[129]

For assessing the risk of exposure to cyanobacterial toxins it is crucial to understand the occurrence of the toxins in surface waters. For predicting the occurrence of cyanobacterial toxins, the following factors have to be considered:

- Conditions leading to cyanobacterial dominance
- Temporal dynamics of toxigenic cyanobacteria and toxins
- Spatial variability of toxin concentrations
- The environmental fate of the toxins.

Cyanobacterial dominance and bloom formation in inland waters depends mainly on the carrying capacity of particular water bodies, that is, the amount (concentration) of nutrients that can be transformed into biomass. A wealth of literature is available highlighting several factors that lead to seasonal mass developments of cyanobacteria in nutrient rich waters instead of other phytoplankton taxa like green algae or diatoms.[130–132]

One main characteristic of bloom forming freshwater cyanobacteria is their ability to regulate buoyancy with the aid of gas vesicles and thus to avoid light limitation in turbid waters.[133] This provides a clear competitive advantage over other phytoplankton that may sediment when the water column is stable during periods of thermal stratification.[134] Buoyancy due to gas vesicles frequently induces the formation of surface blooms, so-called scums, thus causing a high variability in spatial distribution of cyanobacteria.[135]

Although cyanobacteria, including toxigenic ones, are principally present in all surface waters from oligo- to hypertrophic states, biomasses that have to be considered critical with respect to cyanotoxins occur primarily in eutrophied waters. A major factor of eutrophication is the concentration of phosphorus[136] and it can be used to estimate the maximal cyanobacterial biomass that can be reached in a given water body. The most common and frequently encountered toxigenic genera are *Microcystis*, *Planktothrix*, *Anabaena*, *Cylindrospermopsis*, and *Aphanizomenon*. Whenever blooms of these genera are present in a water body, the presence of cyanotoxins is very likely.[137,138]

Although a high number of MC structural variants have been reported from commonly occurring taxa, only a few variants contribute substantially to the total MC pool in the majority of samples.

Which variants are predominating depends primarily on the dominant species: while in *Microcystis* blooms major MCs are the LR-, RR-, and YR-variants, in *P. agardhii* blooms the corresponding [Asp^3]-variants are most abundant, and in *Planktothrix rubescens* blooms [Asp^3,Dhb^7]-variants.[139,140] For analytical considerations, it is important to know which structural variants are produced by which species and a taxonomic analysis of water samples is therefore strongly recommended.

Cyanotoxin concentration is generally correlated to the cyanobacterial abundance and since the latter is depended on nutrient availability, it also correlates to nutrient concentrations.[137,141–143] The correlations, however, can show a considerable residual scatter and thus the concentration of toxins can vary considerably among water bodies or different sampling sites within a water body despite similar cyanobacterial abundances.[144]

Reported toxin contents of field samples range from undetectable to about 1% of DW, which is also the maximum toxin content reported for isolates, both for MC and CYN. The variation in toxin content of field samples can be attributed to the composition of the community that generally consists of multiple species and clones, each with a different toxin content.[27,87,145] Clonal composition can be highly dynamic and consequently the toxin content of an apparently monospecific bloom can change markedly in the course of a season.[146,147] A recent study[148] showed a significant relationship of the MC content of seston samples with the relative share of MC-producing *Microcystis* colonies in a shallow reservoir (Figure 39.5). The *Microcystis* population was dominated by MC-producing strains at the onset of the bloom and MC content of seston samples was close to 1 mg/g DW. In the course of the season, the percentage of MC-producing colonies steadily declined, as did the MC content of seston samples. A declining MC content of *Microcystis* blooms in the course of the season was also observed for Dutch lakes and might be a general pattern.[149]

To date, little is known about dynamics of individual clones and factors driving shifts in clonal composition. Therefore, the toxin content of cyanobacterial blooms remains unpredictable and patterns found for particular water bodies should be generalized only with care.

39.5.2 Toxin Concentrations Reported in Field Samples

In the following, toxin concentration is used to describe the amount of toxin per unit of water volume while toxin content refers to the amount of toxin per unit of biomass or seston. Data on toxin concentrations are a much better base for the assessment of health risks compared to toxin contents

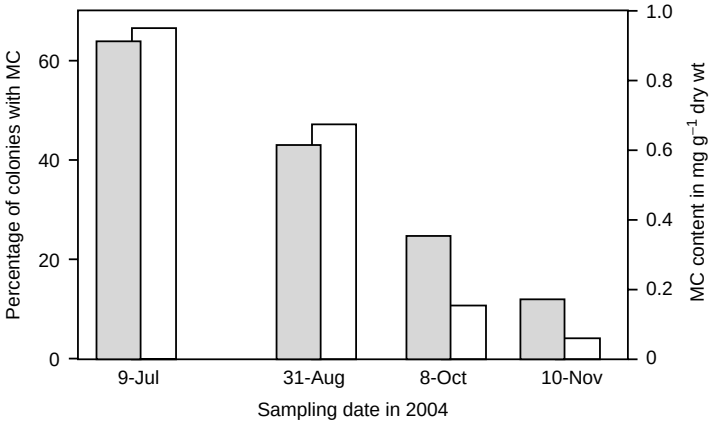

FIGURE 39.5 Microcystin (MC) content of seston samples (> 42 µm) taken in Brno reservoir (Czech Republic) at four sampling dates (open bars, right scale) and percentage of *Microcystis* spp. colonies in the same samples in which microcystins could be detected by MALDI-TOF mass spectrometry (grey bars, left scale).[148] (Redrawn from Welker et al., *Limnol. Oceanogr.*, 52, 609, 2007. Permission granted by American Society of Limnology and Oceanography, Inc.)

of net samples. Nonetheless, the majority of studies on cyanobacterial toxicity have reported the latter parameter. For MC, a number of studies have been published reporting toxin concentrations while for CYN the data are still scarce. Therefore, the following is focused mainly on MC but generalizations likely apply also to CYN.

Reported toxin concentrations range from just above detection limit (which is strongly dependent of the applied analytical techniques) to several tens of milligrams per liter. Concentrations, however, depend to a very high degree on the spatial heterogeneity of cyanobacteria.

Average toxin—MC and CYN—concentrations of whole water bodies generally are in the low micrograms per liter range.[119,142,143,150–154] In all studies, the major share of the toxins was found cell-bound and concentrations of dissolved MC—if detected at all—were found one to several orders of magnitude lower.[150,151] A release of the toxins from the cells is thought to happen primarily when the cells are lysing, either naturally or after algicide treatment,[35,155] while for CYN, larger fractions have been reported to occur dissolved without an apparent lysis event.[156]

With respect to the guideline value for drinking water, average concentrations of cyanobacterial toxins in eutrophied water bodies seemingly are often not alarming—provided the drinking water production technique operates properly and cells are not disrupted. A risk assessment based on average toxin concentration, however, is not realistic because it ignores the fact that cyanobacteria and hence cyanotoxins are most often not distributed homogeneously in a water body.

Owing to the ability of the most abundant bloom forming taxa to regulate buoyancy, a markedly heterogeneous distribution in the water column is common.[135] While *Microcystis* spp. tends to accumulate at the surface, other species are either more evenly distributed (e.g., *Planktothrix agardhii*), or form metalimnetic maxima (e.g., *P. rubescens*[157]). The latter species generally has a high percentage of MC producing genotypes[158] and thus a metalimnetic concentration maximum of MC is to be expected wherever this species is abundant. This is frequently the case in mesotrophic, deep lakes, and reservoirs[159] that are often used as drinking water reservoirs.

All toxigenic taxa have been reported to form surface blooms that can be displaced by wind action to the downwind side of lakes.[160] As a result, MC concentrations can vary considerably at different sampling sites. In the example shown in Figure 39.6, MC concentrations at central sites are

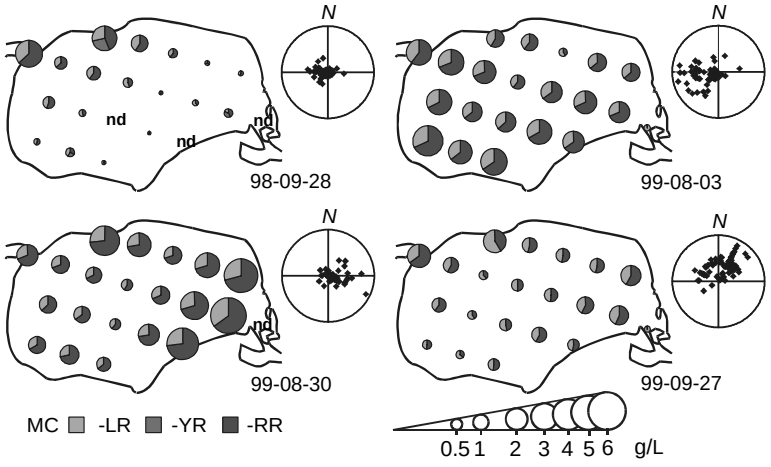

FIGURE 39.6 Horizontal variability of microcystin concentration at 21 sampling sites in Müggelsee (Germany) on four sampling dates.[152] For analyses, the first meter of the water column was sampled. The area of the circles is proportional to the MC concentration in individual samples. Polar plots show hourly average values for wind direction and wind speed during the 72 h preceding the sampling. The outer circle represents 5 m/s wind speed; for clarity, only the endpoints of vectors originating in the centre are plotted. n.d. MC concentration <0.05 µg/L. (From Welker et al., *Arch. Hydrobiol.* 157, 227, 2003. Permission granted by E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.)

among the lowest determined on specific sampling dates.[152] Concentrations in open water samples (taken at a minimum distance to the shore of 140 m) varied for more than an order of magnitude in the topmost meter of the water column. The gradient was correlated to the predominant wind direction during the 3 days preceding the sampling and concentrations increased toward the downwind side on particular sampling dates.

Surface bloom formation depends to a high degree on recent meteorological conditions[161] allowing buoyant cyanobacteria to reach the surface layers during periods of water column stability. In turn, nuisance blooms can be prevented to a certain degree by artificial mixing of the water column.[162] Such a technology, however, is applicable only in smaller water bodies. For larger water bodies, models based on satellite images have been developed, predicting surface bloom formation based on weather forecasts for water and health management purposes.[163]

At near shore sites, dense surface scums can be formed or washed ashore. In consequence, close to the shoreline toxin concentrations can be measured that are orders of magnitudes higher than those measured in open water samples. For example, MC concentrations of up to 25,000 µg/L were reported from river Havel and Wannsee (Germany) at sites that are frequented at the same time by the local population for recreation.[137] Swallowing even small amounts of such dense blooms constitutes a serious health risk to which especially children are exposed.

From dense scum material toxins can be released when cells start to lyse.[35] It can happen that scums are washed ashore where they dry forming crusts along the shoreline. A release of toxins can then occur with a time delay when the cells get submerged or rehydrated again.[164]

Although dense scums along the shorelines are the most visible (and often the most smelly) sign of a cyanobacterial bloom, the biomass accumulated there represents only a minor part of the entire population in the water body. Under changing weather conditions accumulations and scums can occur at other sites within a short time.

For a risk assessment it is therefore crucial to define accurately the part of a water body concerned by the specific water use. Raw water abstraction for drinking water production, for example, requires the monitoring of toxin concentration close to the off take site. Data on average concentration in the entire water body are valuable to judge whether cyanobacterial toxins are present at all but likely provide only insufficient or even misleading information for drinking water safety.

The same has to be considered for monitoring programs of recreational waters that have to account not only for the spatial heterogeneity but also for temporal fluctuations of toxin concentrations. Toxin concentrations can change dramatically within hours from high to low and reverse, potentially requiring constant monitoring of sensitive sites, that is, at frequented downwind sites during the blooming season.

Toxic cyanobacteria occur naturally in eutrophied waters, and toxin production is a common feature among bloom forming genera and species. A sustained control or reduction of cyanotoxin concentration can therefore only be reached by reducing the potential of a water body to support cyanobacterial blooms. Nutrient load reduction and catchment management are therefore the most appropriate long-term measures to diminish the health risks of cyanobacterial toxins.

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40 Hepatotoxins: Context and Chemical Determination

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CONTENTS

40.1 INTRODUCTION

40.1.1 Toxic Cyanobacteria

Many names are used for the organisms that produce freshwater toxins and these include blue-greens, blue-green algae (BGA), myxophyceans, cyanophyceans, cyanophytes, cyanobacteria, cyanoprokaryotes, and so forth. This apparent confusion in the naming highlights the important position that these organisms occupy in the development of biology as a science. From their earliest observation and recognition by botanists[1,2] to their treatment more recently in modern text books [3,4] the amazing combination of properties found in algae and bacteria that these organisms exhibit have been a source of fascination and attraction for many scientists. The majority of cyanobacteria are aerobic photoautrotrophs. Their life processes require only water, carbon dioxide, inorganic substances, and light. Photosynthesis is their principal mode of energy metabolism. In the natural environment, however, it is known that some species are able to survive for long periods in complete darkness, in extremely high and low temperatures, in water that is salty, brackish, or fresh, in cold and hot springs, and in environments where no other microalgae can exist. They also form symbiotic associations with animals and plants such as fungi, bryophytes, pteridophytes, gymnosperms, and angiosperms [5,6].

Cyanobacteria provide an extraordinarily wide-ranging contribution to human affairs in everyday life [7] and are of economic importance [8]. Both the beneficial and detrimental features are of considerable significance. They are important primary producers and their general nutritive value is high. The nitrogen-fixing species contribute globally to soil and water fertility [9]. The use of cyanobacteria in food production and in solar energy conversion holds promising potential for the future [10]. Since the 1970s many types of compounds have been isolated from cyanobacteria that show characteristic biological activities: cytotoxic, immunosuppressive, antifungal, cardioactive, and enzyme inhibitory. The cytotoxic compounds such as scytophycins [11] and tantazoles [12] have been the focus of much attention, increasing their importance as sources for bioactive substances. However, cyanobacteria may also be a source of considerable nuisance in many situations. Abundant growth of cyanobacteria in water reservoirs creates severe practical problems for water supplies. The development of strains containing toxins is a common experience in polluted inland water systems all over the world, as well as in some coastal waters.

40.2 HARMFUL CYANOBACTERIAL BLOOMS

The toxicity of cyanobacterial blooms was originally brought to the attention of scientists through reports of animal poisonings by farmers and veterinarians. The first well-documented case; was reported in Australia in 1878 [13] (40.1). Since then, harmful algal blooms (HABs) have been reported worldwide, this is atributed to several factors, primarily eutrophication and climate change [14]. Cyanobacterial toxins have been found to occur in fresh (lakes, ponds, rivers, and reservoirs) and brackish (seas, estuaries, and lakes) waters throughout the world. Organisms responsible include an estimated 40 genera, but the main ones are *Anabaena*, *Aphanizomenon*, *Lyngbya*,

TABLE 40.1
Global Occurrences of Cyanobacterial Toxins [21]

Country	Cyanobacteria	Cyanotoxin	Reference(s)
Africa			
Egypt	*Oscillatoria tenuis*	Microcystins	[208]
Kenya	*Oscillatoria willei* and *Phormidium terebriformis*	Microcystins	[209]
Morocco	*Microcystis aeruginosa*	Microcystins	[210]
America (South)			
Brazil	*Microcystis* spp.	Microcystins	[211]
Brazil	*Microcystis aeruginosa*	Microcystins	[212]
Brazil	*Microcystis aeruginosa* and *Oscillatoria*	Microcystins	[213]
Brazil	*Cylindrospermopsis raciborskii*	Saxitoxins	[214,215]
America (North)			
Canada	*Anabaena flos-aquae*	Microcystins	[200,216]
Canada	*Anabaena flos-aquae*	Anatoxin-a	[19,217]
Canada	*Anabaena flos-aquae*	Anatoxin-a(S)	[218,219]
USA	*Haphalosiphon hibernicus*	Microcystins	[153]
USA	*Microcystis* spp.	Microcystins	[83]
USA	*Microcystis* spp.	Microcystins	[220]
USA	*Aphanizomenon flos-aquae*	Saxitoxins	[221,222]
USA	*Lynbya wollei*	Saxitoxins	[60,223]
Asia			
China	*Microcystis aeruginosa*	Microcystins	[224]
China	*Oscillatoria agardhii*	Microcystins	[225]
China	*Oscillatoria* and *Anabaena*	Microcystins	[226]
Japan	*Microcystis viridis*	Microcystins	[227]
Japan	*Microcystis viridis*	Microcystins	[228]
Japan	*Anabaena* spp.	Anatoxin-a	[229]
Japan	*Microcystis* spp.	Anatoxin-a	[229]
Japan	*Umezakia natans*	Cylindrospermopsin (CYN)	[15]
Japan	*Microcystis* spp.	Microcystins	[230]
Thailand	*Cylindrospermopsis raciborskii*	CYN and deoxy-CYN	[231]
Australia			
Australia	*Nodularia spumigena*	Nodularins	[107]
Australia	*Cylindrospermopsis raciborskii*	Cylindrospermopsin	[232,233]
Australia	*Anabaena circinalis*	Saxitoxins	[234–236]
New Zealand	*Microcystis aeruginosa*	Microcystins	[17]
New Zealand	*Nodularia spumigena*	Nodularins	[96,237]
New Zealand	*Cylindrospermopsis raciborskii*	Cylindrospermopsin	[17]
Europe			
Denmark	*Anabaena* spp.	Microcystins	[238]
Denmark	*Microcystis botrys*	Microcystins	[238]
Denmark	*Oscillatoria agardhii*	Microcystins	[238]
Denmark	*Oscillatoria mougeotii*	Microcystins	[238]
Denmark	*Anabaena* spp., *Microcystis* spp. and *Planktothrix* spp.	Microcystins	[239]
Denmark	*Anabaena lemmermannii*	Anatoxin-a(S)	[240,241]
Denmark	*Anabaena lemmermannii*	Saxitoxins	[242]
England	*Microcystis aeruginosa*	Microcystins	[50]
England	*Nostoc* spp.	Microcystins	[73]
Finland	*Nostoc* spp.	Microcystins	[72,243]

Continued

TABLE 40.1 (Continued)

Country	Cyanobacteria	Cyanotoxin	Reference(s)
Europe (*Continued*)			
Finland	*Anabaena* spp.	Microcystins	[244]
Finland	*O. agardhii*	Microcystins	[64, 245]
Finland	*Anabaena* spp.	Anatoxin-a	[246]
Finland	*Aphanizomenon* spp.	Anatoxin-a	[246]
Finland	*Cylindrospermum* spp.	Anatoxin-a	[246]
Finland	*Oscillatoria* spp.	Anatoxin-a	[246]
France	*A. circinalis*	Microcystins	[247]
France	*A. flos-aquae*	Microcystins	[248]
Germany	*Planktothrix* spp.	Microcystins	[249]
Germany	*Anabaena* spp.	Anatoxin-a	[250]
Germany	*Aphanizomenon* spp.	Anatoxin-a	[250]
Greece	*A. millerii*	Microcystins	[74]
Hungary	*C. raciborskii*	Cylindrospermopsin	[251]
Ireland	*Microcystis* spp.	Microcystins	[125]
Ireland	*Anabaena* spp.	Anatoxin-a	[252]
Ireland	*Oscillatoria* spp.	Anatoxin-a	[252]
Ireland	*Anabaena* spp. and *M. aeruginosa*	Homoanatoxin-a	[253]
Israel	*A. ovalisporum*	Cylindrospermopsin	[16]
Italy	*A. planctonica*	Anatoxin-a	[254]
Latvia	*Microcystis aeruginosa*, *Aphanizomenon flos-aquae and Anabaena flos-aquae*	Microcystins	[255]
Norway	*Anabaena flos-aquae*	Microcystins	[244]
Norway	*O. agardhii*	Microcystins	[216,256]
Norway	*O. formosa*	Homoanatoxin-a	[257]
Portugal	*Microcystis* spp., *Anabaena flos-aquae* and *Nostoc* spp.	Microcystins	[258]
Portugal	*O. mougeotii*, *M. aeruginosa* and *P. muciolla*	Microcystins	[259]
Portugal	*Aphanizomenon flos-aquae*	Saxitoxins	[260, 261]
Scotland	*Oscillatoria* spp.	Anatoxin-a	[262]
Slovakia	*Microcystis* spp., *O. rubescens*, *A. flos-aquae* and *A. flos-aquae*	Microcystins	[263]
Switzerland	*O. limosa*	Microcystins	[264]

Microcystis, *Nostoc*, and *Oscillatoria (Planktothrix)*. Cyanobacteria toxins (cyanotoxins) include cytotoxins and biotoxins (neurotoxins: anatoxin-a, anatoxin-a(s) and saxitoxins, and the hepatotoxins microcystins MCs, and nodularins), with biotoxins being responsible for acute lethal, acute chronic, and subchronic poisonings of wild/domestic animals and humans. In most of the reported cases, afflicted animals consumed water from water bodies where there was an obvious presence of cyanobacterial scum on the water surface. More recent measurements of cyanobacterial toxins using sensitive modern analytical methods have often revealed high frequencies of toxic blooms even when animal poisonings have not been reported.

Throughout the world, it appears that hepatotoxic, microcystin-containing freshwater blooms of cyanobacteria are more commonly found than neurotoxic blooms (Table 40.1). Liver toxic blooms have been reported from all continents where samples were collected for analysis. Nevertheless, mass occurrences of neurotoxic cyanobacteria are common in some countries and these have been reported from North America, Europe, and Australia, where they have caused

several animal poisonings. Blooms that have caused liver and kidney damage due to cylindrospermopsin have been reported in Australia, Japan, Israel, Hungary, Thailand, and New Zealand [15–18].

40.3 OCCURRENCE OF CYANOTOXINS

In short, cyanobacterial toxins or "cyanotoxins" have become a major concern for human health. George Francis reported the first toxic cyanobacterial bloom on May 2, 1878 [13,19,20] in lake Alexandria, Australia. *Nodularia spumigena* had so proliferated in the estuary that it formed "a thick scum, like green oil paint, some 2–6 in. thick, and as thick and pasty as porridge." Cattle, sheep, pigs, and dogs died within a few hours after consuming the toxic scum that had collected on the leeward shoreline. However, a number of reports predate this, including one from a Chinese general on military maneuvers over 1000 years ago who reported deaths of his troops after they had consumed water from a river, which was green in color [21]. Codd (1996) [22] reported that human awareness of toxic blooms existed in the twelfth century at the former Monasteries Virdis Stagni (Monastery of the Green Loch), located near the eutrophic, freshwater Soulseat Loch in southwest Scotland. Since then both human and animal intoxications have been reported with increasing frequency throughout the world (Table 40.1) [23–29]. Of particular concern is the number of documented public health incidences associated with microcystin toxin poisoning, in particular through drinking water and also through haemodialysis water.

40.4 MORPHOLOGY

Cyanobacteria tend to be either single-celled or colonial and may form filaments, sheets, or hollow balls depending on their species colonies. Some filamentous colonies have the ability to differentiate into three different cell types, vegetative, akinetes, or heterocysts. Vegetative cells are the normal, photosynthetic cells formed under favorable growing conditions. Akinetes are thick-walled spores formed when environmental conditions are harsh; this enables them to survive periods of adverse conditions such as cold and drought. Heterocysts are special cells formed for nitrogen fixation; these are only formed in certain filamentous genera, such as *Anabaena*, *Aphanizomenon*, *Gloeotrichia*, *Nostoc*, and *Nodularia* [30].

Cyanobacterial cells may also possess gas vesicles, which enable them to regulate their buoyancy in water. This adaptive feature is especially important in photosynthesis as it allows the cyanobacteria to float to the photic zone for optimum exposure to light [31–35].

40.5 NATURAL ENVIRONMENTS

Cyanobacteria are found in many different habitats, ranging from freshwater to hot springs and they have the ability to flourish where no other microalgae can exist. They are often the first plants to colonize bare areas of rock and soil, and other infertile areas such as volcanic ash and desert sand [36]. Their resilience to adverse conditions is due to the presence of ultraviolet (UV) absorbing sheath pigments, which allows these organisms to adapt to exposed land environments. Apart from the ability to withstand very high temperatures, cyanobacteria can also survive extremely cold temperatures such as in snow and ice [37]. Numerous cyanobacterial species are capable of living in the soil and other terrestrial habitats, where they are important in the functional process of ecosystems and the recycling of nutrient elements [38].

Most marine forms of cyanobacteria grow along the shore as benthic vegetation [39], encompassing a large portion of marine plankton worldwide [40]. Freshwater environments with diverse tropic states are the prominent habitats for cyanobacteria. Many species inhabit both near-surface epilimnic and deep, euphotic, hypolimnic waters of lakes [41]. Others colonize surfaces by attaching to rocks or sediments, sometimes forming mats that may tear loose and float to the surface.

40.6 FAVORABLE CYANOBACTERIA GROWTH CONDITIONS

In temperate regions cyanobacterial blooms dominate during a specific period of the year, with most blooms tending to occur during the summer months. These cyanobacterial blooms are due to a combination of interacting factors. High temperature and increased nutrient loading into the water bodies have been considered the most important environmental factors that control the dominance of cyanobacterial bloom [6]. Cyanobacteria have relatively slow growth rates; therefore a long retention time of water within the water body is necessary for their dominance [42]. Cyanobacteria have a generally higher optimum water temperature for growth than other phytoplankton, although this alone cannot explain the dominance of cyanobacteria as they can occur during periods of cold-water temperature. Therefore the direct effects of temperature seem to be secondary to its indirect effects. For example, stratified water columns and their stability create favorable conditions for the dominance of cyanobacteria that can regulate their buoyancy by gas vesicles [43].

Eutrophication, the enrichment of waters with plant nutrients, is a contributing factor in the growth of cyanobacterial blooms and this association is common throughout the world [44,45]. In recent years industrial, farm, and domestic run-offs containing high levels of fertilizers and detergents have altered the chemistry of many water supplies, thus increasing the concentration of nitrogen and phosphorus. Low ratio (<29) of total nitrogen to total phosphorus has been implicated as a major factor favoring cyanobacteria; this is because certain cyanobacteria are able to fix atmospheric nitrogen. Cyanobacteria; also have the ability to store large amounts of phosphorus within their cells that allows them to survive when phosphorus may become limiting in late summer [46]. Although eutrophication is thought to be the primary cause for the increase in cyanobacterial blooms globally, it is not solely responsible, as many blooms predate intensive agriculture and industry, as well as high human populations. Cyanobacterial blooms have also been present in the least polluted of water bodies. Water temperature and nutrient enrichment are the principal factors associated with cyanobacterial bloom formation but many indirect effects of these factors are also significant [42].

40.7 CYANOBACTERIAL BLOOMS (AESTHETIC AND ODOR ISSUES)

Cyanobacterial bloom formation, the accumulation of nuisance scum onto the surface of the water, may result owing to a change in weather conditions [42,43]. Blooms of cyanobacteria, if driven onshore by wind, can result in copious, extensive, and objectionable accumulations of decaying matter along the shoreline. Buoyancy regulation of cyanobacteria is a key role in the phenomenon. As a large dominant cyanobacterial population has evolved in a lake, continuous turbulence, mixing of the water body and decrease of the light penetration cause cyanobacteria to increase their buoyancy. When the wind subsides, cyanobacteria float quickly toward the surface owing to "overbouyancy"; this is the inability to reverse the buoyancy quickly. If the wind is not strong enough to redisperse the cells into the water column, then the cyanobacteria accumulate on the surface of the water. The floating scum then tends to drift downwind to lee shores and calm bays [32–35].

The cyanobacterial cells themselves are microscopic, typically less than 10 μm in length or diameter. One colony can contain hundreds or thousands of cells and grow to only millimeters in diameter. Thus, a cyanobacterial bloom, scum, and mat are formed when millions of these colonies are produced in the water. Under favorable conditions, cyanobacteria numbers may double in 1 day or less and the bloom can persist for several weeks [32–35].

The macroscopic appearance of these colonies has been compared to green oily paint on the surface of the water and is thus aesthetically undesirable. This offensive cyanobacterial mat also causes turbidity in recreational and amenity waters. As the cyanobacterial colonies begin to deteriorate a blue pigment forms, and this gives rise to the name cyanobacteria [47,48].

Cyanobacteria have the potential to synthesize a large number of low molecular weight compounds that cause taste and odor problems. These substances, including geosmin and methyl isoborneol often result in complaints regarding recreational and amenity water bodies and the quality of raw and treated drinking water [49].

40.8 BENEFITS OF CYANOBACTERIA

40.8.1 Nitrogen Fixation

Cyanobacteria, such as *Nostoc*, are very important for the health and growth of many plants as it has the ability to convert atmospheric nitrogen into an organic form, that is, ammonia or nitrate, in the presence of the enzyme nitrogenase. This nitrogen fixation occurs in the specialized heterocysts, and these larger cells are clearly visible in the filamentous *Nostoc* species. The addition of phosphorus and the limitation of nitrogen often increase the proportion of cyanobacteria that are able to fix atmospheric nitrogen [34,50].

Many symbiotic relationships occur between cyanobacteria and plants for example, the relationship between the cyanobacterium *Anabaena azollae* and the tiny aquatic water fern, *Azolla*. The fern provides specialized oval cavities inside their leaves to house the cyanobacteria, in return for organic nitrogen. This has proved very useful in the fertilization and cultivation of rice, where the floating fern, *Azolla* accompanied by the symbiotic cyanobacterium, *Anabaena* is distributed across the rice paddy fields [9].

40.8.2 Nutrition Source

Spirulina and other cyanobacteria are well known for their high nutritive value, in particular for their high protein and vitamin content [51]. The African flamingos get their pink color from eating the cyanobacterium. The Aztecs ate it regularly and it still remains popular in several oriental dishes. As a result of its high nutritive value and its ability to stimulate the immune system, cyanobacteria are commonly sold as a "health food," especially in dried powder or tablet form. In Autumn 1996, the Oregon Health Division (OHD) in Portland, Oregon, discovered that Upper Klamath Lake had a massive *Microcystis aeruginosa* bloom, and that MC levels in the lake were high, and the OHD issued a warning advising people to avoid all contact with the water. Consumers of blue-green alga (BGA) expressed concern regarding cyanobacterial products, in response the OHD established a limit of 1 ppm for MC toxins in BGA-containing products: (tablets and capsules) and carried out laboratory testing of supplements from four different harvesters during the years 1996–1999. MC toxins were detected in 85 of 87 Upper Klamath Lake *Aphanizomenon flos-aquae* (AFA) samples tested, with 63 samples (72%) containing concentrations greater than 1 mg/kg [52]. The average MC levels in samples collected by OHD investigators from harvesters between 1996 and 1998 ranged between 1.76 and 16.42 mg/kg. Gilroy et al. [52] used enzyme-linked immunosorbent assay (ELISA) and HPLC-ELISA to determine primarily MC-LR, therefore the specificity of the analysis was severely limited.

In 1999, many BGA derived products were recalled from retail outlets across Canada and were analyzed for MC toxins [53]. The results of the study report combined levels of MC-LR and MC-LA ranging between not detected (N.D.) and 35.7 mg/kg. These results emphasize the requirement for rigorous testing of all BGA nutriceuticals.

40.8.3 Pharmaceutical Products

Cyanobacteria are also sources of substances of pharmaceutical interest, for example, antibiotics [54]. Moore et al. isolated structurally unique cytotoxins from a lipophilic extract of *Nostoc* spp. GV 224. These cyclic depsipeptides called cryptophycins showed both solid tumor and tumor-selective cytotoxicity in the Corbett assay and *in vivo* studies revealed them to be very active against a number of tumors implanted in mice [55]. These studies may lead to the discoveries of new antitumor drugs [56].

40.9 CYANOBACTERIAL TOXINS

40.9.1 Classification of Cyanobacteria Toxins

Cyanotoxins are a diverse group of natural toxins, both from the chemical and the toxicological points of view. They fall into three broad groups of chemical structure: cyclic peptides, alkaloids, and lipopolysaccharides (LPS). In spite of their aquatic origin, most of the cyanotoxins that have been identified to date appear to be more hazardous to terrestrial mammals than aquatic biota. Cyanobacteria produce a variety of unusual metabolites, the natural function of which is not clear, although some exert effects upon other biota when exposed to the cells or free toxins in water. Research has primarily focused on compounds that impact on humans and livestock, either as toxins or as pharmaceutical substances. Further ranges of nontoxic products have been found in cyanobacteria and the biochemical and pharmacological properties of these are largely unknown. Mechanisms of cyanobacterial toxicity currently described and understood are very diverse and range from hepatotoxic, neurotoxic, and dermatotoxic effects to general inhibition of protein synthesis. To assess the specific hazards of cyanobacterial toxins it is necessary to understand their chemical and physical properties, their occurrence in waters used by people, the regulation of their production, and their fate in the environment.

The toxicity of hepatotoxins typically refers to animal testing data, and is expressed as the amount of cyanobacterial toxin lethal to an animal. The commonly reported LD_{50} value, a measure of toxin potency, is the amount of pure toxin needed to kill 50% of animals in a trial, per kilogram of body weight [21]. Thus, the lower the LD_{50} value, the more potent the cyanobacterial toxin. Table 40.2 illustrates the LD_{50} values (μg/kg) for the most common cyanobacterial toxins using various exposure routes including intravenous (i.v.), intraperitoneal (i.p.), intranasal (i.n.), and oral. It also details the LD_{50} values associated with the Lowest Observed Adverse Effect Level (LOAEL) and the No Observed Adverse Effect Level (NOAEL), which are important, along with other factors, in calculating the Tolerable Daily Intake (TDI) for humans [21].

Studies on the occurrence, distribution, and frequency of toxic cyanobacteria were conducted in a number of countries during the 1980s using mouse bioassay [34,57]. Analytical methods suitable for quantitative analysis for toxin determination only became available in the 1980s, but studies of specific cyanotoxins have been included since then. The result indicates that neurotoxins are generally less common. In contrast MCs and nodularin that primarily cause liver damage are more widespread and very likely to occur if certain taxes of cyanobacteria are present [32–35].

TABLE 40.2
Toxicity of Common Cyanobacterial Toxins by Various Administration Routes [49]

	LD_{50} (μg/kg)					
Cyanobacterial Toxin	**i.v.**	**i.p.**	**i.n.**	**Oral**	**LOAEL**	**NOAEL**
Microcystins	NR	25–150	36–122	5000–10900	100	40
Nodularin	NR	50	NR	NR	NR	NR
Cylindrospermopsin	NR	200–2000	NR	4400–6900	NR	30
Anatoxin-a	<100	375	2000	>5000	NR	100
Homoanatoxin-a	NR	250	NR	NR	NR	NR
Anatoxin-a(S)	NR	20	NR	NR	NR	NR
Saxitoxin	3.2–3.6	7.6–10.5	NR	251–267	NR	NR

Notes: i.v. = intravenous, i.p. = intraperitoneal, i.n. = intranasal, LOAEL = lowest observed adverse effect level, NOAEL = no observed adverse effect level, NR = not reported.

40.10 MICROCYSTINS AND NODULARIN

The water quality problems caused by dense populations of cyanobacteria are intricate, many, and various [58,59] and can have negative health and economic impacts. As a result of this, the negative aspects of cyanobacteria have gained research attention and public concern. Cyanobacteria are capable of producing a number of secondary metabolites, which are not essential for primary metabolism. Certain secondary metabolites inhibit the growth and development of other organisms and can be lethally toxic to wildlife, domestic livestock and to humans [60].

Approximately 75% of the world's cyanobacteria samples were found to contain toxins but this toxicity is difficult to assess since both toxic and nontoxic strains of a single species can be found within a bloom and toxicity can also vary between blooms [32]. Cyanobacterial toxin production is highly variable between strains and there are significant temporal variabilities in even the same strain [45]. Even among toxigenic strains, some always produce more toxins than others [61]. The toxicity of individual strains can diminish for no apparent reason, thus cyanobacteria are a complex and challenging group of organisms.

There are many environmental factors that affect cyanobacterial toxin production: temperature, light intensity, phosphorus, nitrogen, micronutrients, and salinity. In general toxin production is greatest between 18°C and 25°C with production lowest at ≤10°C or ≥30°C. The impact of light intensity had varying effects, with maximum toxin production occurring between 12 and 142 μE/m^2/s [62–64] and the effect of phosphorus concentration on cyanotoxin production was also variable, ranging from 0 to 5.5 mg/L for maximum production. Hence, it is difficult to determine the optimum environmental conditions for cyanobacterial toxin production.

Globally the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the cyclic peptide MCs and nodularin family [65,35]. The MCs are geographically most widely distributed in freshwaters and recently they have been identified in marine waters [5, 66] as a cause of liver disease in net-pen reared salmon, although it is not clear which organism in marine environments contains these toxins. Microcystins and nodularin pose a major challenge for the production of safe drinking water from surface waters containing cyanobacteria with these toxins.

Microcystins have been characterized from planktonic *Anabaena* [67], *Microcystis* [68–70], *Oscillatoria* (*Planktothrix*) [71], *Nostoc* [72,73], and *Anabaenopsis* species [74], and from terrestrial *Hapalosiphon* genera [75,76]. Nodularin has been characterized from *N. spumigena* and in the marine sponge *Theonella swinhoei* [77]. The cyclic peptides are comparatively large natural products, molecular weight (MW) ≈ 800–1100, although small compared with many other cell oligopeptides and polypeptides (MW > 10000). They contain either five (nodularins) or seven (microcystins) amino acids, with the two terminal amino acids of the linear peptide being bonded to form a ring. Microcystins and nodularins act by inhibiting protein phosphatases, which leads to hyperphosphorylation of cellular proteins such as cytokeratin 8 and 18 [78,79]. Eutrophication of water bodies has been implicated in toxin synthesis [44], the environmental conditions under which cyanobacteria produce toxins remain largely unclear. The functional roles of these toxins for cyanobacteria are also however, uncertain. They have been suggested to be defense mechanisms to protect against grazers or to provide an ecological advantage over algae [80], but not over other cyanobacterial species [81], to regulate endogenous protein phosphatases [81] or even as storage substances [82].

40.10.1 Structure of Microcystins

The first chemical structures of cyanobacterial cyclic peptide toxins were identified in the early 1980s and the number of fully characterized toxin variants has greatly increased since the 1990s. To-date there are 77 different microcystins isolated from cyanobacteria (Table 40.3). The first such compounds found in freshwater cyanobacteria were cyclic heptapeptides with the general structure of cyclo-(D-alanine^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glutamate^6-Mdha^7) in which X and Z are variable amino acids, D-MeAsp^3 is D-*erythro*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine (Figure 40.1a). The amino acid Adda, (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8- trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual structural moiety in

TABLE 40.3
Microcystins Cited in the Literature (Update on table in WHO, Toxic Cyanobacteria in Water) [21]

Microcystin	Molecular Weight	Toxicity LD_{50}	Organism	Reference(s)
MC-LA	909	50	*Microcystis aeruginosa Microcystis viridis*	[66,228]
MC-LAba	923	NR	*Microcystis aeruginosa*	[265]
MC-Lbu	923		*Microcystis aeruginosa*	[266]
MC-LL	951	+	*Microcystis aeruginosa*	[159]
MC-AR	952	250	*Microcystis* spp.	[83]
MC-RA	952	+	*Microcystis aeruginosa*	[149]
MC-YA	959	NR	*Microcystis aeruginosa*	[199]
[D-Asp^3,Dha^7]MC-LR	966	+	*Microcystis aeruginosa Anabaena* spp.	[200,244]
[D-Asp^3,Dha^7]MC-EE(OMe)	969	+	*Anabaena* spp.	[267]
MC-VF	971	NR	*Microcystis aeruginosa*	[268]
[D-Asp^3]MC-LF	971		*Microcystis aeruginosa*	[268]
[D-Asp^3]MC-LR	980	160–300	*A. flos-aquae Microcystis aeruginosa Microcystis viridis O. agardhii*	[150,216–245, 270,271]
[Dha^7]MC-LR	980	250	*Microcystis aeruginosa Anabaena* spp. *O. Agardhii*	[200,244,245]
[$DMAdda^5$]MC-LR	980	90–100	*Microcystis* spp. *Nostoc* spp.	[72,83]
[D-Asp^3, (E)- Dhb^7] MC-LR	980	70	*P. agardhii*	[151]
[D-Asp^3, (Z)- Dhb^7] MC-LR	980	+	*P. agardhii*	[272]
MC-AW	982		*Microcystis aeruginosa*	[273]
[Dha^7]MC-EE(OMe)	983	+	*Anabaena* spp.	[267]
[D-Asp^3,Dha^7]MC-E(OMe)E(OMe)	983	+	*Anabaena* spp.	[267]
MC-LF	985	+	*Microcystis aeruginosa*	[274]
MC-LR	994	50	*Microcystis aeruginosa A. flos-aquae Microcystis viridis*	[96,199,216 275]
[D-Asp^3,D-Glu$(OCH_3)^6$]MC-LR	994	NR	*A. flos-aquae*	[276]
[(6Z)-$Adda^5$]MC-LR	994	>1200	*Microcystis viridis*	[84,270]
[Dha^7]MC-E(OMe)E(OMe)	997	+	*Anabaena* spp.	[267]
[L-Ser^7]MC-LR	998	+	*Anabaena* spp.	[277]
MC-LY	1001	90	*Microcystis aeruginosa*	[278]
[L-Ser^7]MC-EE(OMe)	1001	+	*Anabaena* spp.	[267]
[D-Asp^3,Ser^7]MC-E(OMe)E(OMe)	1001	+	*Anabaena* spp.	[267]
MC-HilR	1008	100	*Microcystis* spp.	[152]
MC-LHar	1008	NR	*O. tenuis*	[208]
[D-Asp^3, $ADMAdda^5$] MC-LR	1008	160	*Nostoc* spp.	[243,279]
[D- Glu$(OCH_3)^6$] MC-LR	1008	>1000	*A. flos-aquae*, *Microcystis* spp.	[268,276,280]
[D- Asp^3, Dha^7] MC- RR	1009	+	*P. agardhii*, *Anabaena* spp., *Microcystis aeruginosa*	[216,244,281]
[D-Asp^3, $ADMAdda^5$, Dhb^7] MC- LR	1009	+	*Nostoc* spp.	[73]
Demethyl-MC-LW	1010		*Microcystis aeruginosa*	[269]
[L-$MeSer^7$] MC-LR	1012	150	*Microcystis* spp.	[83,152]
[D-Asp^3] MC-FR	1014	NR	*Microcystis aeruginosa*	[282]
[Dha^7] MC-FR	1014	NR	*Microcystis* spp.	[281]
[L-Ser^7] MC-E(OMe)E(OMe)	1015	+	*Anabaena* spp.	[267]
[$ADMAdda^5$] MC-LR	1022	60	*Nostoc* spp.	[243,279]
[D-Asp^3, $ADMAdda^5$] MC- LHar	1022	+	*Nostoc* spp.	[72]

TABLE 40.3 (Continued)

Microcystin	Molecular Weight	Toxicity LD_{50}	Organism	Reference(s)
[D-Asp^3] MC-RR	1023	250	*P. agardhii*, *Anabaena* spp., *Microcystis aeruginosa*	[244,256, 281]
[Dha^7] MC-RR	1023	180	*P. agardhii*, *Anabaena* spp., *Microcystis aeruginosa*	[244,245]
[D-Asp^3, (E)-Dhb^7] MC-RR	1023	250	*P. agardhii*	[283]
MC-LW	1024	NR	*Microcystis aeruginosa*	[268]
MC-FR	1028	250	*Microcystis* spp.	[83]
MC-M(O)R	1028	700–800	*Microcystis* spp.	[83]
[Dha^7] MC-HphR	1028	+	*Anabaena* spp.	[203]
[D-Asp^3, Dha^7] MC-HtyR	1030	+	*Anabaena* spp.	[203]
[Dha^7] MC-YR	1030	+	*Microcystis aeruginosa*	[69]
[D-Asp^3] MC-YR	1030	+	*Microcystis* spp.	[284]
MC-YM(O)	1035	56	*Microcystis aeruginosa*	[199,285]
[D- Leu^1] MC-LR	1036	+	*Microcystis aeruginosa*	[211]
[ADMAdda] MC-LHar	1036	60	*Nostoc* spp.	[243,279]
[D-Asp^3,D-Glu(OCH$_3$)^6]MC-RR	1037	NR	*P. rubescens*	[286]
MC-RR	1037	600	*Microcystis aeruginosa*, *Microcystis viridis*, *Anabaena* spp.	[227,244, 275,287]
[6(Z)- Adda^5] MC-RR	1037	>1200	*Microcystis viridis*	[84,270]
[D- SerP^1- ADMAdda^5] MC-LR	1038	+	*Nostoc* spp.	[72]
[ADMAdda^5, MeSer^7] MC-LR	1040	+	*Nostoc* spp.	[72]
[L- Ser^7] MC- RR	1041	+	*Anabaena* spp., *Microcystis aeruginosa*	[277,281]
[D- Asp^3, MeSer^7] MC-RR	1041	+	*P. agardhii*	[245]
seco[D- Asp^3] MC-RR	1041	NR	*P. rubescens*	[286]
MC-YR	1044	70	*Microcystis aeruginosa*, *Microcystis viridis*	[199,275]
[D-Asp^3] MC-HtyR	1044	160–300	*A. flos-aquae*	[271]
[Dha^7] MC-HtyR	1044	+	*Anabaena* spp.	[203]
[D-Asp^3, (E)- Dhb^7] MC-HtyR	1044	70	*P. agardhii*	[151]
[D-Asp^3, (Z)- Dhb^7] MC-HtyR	1044	+	*P. agardhii*	[272]
MC-(H_4)YR	1048	NR	*Microcystis* spp.	[152]
[D-Glu-OC$_2$H$_3$(CH$_3$)OH^6] MC-LR	1052	>1000	*Microcystis* spp.	[83]
[D- Asp^3, ADMAdda^5, Dhb^7] MC-RR	1052	+	*Nostoc* spp.	[73]
[D-Asp^3] MC-WR	1053	NR	*Microcystis aeruginosa*	[282]
MC-HtyR	1058	80–100	*A. flos-aquae*	[271]
[L- Ser^7] MC-HtyR	1062	+	*Anabaena* spp.	[203]
MC-HarHar	1065	NR	*Microcystis* spp.	[288]
MC-WR	1067	150–200	*Microcystis* spp.	[83]
[D-Asp^3, ADMAdda^5, Dhb^7] MC-HtyR	1073	+	*Nostoc* spp.	[73]
[L- MeLan^7] MC-LR	1115	1000	*Microcystis* spp.	[152]

Aba	Aminoisobutyric acid	Hil	Homoisoleucine
ADMAdda	*O*-Acetyl-*O*-demethylAdda	Hph	Homophenylalanine
Dha	Dehydroalanine	Hty	Homotyrosine
Dhb	Dehydrobutyrine	Hty	Homotyrosine
DMAdda	*O*-DemethylAdda	MeLan	*N*-Methyllanthionine
E(Ome)	Glutamic acid methyl ester	M(O)	Methionine S-oxide
H_4Y	1,2,3,4-tetrahydrotyrosine	MeSer	*N*-Methylserine
Har	Homoarginine	NR	Not Reported

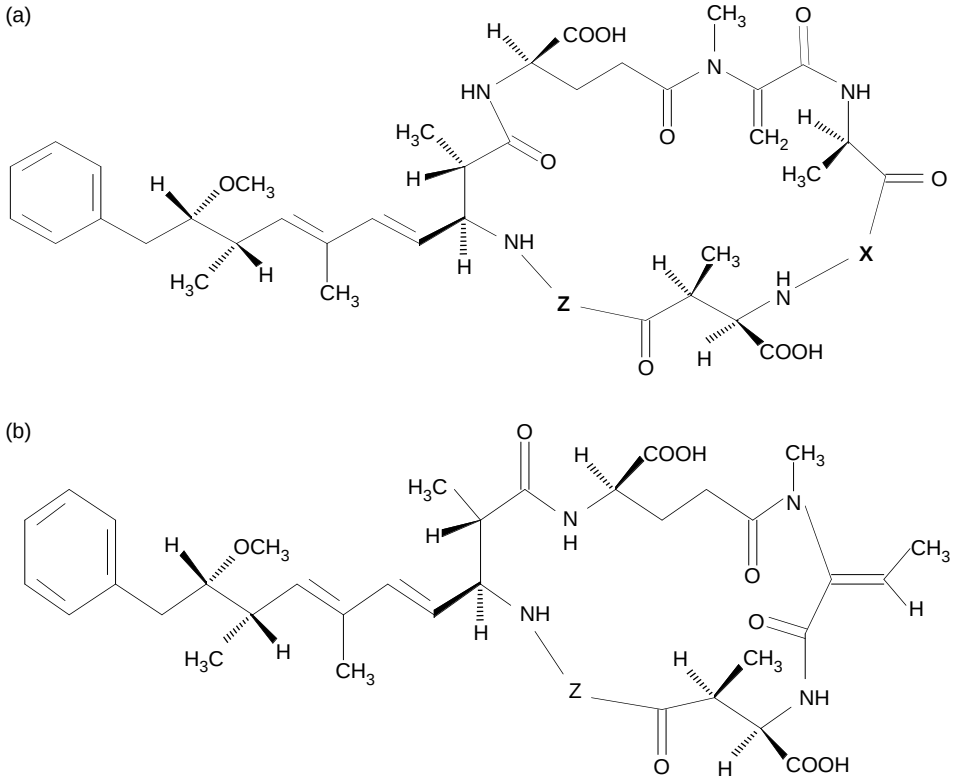

FIGURE 40.1 (a) General microcystin structure; MC-RR (Z = Arg, X = Arg); MC-YR (Z = Tyr, X = Arg); MC-LR (Z = Leu, X = Arg); MC-LA (Z = Leu, X = Ala); MC-LW (Z = Leu, X = Trp); MC-LF (Z = Leu, X = Phen). (b) Structure of nodularin-R (Z = Arg).

this group of cyanobacterial peptide toxins. The Adda moiety is essential for the toxicity of MCs and the Mdha is responsible for covalent binding of the toxin to protein phosphatases [82]. Changes in the stereochemistry at the diene of Adda result in a loss of toxicity [83], while slight modifications, for example, demethylation or acetylation at the C-9 position of Adda retain toxicity [84]. Other modifications include replacement of D-alanine or methyldehydroalanine by D-serine and *N*-methylserine, respectively. These compounds were first isolated from the cyanobacterium *M. aeruginosa* and therefore the toxins were named microcystins [85]. Structural variations have been reported in all seven amino acids, but most frequently with substitution of L-amino acids at positions × and Z, and demethylation of amino acids at positions 3 and/or 7.

40.10.2 Toxicology of Microcystins

Symptoms of microcystin intoxication are diarrhea, vomiting, piroerection, weakness, and pallor [86]. MCs targets the liver causing cytoskeletal damage, necrosis, and pooling blood. As a consequence, the weight of the liver may be increased up to 100% [87]. MCs exhibit their toxicity by uptake into liver hepatocytes through multispecific bile acid transport systems, by inhibition of serine/threonine protein phosphatases 1 and 2A [78,79,88], by depolymerization of intermediate filaments and microfilaments, and by disruption of the liver cytoskeleton. This leads to loss of cell morphology, loss of cell-to-cell adhesion, and finally cellular necrosis [89–91]. At acutely toxic doses, MCs cause rounding or shrinkage of the hepatocytes and loss of normal hepatocyte structure. This disorganization of the structure leads to massive hepatic hemorrhage followed by hypovolemic

shock or hepatic insufficiency and, as a consequence, death [78,87,89,92]. Death can occur within a few hours after a high dose [86,87,92].

Microcystins are also potent tumor promoters that are mediated through the inhibition of the protein phosphatase type 1 and 2A activities. Their mode of action appears to be different from that of other protein phosphatase inhibitors, such as okadaic acid for, example [93], and their effects are organ specific [70,94,95]. The incidence of primary liver cancer in China, where people drink pond and ditch water, is about eight times higher than in populations that drink well water [25], and MCs were found in areas were cancer incidence was high [26].

40.10.3 Structure of Nodularin

In a species of brackish water cyanobacterium, a similar acting and structurally related, cyclic pentapeptide was found (Figure 40.1b). It was called nodularin after its producer, *N. spumigena* [96]. The proper name for nodularin-R would be cyclo-(D-MeAsp^1-L-Arginine^2-Adda^3-D-glutamate^4-Mdhb^5), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid; the presence of this aminoacid instead of the Mdha in MCs does not allow nodularin to form covalent bonds with protein phosphatases [97]. Most nodularins found so far contain arginine at the Z position but a few naturally occurring variations of nodularins have been identified. A nontoxic 6(Z)-Adda stereoisomer of nodularin has been discovered [98]. Demethyl variations at positions 1 and 3 are also present. Nodularin-V is structurally identical to nodularin-R except that it contains Valine in the Z position [99]. This toxin was isolated from *Theonella swinhoey Gray*, a marine sponge [77]. It is suspected that the toxin is of cyanobacterial origin, as the sponge is known to harbor cyanobacterial symbionts. This toxin was named motuporin but it is structurally a nodularin [77,98,100]. With the exception of *T. swinhoey* marine sponge, *N. spumigena* was, up until recently, the only source of nodularins. A new homoarginine containing nodularin has been identified in *Nodularia* PCC 7804 along with nodularin-R [101].

40.10.4 Toxicology of Nodularin

Both in experimental animals and based on observations in field reports, the toxicity and pathogenicity of nodularin is very similar to that of MCs [91,102]. Nodularin induces enlarged hemorrhagic livers, centrilobular necrosis, lysis of hepatocytes, and death [102]. Nodularin promotes liver tumors [88] and is a direct liver carcinogen [103]. *N. spumigena* has been associated with several livestock, canine and wildlife mortality events, principally occurring in brackish waters in Australia, New Zealand, and Baltic Sea [104–107]. A few studies have demonstrated effects of nodularin on zooplankton, showing that, depending on the species, they differ markedly in their physiological sensitivity to nodularin [108].

Few reports have associated nodularin with aquatic animal mortalities, although recently, fish mortalities in the Black Sea, Georgia, coincided with *N. spumigena* blooms. However, it was noted that other plankton, such as *Anabaenopsis* or *Rhizosolenia*, were also present in the bloom, so it is unclear which species were responsible for these mortalities [109].

Along with MCs, nodularin has been found in marine mussels but the bioorigin of these toxins is currently unknown [110]. There are potential human health risks associated with shellfish that have consumed *Nodularia*. Some studies have demonstrated that retained in the gastrointestinal tracts of mussels, retained in the toxicity declined after the algal bloom ended [111]. Low concentrations of nodularin were also found in livers of flounders and cod in the Baltic Sea [112–114]. The potential chronic effect of nodularin in aquatic animals requires further research.

40.10.5 Cyanotoxin Legislation

The danger of tumor promotion by chronic exposure of microcystins in drinking water was the main reason for the definition of guidelines for these toxins by the World Health Organization (WHO).

A guideline provisional limit of 1 μg/L for MC-LR was proposed on the basis of animal studies of MC-LR orally administered to pigs and mice [100,115]. This limit has been adopted by many countries including the United Kingdom, New Zealand, Brazil, and so forth, as a guideline for MC-LR in drinking water. Canada has proposed a guideline limit of 1.5 μg/L for MC-LR in drinking water and a limit of 10 μg/L for short-term exposure [115]. In Australia the proposed values range from 1.3 to 10 μg/L. Poland has adopted a drinking water legislation for MC-LR (1 μg/L), whereas Brazil and Spain have a drinking water legislation for all microcystins (1 μg/L) [116,117]. An antiterrorism legislation has been implemented by both the United Kingdom and Czech Republic for microcystins [117].

For recreational waters with cyanobacterial blooms, WHO has established three health hazard alert levels, depending on the risk of adverse health effects [118] and these are based on cyanobacterial intensities [76]. Finally for cyanobacterial food supplements, there is a MC-LR proposed guideline of 10 μg/g [119] and, in Oregon, USA, a maximum limit of 1 μg/g was established for food [52,53].

Cyanotoxins are increasingly being categorized in terms of their toxicity, and national and international organizations are documenting them in terms of their potential as agents of bioterrorism [120]. Other organizations to which cyanotoxins are relevant include the Australia Group, a consortium of governments dedicated to minimizing the proliferation of such weapons, which includes microcystins [117].

40.11 ISOLATION OF MICROCYSTINS AND NODULARIN

Natural cyanobacterial blooms are typically sampled at sites where the accumulation of the cyanobacteria is likely to affect both humans and livestock, or at drinking water reservoirs. Samples are taken for quantitative and structural analysis and also for microscopic identification. The samples for microscopic identification are normally collected in brown glass bottles and preserved in Lugol's iodine solution or formaldehyde solution. Samples for quantitative and structural analysis are normally collected in large wide neck glass or plastic bottles [121].

40.12 EXTRACTION OF MICROCYSTINS AND NODULARIN

40.12.1 Intracellular Toxins

The extraction of toxins from cyanobacterial samples requires the disruption or perforation of the strong cyanobacterial cell wall structure, which is necessary for effective toxin recovery. Freeze-drying [122,123] or freeze-thawing [124,125] have been employed for this purpose. Toxins can be extracted from filter papers containing harvested cyanobacterial cells or from weighed lyophilized bloom material. A number of studies have illustrated the enhancement of cell disruption when using sonication, especially ultrasonication or more recently probe-type sonication [114,126,127]. However, Spoof et al. highlighted the limitations of prolonged probe sonication; (a) small samples may reach high temperatures and evaporate or degrade, and (b) glass-fiber filters, used for biomass concentration disintegrate and release fibers that can clog high performance liquid chromatography (HPLC) systems [114]. According to Ramanan et al., sonication yields higher amounts of MC-LR, but degrades some of the toxin, therefore resulting in comparable amounts of MC-LR in both sonicated and unsonicated samples [128].

After cell lysis, the hepatotoxins can be extracted using several different solvent combinations. The differences in extraction efficiency are not solely dependent on the solvent used but also on the morphological differences of cyanobacteria and on the microcyst variants present in the cells [129]. However, many studies have investigated the most efficient extraction solvents for hepatotoxins [124,129–131], these indicate that the selection of solvents will vary depending on the sample type and its microcystin content. More recently, Barco et al. evaluated a wide range of extraction volumes

and solvents, over a range of pH and extraction times in order to optimize a suitable method for the extraction of a wide range of microcystins [132]. Eighty percent aqueous methanol was selected as the most suitable mixture for the extraction of both hydrophobic and hydrophilic microcystins, which is consistent with those previously reported in the literature [124,130], however, acidified methanol led to the highest yields of most microcystins, since the hydrophobic character of microcystins improves as the pH value decreases [132].

Barco et al. concluded that sonicating with acidified methanol (pH ~ 2) has been shown to be a rapid and efficient method for the routine analysis of a wide range of intracellular microcystins as well as nodularins with no recorded degradation [132]. Nonetheless, methanol has been shown to be a suitable solvent because it gives good extraction efficiency and has the added advantage of allowing sample concentration through evaporation.

40.12.2 Extracellular Toxins

Extracellular toxins are typically present in very low concentrations and thus are difficult to analyze directly. Therefore the use of solid-phase extraction (SPE) or liquid–liquid extraction (LLE) for sample concentration and clean up are required [114,127,133,134].

40.13 ANALYTE CONCENTRATION

Table 40.4A outlines the main publication that describe the extraction of MCs from an array of different biological matrices. As previously discussed, low concentrations, direct determination of hepatotoxins is difficult owing to the lack of sensitivity of most detection procedures, therefore the requirement of a sample preconcentration step is essential. This step may also serve as a cleanup step by allowing removal of coextracted material and proteins, which may interfere in the analysis. SPE is the most widely used technique in sample concentration and cleanup of microcystins and nodularin [124,125,127,135–138]. It has almost completely replaced the LLE method [139]. Alkyl-bonded silicas are most often used but polymeric materials are becoming increasingly popular.

SPE is based on the principles of chromatography and involves passing a sample through a cartridge, which retains the toxins, by adsorption. Toxins can then be eluted using organic solvents such as methanol, which may be reduced in volume by evaporation resulting in increased toxin concentration. The correct choice of SPE cartridge is essential. The most important parameter to consider is pore size, as it should be small enough to exclude interfering proteins [140–142]. C_{18} cartridges are commonly used [124,125,127,137,138,143] for the extraction of microcystins and nodularin, but these cartridges also extract the naturally occurring organic matter (NOM) present in water. This coextracted NOM can interfere in chromatographic separation by producing peaks, which may obscure the toxin peaks, thus making quantitation imprecise. Tsuji et al. studied different types of SPE chemistries and developed a two-step cleanup method, with SPE on a C_{18} cartridge followed by a silica gel cartridge [144]. The nonpolar poly (divinylbenzene-co-*N*-vinylpyrrolidone) material (Oasis HLB cartridges from Waters) has been shown to concentrate microcystins from water samples effectively [126].

The limitations of SPE are many-fold: a validation of the chosen SPE method is necessary; validation requires spiking a blank water sample with a known amount of cyanotoxin and then determining the recovery, methods are both tedious and time-consuming and give variable rates of recovery that may be due to, among other things, batch-to-batch variation amongst catridges. Efficient extraction of microcystins is also problematic because of the differing polarities of these toxins, therefore using a generic SPE method for all microcystins is impossible. Lawton et al. reported recovery problems with MC-LW, especially from tap water [124] and Moollan encountered serious problems with this procedure in the analyses of chlorinated waters [145]. SPE is also an expensive

TABLE 40.4A
Extraction, LC-MS Parameters and Methods Sensitives for Determination of Microcystins in Complex Biological Matrices

Sample Matrix	Sample Preperation	Sample Introduction	Mobile Phase	Ionization Source and Mode	Mass Analyser	Acquisition Mode	Analytes	LOD (MC-LR)	Linear Range (MC-LR)	Year and Reference
Freshwater mussel and fish tissue	Extraction, SPE	ODS Column	A:H_2O + 0.05% TFA B:MeOH (Isocratic)	FAB	Single-quadrupole	TIC	MC-LR,-RR	NR	NR	1997 [289]
Mussel and flounder tissue	Extraction, SPE	C_{18} Column	A:H_2O + 0.5% FA B:ACN (Gradient)	ESI, positive	Triple-quadrupole Ion-trap	Triple-quad = SIR, MRM Ion-trap = SIR, Product Scan	Nod, Demethyl Nod	NR	0.05–50 ng on column	2003 [290]
Human serum	Extraction, SPE	C_{18} Column	A:H_2O + 0.02% HFBA B:ACN + 0.02% HFBA (Gradient)	ESI, positive	Ion-trap	SRM	MC-LR	<10 pg on column	NR	2005 [291]
Fish and bird liver, and mussel tissue	Extraction, SPE	C_{18} Column	A:H_2O + 0.5% FA B:ACN + 0.5% FA (Gradient)	ESI, positive	Triple-quadrupole	SIR, MRM	Demethyl MC-LR,-RR, MC-LR,-RR,-LW,-LF, Nod	11–24 pg on column	NR	2005 [292]
Fish muscle tissue	MSPD extraction	C_{18} Column	A:H_2O + 0.01M FA B:ACN + 0.01M FA (Gradient)	ESI, positive	Triple-quadrupole	MRM	MC LR,-RR,-YR,-LA,-LW	0.0023 µg/g	0.2–400 ng on column, $R^2 \geq 0.9957$	2005 [293]
Liver and kidney tissue	MSPD extraction	C_{18} Column	A:H_2O + 0.05% TFA B:ACN + 0.05% TFA (Gradient)	ESI, positive	Single-quadrupole Ion-trap	Scan, SIM using single-quad MRM using ion-trap	MC LR,-RR,-YR	0.01 µg/g	0.15–25 µg/g, R^2 >0.9900	2005 [294]
Rat or chicken liver	MMPB oxidation, SPE	C_{18} Column	A:H_2O (95%) + MeOH (5%) + HOAc (0.05%) B:MeOH (95%) + H2O (5%) + HOAc (0.05%) (Gradient)	ESI, negative	Ion-trap	SIM	MC-LR	5 µg/g	5–50 µg/g, $R^2 = 0.9963$	2006 [192]
Human liver and human sera	SPE, MMPB	C_8 Column	A:H_2O + 0.05% TFA B:ACN + 0.05% TFA (Gradient)	ESI, positive	Ion-trap	SRM	MC-LR	16 pg on column	NR	2006 [196]

method as cartridges are disposed of after each extraction and large amounts of organic solvents are required. Allis et al. describes a novel method for the analysis of hepatotoxins; microcystins and nodularin (Nod) in lake water and domestic chlorinated tap water are determined by electrospray ionization triple quadrupole mass spectrometry (LC-ESI-MS/MS) without the need for SPE clean up before analysis. The method employs a reversed-phase guard cartridge system which effectively pre-concentrates the toxins onto the cartridge elution by a gradient solvent system thereby elimating sample pre-concentration procedures. The cartridge system can be reused >100 times to pre-concentrate the hepatotoxin samples online providing for a rapid analysis method. Excellent limits of detection (LOD) and limits of quantitation (LOQ) were achieved with spiked MCs and Nod samples; LOD = 0.08 μg/L and LOQ = 0.25 μg/L in both lake and chlorinated tap water (Table 40.7). The application of this LC-ESI-MS/MS method for routine screening of hepatotoxins in lake and chlorinated tap water was achieved and this study represented the first direct method for the screening of hepatotoxins in chlorinated tap water [146].

40.13.1 Separation and Purification

The first *Microcystis* toxin purification approach combined dialysis, solvent extraction, and column chromatography using diethylaminoethyl-Sephadex (DEAE-Sephadex) A-25 [147], but it took until 1982 before sufficient pure material was obtained for complete structural analysis by nuclear magnetic resonance (NMR) [148]. There has been no reports in the literature of a single successful purification step; all methods described include numerous chromatographic purification steps—size exclusion chromatography (SEC), ion exchange chromatography (IEC), thin layer chromatography (TLC), flash/column chromatography, and/or preparative liquid chromatography (LC).

40.14 SIZE EXCLUSION CHROMATOGRAPHY

Botes et al. used size exclusion (Sephadex G-50 column) as the first chromatography step followed by ion-exchange (DEAE-cellulose column) chromatography, isolating two pure microcystins [148]. Sephadex LH-20 from Pharmacia is commonly used as an initial separation technique that allows the removal of larger compounds such as pigments and large interfering molecules [148]. SEC is a particularly useful step as it can be used with organic solvents, facilitating normal-phase partition chromatography as well as size exclusion. Toyopearl HW-40F have often been used as the final purification step, it is reported to be more efficient than LH-20 for separating individual microcystins [83].

40.14.1 Ion Exchange Chromatography

Cremer and Henning used IEC anion exchangers (quaternary methylamine anion-exchange resins) as a preliminary step in the purification of microcystins [150]. While Botes et al. [148] used IEC for the final purification of several microcystins from natural cyanobacterial samples.

40.14.2 Thin Layer Chromatography

Analytical TLC or high-performance TLC (HPTLC) was commonly reported in the literature for final purification after column chromatography [67,73,83,151]. Most reports use silica-coated plates and solvents such as chloroform, methanol, and water in varying proportions with the separated components visualized using short wave UV irradiation.

40.14.3 Flash/Column Chromatography

Flash/column chromatography typically involves the addition of large quantities of cyanobacterial cells through a silica column under positive pressure/gravity. The partial purification of MCs using a silica gel column after the removal of pigments using SEC has been regularly reported [149,152].

TABLE 40.4B
Typical Semi-preparative/Preparative HPLC Columns Used in the Isolation and Purification of Microcystins from Cyanobacterial Extracts

Stationary Phase	Column Dimensions	Particle Size (μm)	Reference
Nucleosil C_{18}	250 mm × 10 mm I.D.	7	Meriluoto et al. [256]
Silica based bonded to C_2 and C_{18}		15	Cremer et al. [150]
Bondapak C_{18}	300 mm × 47 mm I.D.	55–105	Namikoshi et al. [277]
Mightysil RP-18 column	250 mm × 20 mm I.D.		Sivonen et al. [72]
μBondapak C_{18}	150 mm × 19 mm I.D. or 300 mm × 19 mm I.D.		Sivonen et al. [244]
Alltech C_{18}	250 mm × 4.6 mm I.D.		
Bondapak C_{18}	300 mm × 19 mm I.D.	15–20	Azevedo et al. [274]
Nova-Pak C_{18}	100 mm × 25 mm I.D.		Lawton et al. [50]
Nucleosil C_{18}	250 mm × 10 mm I.D.	7	Namikoshi et al. [152]
Hyperprep HS BDS C_{18}	150 mm × 7.5 cm I.D.	8	Edwards et al. [140,141]

The use of bonded silica, usually C_{18}, is also reported for initial separation of crude MC extracts [153]. Reverse-phase flash chromatography using a simple step gradient has been found to be particularly suitable for the rapid extraction of microcystins [140,141]. This concentration and cleanup step yields very high percentage recoveries of microcystins, but has its limitations regarding subsequent concentration of microcystin extracts as these extracts typically contain high percentages of water. Harada et al. used the complimentary selectivities of normal and reversed-phase chromatography to obtain purified compounds [84]. Lawton et al. also used the combination of reversed-phase flash chromatography, followed by normal-phase chromatography to provide a simple and cost-effective method for obtaining MC-LW and MC-LF, two closely eluting hydrophobic microcystins, which had only previously been purified by HPLC [154].

40.14.4 Preparative High Performance Liquid Chromatography

Reversed-phase HPLC (RP-HPLC) is widely used in the purification of MCs with most isolation protocols containing at least one RP-HPLC step. The literature typically reports the use of C_{18} bonded to silica as the stationary phase and using 1 cm ID columns. Table 40.4B gives a selection of references for semipreparative/preparative HPLC columns used in the purification of microcystins.

40.15 ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF MICROCYSTINS

There have been many biological methods developed for cyanotoxins that use the bioactivity of these toxins, such as potent hepatotoxicity, neurotoxicity, cytotoxicity, enzymatic activity, and immunological interactions. All these methods, although they are useful for sensitive screening of cyanotoxins, cannot detect and quantitate individual cyanobacterial toxins. Bioassays are recommended if any of these conditions are fulfilled [5]:

- A laboratory can easily establish them, but has little or no access to adequate equipment and expertise for conducting physicochemical analysis, or little means of subcontracting analysis.
- There is indication of cyanotoxins other than (or in addition to) the known toxicants.

- Cyanobacteria taxa, the toxins of which have not yet been well studied and which may therefore contain MC toxic metabolites, dominatae.
- Confirmation of results from physicochemical analysis is required, especially to confirm.
- Bioactivity.
- Validation of physicochemical methods by an alternative method is desired.

A summary of the different biological techniques used and their main characteristics is provided (it is necessary to examine these methods as they are often used as complimentary techniques to analytical chemical determination methods).

40.15.1 Mouse Bioassay

For many years, the mouse bioassay alone has been used to determine bloom toxicity by intraperitoneal (i.p.) injection of a lysate of cyanobacteria prepared either by sonication or by free thawing of a cell suspension that has been sterilized by membrane ultrafiltration. Rapid death within 30 min with no observable signs of organ damage is regarded as neurotoxic response. The liver toxins cause death 1–4 h after i.p. injection by hypovolemic shock after massive hemorrhage and necrosis of the liver. Although this bioassay provides a measure of the total toxicity, it is generally not very sensitive or specific. Also, the ethical reasons about animal testing have encouraged development of alternative procedures and many novel and sensitive methods have become available in recent years. Unfortunately, no single method is currently available to replace the mouse for the detection of all cyanotoxins using a simple assay, and further validation and comparison of methods are needed before general recommendations on their application can be given [34,57,155].

40.15.2 Invertebrate Bioassays

The amount of zooplankton is known to decrease under cyanobacterial bloom conditions. Most studies on the effects of cyanobacteria on zooplankton have dealt with *Daphnia* spp. Fewer studies have been done with crustaceans and rotifers. Out of the invertebrate biotests commonly used, the *Artemia salina* test seemed attractive [156]. One advantage of *A. salina* as a test organism is that it is easy to grow from dried eggs available in any aquarium shop.

40.15.3 Bacterial Bioassays

Bacterial bioassays have been also investigated to determine if they can provide simple routine methods for the analysis of cyanotoxins. The one that has received more attention is the Microtox bioluminescence assay, which indicates toxicity by a reduction of the light, emitted by the test bacterium (*Photobacterium phosphoreum*). Further analysis revealed that there is no correlation between response in the Microtox assay and cellular content of the known cyanotoxins [157].

A second bacterial bioassay that used the inhibition of pigment formation is using *Serratia marcescens* [158]. This bioassay was thought to be useful for saxitoxins and microcystins but, as it happened before, no correlation was found between actual content of known cyanotoxins and inhibition of pigment formation [157].

40.15.4 Biochemical Assays

Mackintosh et al. [79] demonstrated that MCs inhibit enzymes responsible of the dephosphorylation of intracellular phosphoproteins, the protein (serine/threonine) phosphatases, in particular PP1 and PP2A. The degree of inhibition can therefore be used as a measure of toxin concentration.

The basis of this type of procedure is the measurement of phosphate release from a phosphorylated protein substrate in the presence of a phosphatase enzyme preparation and an inhibitor such

as microcystin. This approach using ^{32}P-radiolabeled substrates has been used for the measurement of microcystins in environmental samples [104,159,160]. The method is sensitive to subnanogram levels and is a rapid assay allowing the analysis of many samples in a few hours. However, it suffers from a major drawback that the ^{32}P isotope has a short life, and radiolabeled proteins for the assay have to be prepared on a regular basis using relatively long reasonable procedures. In addition, many routine laboratories are not set up to carry out radioactive determinations.

Colorimetric assays for the determination of microcystins are therefore attractive, provided that sufficient specificity and sensitivity can be achieved. Methods based on using *p*-nitrophenyl phosphate (*p*-NPP) as substrate and measuring the colored *p*-nitrophenol released have been reported [126,130,161–163]. More recently, the method has been adapted for fluorescence measurements using the substrates methylumbelliferyl phosphate (MUP) and difluoromethyllumberlliferyl phosphate (DiMUP) [164,165].

Another variant of PP2A assay is the one reported by Isobe et al. [166] where a firefly bioluminescence system is used for the detection of protein phosphatase 2A inhibitors, in which luciferin phosphate is hydrolyzed to luciferin and inorganic phosphate by protein phosphatase 2A. The recent commercial availability of the phosphatase enzymes, which obviates the need to isolate them from animal tissues, also makes this approach very attractive. However, not all microcystins variants react with protein phosphatase enzymes to a similar extent [161,163] and the assay is sensitive to protein phosphatase inhibitors other than microcystins, such as okadaic acid, tautomycin, and calyculin A. In addition, the cyanobacterial sample itself may contain phosphatase activity that masks the presence of toxins [160]. As a consequence, the lack of specificity of the protein phosphatase inhibition assays requires that additional confirmatory analytical methods be employed for specific analysis of cyanobacterial toxins.

40.15.5 Immunological Detection

The ELISA is currently the most promising method for rapid sample screening for MCs because of its sensitivity, specificity, and ease of operation. These assays are based on the use of monoclonal or polyclonal antibodies. These assays show greater specificity than protein phosphatase inhibition assays but do not indicate the relative toxicities of microcystin and nodularin variants; instead, ELISAs rely on the structure of toxins for detection. Therefore cross-reactivities of the different toxins may vary and sensitivity depends on the structure rather than toxicity.

One approach to improving the sensitivity and accuracy of ELISA is changing the assay format. ELISA can be classified into two groups, competitive and noncompetitive; the latter is theoretically superior in sensitivity and accuracy [167]. Various types of noncompetitive ELISA have been developed, the most widespread being the sandwich ELISA that uses two different antibodies to link the analytes. However, it is generally impossible to establish sandwich ELISA for small molecules such as microcystins because small analytes usually possess only one epitope or spatially overlapped epitopes. As a way of overcoming these limitations Nagata et al. have proposed the use of a secondary antibody that recognizes the newly formed epitope on the immune complex (IC) of MCs and the primary antibody [168]. The anti-IC antibody may recognize parts of the structure of both MCs and the primary antibody at the same time or the conformational change on the primary monoclonal antibody induced by microcystin binding.

Recently, a highly sensitive immunoassay based on the time resolved fluorimetry was developed for the analysis of MCs in water [169]. In order to improve the sensitivity and to simplify the procedure, this method uses a fluorescent lanthanide (Europium) for labeling the MC and the product of the reaction is detected with a time resolved fluorimeter. This method showed a more consistent response at the lowest concentrations and an equal sensitivity compared to the ELISA methods.

To solve the problems arising from the use of protein phosphatases and ELISAs, a new colorimetric immunoprotein phosphatase assay (CIPPIA) for specific detection of microcystins and nodularins of cyanobacteria was developed by Metcalf et al. [170]. The principle of this method is based

on the combination of the immunoassay and a colorimetric protein phosphatase inhibition system in a single assay that is specific for MCs.

40.15.6 Mammalian Cells

Bioassays, using mammalian cells, have received attention as suitable replacement for mouse toxicity tests. The well-documented fact that microcystins have caused acute liver damage has prompted studies using hepatocytes. Freshly isolated rat hepatocytes were first investigated by Aune and Berg who reported good correlation between toxicity measured by leakage of the enzyme lactate deshydrogenase (LDH) from hepatocytes and results from mouse bioassay [171]. More recently, studies have shown that different LC_{50} values were found for microcystin variants (Table 40.3), which is consistent with *in vivo* toxicity data. Other cells have been also investigated, such as fibroblasts [172] and blood cells [172], but the results obtained were not satisfactory.

40.16 ANALYTICAL CHEMISTRY METHODS FOR CYANOTOXINS

Analytical methods use the physicochemical properties of cyanotoxins such as molecular weight, chromophores, and reactivities owing to the functional groups in the molecules. These types of analyses are recommended [5] under the following circumstances:

- Cyanobacterial species composition or bioassay results indicate which toxins to look for
- Unambiguous identification of toxins is required
- Rapid screening and a requirement for accurate quantitation of a large number of samples is required, especially for regular monitoring of sites where the toxin patterns are well established
- Low toxin concentrations that may not be detected by bioassay are expected
- New toxic cyanobacterial metabolites are to be identified

40.16.1 Liquid Chromatography for the Separation of the Microcystins

There exists in the peer-reviewed literature a wide variety of LC based methods for the determination of the MCs and nodularins (refer to 40.5 for a comprehensive summary of LC methods); in the earliest papers the principle detection methods relied upon UV or photodiode array (PDA) detection.

By the mid to late 1980s several LC methods had been described utilizing reverse phase (RP) C_{18} columns for the determination of the MCs [173–176]. However, there existed one severe problem associated with the use of C_{18} columns for MC separation, the irreparable damage inflicted on C_{18} stationary phases from proteins in samples, necessitating rigorous sample cleanup before analysis, a problem that exists to date. This problem was addressed by Meriluoto and Eriksson [177] in 1988 when they deployed an internal surface reversed phase (ISRP) column to overcome the problems inherent in the LC separation of MCs. The ISRP column was essentially a hybrid of RP and SEC; large interferant proteins traversed the column without retention while the MCs were sieved through the pores and partitioned on the RP and detected by UV. The method was employed to determine MC levels in algae following a simple 2–3 step liquid–liquid sonication-extraction procedure of lyophilized samples, the limit of detection (LOD) was quoted as 50–100 μg toxin per gram of freeze-dried cyanobacteria material.

Spoof et al. assessed four C_{18} RP columns and an amide C_{16} column to compare their efficacy in separating MCs and Nod; also appraised was the efficiency of acid and basic mobile phases [178]. The results indicated that the amide column combined with an acidic stationary phase provided by far the best selectivity. LC-UV/PDA methods were published from the early 1990s to date to determine MCs in diverse sample types from fish to water to algae (Table 40.5).

TABLE 40.5
HPLC Methods for the Determination of Microcystins in Complex Matrices

Sample Matrix	Sample Preparation	Stationary Phase	Mobile Phase	Detector	Complementary Methods	Analytes	LOD (MC-LR)	Linear Range (MC-LR)	Year and Reference
Water and Cyanobaterial Matrices									
Cyanobacteria	Extraction, SPE, MMPB	C_{18} Column	A:H_2O , B:MeOH (Isocratic)	Fluorescence	GC, MS	MC-LR,-RR	NR	NR	1992 [179]
Water (Raw and treated)	Extraction, SPE	µ-Bondapak C_{18} Column	A:H_2O + 0.05% TFA B:ACN + 0.05% TFA (Gradient)	PDA at 200–300 nm	NR	MC-LR,-RR,-LW,-LF,-LY, Nod	NR	0.089–8.880 µg/L in treated H_2O, 0.444–8.880 µg/L in raw H_2O	1994 [124]
Dried *Microcystis aeruginosa* crusts	Extraction, Preparative Chromatography	µ-Bondapak C_{18} Column	A:H_2O + 0.008M ammonium acetate, B:ACN + 0.008M ammonium acetate (Gradient)	Optical scanning from 200–300 nm	ESI-MS (Scan)	MC-LR equiv.	NR	NR	1995 [180]
Cyanobacteria	Not detailed	ODS-80Ts Column	A:H_2O + 0.05% TFA B:ACN (Gradient)	Chemi-luminescence	NR	MC-LR,-RR-YR	Dns-Cyc LR< 15 Fmol	Dns-Cys-LR 15–1670 Fmol r = 0.999	1995 [181]
Cyanobacteria	Extraction, SPE	Monoliltic silica C_{18} Column	A:H_2O + 0.05% TFA B:ACN + 0.05% TFA (Gradient)	PDA at 200–300 nm	NR	MC-LR,-RR,-LW, -LF,-LY, Nod, Demethyl MC-LR,-RR	3 ng per 10 µL injection	NR	2002 [182]
Biological Matrices									
Human liver	Extraction, SPE	µ-Bondapak C_{18} Column	A:H_2O + 0.05% TFA B:MeOH (Isocratic)	PDA	ELISA, LC-MALDI, LC-MS	MC-LR,-YR,-AR, CYN	NR	NR	2001 [194]
Fish	Extraction, SPE	C_{18} Column	A:H_2O + 0.02M ammonium acetate (pH 5), B:ACN (Isocratic)	UV at 190–300 nm	NR	MC-LR equivalent	NR	NR	2001 [213]
Bird stomach (content) and fecal pellets	Extraction	C_{18} Column	A:H_2O + 0.05% TFA B:ACN + 0.05% TFA (Gradient)	PDA	MALDI-TOF-MS	MC-LR,-RR,-YR,-LF, AN-a	NR	NR	2002 [209]
Fish (sea trout)	Extraction, SPE	C_{18} Column	A:HB2BO + 0.01M ammonium dihydrogen phosphate (pH 2), B:ACN (Isocratic)	PDA	ELISA	Nod	NR	NR	2002 [295]
Fish (Planktivorous)	Extraction, SPE	C_{18} Column	A:H_2O + 0.05M phosphate (pH 3), B:MeOH (Isocratic)	PDA	LC-ESI-MS	MC-LR,-RR	NR	NR	2004 [296]

Sano et al. employed a C_{18} column and fluorescence detection to determine MMPB (methyl-3-methoxy-4-phenylbutyric acid) derivatized MC-LR and MC-RR following liquid extraction and SPE cleanup of cyanobacterial samples; the samples were also verified by gas chromatography–mass spectrometry (GC-MS) [179]. Lawton et al. describes an LC-PDA method using a C_{18} column with gradient elution on SPE pretreated water samples that was successful in determining MC-LR, MC-RR, MC-LW, MC-LF, MC-LY, and nodularin [124]. Jones et al. used a similar approach in 1995, to determine MC-LR in dried *Microcysis aeruginosa* samples; however sample clean up involved using preparative chromatography [180].

In 1995, Murata et al. described an LC method that employed a chemiluminescence detector that could determine the danysl-cisteine adducts of MC-RR, YR, and LR, that had been derivatized using Danysl Chloride [181].

Spoof et al. devised an LC-PDA method using a silica C_{18} monolithic column to determine seven MCs and nodularin from cyanobacterial samples in 2002, with a LOD of 3 ng per 10 μL injections [182]. Monolithic columns are a new generation of RP separations and offer many advantages over conventional RP chromatography, including very fast run times, very fast equilibration times in gradient runs, high sample loading capacity, and reproducible retention times.

Gradient elution is the norm for the analysis of the MCs, the reason is that the MCs differ significantly in their polarities and therefore in their retention characteristics; however, there are a number of limitations inherent to gradient chromatography, namely, long equilibration times between analyses and retention time shift. As a consequence of the difficulties associated with gradient chromatography several isocratic methods have been developed to determine the MCs, many with complimentary, confirmatory mass spectral data (Tables 40.5 and 40.7).

Owing to the lack of commercially available standards (there are only seven standards and there are 77 known MCs; Table 40.3), it is virtually impossible to identify all toxins using the aforementioned methods. The use of PDA detection has undoubtedly added a degree of specificity to the method by virtue of the characteristic UV spectra obtained for MCs [124]. It is not, however, possible to differentiate between individual MCs on the basis of their UV spectra alone. While recent developments in detector hardware, combined with increased sophistication of spectral matching software, may assist in the identification of MCs by spectral match data in conjunction with retention time, the lack of the standards makes the job of creating a thorough spectral library virtually impossible [21].

There have been some attempts to enhance sensitivity using ninhydrin derivatization of the guanidine moiety on the arginine residue with fluorescence detection [183]. However, the inability to detect microcystin variants, which do not contain arginine, is a major disadvantage for this approach. Another attempt to increase the sensitivity has been the derivatization of the diene on the Adda moiety using DMEQ-TAD (4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3, 4-dihydroquinoxalinyl) ethyl] -1,2,4-triazoline-3,5-dione). Micellar electrokinetic capillary chromatography (MECC) [184], where the solutes partition themselves between the micellar phase and aqueous phase during the normal course of electrophoresis is in a manner analogous to RP-HPLC. However, due to the very closely related structures among microcystins, the small differences in their electrophoretic mobilities do not allow for complete resolution.

But the biggest disadvantage of these methods lies in the fact that the fundamental problem still remains: the lack of availability of MC reference materials. However, LC combined with mass spectrometry has offered an unparalleled approach to the accurate determination of the MCs.

40.16.2 The Use of Mass Spectrometry in the Analysis of Microcystins and Nodularins

Liquid chromatography-mass spectrometry (LC-MS) is a very promising analytical method for the MCs because it enables simultaneous separation and identification of cyanobacterial toxins in a mixture (Figure 40.2). Mass spectrometry, unlike UV spectroscopy, has the advantage that it can handle compounds that lack the UV chromophore. In the case of MCs, the characteristic ion *m/z* 135 derived

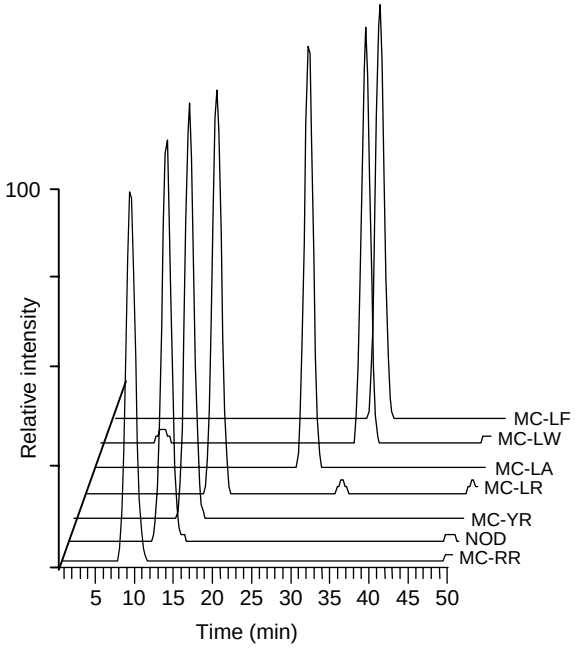

FIGURE 40.2 LC-MS^2 analysis of six standard microcystins and nodularin-R using the following target parent and product ions: MC-RR (8.57 min) Full MS^2 *m/z* 1038, 1020; NOD (12.63 min) Full MS^2 *m/z* 825, 807; MC-YR (14.92 min) Full MS^2 *m/z* 1045, 1027; MC-LR (16.69 min) Full MS^2 *m/z* 995, 977; MC-LA (27.32 min) Full MS^2 *m/z* 910, 892; MC-LW (34.09 min) Full MS^2 *m/z* 1024, 1006; MC-LF (34.12 min) Full MS^2 *m/z* 987, 969 [125].

from Adda has proved to be useful for the discrimination of microcystins from other types of compounds [185]. Different ionization methods have been used for the analysis of MCs; among them are fast atom bombardment (FAB), electrospray source ionization (ESI), and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF). FAB-MS requires matrices to assist ionization, so it is necessary either to develop a HPLC method with the matrix being incorporated into the mobile phase, or to introduce the matrix coaxially into the mass spectrometer ion source [186]. Care has to be taken to ensure a steady film of liquid being delivered to the FAB target. But not all the matrix components are able to form this film, as a consequence of high matrix backgrounds that often hinder the detection of compounds of interest and severely decrease the LOD. The amount of sample required by FAB to acquire a spectrum of the purified toxin is in the nanogram range.

On the other hand, ESI-MS has proved invaluable in the analysis of thermally labile polar molecules, especially peptides and proteins [187]. ESI-MS was developed primarily for molecular weight determination of proteins and other large biomolecules but it has been demonstrated that it is also applicable for the analysis of low molecular weight compounds with good sensitivity. It is a simple, specific, reliable, and rapid technique to monitor precisely and simultaneously various algal toxins in extracts from laboratory cultures or algal blooms present in eutrophic waters [188]. An LC-ESI-MS method has been developed for the simultaneous determination of various algal and cyanobacterial toxins. This method enables the identification of saxitoxin, anatoxin-a, nodularin, and MCs among other marine water and lake toxins [189].

New and innovative approaches utilizing MS have been implemented to determine the MCs; Yuan and Carmicheal in 2004 describe the application of surface-enhanced laser desorption-ionization time-of-flight (SELDI TOF-MS) to detect MCs and nodularin with femtomolar sensitivity [190]. SELDI TOF-MS involves trapping the analyte of interest on to a chip usually with an antibody that has a high affinity for the analyte of interest and then using a laser beam to desorb and

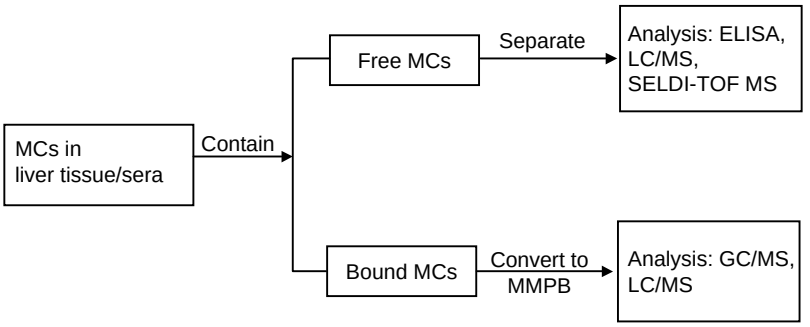

FIGURE 40.3 The assay methods used in the analysis of free and protein bound microcystins from liver samples implicated in human fatalities in Brazil in 1996 [196].

ionize the analyte for direct introduction into the MS. In 2006 Gregson et al. [191] described the simultaneous determination of four MCs using SELDI TOF-MS reporting LODs of 0.025 μg/L.

Ott and Carmicheal in 2006 described a method for the determination of total MCs in animal tissues using LC-ESI MS; this was an important innovation as MC determination by LC-MS is normally restricted to the determination of free MCs. But the MCs form covalent linkages with PP1 and PP2A *in vitro*; the authors describe a method involving an oxidation procedure on liver tissue using MMPB (2-methyl-3-methoxy-4-phenylbutric acid) followed by LC-negative ESI ion trap MS [192].

In 1996, 131 patients at a haemodialysis centre in Brazil were exposed to MC contaminated water; 100 patients developed liver failure, and 52 were confirmed as having been exposed to lethal levels of the toxin [193–195]; 10 years on, Yuan et al. [196] analyzed protein-bound MCs using the MMPB (erythro-2-methy-3-methoxy-4-phenylbutyric acid) method and determining MC content by GC-MS. The method proved more sensitive than the original, ELISA, LC/MS, and GC-MS methodologies [204] (Figure 40.3).

It is fair to say that MS has expanded the capacity to determine selectivity, qualitatively, quantitatively, and semiquantitatively the MCs in real samples. Invaluable information has been provided by both direct collision induced dissociation (CID), (refer to Table 40.6) and by LC-MS/MS and LC-MS^n methods (refer Table 40.7).

Muniz Ortea et al. [125] determined six MCs and nodularin using a variety of methods, including LC-PDA UV, protein phosphate assay, and LC-QIT MS^n for comparison purposes and demonstrated that optimum calibration and reproducibility were obtained for MC-LR using the LC-QIT MS^2 data (Figure 40.4). Zeck et al. [197] used HPLC/ELISA coupling and compared sample data with data obtained from HPLC/UV and electrospray ionization mass spectrometry (ESI-TOFMS) for the screening of microcystins in freeze-dried cyanobacterial extracts; however this methodology was not applied to water samples devoid of cyanobacterial cells. Also this methodology is quite time-consuming as approximately ten fractions must be collected for each milliliter of a chromatographic run and tested by ELISA or protein phosphatase with resulting plots being compared with the LC-UV chromatograms. For large water screening programs this testing protocol would be both uneconomical and time restrictive as the method requires numerous tests to be carried out on each sample and is not automated [125]. However, the method developed by Allis et al. (refer to Section 40.13) has revolutionised the ability to directly analyse microcystins and nodularin toxins in 1 mL water sample quantities by LC-MS/MS, with method detection limits below the WHO provisional guideline limit of 1 μg/L MC-LR [146].

40.16.3 Exploiting Mass Spectrometry to Overcome the Problem of Lack of Reference Standards

To date, over 65 MCs have been identified and fully structurally elucidated while a number of others have been identified, but only partially elucidated (Table 40.3) [198]. The structural determination of MCs has been carried out using a number of techniques including ^{1}H-NMR, ^{13}C-NMR

TABLE 40.6
Direct Introduction MS Methods to Provide Structural Data

Sample Preparation	Sample Introduction	Ionization Source and Mode	Mass Analyser	Acquisition Mode	Analytes	Complementary Methods	Year and Reference
NR	Direct infusion or flow injection	ESI, positive	Triple-quadrupole	Scan, CID	MC-LR, [ADMAdda^5] MC-LR, [ADMAdda^5] MC-LHar, [D-Asp^3,ADMAdda^5] MC-LR, [D -Asp^3,ADMAdda^5] MC-LHar, [D -Ser^1,ADMAdda^5] MC-LR, [ADMAdda^5, MSer^7] MC-LR	NR	1998 [297]
NR	Direct infusion or flow injection	ESI, positive	Triple-quadrupole	Scan, CID	MC-LR,-RR,-YR,-LA, [D -Asp^3] MC-LR,-RR, [Dha^7] MC-RR, [D -Asp^3, Dha^7] MC-RR	NR	1999 [298]
NR	Direct infusion or flow injection	ESI, postive and ESI, negative	Triple-quadrupole	Scan, SIM = flow injection, MS^2 using CID	MC-LR,-RR,-YR,-LA	NR	1999 [299]
Extraction, HPLC	Direct infusion	ESI, negative	TOF	Scan	MC-LR,-RR,-YR, Nod	HPLC/UV, HPLC/ELISA, PP1A	2001 [197]
Extraction, flash chromatography (LC-UV-DAD)	MALDI and nanospray	ESI, positive	TOF	Scan, CID	*seco*[D -Asp^3] MC-RR, [D -asp^3,G-Glu (OCH$_3$)^6] MC-RR, [D -Asp^3] MC-RR	NMR, PP1A	2004 [286]

TABLE 40.7
Liquid Chromatography-Mass Spectrometry Methods for the Determination of Microcystins

Sample Preparation	Sample Introduction	Mobile Phase	Ionization Source and Mode	Mass Analyser	Acquisition Mode	Analytes	LOD (MC-LR)	Linear Range (MC-LR)	Year and Reference
Extraction, SPE	C_{18} Column	A:H_2O + 0.01% TFA B:MeOH (0.8% glycerol) (Isocratic)	Frit-FAB, positive	Single-quadrupole	Scan, SIR	MC-LR,-RR,-YR, Demethyl MC-LR,-RR	NR	NR	1992 [185]
Extraction, SPE	Direct injection/ C_{18} Column	A:H_2O + 0.1% AA B:ACN + 0.1% AA (Gradient)	ESI, positive	Triple-quadrupole	Scan	MC-LR,-RR, AN-a, SAX, Nod, Demethyl MC-RR	NR	NR	1993 [188]
Extraction, SPE	C_{18} Column	A:H_2O + 0.05% TFA B:ACN + 0.05% TFA (Gradient)	ESI, positive	Triple-quadrupole	Scan, parent scan and SIR	MC-LR,-RR,-YR,-LA,-LY, Nod, [D-Asp^3] MC-RR	NR	NR	1993 [134]
Extraction, SPE	C_{18} Column	A:H_2O + 0.05% TFA B:MeOH (0.8% glycerol) (Isocratic)	Frit-FAB, positive	Single-quadrupole	SIM	MC-LR,-RR,-YR, [D-Asp^{3}P] MC-LR, [DhaP7P] MC-LR	400 pg	2–50 ng	1995 [300]
Extraction, SPE	Silica column/ CE	ACN + 0.1% TFA (Gradient)	ESI, positive	Triple-quadrupole	Scan, SIR, MRM, SIM	MC-LR,-LW,-LF,-LY,-VF,-AR, [D-Glu$(OCH_3)^6$] MC-LR, Demethyl MC-LR	50 pg	LC-ESI-MS 50–100,000 µg/L, $R^2 = 0.9992$	1995 [268]
Extraction, on-line column conc.	Microflow C_8/ C_{18} Column	A:ACN B:MeOH C:Formic Acid (Gradient)	ESI, positive	Ion-trap	Scan, MS^n	MC-LR,-LW, methyl MC-LR, [Asp^3, DhbP7P] MC-LR,-HtyR	250 pg	NR	2000 [139]
Extraction, SEC or SPE	C_{18} Column	A:H_2O + 0.05M FA B:ACN + 0.05M FA (0.002M ammonium formate) (Gradient)	ESI, positive	Single/Triple quadrupole	Scan, SIM and daughter scan	MC-LR,-RR,-YR	NR	NR	2001 [127]
Extraction, SPE	C_8 Column	A:H_2O + 0.08% HCOOH, B:ACN (Gradient)	ESI, positive	Triple-quadrupole	SIM, MRM	methylated MC-LR, MC-LR,-RR,-YR,-LA,-LW,-LF,-LY, Nod(IS)	0.04–0.36 µg/g	NR	2001 [53]

Continued

TABLE 40.7
(Continued)

Sample Preparation	Sample Introduction	Mobile Phase	Ionization Source and Mode	Mass Analyser	Acquisition Mode	Analytes	LOD (MC-LR)	Linear Range (MC-LR)	Year and Reference
SPE	C_{18} Column	A:H_2O + 0.002M HFBA (pH 3.5) B:ACN + 0.002M ammonium acetate & 0.1% AA C:H_2O + 0.002M ammonium acetate & 0.1% AA (Gradient)	ESI, positive	Triple-quadrupole	Scan, daughter scan, SIM and MRM	MC-LR,-RR,-YR,-LA, Nod, AN-a	0.027 µg/L	0.020–1.00 µg/L, $R^2 = 0.9998$	2001 [189]
SPE	Microflow C_{18} Column	A:H_2O + 0.08% FA B:ACN + 0.08%FA (Gradient)	ESI, positive	Single-quadrupole	Scan, SIR	MC-LR,-RR,-YR, Nod	Scan = 80 pg, SIR = 10 pg	NR	2002 [138]
Extraction	C_{18} Column	A:H_2O + 0.01M TFA & 0.01% HFBA B:ACN (Gradient)	ESI, positive	Single-quadrupole/ Triple-quadrupole-TOF	Scan, SIR	MC-LR,-RR,-YR,-LW,-LF,-LA, Nod, SAX, AN-a, DA, OA, DTX-1	1.0 ng on column	2.00–100 ng on column, $R^2 = 0.9830$	2003 [301]
Extraction	C_{18} Column	A:H_2O + 0.5% FA B:ACN (Gradient)	ESI, positive	Triple-quadrupole	Scan, SIR, MRM	Demethyl MC-LR,-RR,-YR, Didemethyl MC-LR,-RR,-YR, MC-LR,-RR,-YR,-LW,-LF,-LY, Nod	SIR = 50 pg	NR	2003 [114]
Extraction, SPE	C_{18} Column	A:H_2O + 0.01% TFA B:ACN (Isocratic)	ESI, positive	Ion trap	Scan, parent scan	MC-LR	0.0026 µg/L (with- 1000 fold pre-contration)	0.1–10 ng, $R^2 = 0.9992$	2004 [204]
Extraction	C_{18} Column	A:H_2O + 0.5% FA B:ACN (Gradient)	ESI, positive	Triple-quadrupole	SIM	Demethyl MC-LR,-RR, Nod, MC-LR,-RR,-YR,-LW,-LF,-LY, Nod	50–100 pg per injection	NR	2004 [302]
Extraction	C_{18} Column	A:H_2O + 0.02M ammonium hydroxide (pH 9.7) B:ACN (Gradient)	ESI, positive	Single-quadrupole	Scan, SIM	MC-LR,-RR,-YR	Scan = 1000 µg/L SIM = 100 µg/L	2500–20000 µg/L, $R^2 = 0.9998$	2004 [303]

SPE	C_{18} Column	A:H_2O + 0.05% TFA B:ACN + 0.05% TFA (Gradient)	ESI, positive	Ion Trap	Scan, MS^n	MC-LR,-RR,-YR,-LW,-LF,-LA, Nod	<0.1 ng on column	100–5,000 µg/L, R^2 = 0.9920	2004 [125]
Extraction using C_{18} disks	Microflow C_{18} Column	A:H_2O + 0.006% AA B:MeOH + 0.006% AA (Gradient)	ESI, positive	TOF	TIC	MC-LR,-RR,-YR,-LW,-LF, Nod, CYN, AN-a	<0.5 ng on column (<1 µg/ L)	NR	2004 [304]
NR	NR	NR	ESI, positive	LT-FT-ICR	Scan, MS^n	MC-LR,-RR,-YR, Nod	NR	NR	2005 [198]
SPE	Microflow C_{18} Column	A:H_2O + 0.1% TFA B:ACN (Gradient)	ESI, positive and ESI, negative	Triple-quadrupole	Scan, in-source CID	MC-LR,-RR, MC-YR, MC-LW, MC-LF, MC-LA	NR	NR	2005 [305]
SPE (C_8)	C_8 Column	A:30% MeOH + 0.1% FA B: 95% MeOH + 0.1% FA (Gradient)	ESI, positive	Triple-quadrupole	Scan, MRM	MC-LR, Nod	2 pg on column (0.002 µg/L)	0.002–0.040 µg/L, R^2 = 0.9983	2006 [306]
SPE for samples ≤ 0.5 µg/L MC-LR	C_{18} Column	A:H_2O + 0.1% FA B: MeOH + 0.1% FA (Gradient)	ESI, positive	Triple-quadrupole	MRM	MC-LR, MC-RR, MC-LW, MC-LF	0.012 µg/L	0.1–1.0 µg/L	2006 [135]
SPME	Microflow C_{18} column	A:H_2O + 0.01% FA B:MeOH + 0.01% FA (Gradient)	ESI, positive	Triple-quadrupole-TOF	Scan, SIM	MC-LR, MC-RR	1.6 pg on column	1.0–250 µg/L	2006 [307]
SPE	C_{18} Column	A:H_2O + 0.01M FA B:ACN + 0.01M FA (Gradient)	ESI, positive	Triple-quadrupole	MRM	MC-LR, MC-RR, MC-YR, MC-LW, MC-LA	0.002 µg/L	NR	2006 [308]
Not required	C_{18} ODS octadecyl cartridge	A:H_2O + 0.001M ammonium acetate B:MeOH + 0.001M ammonium acetate (Gradient)	ESI, positive	Triple-quadrupole/ QTOF	MRM, in-source CID	MC-LR,-RR,-YR,-LW,-LF,-LA, Nod	0.08 µg/L	0.3–1.5 µg/L $R^2 \geq 0.9990$	2007 [146]

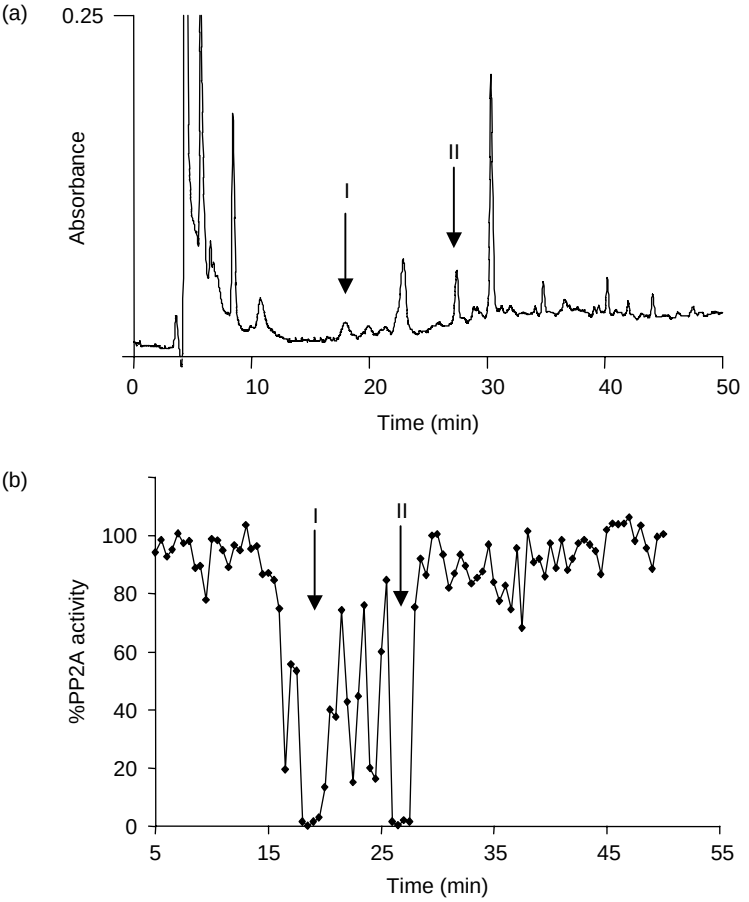

FIGURE 40.4 (a) Chromatogram of water sample from Long Lake, Co. Cavan, Ireland, obtained using LC-UV; 238 nm. Chromatographic conditions: Atlantis C-18 column (3.2 × 250 mm, 5 µm, Phenomenex). Gradient elution of acetonitrile/water (30/70 with 0.05% TFA) to 100% acetonitrile (with 0.05% TFA) over 42 min; flow rate was 0.5 mL/min [125]. (b) Protein phosphatase chromatogram obtained using the chromatographic fractions in (a) and diluted 1:1000 prior to the determination of inhibition of PP2A [125].

[66,199,200], and mass spectrometry. Mass spectral analyses have been used to obtain additional structural information for the MCs [201,202], especially low abundance MCs where NMR data cannot easily be obtained. Some of the MS techniques that have been applied to MCs include FAB-MS [203], ESI-MS [201,204], FAB-MS/MS [72], TSP-LC-MS [205], ESP-LC-MS [202], API-LC-MS [202], LC-ESI-ITMS [139], and EI-GC-MS [205].

MS fragmentation pathways of peptides yield two main fragment ion series, one containing N-terminal residues and the other containing C-terminal residues. Type *a* ([M]-CO), *b* ([M]), and *c* ([M]+NH_3) ions are produced when the charge remains on the C-terminal residue; refer to Table 40.5 ([M], in this case corresponds to the amino acid sequence of the fragment under examination). Essentially, these two series of ions are complementary, for example, the *a* and *x* emanate from the breaking of the same bond; the difference being where the charge is located. These ions are often associated with losses of water or ammonia, and observation of these losses aids in the confirmation of the ions. Also, it is known that arginine-containing peptides undergo facile, preferential cleavage adjacent to amino acid residues with acidic side chains [206]. This approach to naming peptide fragment ions is most appropriately applied to linear peptides. In the ensuing treatment of MCs, all these ions types were observed. In cyclic peptides, two bonds must be broken in order

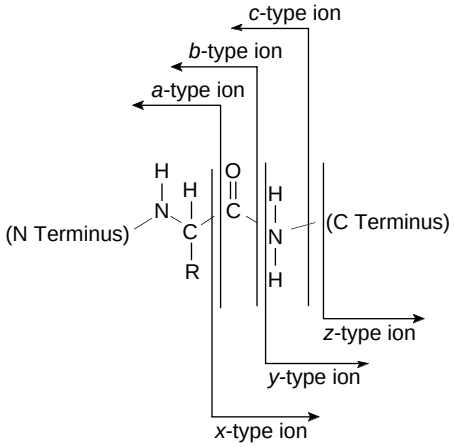

FIGURE 40.5 The main cleavages observed in the MS/MS spectrum of peptides.

to fragment the original molecule, and the most common occurrence was the breaking of amide bonds producing an *y* type process at the N terminus and *a b,* type process at the C terminus. Also the observation of losses of CO and NH produced *a*- and *z*- type ions, respectively. In addition to this type of fragmentation, fragments with an extra CO (i.e., a CO at both ends, and denoted "_CO" in structural assignments) were observed; this can be interpreted as a *b*-type process at the C terminus and a *x*-type process at the N terminus. Another observation was fragments with an extra NH (i.e., a NH at both ends of the fragment, and denoted "_NH" in structural assignments) were observed; this can be interpreted as a *c* type process at the N terminus. In addition to these processes, there were observed losses from the side chains that have been used in the assignment of fragments. The groups involved were CO_2, $C_9H_{11}O$ (the important *m/z* 135 ion from the Adda group, Figure 40.6), $C_{11}H_{16}NO$ (Adda-135), $C_{11}H_{15}O$ (Adda-135-NH), guanidine, and $CH(NHNH_2)$ from the guanidine group.

MCs typically lose one or more water molecules during MS/MS experiments, providing very little in terms of structural information; water-loss peaks provide no greater specifity than monitoring the molecule-related ion by MS. However, successive MS stages improve sensitivity by the elimination of matrix interference. Quadrupole ion trap (QIT) MS has the facility to generate MS^n spectra that provide multigenerational fragment ions [125]. High mass accuracy MS/MS like those typically generated by quadrupole time-of-flight (QqTOF) MS instruments have further expanded the ability to assign molecular formulae and to aid in the structural elucidation of new compounds by offering a high degree of specifity (REF QqTOF methods). QqTOF MS has a much wider *m/z* range than quadrupole analysers, as well as high efficiency, making them suitable for the analysis of a wide range of biomolecules. MCs are peptides and as such are readily susceptible to detection with QqTOF-MS [207] because fragmentation occurs preferentially at the peptide bonds and, also, because of the amino groups in the molecule there are multiple sites for possible protonation and deprotonation.

In fact, MS/MS studies can be used to circumnavigate the difficultly of lack of purified reference standards as many of MCs and nodularins present common fragmentation ions, as shown in Table 40.8, that can be useful for the identification of unknown analogues, this is due to the similar structural characteristics present in these molecules. The variant of the amino acid at position 7, Mdha or Mdhb, on MCs, can also be determined using high mass accuracy MS/MS fragmentation data. The presence of fragment ions indicating the presence of Mdhb instead of Mdha in the spectrum of an unknown cyanotoxin would strongly indicate the presence of a nodularin. MC's ion fragmentation characteristic are of interest owing to the cleavage of the Adda region of the molecule and the presence of a prominent fragment ion at *m/z* 135 that corresponded to the Adda

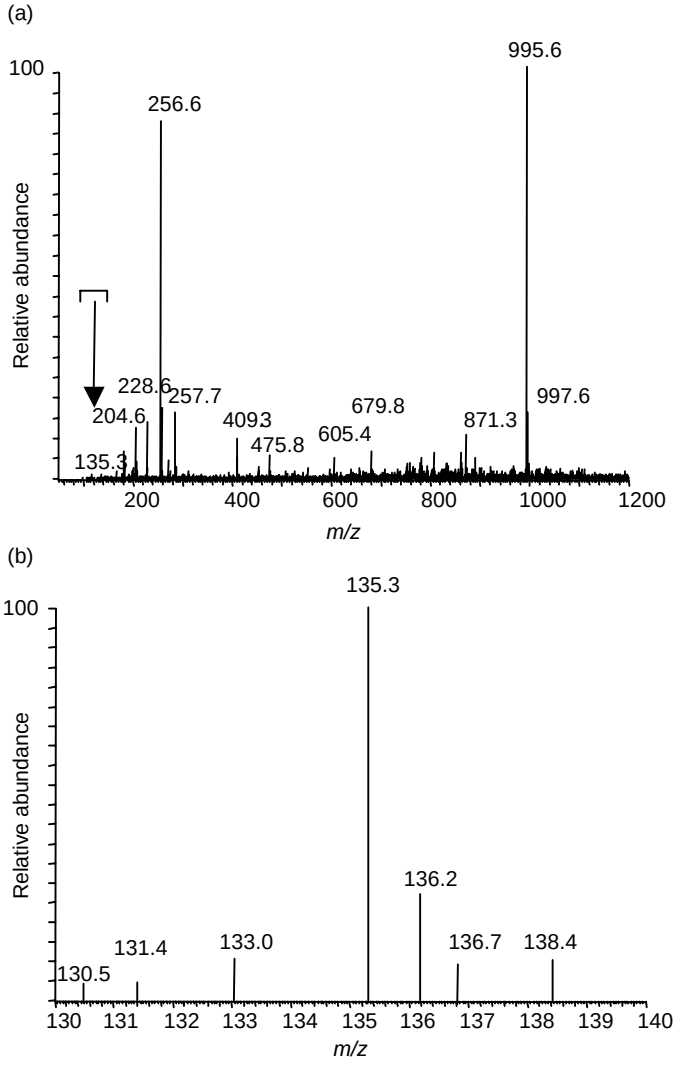

FIGURE 40.6 Electrospray ionization spectrum of MC-LR obtained using a quadrupole ion-trap mass spectrometer. (a) Source CID-MS spectrum (b) Expanded MS region of spectrum A; *m/z* = 130–140 [125].

group cleavage after the methoxy substituent, $[C_9H_{11}O]^+$ and at *m/z* 163 $[C_{11}H_{15}O]^+$, which is the remnant peptide missing an ammonium group. Other common ions associated with the MCs were found at *m/z* 155 that correspond to $[AlaMdha+H]^+$ and at *m/z* 127 due to $[AlaMdha-CO+H]^+$, its CO loss (Figure 40.7).

Diehnelt et al. [198] presented a detailed review on the structural characteristics of 67 MCs and of nodularin; with the objective of finding structural characteristics common to most MCs, the authors obtained high mass accuracy MS/MS data on hybrid linear ion trap-Fourier transform ion cyclotron resonance (LT-FT-ICR) using three commercially available MCs and nodularin.

The search for typical fragment ions that may form part of a spectral library can easily be integrated into the analyses of samples to increase the probability of detecting new or unknown MCs and nodularins.

TABLE 40.8
Product Ions Containing Amino Acids Which Are Present in the Structure of Both MCs and Nodularins

m/z	Proposed Ion Assignments	MC-LA	MC-LF	MC-LW	MC-LR	MC-YR	MC-RR	NOD
105.0704	$[PhCH_2CH(OMe)\ -CH_3OH+H]^+$	√	√	√	√	√	√	√
130.0504	$[Glu/MeAsp+H]^+$	√	√	√	√	√	√	√
135.0810	$[PhCH_2CH(OMe)]^+$	√	√	√	√	√	√	√
161.0926	$[H_3CHN\_Glu+H]^+$	√	√	√	√	√	√	√
163.1123	$[C_{11}H_{15}O]^+$	√	√	√	√	√	√	√
237.1643	$[Adda\text{-}CO\text{-}CH_3OH\text{-}NH_3+H]^+$	√	√	√	√	√	√	√
246.1494	$[Glu\_C_{11}H_{16}NO\text{-}CO\text{-}H_2O\text{-}NH_3+2H]^+$	√	√	√	√	√	√	√
264.1510	$[Glu\_C_{11}H_{16}NO\text{-}CO\text{-}NH_3+2H]^+$	√	√	√	√		√	√
282.1858	$[Adda\text{-}CH_3OH+H]^+$	√	√	√	√	√	√	√
292.1549	$[Glu\_C_{11}H_{15}O]^+$	√	√	√	√		√	√
297.1855	$[Adda\text{-}NH_3+H]^+$	√	√	√	√	√	√	√
320.1974	$[H_3CN\_Glu\_C_{11}H_{16}NO\text{-}H_2O+H]P^+$	√	√	√	√	√	√	
366.2029	$[H_3CN\_Glu\_C_{11}H_{16}NO\_CO+H]^+$	√	√	√	√	√	√	
426.2280	$[GluAdda\text{-}NH_3+H]^+$	√	√	√	√	√	√	

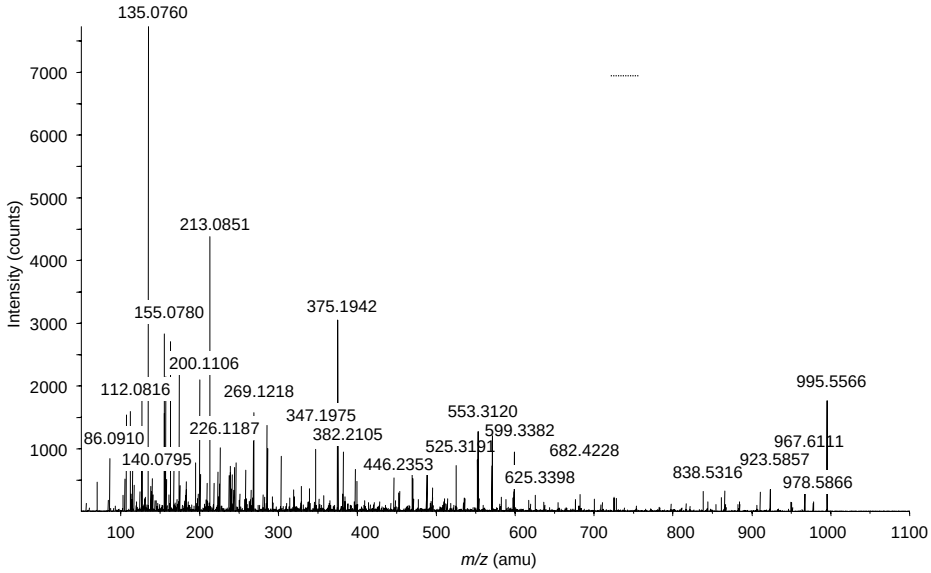

FIGURE 40.7 High mass accuracy spectrum of isolated MC-LR obtained using QqTOF MS [146].

40.17 CONCLUSION

The MCs provide a compelling array of similar yet diverse chemical structures that present difficult analytical challenges for their extraction, separation, and detection. The methods presented in this review demonstrate the difficulties inherent in determining the MCs and their many variants in different matrices, and new approaches continue to emerge.

Undoubtedly, LC-MS provides the most powerful tool to date, often used in parallel with other chemical or biochemical detection methods for the determination of MCs and their many variants.

It is certain, however, that to ensure the health and safety of humans and animals, both wild and domestic, adequate monitoring programs must be initiated to prevent the consumption of water contaminated by these potent cyanobacterial toxins.

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Part XIII

Toxins as a Starting Point to Drugs

41 Marine Neurotoxins as a Starting Point to Drugs

Michael R. Watters

CONTENTS

41.1 INTRODUCTION

Marine neurotoxins fall into two general categories, ingestible polyether toxins and contact peptide neurotoxins. The *polyether toxins* are associated with clinical syndromes (Stommel, 2004) after ingestion of seafoods containing the toxins or the microorganisms that produce the toxins, and include the ciguateric toxins (ciguatoxin, maitotoxin, palytoxin, and scaritoxin), tetrodotoxin (TTX) (pufferfish or *fugu* syndrome), saxitoxin (paralytic shellfish syndrome), and brevetoxins (BTX) (nonparalytic neurotoxic shellfish poisoning). The actions of these nonprotein toxins are principally directed toward the sodium channel, which can be subdivided into TTX-sensitive channels and BTX-sensitive channels. TTX-sensitive sodium channels are present in peripheral sensory neurons, some motor axons, and along muscle membranes. Along axons, these channels are clustered in high densities at the nodes of Ranvier, with up to 1000 channels per μm^2 compared to 25 per μm^2 internodally, and are responsible for the propagation of the axonal action potentials. The slower BTX-sensitive sodium channels are found on nociceptive sensory neurons. Alterations of either type of sodium channel may contribute to remodeling, ectopic action potentials, and ephaptic transmissions, resulting in spontaneous sensory disturbances, misperceptions of sensation, and neuropathically mediated pain. Many of the medications we utilize for such pain states modulate neural sodium channels, to include lidocaine, mexiletine, carbamazepine, oxcarbazepine, phenytoin, lamotrigine, topiramate, zonisamide, and tricyclic antidepressants. The ubiquity of such channels make these toxins also suitable for diagnostic purposes.

Peptide neurotoxins include both the *contact marine toxins* associated with passive stinging apparatuses (jellyfish, sea anemone, venomous fish) as well as the *envenomation toxins* (cone snail, sea snakes) found in creatures that actively participate in envenomation of prey or victim (Watters, 2005). These protein toxins are not produced by microorganisms, as are the polyether toxins, but in venom glands found within these marine animals. The larger molecular size of the peptide toxins, compared to the polyether ingestible toxins, contributes to heat instability, thus affording a potential therapeutic intervention to degrade these toxins and reduce their effect on the channels, which they target. Characteristics of the cell membranes from animals resistant to envenomation with contact toxins afford potential for drug development in reducing the risk of envenomation.

41.2 UTILIZING DATABASES AND GENETIC MINING

Many venoms contain a cocktail of molecules. Researchers must identify all of the genes active in venom gland cells and read the DNA sequences, particularly the peptides. Some of these peptide neurotoxins can discriminate between closely related intracellular targets to include ion channels and G-protein coupled receptors, differentiating between closely related homologues of the same protein, which makes them useful to define cation channels and attractive for drug development. Peptide marine neurotoxins may show select affinity for specific subtypes of calcium channels, potassium channels, sodium channels, and acetylcholine receptors (AChRs). Several novel approaches have been developed for direct analysis of venomous proteins, to include polymerase chain reaction (PCR), high performance liquid chromatography (HPLC), mass spectrometry, micro- and nanotechnologies, sequencing of cDNA from venom glands, and proteomics of venom components, to help establish biologic and biochemical databases on venomous animals and peptide mapping of their venoms, and to provide domain imaging and query tools to facilitate therapeutic modeling (see 2006 weblinks to *Institute for Molecular Bioscience* and *Pfam*). Marine cone snail toxins have been particularly attractive to researchers, given that there are more than 500 species of marine cone snails, with up to 200 compounds being isolated from a single venom (Gayler, 2005). Although the disulfide bonding regions are highly conserved, the amino acids comprising the intercysteine loops are hypervariable, which provide the specificity of the conopeptides. Modifications of the more conserved disulfide connectivity may have profound effects on the potency of the conopeptide. Consequently, even small changes at either site in the amino acid sequencing of a conopeptide can result in large changes in the specificity and efficacy at receptors (Livett, 2004).

Researchers at Clarkson University in New York have shown that *cone snails* have rapidly mutating genes compared to mammalian genes, to result in venoms that have been optimized through *accelerated evolution*, to target specific ion channels (Moczydlowski, 2006). Using genes as a starting point in the analysis, genetic mining techniques have lead to the synthesis of conopeptide analogs solely from the DNA sequence, to provide *unique fingerprints* for potential new compounds (Jakubowski, 2004). Targeted screening utilizing *in vitro* platforms and selected natural sources have reduced the number of samples needed to study the actions of these conopeptides. Cone snail researchers have estimated their collective need for approximately 5000 snails per year (Duda, 2004). The first drug derived from conopeptides was ziconotide, a synthetic *omega* conopeptide that binds to the external side of the alpha-2-delta subunit of the neuron-specific voltage-gated calcium channels. At the spinal level, this type of calcium channel is found only at the dorsal root entry zone. Similar to other alpha-2-delta ligands (gabapentin, pregabalin) now approved in the United States and other countries, ziconotide is useful in the control of neuropathic pain. Clinical data is emerging with therapeutic usage of the *alpha* conotoxins, to accelerate the rate of recovery from peripheral nerve injury, as well as to reduce neuropathic pain (Livett, 2006).

Marine clinical trials are time consuming and expensive. Computerized databases, domain imaging, and query tools are powerful and cost-efficient allies in this quest. In 1995, approximately one drug patent was issued for every $1.1 million spent on marine biotechnology (National Academy of Science, 1999). Most *venom-derived peptides* have poor oral bioavailability, owing to their relative large size, hydrophilic nature, and susceptibility to hydrolysis in the stomach (Sherman 2005). This necessitates more costly administration by intravenous, intrathecal, intraperitoneal, intramuscular, subcutaneous, or epidural injections. Such parenteral administration to the site needed may limit adversities. *High receptor affinity* can limit diffusion from the injection site, and hence limit systemic spread of the agent, while allowing regionally restrictive targeting of the cellular domains afforded by the toxin-derived compounds. Ziconotide, the first drug derived from a peptide in the venom of a marine creature (cone snail), is given intrathecally for intractable neuropathic pain. A review of the process of necessary clinical studies preceding approval of ziconotide will illustrate the complexity of such drug development (Watters, 2004b).

41.3 CUTANEOUS MEASURES TO REDUCE ENVENOMATION MANIFESTIONS

Coelenterate (cnidarian) toxins are found in venomous jellyfish and sea anemones. They produce not only severe pain, but also contact dermatitis that may include hemolysis and scarification. Some jellyfish envenomations are fatal, to include stings by both large (*Chironex fleckeri*) and small (*Carukia barnesi*) Australian box jellyfish (Watters, 2006a). Anaphylaxis may occur with species that have less toxic venoms. Prevention from envenomation by those who enter the marine environment is therefore clinically important.

Clown fish do not activate the stinging nematocysts of sea anemones, despite active physical contact, suggesting protection by the clown fish's integument. This observation has spurred interest in the development of topical sting inhibitors to afford protection from envenomation by coelenterates. Physical properties of the venom, to include membrane binding and thermostability, have been useful in preventing and treating envenomations with cutaneous measures. Topical *jellyfish sting inhibitors* are now available to prevent envenomation by coelenterates (Kimball, 2004). Proposed mechanisms include the use of hydrophobic emollients that limit tentacle attachment with skin, as well as the use of amino acids and sugars that nonselectively compete for and inhibit the binding receptors on the jellyfish membrane.

Work by Yanagihara and colleagues at the Bekesy Neurobiology Lab of the University of Hawaii has identified the impact that heat and sugars have on the Hawaiian box jellyfish (*Carybdea alata*) venom's induction of hemolysis, a common measure of venom potency (Watters, 2003). Cytolytic components of venom bind to glycosylated residues of membrane proteins. By testing whether the presence of sugars that compete for such binding sites might protect red blood cells from the hemolytic proteins in the venom, it has been shown that D-lactulose completely inhibits hemolysis, while greater than 70% inhibition is observed with D-galactose and two other less common sugars. Topical preparations containing such carbohydrates therefore would be expected to limit binding by these peptide toxins. As previously stated, heat denatures the peptides within the venom, to afford a therapeutic intervention to deactivate the toxin once envenomation has occurred. The effect of heat on *Carybdea* venom's hemolytic properties is shown in Figure 41.1. Three clinical trials have substantiated the usefulness of the analgesic effect of *heat therapy* in envenomations by Hawaiian box jellyfish (Thomas, 2001; Normura, 2002; Yoshimoto, 2002). Anecdotal reports indicate that heat therapy is also useful to reduce symptoms from envenomation by venomous fish (Watters, 2004a).

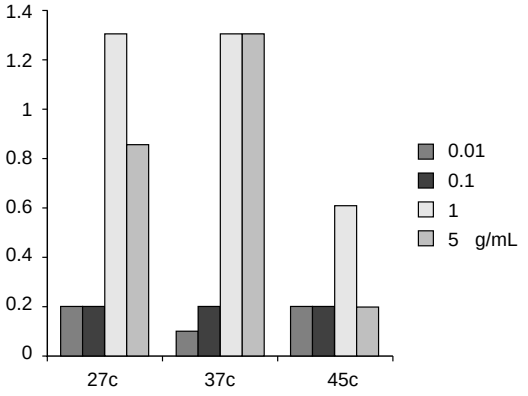

FIGURE 41.1 Effect of heat on venom hemolysis. Sheep RBCs are incubated in various concentrations of Carybdea alata venom (bars). Hemolysis is determined spectrophotometrically as the absorbance of supernatant at 414 nm (vertical axis). Hemolysis, expressed as the percentage change of absorbance relative to that observed when exposed to buffer alone, is significantly reduced by heating concentrated venom to 45°C. Bekesy Neurobiology Lab.

41.4 MARINE NEUROTOXINS AS DIAGNOSTICS

Ciguatoxin can be detected in fish by commercially available test kits, using a stick-enzyme immunoassay developed at the University of Hawaii in the 1980s (Hokama, 1985). Acute fatigue and asthenia, with or without objective weakness, were noted in over half of 327 cases of ciguatera in Hawaii (Watters, 1995). Most patients with chronically persistent or relapsing symptoms of ciguatera will complain of fatigue. High levels of acetone-soluble lipids have been detectable in the sera of patients with chronic ciguatera fish poisoning, chronic fatigue syndrome (CFS), hepatitis B, and some cancers. These have been designated as *chronic phase lipids* (CPLs), comparable to the acute phase proteins found in many inflammatory diseases (e.g., c-reactive protein). Membrane immunobead assay using a *monoclonal antibody to ciguatoxin* (MAb-CTX) has been used at the University of Hawaii for analysis of these CPLs. Significant titers (>1:40) were detectable in 96% of 115 patients carrying the diagnosis of CFS, compared to only 11% of 37 normal subjects (Hokama, 2003). Whether the immunological interaction between MAb-CTX and CFS lipids suggests common structural epitopes for ciguatera and CFS, which might contribute to clinical fatigue, or merely a cross-reaction, requires more study. This data suggests that MAb-CTX offers supplemental laboratory testing for the clinical diagnosis of CFS. The applicability for other chronic disorders has yet to be established.

Assays to identify *in vivo* marine toxins are needed by clinicians. Although there are some efforts aimed at developing human biomarkers for ingested marine toxins (Becker, 2004), more work is needed. The diagnoses remain clinically based (Watters, 1999). There can be considerable overlap of symptoms from ingested neurotoxins, which typically manifest gastrointestinal prodromes before sensorimotor symptoms. Misdiagnoses are common. The source of the ingestible toxins are microorganisms, with the toxins being passed up the marine food chain to man. Algae blooms are normally in a balance that favors nontoxic algae, but environmental factors are increasing toxic blooms. The collapse of the large fish stocks more than a decade ago has affected the entire marine food chain off Atlantic Canada and New England. After populations of larger predators like cod declined, smaller herring, shrimp, and snow crab have undergone a population explosion (Watters, 2006b). These smaller surviving fish feed on nontoxic zooplankton and algae, which leads to a depletion of normal concentrations of the nontoxic algae. Loss of nontoxic algae allows blooms of toxic phytoplankton and algae to increase in these areas, which can result in red tides, dead zones lacking fish or sea mammals, and emergence of outbreaks of the clinical syndromes associated with the polyether toxins elaborated by toxic algae. A 2002 study funded by NASA concluded that dust clouds from the Sahara dessert have deposited mineral-rich dust into the Gulf of Mexico and Caribbean Sea, which has further stimulated the growth of *toxic algal blooms*. The American National Weather Service has created satellite maps and drift models for SLOSH (Sea, Lake, and Overland Surge from Hurricanes) for the Gulf of Mexico, to monitor the movement of toxic blooms (http://synergyx.tacc.utexas.edu/Spotlight/Hurricane/hurricane_flood.html). Nutrient-rich runoff, particularly when containing phosphorus and nitrogen, has contributed further to blooms of toxic algae, which after dying are consumed by bacteria that use up the oxygen in the water, making it difficult for fish, oysters, and other marine creatures to survive. A report released by the United Nations in 2006 showed a 34% increase in the number of dead zones in our seas and oceans over the preceding 2 years (www.gpa.unep.org/bin/php/igr/igr2/supporting.php). Hence, it is quite likely that we will see a significant increase in clinical disorders resulting from toxic marine organisms and harmful algae blooms, resulting in a pressing clinical need for accurate human biomarkers, which will have broad commercial applicability.

41.5 MARINE NEUROTOXINS TARGETING VOLTAGE-GATED ION CHANNELS

Ion channels triggered by voltage changes along the cell membrane are termed voltage-gated ion channels (VGICs), and include channels that conduct sodium, calcium, and potassium. Activation of

sodium or calcium VGICs produce cell stimulation by membrane depolarization, while potassium channel activation results in inhibitory hyperpolarization. Ligand-gated ion channels (LGICs) can modulate the passage of cations or anions. These important channels influence physiological functioning throughout the body, as well as pathological disturbance in neuronal diseases. Toxins that bind VGICs can serve as powerful *molecular probes* to study the channel structure and function, as well as serve as models for studying evolutionary targets and drug development.

As the nerve action potential reaches the nerve ending, voltage-gated sodium channels are activated to depolarize the *presynaptic membrane*. This process activates calcium VGICs to trigger the membrane-vesicle fusion process that results in the release of neurotransmitter at the synapse, which is terminated by the membrane hyperpolarizatioin triggered by activation of the potassium VGIC (Arias, 2006). Some LGICs with high permeability for calcium (*N*-methyl-D-aspartic acid (NMDA) glutamate receptor, neuron-specific voltage-gated calcium channel, alpha-7 nicotinic acetycholine receptor) can also induce the release of synaptic neurotransmitters (Miller, 1998). Marine toxins can potentially be used to develop modulatory drugs that inhibit or prolong ion channel functioning in specific channelopathies, as well as interruption of neuropathic signaling (e.g., neuropathic pain).

The presynaptic *sodium VGIC* has at least six binding sites. Tetrodotoxin, saxitoxin, and mu-conotoxin each bind to site 1 to block sodium influx, while brevetoxin and ciguatoxin bind to site 5 to shift the voltage-dependent activation toward more negative potentials and open the sodium channel (Arias, 2006). Antillatoxin, from marine cyanobacterium, also opens sodium channels, but through binding to site 2 (Li, 2001). Opening of the neuronal sodium VGIC induces spontaneous and repetitive action potentials in response to a single electrical stimulation, causes trains of repetitive endplate potentials that can trigger muscle action potentials, and results in a time-dependent swelling of the nodes of Ranvier where the channels are clustered along myelinated axons (Benoit, 2002). Coelenterate toxins found in jellyfish and sea anemones bind to both sodium and potassium channels (Messerli, 2006). Coelenterates represent the earliest extant creature with a neuromuscular system. Toxins from the sea anemone are the best characterized cnidarian toxins in terms of mechanism of action, and more than 50 different toxins that target sodium VGICs have thus far been isolated or cloned (Honma, 2006). Each of these toxins can serve as a probe of sodium VGIC structure and function, and provide selectivity for sodium-dependent mechanisms in cardiac and neuronal tissues, to include autonomic nerves that mediate gastrointestinal symptoms in a variety of disease states.

Neuronal calcium channels are found in cell bodies, dendrites, and presynaptic terminals of neurons, as well as skeletal muscle, and have been implicated increasingly in neurologic disorders, to include Lambert-Eaton myasthenic syndrome, hypokalemic periodic paralysis, congenital night blindness, and certain ataxic disorders (Ackerman, 1997; Catterall 2003). *Calcium VGICs* are normally closed at resting membrane potentials, and open in response to depolarization of the membrane. Physical properties can be studied by use of voltage-clamp and patch-clamp methods. Structural subunits include the alpha-1, alpha-2, delta, beta, and gamma subunits (Yamakage, 2002). The alpha-1 and gamma subunits span the membrane, and contain the ion channel, voltage sensor, and gate. The extracellular alpha-2 subunit is attached by two disulfide bonds to the delta subunit, which spans the membrane. Collectively the alpha-2-delta subunits provide structural support and modulate inactivation of the opening of the channel. The extracellular site of the alpha-2-delta complex makes it accessible by diffusion of extracellular substances, and is the site of action for intrathecal ziconotide. Calcium channels are found in cardiac myocytes, which serve as targets for phenlaklylamines (e.g., verapamil), dihydropyridines (e.g., nifedipine, nicardipine, nimodipine), and benzothiazepines (e.g., diltiazem) used commonly for management of cardiovascular disorders. Biotoxins from cone snails, scorpions, spiders, snakes, and some sea anemones show potent and selective affinity for specific subtypes of calcium channels (Arias, 2006). Equinatoxin-II isolated from the sea anemone *Actinia equina* is a cation-selective pore-forming protein, which can induce the formation of calcium VGICs in myelinated axons when extracellular calcium is available (e.g., following neuronal cell death from apoptosis or injury), and may further contribute to nodal

swelling of myelinated axons independent of sodium VGIC opening (Benoit, 2002). This knowledge could lead to a new neuroprotective strategy for models of excitatory neurotoxicity.

Potassium VGICs principally inactivate by a "ball and chain" mechanism, whereby a long chain of amino acids with a spherical "ball" amino terminal swings into the cytoplasmic side of the ion channel, to control potassium efflux out of the cell (Arias, 2006). Dysfunctioning of these channels has been implicated in several common medical disorders, to include long-QT syndrome, congenital deafness, diabetes, epilepsy, Alzheimer's disease, migraine, anxiety, and bipolar disorder (Miller, 2000). Potassium channels are the targets of both synthetic and natural drugs, to include oral hypoglycemic agents (glipizide, glyburide, tolazamide), cardiovascular agents (amiodarone, diazoxide, minoxidil), and mood stabilizers (lithium). Biotoxins from several creatures bind potassium channels, to include toxins from scorpions, spiders, several snakes, honeybee, sea anemone, and the *kappa* conotoxins (Arias, 2006). Ciguatoxin also blocks voltage-gated potassium channels, while opening sodium channels through binding at sodium site 5.

The *postsynaptic membrane* has two main classes of receptors, ionotropic and metabotropic, which are lipid-embedded proteins. Binding of a neurotransmitter to its specific metabotropic receptor produces a slow biological response indirectly by the enzymatic interaction with a G-protein. The binding of a ligand to its specific inotropic receptor induces a faster opening of the ion channel that is intrinsically coupled to the receptor complex (Arias, 2006). The sites and modes of action of some of the many conopeptides studied thus far have already been identified as presynaptic and postsynaptic VGICs and LGICs (Livett, 2004).

41.6 MARINE TOXINS TARGETING LIGAND-GATED ION CHANNELS

Four different superfamilies of LGICs have been identified, and are available online (www.pasteur.fr/recherche/banques/LGIC/LGIC.html) from a French website (Le Novere, 2001). These include the *cys*-loop (nicotinicoid) receptor superfamily, the glutamate receptor (ionotropic) superfamily, the ATP-activated receptor superfamily, and the transient receptor potential-canonical channels super family (Arias, 2006). Although structurally different, all LGIC receptor members have at least some common features. Common features include an *extracellular domain* containing the neurotransmitter binding sites and sequences involved in glycosylation, a *transmembrane domain* forming an aqueous pore interacting externally with the lipid membrane, and a *cytoplasmic domain* serving as a structural link to cytoskeletal proteins and modulatory phosphorylative processes (e.g., protein kinases) important for receptor functioning and cell surface expression needed for receptor trafficking (Arias, 2006).

LGICs also share common functional properties. Receptors recognize a specific neurotransmitter, which leads to ion channel opening and crossing of the lipid membrane by charged particles to alter the intracellular ionic concentration. Increasing cation concentrations within the cytoplasm results in activating membrane depolarization, whereas increases in anion concentrations induces inhibitory membrane hyperpolarization. In general, LGICs are functionally involved in higher cortical functioning (learning, memory, addictive behavior), in both mature and immature neurons, to influence embryonic development, cognitive growth and decline, and behavior. They play significant roles in both normal and disease states. Prolonged presence of neurotransmitter promotes the activated receptor into a state of *desensitization***,** which in turn contributes to synaptic inefficacy by controlling the number of available receptors (Quick, 2002). Prolonged exposure of the LGIC receptors to agonists, as well as some receptor antagonists, produces conformational protein changes leading to a refractory state where the LGIC fails to open in the presence of receptor agonists (Quick, 2002). Promotion of desensitization of LGICs potentially could be important in dealing with *excitotoxicity* of neurons, a process common to many paroxysmal, inflammatory, and neurodegenerative disorders.

For example, the substitution of an amino acid in the pore domain of a neuronal nicotinic AChR facilitates desensitization and promotion of partial-onset seizure activity (Weiland, 1996).

Activation of AChRs increases cation permeability for sodium, potassium, or calcium VGICs, which in turn affects membrane polarization. When summated, sufficiently large depolarization potentials elicit an action potential at the innervated cell (e.g., along muscle membrane). Several natural marine toxins target AChRs, to include anatoxin-a (from blue-green algae *Anabena flos-aquae*), epibatidine (from the Ecuadorian frog *Epipedobates tricolor*), anabaseine (from *Nemertine* marine worms), phopholipase-A and *Hydrophiidae* bungarotoxins (from sea snakes), laphotoxins (from gorgonian corals), neosurugatoxin (from Japanese ivory shellfish *Babylonian japonica*), and the *alpha* conotoxins (from marine cone snails). Although several of these natural marine toxins bind acetylcholine LGICs nonspecifically, some (e.g., alpha conotoxins, anatoxin, and anbaseine) have very high affinity for specific LGIC binding targets (Arias, 2006).

41.7 SUMMARY

The future human food chain is likely to be impacted significantly by the increasing presence of marine polyether toxins, owing to increasing harmful algal blooms in response to climatic and man-made environmental factors. Diagnostic human biomarkers sensitive to these toxins are needed, which would afford a broad commercial applicability for both acutely and chronically ill patients. Pharmaceutical and diagnostic applications for the more complex peptide toxins are likely to benefit from genetic mining and computerized modeling techniques. Understanding of physical properties has contributed to cutaneous measures useful in the management of clinical envenomations. Venom-derived peptides targeting voltage-gated and LGICs hold true promise for the advancement of our understanding and management of human disease states having neurologic, cardiac, neuromuscular, endocrine, or gastrointestinal manifestations.

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Part XIV

Legal and Economic Views

42 Incidence of Marine Toxins on Industrial Activity

Juan M. Vieites and Ana G. Cabado

CONTENTS

42.1 WORLD PRODUCTION OF BIVALVE MOLLUSCS AND OTHER RELATED SOCIOECONOMIC FACTS

This chapter is focused on the most important facts regarding world production of bivalve molluscs, by species and countries. It also provides related socioeconomic information that will show the importance of the productive–extractive sector in different countries, as well as the impact of toxic episodes on the industrial activity. A second section is related to the main detoxification processes of shellfish.

Total production of bivalves, including aquaculture and capture, increased almost 117% from that in 1992. Total production in 2005 was 13.5 million tons. Aquaculture is growing much faster than capture production. In 2005, aquaculture accounted for over 87% of total bivalve production. Among species, the most important bivalve species are clams/cockles and oysters with 4.9 and 4.8 million tons, respectively, while scallops and mussels are less important with 2.0 and 1.9 million tons, respectively. Currently the aquaculture production of bivalves is centered on China [1]. Next, a brief description of main bivalve species production is reported.

42.1.1 Scallops

In recent years, trend in world scallop production has been positive in spite of declines during 1998 and 1999. Total production for 2005 was 1,986,185 tons. In 2005, aquaculture production was around 1,274,843 and captures were 711,342.

Aquaculture, essentially in China and Japan, accounts for more than half of total production although the aquaculture has declined recently, falling from 71% in 1996 to less than 64% in 2005.

Among countries, Asia accounted for over 77% of total production of scallops. The total production of scallops in Asia was 1,537,300 tons (aquaculture production was 1,248,602 and capture production was 288,698 tons) in 2005. As mentioned above, the most significant country is China, being the major world producer of scallops (1,045,035 tons in 2005). All of China's production comes from aquaculture. Japan is the second largest producer of scallops; almost half of the production also comes from aquaculture.

The United States is the third leading world producer of scallops—215,564 tons in 2005—and this country did not use aquaculture methods; all of its production comes from its captures. Canada is also an important producer of scallops with a total production of 57,125 tons from capture and 237 tons from aquaculture in 2005.

As for European production of scallops, this has decreased during the last 5 years. France was the main European producer in 2005 (32,126 tons), followed by United Kingdom with 21,720 tons the same year. Spain is not among the leading scallop producers [1].

42.1.2 Clams, Cockles, and Arkshells

World production of clams, cockles, and arkshells showed an annual increase during the last few years. In 2005, total production of clams was 4,881,556 tons. Aquaculture production accounted for over 85% of total production of clams in 2005 and showed a constant increase during the last few years, reaching 4,175,907 tons in 2005. On the contrary, capture production of clams is showing a reduction in the last 3 years, having values of 899,362 tons in 2003, 835,150 tons in 2004, and 705,649 tons, in 2005. These data are shown in the following tables.

Among continents, Asia accounted for over 88% of total world production of clams in 2005. Asian aquaculture production of clams was 4,054,547 tons and its capture production was 245,039 tons. China is the first among the Asian countries in aquaculture production. In 2005, its aquaculture production was 3,874,949 tons and accounted for over 95% of total Asian aquaculture production approved change.

North America is the fifth aquaculture producer of clams (42,569 tons), the first capturer of clams (274,205 tons), and the second global producer in 2005. The United States is the first North American capturer and aquaculture producer. Canada, another important producer of clams, captured 26,992 tons in 2005. Canadian aquaculture production was 1788 tons in 2005.

In South America, Venezuela, and Chile are the countries with more captures (47,074 and 38,984 tons, respectively) in 2005.

As for European countries, the most important producer of clams was Italy, which reached 84,624 tons in 2005 (Italy captured 14,946 tons and its aquaculture production was 69,678 tons in 2005). United Kingdom capture production reached 13,421 tons in 2005 [1].

42.1.3 Oysters

Oyster production shows a pattern similar to the rest of bivalve molluscs. Oyster aquaculture production is growing much faster than capture production corresponding to 96.5% of total production. While the oyster aquaculture production showed a constant increase during last years, the oyster capture production reached a plateau as the following tables show. In 2005, aquaculture production increased, reaching values of 4,615,400 tons, the total production being 4,781,545 tons.

Among continents, Asia is the main aquaculture producer of oysters. China is the first among aquaculture producers in Asia. In 2005, its aquaculture production was 3,826,363 tons and accounted for over 87% of total Asian aquaculture production. The second major Asian aquaculture producer of oysters was Korea (251,706 tons) followed by Japan (218,896 tons).

North America is the third aquaculture producer of oysters and the first capturer of oyster. The United States is the first North American aquaculture producer (76,511 tons). As for capture production, the United States capture production was 85,224 tons in 2005. The other great American producer of oysters, Mexico, reached 46,827 tons in 2005.

Yearly World Total Production of Scallops (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	0	0	0	0	0	28	2	1	4	0	0	0	0	0
N. America	215,468	164,110	243,310	151,272	143,225	119,133	114,978	139,899	209,913	261,589	295,412	309,032	335,024	277,811
S. America	9,894	9,247	13,298	25,813	38,926	47,710	74,594	94,582	77,371	71,820	80,402	90,445	92,040	82,206
Asia	739,833	1,194,267	1,297,279	1,421,237	1,538,435	1,518,710	1,145,937	1,229,990	1,438,515	1,487,862	1,515,870	1,543,256	1,447,637	1,537,300
Europe	68,109	68,632	67,942	66,917	61,274	78,303	80,429	79,604	80,949	84,051	77,319	68,115	67,732	70,740
Oceania	39,691	42,668	33,725	27,598	17,524	27,638	14,557	17,854	14,926	16,188	10,133	12,152	11,246	18,128
Total	**1,072,995**	**1,478,924**	**1,655,554**	**1,692,837**	**1,799,384**	**1,791,522**	**1,430,497**	**1,561,930**	**1,821,678**	**1,921,510**	**1,979,136**	**2,023,000**	**1,953,679**	**1,986,185**

Source: FAO.

Yearly World Aquaculture Production of Scallops (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	0	0	0	0	0	28	2	1	4	0	0	0	0	0
N. America	68	80	86	51	177	61	54	55	70	255	109	148	90	240
S. America	2,312	4,983	11,231	8,695	10,806	11,947	18,381	23,310	22,936	22,450	20,827	21,521	30,763	25,385
Asia	546,072	969,778	1,025,023	1,144,374	1,265,228	1,256,199	855,867	928,724	1,132,665	1,195,973	1,207,582	1,196,501	1,131,291	1,248,602
Europe	391	386	1,203	345	511	1,061	862	422	434	450	173	481	648	616
Oceania	19	110	0	0	0	0	0	0	0	0	0	0	0	0
Total	**548,862**	**975,337**	**1,037,543**	**1,153,465**	**1,276,722**	**1,269,296**	**875,166**	**952,512**	**1,156,109**	**1,219,128**	**1,228,691**	**1,218,651**	**1,162,792**	**1,274,843**

Source: FAO.

Yearly World Captures of Scallops (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N. America	215,400	164,030	243,224	151,221	143,048	119,072	114,924	139,844	209,843	261,334	295,303	308,884	334,934	277,571
S. America	7,582	4,264	2,067	17,118	28,120	35,763	56,213	71,272	54,435	49,370	59,575	68,924	61,277	56,821
Asia	193,761	224,489	272,256	276,863	273,207	262,511	290,070	301,266	305,850	291,889	308,288	346,755	316,346	288,698
Europe	67,718	68,246	66,739	66,572	60,763	77,242	79,567	79,182	80,515	83,601	77,146	67,634	67,084	70,124
Oceania	39,672	42,558	33,725	27,598	17,524	27,638	14,557	17,854	14,926	16,188	10,133	12,152	11,246	18,128
Total	**524,133**	**503,587**	**618,011**	**539,372**	**522,662**	**522,226**	**555,331**	**609,418**	**665,569**	**702,382**	**750,445**	**804,349**	**790,887**	**711,342**

Source: FAO.

Yearly World Total Production of Clams (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	1,417	1,583	1,063	1,587	522	178	237	229	910	676	1,188	834	664	789
N. America	458,161	479,211	438,514	453,296	414,514	388,465	362,206	376,917	381,263	397,149	419,353	429,041	435,368	352,426
S. America	106,148	84,695	84,756	84,811	84,370	83,250	76,753	83,630	86,134	95,018	75,988	85,152	98,333	89,489
Asia	1,366,236	1,734,768	1,942,955	1,983,358	2,047,243	2,135,952	2,442,586	2,934,444	2,785,467	3,312,947	3,626,890	4,041,370	4,280,395	4,299,586
Europe	191,712	162,975	160,071	200,016	150,890	143,450	2,12,285	199,147	169,890	153,933	129,038	152,572	126,255	135,473
Oceania	1,589	1,094	1,111	3,750	2,888	4,227	5,894	7,537	7,859	8,212	5,105	3,479	3,788	3,793
Total	**2,125,263**	**2,464,326**	**2,628,470**	**2,726,818**	**270,0427**	**2,755,522**	**3,099,961**	**3,601,904**	**3,431,523**	**3,967,935**	**4,257,562**	**4,712,448**	**4,944,803**	**4,881,556**

Source: FAO.

Yearly World Aquaculture Production of Clams (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	20	30	16	28	15	24	22	8	8	16	14	2	0	0
N. America	8,787	12,255	10,546	14,366	8,453	21,120	22,649	31,315	27,487	27,067	25,727	37,212	71,851	44,358
S. America	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Asia	1,023,631	1,356,839	1,616,498	1,675,746	1,721,778	1,866,083	2,176,535	2,662,986	2,537,465	3,049,536	3,383,067	3,743,968	4,003,115	4,054,547
Europe	36,020	36,377	52,661	75,947	51,078	54,245	62,630	64,516	67,063	67,377	49,411	31,898	34,679	76,996
Oceania	2	2	3	3	2	2	7	1,421	1,431	1,419	7	6	8	6
Total	**1,068,460**	**1,405,503**	**1,679,724**	**1,766,090**	**1,781,326**	**1,941,474**	**2,261,843**	**2,760,246**	**2,633,454**	**3,145,415**	**3,458,226**	**3,813,086**	**4,109,653**	**4,175,907**

Source: FAO.

Yearly World Captures of Clams (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	1,397	1,553	1,047	1,559	507	154	215	221	902	660	1,174	832	664	789
N. America	449,374	466,956	427,968	438,930	406,061	367,345	339,557	345,602	353,776	370,082	393,626	391,829	363,517	308,068
S. America	106,148	84,695	84,756	84,811	84,370	83,250	76,753	83,630	86,134	95,018	75,988	85,152	98,333	89,489
Asia	342,605	377,929	326,457	307,612	325,465	269,869	266,051	271,458	248,002	263,411	243,823	297,402	277,280	245,039
Europe	155,692	126,598	107,410	124,069	99,812	89,205	149,655	134,631	102,827	86,556	79,627	120,674	91,576	58,477
Oceania	1,587	1,092	1,108	3,747	2,886	4,225	5,887	6,116	6,428	6,793	5,098	3,473	3,780	3,787
Total	**1,056,803**	**1,058,823**	**948,746**	**960,728**	**919,101**	**814,048**	**838,118**	**841,658**	**798,069**	**822,520**	**799,336**	**899,362**	**835,150**	**705,649**

Source: FAO.

Yearly World Total Production of Oyster (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	1,279	667	783	797	636	857	811	784	924	938	1,044	980	540	769
N. America	245,040	240,554	243,883	283,281	257,584	253,652	238,556	236,821	316,665	290,083	272,148	295,331	282,718	224,036
S. America	2,699	4,029	4,876	6,098	5,969	6,253	8,026	10,277	9,569	13,923	8,314	9,048	8,148	8,268
Asia	1,299,257	1,616,895	2,390,800	2,780,757	2,777,953	2,821,596	3,277,393	3,448,929	3,757,108	3,965,261	4,091,062	4,218,200	4,316,005	4,398,309
Europe	149,968	157,537	160,792	158,970	166,073	162,443	154,171	157,534	149,971	125,516	132,852	132,339	134,996	134,982
Oceania	11,494	12,669	11,662	13,246	15,543	19,962	23,233	26,795	13,951	13,942	12,122	13,299	15,247	15,181
Total	**1,709,737**	**2,032,351**	**2,812,796**	**3,243,149**	**3,223,758**	**3,264,763**	**3,702,190**	**3,881,140**	**4,248,188**	**4,409,663**	**4,517,542**	**4,669,197**	**4,757,654**	**4,781,545**

Source: FAO.

Yearly Aquaculture Production of Oysters (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	692	631	689	560	504	732	713	651	820	785	879	842	450	567
N. America	122,234	120,771	110,530	119,311	99,090	96,662	97,582	99,662	89,680	110,200	108,212	125,828	165,440	91,774
S. America	318	1,119	1,279	1,572	2,396	3,710	4,682	6,673	7,053	9,067	6,613	6,130	4,782	4,617
Asia	1,277,999	1,587,143	2,367,521	2,758,997	2,756,667	2,801,061	3,264,320	3,434,360	3,738,659	3,953,901	4,074,019	4,197,548	4,289,913	4,370,640
Europe	142,239	154,573	157,448	156,314	163,567	160,223	152,383	155,860	149,116	124,468	131,391	129,978	132,199	133,167
Oceania	10,458	11,798	11,078	12,161	13,610	17,788	22,233	25,738	13,185	13,110	11,306	12,447	14,782	14,635
Total	**1,553,940**	**1,876,035**	**2,648,545**	**3,048,915**	**3,035,834**	**3,080,176**	**3,541,913**	**3,722,944**	**3,998,513**	**4,211,531**	**4,332,420**	**4,472,773**	**4,607,566**	**4,615,400**

Source: FAO.

Yearly World Captures of Oysters (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	587	36	94	237	132	125	98	133	104	153	165	138	90	202
N. America	122,806	119,783	133,353	163,970	158,494	156,990	140,974	137,159	226,985	179,883	163,936	169,503	117,278	132,262
S. America	2,381	2,910	3,597	4,526	3,573	2,543	3,344	3,604	2,516	4,856	1,701	2,918	3,366	3,651
Asia	21,258	29,752	23,279	21,760	21,286	20,535	13,073	14,569	18,449	11,360	17,043	20,652	26,092	27,669
Europe	7,729	2,964	3,344	2,656	2,506	2,220	1,788	1,674	855	1,048	1,461	2,361	2,797	1,815
Oceania	1,036	871	584	1,085	1,933	2,174	1,000	1,057	766	832	816	852	465	546
Total	**155,797**	**156,316**	**164,251**	**194,234**	**187,924**	**184,587**	**160,277**	**158,196**	**249,675**	**198,132**	**185,122**	**196,424**	**150,088**	**166,145**

Source: FAO.

Yearly World Total Production of Mussels (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	2,256	2,509	2,767	2,407	1,868	2,591	2,775	2,343	2,094	2,005	1,981	1,936	1,418	3,644
N. America	32,282	26,931	25,942	26,922	22,971	23,584	23,460	23,972	31,405	31,179	35,376	32,279	35,933	36,329
S. America	29,204	25,962	28,413	35,323	32,518	38,412	53,603	52,852	61,708	78,599	76,167	95,200	102,064	112,239
Asia	634,262	659,038	543,290	597,294	530,454	554,928	648,814	735,945	716,224	760,774	998,159	998,751	1,035,165	1,114,485
Europe	590,538	552,812	600,128	627,281	636,656	660,022	759,170	762,484	738,676	744,380	664,822	687,656	695,205	573,933
Oceania	49,866	48,566	46,337	63,787	66,432	66,623	77,147	75,906	82,485	68,782	82,516	82,875	88,930	98,331
Total	**1,338,408**	**1,315,818**	**1,246,877**	**1,353,014**	**1,290,899**	**1,346,160**	**1,564,969**	**1,653,502**	**1,632,592**	**1,685,719**	**1,859,021**	**1,898,697**	**1,958,715**	**1,938,961**

Source: FAO.

Yearly World Aquaculture Production of Mussels (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	2,232	2,508	2,765	2,399	1,858	2,585	2,768	2,329	1,633	1,871	1,973	1,659	824	850
N. America	5,516	5,839	7,853	9,592	12,248	13,118	16,274	18,719	23,630	23,036	24,137	22,081	24,243	25,418
S. America	3,839	3,162	3,983	9,245	11,593	15,499	20,333	26,758	35,786	46,556	52,859	74,667	84,982	95,717
Asia	589,753	620,299	499,373	564,076	503,706	523,152	619,555	719,406	666,829	753,954	988,426	987,337	1,026,336	1,097,893
Europe	413,347	368,851	425,441	459,956	500,048	494,213	602,360	605,892	565,062	553,075	486,096	546,164	546,394	478,056
Oceania	47,229	47,565	45,833	63,349	65,907	66,622	76,482	72,928	78,017	66,509	80,789	80,727	87,577	97,845
Total	**1,061,916**	**1,048,224**	**985,248**	**1,108,617**	**1,095,360**	**1,115,189**	**1,337,772**	**1,446,032**	**1,370,957**	**1,445,001**	**1,634,280**	**1,712,635**	**1,770,356**	**1,795,779**

Source: FAO.

Yearly World Captures of Mussels (in Tons)

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Africa	24	1	2	8	10	6	7	14	461	134	8	277	594	2,794
N. America	26,766	21,092	18,089	17,330	10,723	10,466	7,186	5,253	7,775	8,143	11,239	10,198	11,690	10,911
S. America	25,365	22,800	24,430	26,078	20,925	22,913	33,270	26,094	25,922	32,043	23,308	20,533	17,082	16,522
Asia	44,509	38,739	43,917	33,218	26,748	31,776	29,259	16,539	49,395	6,820	9,733	11,414	8,829	16,592
Europe	177,191	183,961	174,687	167,325	136,608	165,809	156,810	156,592	173,614	191,305	178,726	141,492	148,811	95,877
Oceania	2,637	1,001	504	438	525	1	665	2,978	4,468	2,273	1,727	2,148	1,353	486
Total	**276,492**	**267,594**	**261,629**	**244,397**	**195,539**	**230,971**	**227,197**	**207,470**	**261,635**	**240,718**	**224,741**	**186,062**	**188,359**	**143,182**

Source: FAO.

The major European producer of oysters is France (119,485 tons in 2005). French aquaculture production of oyster was 119,400 tons in 2005 [1]. Production is oriented toward the domestic market; French oyster exports are somewhat marginal. Sales are destined mainly to neighboring countries, with Italy buying two-thirds of the production. French exports were largely stable during 2004 around 6600 tones. These increases are due to higher sales to both Italy and Belgium, the two leading markets for French oysters [2].

In contrast to France, Italian oyster market depends on imports having a marginal domestic production. In recent years, imports have been more or less stable in volume terms just over 6000 tons. Import values, on the other hand, have increased regularly from less than €12 million in 2001 to almost €15 million in 2004. These trends imply steady import price increases.

Oysters remain like a niche market in Germany with annual consumption of live oysters below the 1000 tons level. Similar to Italy, the German market also depends on the imports [2].

42.1.4 Mussels

Total mussels production has increased during the last 20 years, although 2005 shows a very small decrease (1,938,961) with respect to 2004 (1,958,715). Production was around 700,000 tons in the 1970s, 900,000 tons in the 1980s, around 1.4 million tons in the 1990s, reaching a plateau of 1.8–1.9 million tones currently. In contrast to most other aquatic species, but in line with other bivalves, wild mussel production, (143,182, in 2005) is much smaller than the cultured mussel production (1,795,779, in 2005). It is known that wild mussel production is declining while aquaculture production is increasing. The share of mussel capture fisheries in total mussel production declined in the last few years to 18% in 1995, 10% in 2003, and 7% in 2005. This decline was not only due to an expansion in mussel culture production but also to a drop in the mussel capture fisheries in recent years. The market of mussel fisheries in total bivalve production is limited to 14% and this participation has declined in recent years, since it was still at 18% in 1993. For instance, clam production is growing much faster than mussel production.

Mussel capture fisheries peaked in 1992 (276 tons) and 2000 (262 tons). In 2003, mussel catches dropped drastically to 17% and 23% from 2004 to 2005. The decline was caused by lower production in Denmark. Nevertheless, Denmark continues to be, so far, the main capturing country, accounting for about half of the mussel catch (69,155 tons). Other important producers of wild mussels include Italy (35,065 tons), Turkey (12,362 tons), the United Kingdom (11,158 tons), and the United States (10,566 tons) in 2004.

The blue mussel (*Mytilus edulis*) is the main mussel taken in the wild. This species, mainly taken in the North Sea, accounted for 65% of total mussel capture in 2005. The Mediterranean mussel (*M. galloprovincialis)* is the second major species captured, and capture of this species grew in recent years to reach 22,898 tons in 2005. While the catch of Mediterranean mussel expanded, the capture of green mussel (*M. smaragdinus*) declined from 41,000 tons in 2001 to almost zero at present (41 tons in 2005) [1].

China is so far the main mussel-culturing country with 772,173 tons produced in 2005 and Thailand is the second producing 249,620 tons. The third most important country producing cultured mussel is Spain with 158,059 [1] or 209,314.69 [3] tons. All other produce small amount with approximately 100,000 tons each.

The Chinese mussel production is 100% "sea mussels nei," which explains the high mark of unidentified species among the total mussel aquaculture production. Blue mussel, green mussel, and Mediterranean mussel are the top species among the identified species, mainly produced by Spain, Thailand and Italy, respectively.

A certain quantity of the mussel production is processed into frozen meat products. Total production fluctuated between 6,000 tons and 10,000 tons in the early 1990s. Then, New Zealand as the main producer increased its production, resulting in total frozen mussel production in the year 1996 reaching about 30,000 tons. Total production of frozen mussels reached 40,000 tons in 2003,

with New Zealand accounting for over 60% of total production. Chile is also undergoing a boom in frozen mussel production in recent years [2].

An important processing method for mussel is canning of mussel meat. The main producing country is Spain, which reported huge increases in recent years. Total Spanish production in 2004 was around 41,500 tons, a very important share of Spanish mussels usage. One wonders what happens with all the Chinese mussels. Anyway, Spain accounts for 70% of total production. Denmark is also quite active in canning of mussels. The share of canned mussels in total canned seafood production is rather limited at only 2%. The most important canned seafood is tuna, while small pelagic fish such as sardines and mackerel also play an important role. However, more than 50% of the canned seafood production is unspecified.

Total trade in live, fresh, and frozen mussel is expanding, and reached almost 237,703 tons in volume of exportation in 2004. Of this, 46% are fresh mussels. Main exporting country is Netherlands, followed by Spain, New Zealand, and Denmark. Other countries such as China, Germany, United Kingdom, and Ireland export about 15,000 tons of fresh mussels per year.

Markets for fresh and frozen mussels are almost exclusively in the European Union. France, Italy, Belgium, and Germany are the main importers in quantity terms. Also, Netherlands imported certain quantities in times of low production such as 1991, 1992, 2003, and 2004, to supplement the domestic product. The United States has entered the group of top importers of fresh/frozen or live mussels only in recent years; these imports are almost equally distributed between frozen and fresh imports. Total value of fresh/frozen or live mussels is around US $374 million in volume of exportation, which gives a unit value of around US $1.57/kg.

Canned mussel exports are completely Europe based, and practically all intra-EU trade. Canned exports in quantity fluctuates quite a bit between 26,000 tons and 30,000 tons. Denmark is the most important exporter of canned mussels accounting for about one-third of the total. In value terms, the canned mussel trade represents about 29,608 tons and US $113 million in volume of exportation, which would give a unit value of US $3.82/kg. Spain, being such an important producer, seems to consume the entire canned mussel production inside the country. Main canned mussel importers are France and Italy. Most of these canned mussel products go to the restaurants for antipasti and other preparations where canned mussels are easy to be used [1].

Mussel prices in France generally go up in summer months, to decline again in winter. In recent years, a certain trend for higher prices can be identified on the French market; some of these effects are originated from the change to Euro that increased all prices in the European countries.

Wild mussel prices are about double the price of the cultured mussel in the wholesale market of Barcelona. Prices have gone up in recent years, also an effect of the general price increase again caused by the Euro.

Summing up, aquaculture production of mussels is growing, while capture is undergoing a reduction. China is the major producer, but all the mussel produced is used mainly for own market. Europe is self-sufficient for mussel, and is not importing much from outside. Total trade of mussel is involving 2,82,000 tons of mussels (live weight equivalent), worth about US $537 million. Total trade of mussel is growing, especially inside the European Union. Prices of mussel have increased recently, also as an effect of the introduction of the Euro. Mussel is considered a rather traditional seafood product.

42.1.4.1 Spanish Production of Mussels

Spain is the third world-wide mussel producer and the main supplier to the European market. In 2005, 158,059 tons of mussel were produced in Spain according to FAO [1]. This quantity is even bigger as declared by the Spanish Ministry of Agriculture, Fisheries, and Food, which considers a production of 209,314.69 tons in the same period of time [3]. Thus, 98% of its production comes from Galicia (North West of Spain).

Regarding the data of Spanish foreign trade, the exports of fresh mussel peaked to 27,265 tons in 2003 (82% from Galicia). In 2004, there was an increase to 36,661 tons, but Galicia only accounts

for 76% of the total exports of fresh mussel. As for frozen mussels, there was a decrease of the exports of fresh mussel. Marketing of canned mussels is almost entirely Galician.

The most common destinations of the exports of fresh mussel are countries in the European Union, such as Italy (21,295 tons in 2004) and France (14,464 tons in 2004). On the other hand, Italy (1594 tons in 2004) and Portugal (782 tons in 2004) are the main destinations of the exports of frozen mussel.

Concerning imports, Spain imported 7385 tons of fresh mussel in 2004, mainly coming from France (2784 tons) and Greece (2225 tons). These figures mean an increase in 2003, when the imports of fresh mussel rose to 6584 tons. In 2004, Spain imported 12,913 tons of frozen mussels. The main suppliers were Chile (1822 tons) and New Zealand (1558 tons). The canned mussel imports in 2004 (583 tons) are hardly important. It must be added that from 2004 to 2005 there was an increase of 36% in the canned mussel imports from Chile, the Netherlands, and Ireland [2].

42.2 ECONOMIC IMPACT OF TOXIC EPISODES ON INDUSTRIAL ACTIVITY IN GALICIA

Many industrial sectors are affected by toxic episodes: producers, canning industry, depuration plants, and associated industries that work with fresh or frozen shellfish, restaurants, and tourism among others. In fact, some governments are sensitized to this problem and try to compensate economical losses due to the presence of toxins. For instance, in May 2006, U.S. Senate approved an amount of 20 million dollars to help shellfish industry affected by toxic episodes [3]. Also, Galicia government (NW of Spain, where most of the Spanish mussels culture takes place) approved economical help to producers in cases of 4 months or longer periods of stoppage, during the activity, when these toxic episodes took place, or when sales reach 35% of annual marketing, for instance the Christmas.

In Galicia (see map on page 911), half of the areas dedicated to mussel's culture were closed during the last years due to the presence of diarrhetic shellfish poisoning (DSP) toxins. The maximum record was in 2000 when some areas were closed for 10 months and around 270 days in 2004. In 2005, the presence of toxins led to the closure of more than 50% of the harvesting areas of molluscs, including barnacles, a gastropod that also got affected by paralytic shellfish poisoning (PSP) toxins. For instance, a group of harvesting areas sited in El Grove (Amegrove) produced a total of 15,900,000 kg of mussels during 2004, having an invoicing of 11.6 million Euros. On the other hand, in 2005 the production decreased to 10,325,430 kg of mussels and also the sales decreased to 7,162,089. These data, represented in Figures 42.1 and 42.2, are an example of the mussel production situation in Galicia, during the years 2004 and 2005 [4].

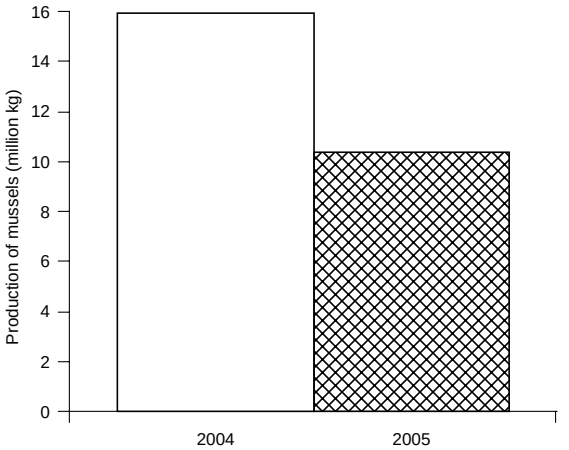


FIGURE 42.1 Comparison of mussels production in a limited harvesting area of Galicia during the years 2004 and 2005.

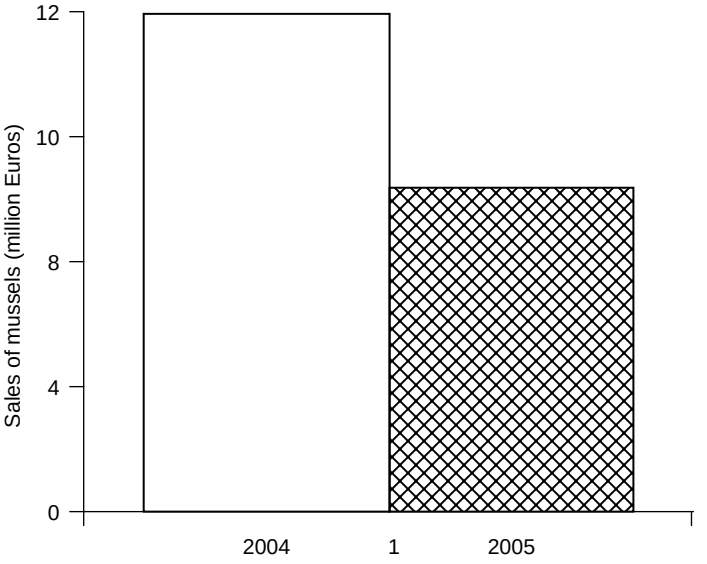


FIGURE 42.2 Comparison of mussels sales in a limited harvesting area of Galicia during the years 2004 and 2005.

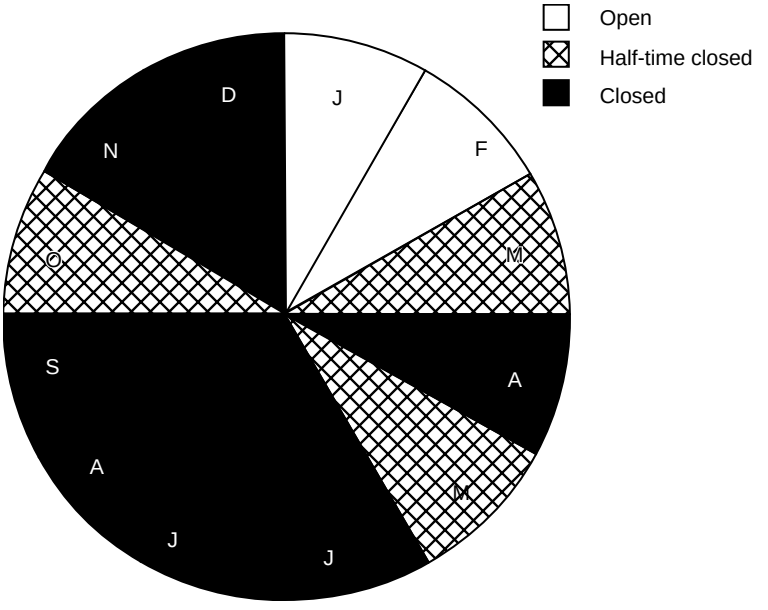
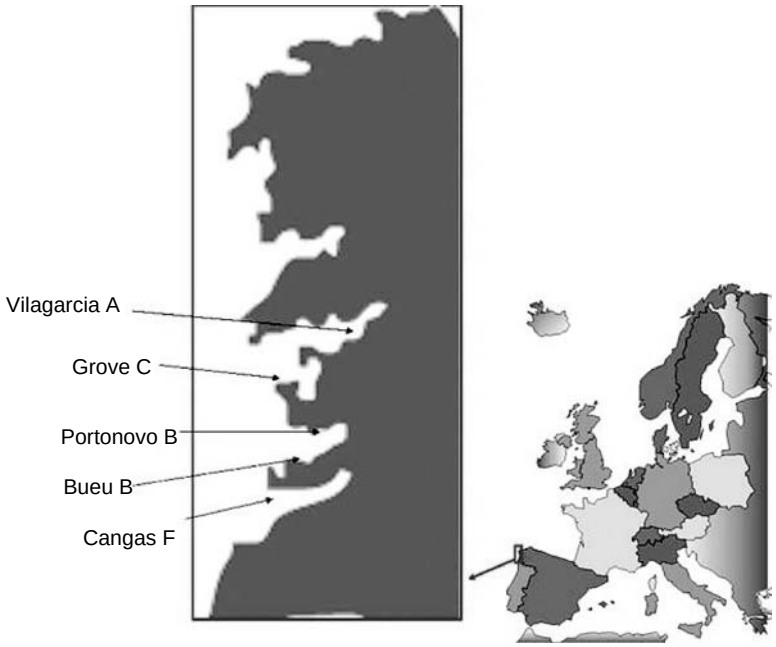


FIGURE 42.3 State of mussels rafts in Galicia during the months of the year 2005 with respect to DSP toxic episodes.

More detailed information related to the duration of toxic episodes is given in Figure 42.3. In 2005, January and February were good months, but in March some areas had to close, in April all the harvesting areas were closed, on some days of May the areas could open, and in June DSP was detected again. Then, from June to October producers could not harvest mussels and during this month some rafts were open several days until the middle of November when DSP and PSP (not very usual in Galician coasts) were detected again and all areas had to close until January 2006.

During the last years, November is a month characterized by the presence of toxins; in 2003 and 2004 most areas were closed on all days of this month.



Geographical distribution of mussel harvesting areas analyzed

An evolution of toxic episodes along the years, from 1998 to 2006 is shown in Figures 42.4, through 42.7 that correspond to different mussel harvesting areas in the same area (O Grove) in Galicia (data from Amegrove and OPMEGA, pers. commun.).

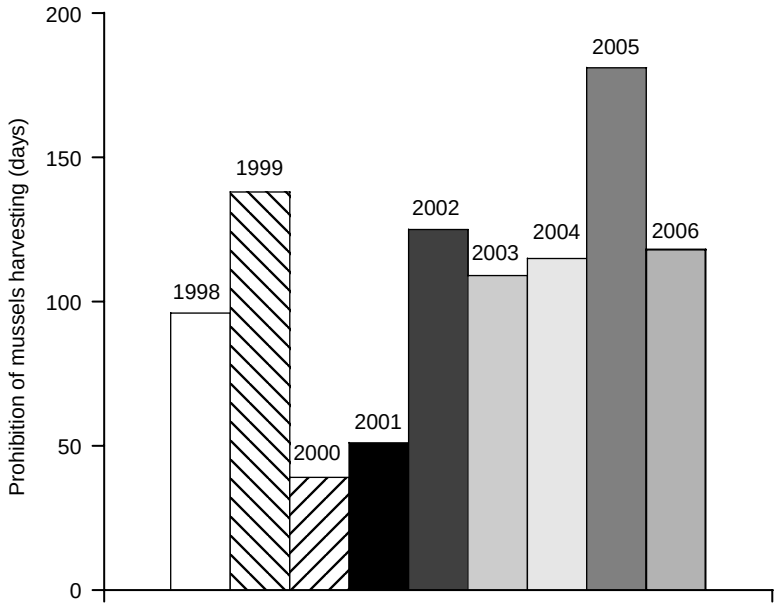


FIGURE 42.4 Days of mussels harvesting area closed from 2000 to 2006 in Grove C1.

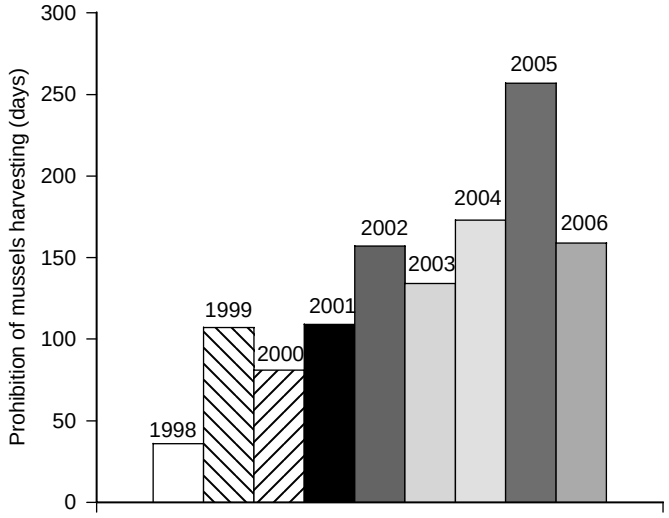


FIGURE 42.5 Days of mussels harvesting area closed from 2000 to 2006 in Grove C2.

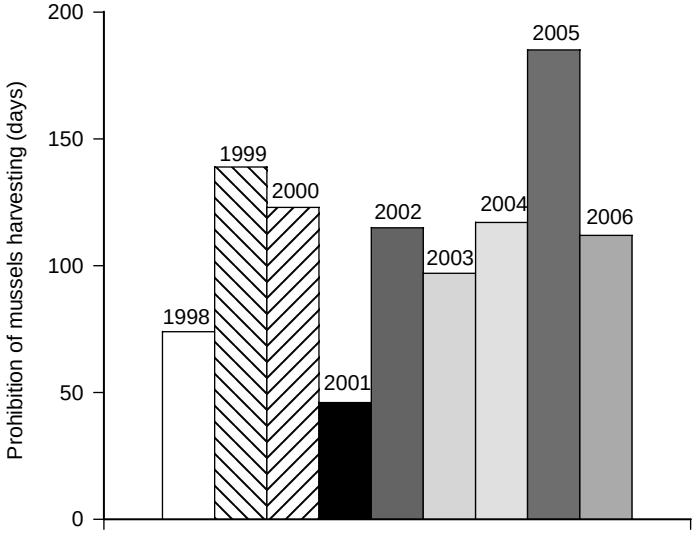


FIGURE 42.6 Days of mussels harvesting area closed from 2000 to 2006 in Grove C3.

Figures 42.8 through 42.11 show the same evolution of toxic episodes in different harvesting areas in Galicia: Vilagarcía A, Bueu B, Portonovo B, and Cangas F, respectively. 2005 was a specially virulent year, the worst of the century, because in Galicia there were toxic episodes caused by PSP toxins (the previous episode was 10 years ago), amnesic shellfish poisoning (ASP) toxins, and also DSP toxins. Christmas production, when producers get around 35–40% of their annual intakes, was in danger. In 2004, Galician mussels sector had an invoicing of 120 million €, in 2005 losses were around 40 million €, and then the invoicing was 80 million € because of the lack of mussels in the markets. This fact was also due to several aspects; when mussels arrived into the market they did in bulk and the prices went low, national and international, specially Italian market, were lost; this also meant loss of clients who decided to buy mussels in other countries. Loss of international markets have a medium or long-term effect since once a market is lost the recovery takes a long time and this consequence of toxic episodes cannot be easily quantified.

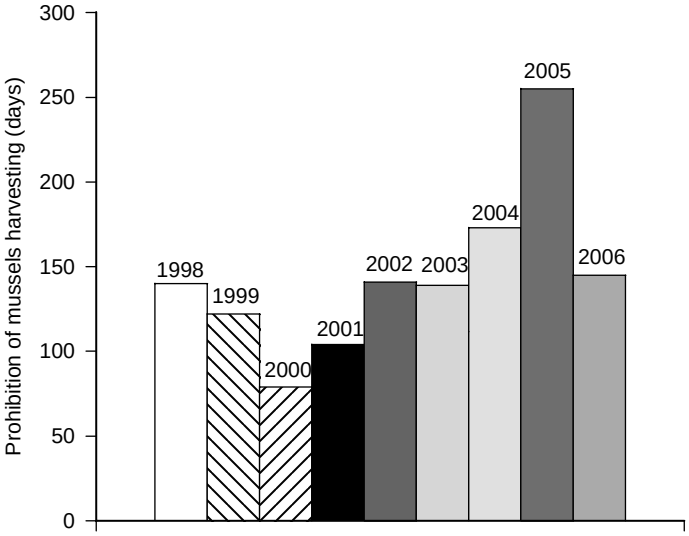


FIGURE 42.7 Days of mussels harvesting area closed from 2000 to 2006 in Grove C4.

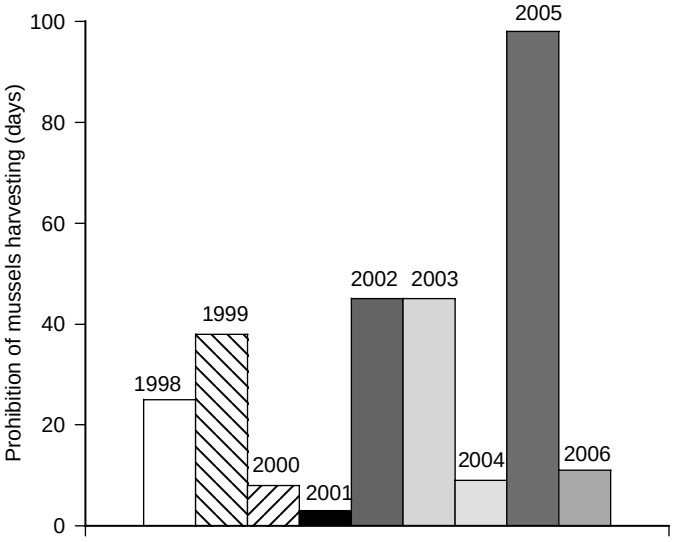


FIGURE 42.8 Days of mussels harvesting area closed from 2000 to 2006 in Vilagarcia A.

In addition, depuration plants, canning and cooking industries were almost with no activity. Some of these industries had to close temporarily, since they had no raw material to work with a lot of people who usually worked in these industries had no employment.

Other consequences of the toxic episodes is that the rafts have plenty of mussels which could not be harvested, so the problem starts with the seed that does have no space in the raft to attach and grow for the next year [4].

42.3 DETOXIFICATION OF SHELLFISH

Mitigation of harmful algal blooms (HABs) has been defined as a means taken to reduce HAB blooms or their effects [5]. Some research is focused on the design and implementation of effective

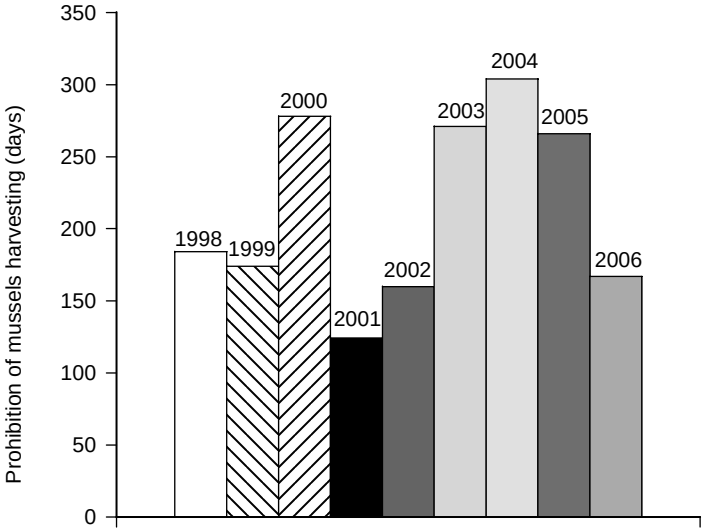


FIGURE 42.9 Days of mussels harvesting area closed from 2000 to 2006 in Bueu B.

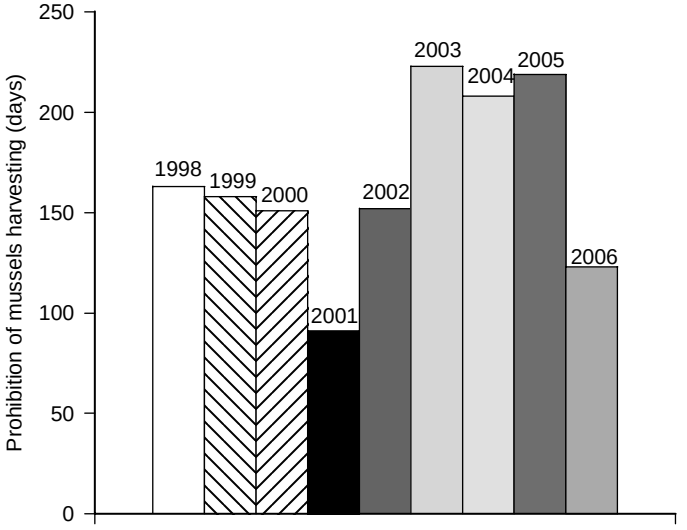


FIGURE 42.10 Days of mussels harvesting area closed from 2000 to 2006 in Portonovo B.

mitigation methods to minimize the economic impact of marine toxins. Several procedures are involved in studies on detoxification of shellfish.

42.3.1 Processing of Seafood

The industrial canning process can decrease the levels of PSP toxins present in some shellfish species, but it was only shown to be practical when the initial toxin levels were relatively low and usually it is necessary to eliminate some toxic tissues. The cockle, *Acanthocardia tuberculatum,* is harvested on the Mediterranean Coast of Spain and marketed exclusively as a canned product. In 1996, the Commission of European Communities (CEC) published a decision (91/492/EEC) that established conditions for harvesting and processing of certain bivalves from areas where PSP levels exceeded the 80 μg STXeq/100 g safety limit [6]. This decision applies specifically to

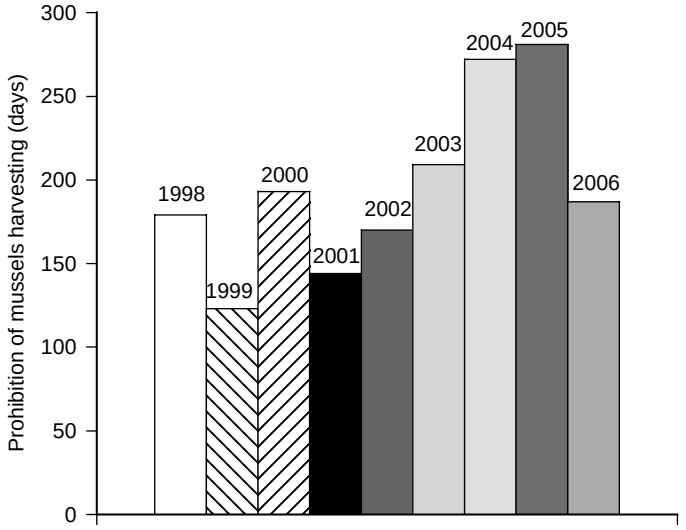


FIGURE 42.11 Days of mussels harvesting area closed from 2001 to 2006 in Cangas F.

A. tuberculatum and authorizes Spain to harvest cockles when toxicities in edible tissues are higher than 80 μg but lower than 300 μg STXeq/100 g. The decision was based on findings that heat treatment effectively reduced toxicity of cockles that must undergo processing defined in the Annex of the decision.

Harvesting of other molluscan shellfish for canning purposes is permitted in areas where scores are between 80 μg/100 g and 160 μg/100 g and the canned packs are tested before release for sale. On the Pacific Coast, butter clam canning has been permitted up to 300–500 μg/100 g, where it is done under permit and the neck has been removed and discarded [7].

The canning process was also shown to reduce toxicity levels in PSP-contaminated Japanese scallops [8,9] and mussels [10]. The high transfer of domoic acid from the scallops to the packing media rather than destruction of DA was also described, which posed a threat to consumers of this processed product [11]. In conclusion, processing methods must be carefully evaluated for each product and specific toxin to determine their effect on total toxicity and toxin distribution among tissues.

42.3.2 Detoxification by Chemical Agents

Ozonation is commonly used to depurate bivalves of bacterial pathogens, and can inactivate toxins from extracts of dinoflagellates and shellfish tissues. It was therefore promoted as a method for elimination of PSP toxins from whole organisms, although one of the main problems with the use of ozonated seawater is that it does not necessarily come into contact with toxins incorporated in tissues [12]. However, recent studies showed reduced toxicity of some toxins after ozone treatment [13,14]. Then, further studies should be done to investigate the applicability of this technique to detoxify shellfish.

A new chemical method for decontamination of PSP toxins was developed by Lagos et al. [15] that involves alkaline immersion of contaminated products followed by heating and washing steps. This method together with elimination of more toxic tissues was reported to yield 99% decontamination of Chilean mussels. However, data do not allow calculation of the relative contribution to decontamination of each processing step (alkaline treatment versus evisceration).

42.3.3 Biological Control

Bacterial flora of shellfish might play a role in their ability to inactivate, eliminate, or transform toxins. In this context, some bacterial isolates from bivalve guts were able to reduce toxicity or degradation of some PSP toxins [16] and ASP toxins [17]. However, these bacteriological methods should take

into account shellfish stocks, because of the significant differences in toxin accumulation rates detected among individuals of the same bivalve species [12].

42.3.4 Supercritical Carbon Dioxide

Supercritical carbon dioxide is an efficient sterilizing method in food and medicinal material, showing inactivating effect in bacteria, viruses, and even heat-resistant proteins.

A recent study describes the partial elimination or inactivation of the DSP toxin, okadaic acid, and its biological toxin activity after exposure to a supercritical mixture of carbon dioxide with acetic acid [18]. This study is the first experimental evidence that the denaturalizing and inactivating effect of some food-compatible supercritical mixtures could be useful in the inactivation of DSP toxins, opening up the possibility to investigate alternative low-temperature treatments to allow safe exploitation of DSP-contaminated shellfish.

42.3.5 Other Procedures

Ultrasonic irradiation, oxidation, or biodegradation of toxins across granular activated carbon filters are new effective and promising methods for shellfish detoxification. Some of these practical procedures combined or alone have shown a great potential to induce degradation or detoxification of several toxins [13,14,19–21].

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43 Toxin Monitoring Programs and Regulatory View

María L. Rodríguez-Velasco

CONTENTS

43.1 INTRODUCTION

As discussed in great detail through previous chapters in this book, different harmful algal species produce toxins that can be accumulated in shellfish causing both human illness and severe economic losses to industry. Once shellfish is contaminated, mitigation strategies are relatively limited. Most countries have implemented monitoring programs to protect public health and reduce economic impacts. The organization of these programs depends on the country but, normally, they include periodic monitoring of oceanographic and environmental conditions, toxic phytoplankton, and presence of toxins in seafood. In that sense, regulatory limits have been laid down for different groups of toxins to prevent the unsafe products being placed on the market. Figure 43.1 illustrates geographical distribution of countries with specific phycotoxin legislation and monitoring for toxic algae and/or shellfish in several coastal areas of the world.

However, as will be showed in this chapter, significant differences between countries are observed in the tolerance limits and in the analytical methods used to ensure they are complied. On the other hand, in most cases, regulatory limits are not based on solid scientific data. These differences should be eliminated for consistency of public health protection and for greater harmonization of international trade. These reasons justify the current efforts of the international community headed for the harmonization of the tolerance limits and the analytical methods for phycotoxins based on the scientific knowledge, and standards and reference materials.

43.2 WORLDWIDE REGULATIONS ON PHYCOTOXINS

Different countries have established food safety regulations that provide guidelines for the control of shellfish contamination by phycotoxins. In most cases, rules have been led by the presence of toxic events in their coasts or to deal with the requirements of the international trade with countries with legislation in force. A considerable number of countries have set legislation for paralytic shellfish

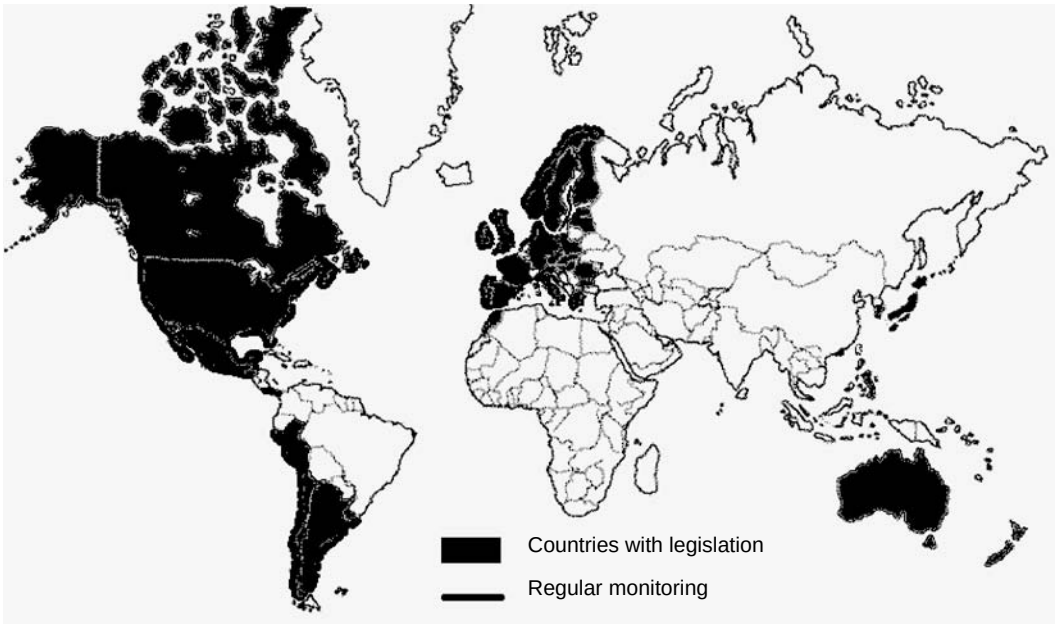

FIGURE 43.1 Geographical distribution of countries with phycotoxin legislation and monitoring for toxic algae and/or shellfish.

poison (PSP) toxins, the most dangerous for public health; a moderate number has established legislation for amnesic shellfish poison (ASP) and lipophilic toxins (including diarrheic shellfish poison (DSP) toxins, pectenotoxins (PTXs), and rarely, yessotoxins (YTXs) and azaspiracids (AZAs)). Those countries with the presence of harmful algae producing ciguatoxins or brevetoxins have provided limits and methods for their determination in seafood.

There are two major regional regulatory systems whose regulations require at least an equivalent system for those countries which export their shellfish into these areas; consequently, a lot of exporting countries, including developing countries, shall implement equivalent control systems.

European Union (EU): Regulation (EC) No 853/2004 of the European Parliament and of the Council [1], laying down specific hygiene rules for food of animal origin, specifies in Chapter V of Section VIII of Annex III, the required health standards for live bivalve molluscs including the maximum content in different marine biotoxins (PSP toxins, ASP toxins, and different lipophilic toxins: okadaic acid (OA), dinophysistoxins (DTXs), PTXs, YTXs, and AZAs). This regulation substitutes the previous Council Directive 91/492/EEC and the subsequent detailed rules for its implementation laid down by the Commission Decision 2002/225/EC. Later on, the Commission Regulation (EC) No 2074/2005 [2] specified in its Annex III the recognized testing methods for detecting marine biotoxins to check compliance with the limits laid down in Regulation (EC) No 853/2004. Identifying that bioassays are the reference methods for detecting certain toxins, this regulation establishes that elements of replacement, refinement, and reduction must be taken into account when biological methods are used, to be in accordance with the animal health protection Council Directive 86/609/EEC [3]. The regulation also states that those methods should be replaced as soon as possible by alternative detection methods, such as chemical methods and *in vitro* assays, once they have been demonstrated to be equivalent to the biological method for public health protection and their performance characteristics have been established through a validation study. Live and processed bivalve molluscs can be imported into EU countries from "approved" countries, whose legislation requirements, power of competent authorities, and inspection services must be at least equivalent to those governing the EU products [4].

Interstate Shellfish Sanitation Conference (ISSC): The ISSC is a tripartite cooperative program formed by the US Food and Drug Administration (USFDA), US state regulators, and shellfish industry representatives who develop policies for the safe harvesting, processing, and distribution of fresh and frozen shellfish. All the US states participating in the ISCC have been certified to harvest, process, and ship shellfish commercially. In addition, five foreign countries, Canada, Chile, South Korea, Mexico, and New Zealand, have a memoranda of understanding with the USFDA in which they agree to abide by the National Shellfish Sanitation Program (NSSP) policy [5].

43.2.1 PSP Toxins

A summary of countries known to have PSP regulations is given in Table 43.1. At present, there are about 45 countries (including the 27 Member States of the European Union) with specific legislation for PSP toxins. Some regulations are set for PSP as a group, although some countries have particular tolerance limits for one of the PSPs, mostly saxitoxin (STX). In some cases, requirements apply to shellfish, but others refer more generally to molluscs or more specifically bivalves as the types of

TABLE 43.1
Worldwide Regulations on PSP Toxins

Country	Toxin(s)	Product	Tolerance Level[a]	Reference Method	Exceptions
Argentina	STX	Molluscs	400 MU/100 g	Mouse bioassay	Snails to be canned: 1.6 mg STX eq/kg
Australia	STX	Shellfish	0.8 mg STX eq/kg	Mouse bioassay	
Canada	PSP	Molluscs	0.8 mg STX eq/kg	Mouse bioassay	Soft shell clams and mussels to be canned: 1.6 mg STX eq/kg Butter clams: with removed entire siphon: 3–5 mg STX eq/kg with removed distal half siphon: 0.8–3 mg STX eq/kg
Chile	STX	Molluscs	0.8 mg STX eq/kg	Mouse bioassay	
European Union	PSP	Bivalve molluscs	0.8 mg STX eq/kg	Mouse bioassay HPLC (alternative)	Spain: *Acanthocardia tuberculatum* to be canned: 3 mg/kg
Guatemala	STX	Molluscs	400 MU/100 g	Mouse bioassay	
Hong Kong	PSP	Shellfish	400 MU/100 g	Mouse bioassay	
Japan	PSP	Bivalves	400 MU/100 g	Mouse bioassay	
South Korea	GTX	Bivalves	400 MU/100 g	Mouse bioassay, HPLC	
Mexico	STX	Bivalve molluscs	0.8 mg STX eq/kg	Mouse bioassay	
Morocco	STX	Molluscs	0.8 mg STX eq/kg	Mouse bioassay	
New Zealand	PSP	Bivalve molluscan shellfish	0.8 mg STX eq/kg	Mouse bioassay	
Panama	PSP	Bivalves	400 MU/100 g	Mouse bioassay	
Philippines	STX		0.4 mg STX eq/kg	Mouse bioassay	
Peru	STX		0.8 mg STX eq/kg	Mouse bioassay	
Singapore	STX	Bivalves	0.8 mg STX eq/kg	Mouse bioassay	
United States	PSP	Bivalves	0.8 mg STX eq/kg	Mouse bioassay	Shellfish to be canned or eviscerated > 0.8 mg STX eq/kg
Uruguay	STX	Molluscs	0.8 mg STX eq/kg	Mouse bioassay	
Venezuela	STX	Molluscs	0.8 mg STX eq/kg	Mouse bioassay	

[a] For consistency of presentation, all action levels are expressed as MU (mouse unit)/100 g or mg STX eq/kg.

products for which tolerance levels of PSP are set [6–8]. Moreover, different concentration units are used to express the limits: mouse unit/100 g (MU/100 g), μg/100 g or mg/kg, normally expressed as equivalents of STX. To be consistent with the last international recommendations on biotoxins tolerance levels [9–11], the units used in Table 43.1 are presented as mg STX equivalents/kg.

Acceptable regulatory levels for PSP toxins range between 0.4 mg STX equivalents/kg and 0.8 mg STX equivalents/kg. A tolerance limit of 400 MU/100 g is observed in some countries, which corresponds to a value around 0.8 mg STX equivalents/kg, the limit agreed by most countries. It is important to remark on the necessity of international agreement on the units that should be used to express PSP toxicity by mouse bioassay (MBA), depending on the standard used for calibration. The tendency is to express results as milligram of STX dihydrochloride/kilogram, as it was agreed at European level by the network of national reference laboratories for marine biotoxins (EU-NRLs) [12].

Some countries have established exceptions to proposed limits depending on the form of consumption of the shellfish and based on the effectiveness of some procedures (mainly evisceration and canning) in reducing PSP toxicity [8,13–15]. Thus, in Argentina, snails to be canned may contain up to 1.6 mg STX equivalents/kg [16]. In the same way, in Canada, products (soft shell calms and mussels) with levels between 0.8 mg STX equivalents/kg and 1.6 mg STX equivalents/kg may be canned [17]. Moreover, butter clams may be marketed containing 0.8–3 mg STX equivalents/kg after removing the distal half of the siphon or 3–5 mg STX equivalents/kg after removing the entire siphon [16]. In the case of EU legislation, there is one exception provided by the Commission Decision 96/77/EC [18] that applies only to the bivalve molluscs belonging to the species *Acanthocardia tuberculatum* (a Mediterranean cockle) destined for canning and allows Spain to authorize harvesting when PSP levels in the edible parts exceed 0.8 mg STX equivalents/kg but are less than 3 mg STX equivalents/kg. In this case, the bivalves must undergo a heat treatment defined in the Annex of the Decision and the final product must not contain a PSP level detectable by the bioassay method. Finally, in the United States of America, shellfish with PSP levels over 0.8 mg STX equivalents/kg destined for canning or subjected to evisceration may be harvested [16].

The main method used for regulatory purposes is the MBA, normally that published as 959.08 Official Method by the Association of Official Analytical Chemists (AOAC) [19]. This method is internationally recognized for quantifying PSP toxicity and has been in use for more than 50 years in many countries.

In the past few decades, different chemical analytical alternatives to the MBA have been developed for most of the common PSP toxin analogues found in shellfish and seafood (Chapter 8). However, among the possible alternatives, just one high performance liquid chromatography method with fluorescence detection (HPLC-FLD) has been validated through a collaborative trial [20] and adopted as AOAC Official method 2005.06 [21]. The "fitness for purpose" of this method for official control of PSP toxins was evaluated at European level during an interlaboratory study organized by the Community Reference Laboratory for Marine Biotoxins (CRLMB) [22]. On the basis of that work, the Commission Regulation (EC) No. 2074/2005 amended by Commission Regulation (EC) No. 1664/2006 [23] allows the use of the described method as an alternative method for the detection of those PSP toxins as published AOAC Official Method 2005.06. European legislation already states that if the results are challenged the reference method shall be the biological method.

43.2.2 ASP Toxins

A summary of the worldwide situation of the legislation on ASP toxins is presented in Table 43.2. Since the first toxic outbreak related to these toxins in Canada in 1987, a few countries have established regulations for this group of toxins. All of them have a uniform tolerance level of 20 mg/kg of domoic acid (DA) in the edible meat.

In order to minimize the economic impact owing to the slow ASP detoxification process for certain pectinidae, the European Union allows, through the Commission Decision 2002/226/EC[24], the harvesting of bivalve molluscs belonging to the scallops species *Pecten maximus* and *Pecten jacobeus* with

TABLE 43.2
Worldwide Regulations on ASP Toxins

Country	Toxin(s)	Product	Tolerance Level (mg/kg)	Reference Method	Exceptions
Australia	Domoic acid	Shellfish	20	LC-MS	
Canada	Domoic acid	Shellfish	20	HPLC-UVD	
Chile	Domoic acid	Bivalve molluscs	20	HPLC-UVD	
European Union	Domoic acid	Bivalve molluscs	20	HPLC-UVD	*Pecten maximus* and *Pecten jacobeus*: 250 mg/kg whole body (to be consumed without hepatopancreas)
Mexico	Domoic acid	Bivalves	20	HPLC-UVD	
New Zealand	Domoic acid	Bivalve molluscan shellfish	20	HPLC-UVD LC-MS	
Peru	Domoic acid	Bivalves	20	HPLC-UVD	
United States	Domoic acid	Bivalves	20 (not official)	HPLC-UVD	Cooked crab (viscera and hepatopancreas): 30 mg/kg

a DA level in the whole body exceeding 20 mg/kg if two consecutive analyses, taken between 1 and 7 days maximum, show that the DA concentration in the whole mollusc is lower than 250 mg/kg, and that the DA concentration in the parts intended for human consumption (adductor muscle and gonads), and analyzed separately, is lower than 4.6 mg/kg. The analyses of the entire body have to be performed on a homogenate of ten molluscs and the analysis on the edible parts on a homogenate of ten individual parts. In addition to the conditions above, supplementary requirements concerning sampling, transport conditions, and authorized establishments have also been stated in the Decision. Finally, after total removal of the hepatopancreas, soft tissues, and any other contaminated parts, the adductor muscle and gonads intended for human consumption must not contain an ASP level above 20 mg DA/kg.

In the United States, the FDA has set a guideline level of 30 mg DA/kg for cooked crabs (viscera and hepatopancreas) [17].

The analytical situation for DA is much better than for other phycotoxins groups and a HPLC method with ultraviolet detection (HPLC-UVD) [25–27] is used in most countries for official control. A liquid chromatography-mass spectrometry (LC-MS) method is being used in Australia and New Zealand for regulatory purposes [28, 29].

A commercial enzyme-linked immunosorbent assay (ELISA) has recently been approved as AOAC Official Method 2006.02 for the detection of DA in shellfish [30].

43.2.3 Lipophilic Toxins (DSP Toxins, Pectenotoxins, Yessotoxins, and Azaspiracids)

Among all the groups of marine biotoxins, the liposoluble toxins group is the subject of greatest controversy. Four groups have been traditionally included in this group: DSP group (OA and DTXs), PTXs, YTXs, and AZAs. These compounds share solubility properties so that neither extraction procedures nor conventional MBAs discriminate between. However, their biological activity and toxicological properties are significantly different. On the other hand, there is no general consensus on which liposoluble toxins should be regarded as DSP toxins, which ones should be monitored and regulated, and which are the most appropriate testing procedures and acceptable levels [8]. For instance, although traditionally PTXs are included in the DSP group they do not belong in this group since they are not phosphatase inhibitors; so experts recommend that a separate regulatory limit for PTXs should be set [31].

Several countries have proposed regulations for some lipophilic toxins groups (Table 43.3). The first country to establish a limit was Japan for DSP, 5 MU/100 g, based on an epidemiological

TABLE 43.3
Worldwide Regulations on Lipophilic Toxins

Country	Toxin(s)[a]	Product	Tolerance Level[b]	Reference Method
Australia	DSP toxins	Shellfish	0.2 mg OA eq/kg	Mouse bioassay LC-MS
Chile	OA, DTXs, and PTXs together YTXs AZAs	Bivalve molluscs	0.16 mg OA eq/kg 1 mg YTX eq/kg 0.16 mg AZA eq/kg	Mouse bioassay
European Union	OA, DTXs, and PTXs together YTXs AZAs	Bivalve molluscs	0.16 mg OA eq/kg 1 mg YTX eq/kg 0.16 mg AZA eq/kg	Mouse bioassay
Japan	DSP toxins	Bivalves	5 MU/100 g	Mouse bioassay
South Korea	DSP toxins	Shellfish	5 MU/100 g	Mouse bioassay
New Zealand	OA, DTXs, and PTXs together YTXs AZAs	Bivalve molluscan shellfish	0.16 mg OA eq/kg[c] 1 mg YTX eq/kg[d] 0.16 mg AZA eq/kg[e]	Mouse bioassay LC-MS

[a] DSP: Diarrhetic shellfish poison; OA: Okadaic acid; DTXs: Dinophysistoxins; PTXs: Pectenotoxins; YTXs: Yessotoxins; and AZAs: Azaspiracids.

[b] For consistency of presentation, all action levels are expressed as mg toxin eq/kg.

[c] LC-MS analysis must include determination of OA and DTXs (including a hydrolysis step in order to detect the presence of esterified forms) and PTXs (that include PTX1 and PTX2).

[d] LC-MS analysis must include determination of YTX, 45 OH YTX, homo YTX, and 45 OH-homo YTX.

[e] LC-MS analysis must include determination of AZA1, AZA2, and AZA3.

study [32]. Most countries applying MBA use survival time for the determination of the toxicity. The commonly agreed criteria is that when "two out of three mice die within 24 h," it constitutes a positive result, which corresponds to a level of 0.16 mg OA equivalents/kg, 1 mg YTX equivalents/kg, or 0.16 mg AZA equivalents/kg. In this case, the MBA behaves as a semiquantitative method.

In the EU, Commission Regulation (EC) No 2074/2005 indicates the biological and alternative detection methods that can be used for the analysis of lipophilic toxins. With regard to biological methods, the regulation states that a series of MBA procedures, differing in the test portion (hepatopancreas or whole body) and in the solvents used for extraction and purification, may be used, indicating that sensitivity and selectivity depend on the choice of solvents used for extraction and purification and that this should be take into account when a decision is made on the method to be used in order to cover the full range of toxins. Legislation does not specify a detailed protocol for the MBA (i.e., mouse strain and weight, volume of extraction solvent, volume of liquid/liquid partition organic solvent) leading to the application of different conditions and a lack of harmonization. Thus, several main protocols are available, mostly based on methods developed by Yasumoto [32–34].

Regulation (EC) No. 2074/2005 also states that alternative detection methods, such as HPLC with fluorimetric detection, liquid chromatography (LC), mass spectrometry (MS), immunoassays and functional assays such as the phosphatase inhibition assay, can be used as alternatives or supplementary to the biological testing methods, provided that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection. The compounds to be detected are OA and DTXs (a hydrolysis step may be required to detect the presence of DTX3); PTX-1 and PTX-2; YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX and AZA1, AZA2 and AZA3. It is declared that biological methods shall be replaced by alternative detecting methods as soon as reference materials for detecting the toxins regulated are readily available and the methods have been validated following an internationally agreed protocol. At the time being, no method has achieved such a status.

TABLE 43.4
Worldwide Regulations on NSP Toxins

Country	Toxin(s)	Product	Tolerance Level	Reference Method
Mexico	Brevetoxin	Shellfish	20 MU/100 g	Mouse bioassay
New Zealand	NSP toxins	Bivalve molluscan shellfish	20 MU/100 g	Mouse bioassay
United States	Brevetoxin	Shellfish	20 MU/100 g	Mouse bioassay

In Australia and New Zealand a multitoxin LC-MS method is used for detecting the lipophilic toxins [28, 29]. Canada and the USA have no official regulation on lipophilic toxins [8].

Finally, it should be borne in mind that the full implementation of chemical and *in vitro* assays for this group of toxins requires a huge effort to develop toxins standards, toxicological studies, and validation of methods, which represent an important step toward harmonizing phycotoxin control approaches.

43.2.4 Neurotoxic Shellfish Poisoning—Brevetoxins

Only those countries affected directly by toxic events related to brevetoxins have established specific regulations (Table 43.4). Currently Mexico, New Zealand, and the United States agree on an acceptable level of 20 MU/100 g.

The testing procedure widely used is the MBA and, specifically, a modified version of that published by the American Public Health Association (APHA) in 1985 [35,36]. ELISA and LC-MS methodologies have been developed to detect a wide range of brevetoxins and identified as alternative candidates to replace bioassay once fully validated [10].

European Union legislation makes no mention of neurotoxic shellfish poisoning (NSP) or brevetoxins, as they have not been reported to occur in significant levels in European coastal waters. However, some countries perform monitoring programs for algal toxic species producing this kind of toxins. This is the case in Denmark, where with a level of 5×10^5 cells/L of *Gymnodinium* spp. (depending on species) fishery products harvesting areas are closed [6,17]. In Italy, the provision of the law is based on the MBA and established "not detectable" in shellfish [6,37].

43.2.5 Ciguatera Toxins

Despite frequent occurrences of ciguatera toxins in many parts of the world, this poisoning is rarely fatal, because of the low concentration of the toxin in fish flesh. Maybe for this reason very few specific regulations exist for ciguatera toxins [6]. In some areas, public health measures have been taken that include bans on the sale of high risk fish from known toxic locations. Such bans have been used in American Samoa, Queensland, French Polynesia, Fiji, Hawaii, and Miami. The bans were apparently with some success but with attendant economic loss [38].

In the European Union, Regulation (EC) No. 853/2004 (Annex III, Section VIII, Fishery Products) establishes that fishery products containing biotoxins such as ciguatoxin or muscle-paralyzing toxins must not be placed on the market. No information about analytical methods is given. In France, there is legislation applicable for products imported from outside the European Union that permits the import of certain marine fish species for which positive list exists [38].

43.2.6 Management of New Toxins

New phycotoxins are regularly discovered, for example, during intoxication events, or known toxins appear in molluscs, which require attention of the scientific community to develop analytical methods and reference materials and of the public health authorities to set regulatory limits for the presence of those toxins.

Advice has been provided by the scientific community [9] on management of new toxins and newly discovered analogues of existing toxins where either there is no epidemiological evidence of illness resulting or where epidemiological evidence exists.

In summary, for new classes of compounds, the recommendation is that either the situation may be identified (human illness outbreak, new species or strain of algae or new symptoms of toxicity in mouse test), toxicological evaluation of the crude toxin extracts made or, ideally, isolated toxic compound should be carried out in order to assess the risk before deciding not to regulate or set a tolerable level.

For toxins for which adequate structure–activity data are available, a decision with regard to regulation can be made on the basis of structure. When no adequate information is available, decision will depend on the concentration in which the new compound is present in the sample related to the parent compound.

The situation described could be applicable to the management of some toxins, like some cyclic imines (gymnodimine and spirolides) or toxins produced by *Ostreopsis* spp. (palytoxins) that have been reported to be found in shellfish products and have demonstrated high toxicity in mice (Parts VIIID and IX).

43.3 MONITORING PROGRAMS

Once having established a regulatory basis, a large number of countries worldwide have organized phycotoxin monitoring programs to prevent risks for public health and minimize economic losses for aquaculture and fisheries [28,39–51].

The organization of the monitoring program and its components will depend on the country or geographical area. Normally, the programs include both surveillance for potential toxic algal species and monitoring of toxins in shellfish. In this case, results obtained by recognized testing procedures are compared with the regulated levels in order to prevent unsafe products being placed on the market. In addition, some countries complement phytoplankton and shellfish toxicity data with the monitoring of different environmental parameters that together with the weather and oceanographic conditions constitute a valuable prediction tool. Among such environmental parameters, there are physical (temperature, current and wind speed and direction, turbidity), chemical (salinity, oxygen content, chlorophyll, nutrients), and biological (phytoplankton, meso-zooplankton, proto-zooplankton, pelagic bacteria, fish, benthos, birds) factors [52]. These variables may indicate that favorable conditions for a toxic event are developing.

A marine biotoxin management program should be described in a marine biotoxin management plan (MBMP). The MBMP should include marine biotoxin action plans for growing areas containing, for instance, sampling strategy and requirements (frequency, sample size, and composition), analysis to be carried out, and management action to be based on monitoring results and expert judgment [9]. In that sense, a microalgal and shellfish sampling protocol over time and space should include adequate location and number of sampling sites. Sampling frequency must be sufficient to address spatial-temporal changes in microalgae, toxins in shellfish, and to cover the risks of rapid rises in shellfish toxicity. Taking into account the changing nature of phytoplankton populations, sampling should be carried out at least weekly, intensifying during the development of a toxic episode [53]. Table 43.5 shows some examples of indicator microalgal species for the different phycotoxins groups.

At this point, it is important to remark that toxicity monitoring in seafood cannot be replaced solely by phytoplankton monitoring. Nevertheless, it is a valuable complementary tool that can be used, in combination with the required monitoring of marine biotoxins in shellfish tissue, to optimize program management and resources. Moreover, it provides complementary information on trends in toxic phytoplankton abundance that may be used as an early warning of impending marine biotoxin accumulation in shellfish and as a guide for determining the frequency of shellfish sampling [10]. It can also focus attention on potential toxins for analysis that might otherwise not be detected or provide information on which algae are responsible if a new biotoxin event occurs. In short, phytoplankton monitoring can guide management decisions on protecting consumer safety.

TABLE 43.5
Examples of Source Indicator Organisms for Some of the Toxins Groups

Toxin Group	Genus	Example Species
PSP toxins	*Alexandrium*	*tamarense, minutum, catenella*
	Gymnodinium	*catenatum*
	Pyrodinium	*bahamense*
Domoic acid	*Pseudo-nitzschia*	*australis, seriata, pungens, multiseries*
DSP toxins	*Dinophysis*	*acuta, acuminate, sacculus, fortii, caudate*
	Phalachroma	*rotundatum*
	Prorocentrum	*lima*
Yessotoxins	*Protoceratium*	*Reticulatum*
	Lingulodinium	*polyedrum*
Azaspiracids	*Protoperidinium*	spp.
Brevetoxins	*Karenia*	*brevis*
Ciguatera toxins	*Gambierdiscus*	*toxicus*
Cyclic Imines	*Alexandrium*	*ostenfeldii, peruvianum (for spirolides)*
Palytoxins	*Ostreopsis*	spp.
	Coolia	spp.

On the other hand, concerning the use in the MBMP of indicator shellfish species for each toxin group, it could be problematic because the rate of toxin uptake and depuration is unique to the combination of species, toxin, and geographic location. For that, it is important to note that using an indicator shellfish species, the absence of toxicity in the indicator species is assumed to imply the absence of toxicity in other species in the growing area. This implication should be verified for each shellfish species and for each group of toxins before defining a particular shellfish species as an indicator for that growing area [9, 10].

43.4 TOWARD HARMONIZATION

When reviewing regulations in force discrepancies in monitoring and testing procedures as well as in action limits indicate a need for harmonization in the interest of an equal consideration for public health protection and international trade of seafood products.

In establishing regulatory criteria and limits for microalgal toxins, various factors play a role such as the availability of survey data, the availability of toxicological data, the distribution of the toxins throughout sampled lots and the stability in the samples, the availability of analytical methods and regulations already in force in several countries.

With respect to toxicity, in most cases, tolerance levels do not seem to be based on risk evaluation. To make such risk evaluations possible, more data on occurrence and toxicity of phycotoxins is needed to evaluate the real risk for consumers in order to set suitable tolerance limits. Moreover, until now only data on the acute oral toxicity both in experimental animals and in humans are available for the majority of the toxins. However, studies on repeated exposure to lower sublethal dose levels are desirable.

Concerning detection methods, there is a general worldwide need for cost-effective, reliable, and sensitive methods to determine marine toxins in shellfish. The present MBA, apart from not being fully harmonized in some cases, is not sensitive enough, shows considerable variation, is time consuming, is vulnerable to interferences, and is unethical in terms of animal welfare. Methodology based on LC-MS, biosensors, or functional assays is being developed but still have not probed its efficiency to protect public health in equal measure than biological methods.

By far, the main difficulties found by researchers and technicians working on the field of marine toxins are the lack of available and enough standards and reference materials. Clearly, the lack of

standards is a limiting factor for everything else, from toxicological and pharmacological evaluation of a toxin to its identification or development of methods for quantitative determination.

One other issue, also linked to the lack of standards, is the need of internationally validated methods. Several approaches can be used to validate a method, but the single-laboratory validation [54] approach is not a reliable procedure for trading and food safety, since it is important to assure the solidity of results and their equivalence among different laboratories [55]. Therefore, international validation with collaborative studies is a demand in phycotoxins monitoring [56,57].

Several international institutions are currently doing significant work to improve the current status of microalgal toxins control:

1. EU-CRLMB (AESAN, Spain, and DG SANCO, EU) carrying out different approaches in the validation of analytical and functional methods for toxins determination as well as initiatives to get standards and reference materials.
2. European Center for the Validation of Alternative Methods (ECVAM, Joint Research Center (JRC), EU) [58].
3. AOAC [59,60].
4. Asia Pacific Economic Cooperation (APEC) [61].
5. Codex Alimentarius, within the Joint FAO/WHO Food Standards Program [5]. The Codex Committee on Fish and Fishery Products (CCFFP) is discussing a Proposed Draft Standard for Live and Raw Bivalve Molluscs [11]. To provide the basis for Committee's actions in this regards, the CCFFP has requested scientific advice from an Expert Consultation [9] and a Working Group [10]. In the hygiene and handling section of the Standard, there are different proposals of maximum levels for different toxin groups and in the sampling, examination, and analyses section, different methodologies are proposed for their determination. Methods are classified as type II (reference method) or type III (alternative approved method), according to Codex classification of methods of analysis. Table 43.6

TABLE 43.6
Proposed Levels and Methods for Marine Biotoxins by Codex Proposed Draft Standard for Live and Raw Bivalve Molluscs at Step 5 of the Procedure

Toxin	Maximum Level	Methodology—Type II	Methodology—Type III
Saxitoxin group	0.8 mg STX 2HCl/kg	LC-FL (AOAC official method 2005.06)	Bioassay (AOAC official method) Receptor Binding Assay Immunochemical LC-MS[a]
Okadaic acid group	0.16 mg OA eq/kg	LC-MS[a]	Bioassay[a,b] PP2A[a] LC-FL ELISA[a]
Domoic acid group	20 mg DA/kg	LC-UVD	ELISA LC-MS LFIC[a]
Brevetoxin group	20 MU or equivalent	LC-MS[a]	Bioassay (APHA mouse bioassay) ELISA
Azaspiracid group	0.16 mg/kg	LC-MS[a]	Bioassay[1]

[a] Further method development needed.

[b] When using the mouse bioassay for detecting lipophilic marine biotoxins, false positives may occur due to the presence of other substances such as yessotoxins, pectenotoxins, and cyclic imines, which are not know to cause human illness. When false positives are suspected, confirmatory testing, using an internationally validated method, can be carried out in order to identify the type(s) of marine biotoxins present.

shows proposed maximum levels and methodologies for different toxin groups by the Codex Proposed Draft Standard for Live and Raw Bivalve Molluscs at step 5 of the procedure.

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Food Science and Technology

Seafood and Freshwater Toxins

Pharmacology, Physiology, and Detection

Encompasses the expanding scope of the field

Marine and freshwater toxins are a rapidly changing problem. Expanding international commerce forces cargo ships into virgin territory, deforestation and pollution violate the natural ecological balance, and a changing climate holds unknown potential to alter current factors and trigger toxic blooms in new forms, at new rates, and in new places. Happily, with notable advances in analysis technology, the body of knowledge in the field is equally dynamic. In just six years since the first edition, toxins that warranted only line listings, including pfiestra, gambierol, and polycavernoside, are now worthy of entire chapters, requiring a new edition to encompass the expanding scope of the field.

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