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Biotoxin contamination and shellfish safety

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Abstract: Some phytoplankton species can produce biotoxins. As molluscan shellfish filter-feed on these toxic species, they bioaccumulate the phycotoxins, which can be transferred to higher trophic levels, resulting in several types of poisoning syndromes. The main shellfish-mediated, phycotoxin-associated, human poisoning syndromes are paralytic, amnesic, neurotoxic, diarrheic and azaspirazid shellfish poisonings; a few other phytoplankton species can also cause harmful effects in humans. This chapter lists the major phycotoxins described, their origins, occurrences, and impact on human health as well as management procedures developed to address them. The trophic dynamics of the phycotoxins are also discussed, as is their bioaccumulation, biotransformation, and detoxification processes.

Key words: shellfish, toxins, harmful algal blooms.

2.1 Introduction

The potential for molluscan shellfish to contain toxins affecting human consumers has been recognized for all of human history. Native American taboos against eating shellfish from waters affected by toxic phytoplankton blooms presaged the often-recounted story of Captain Vancouver's crew's 1793 fatal experience with biotoxin-contaminated shellfish (Dale and Yentsch, 1978). Seasonality in toxicity led to recognition that specific microalgae eaten by molluscs are the origins of many such toxins (Shumway, 1990). Further, monitoring and surveillance programs implemented to protect human consumers of shellfish have led to the recognition of additional toxins not previously known

(e.g., azospiracid) and provided convincing evidence that microalgal blooms leading to biotoxin-contaminated seafood are increasing in geographic distribution and severity (Hallegraeff, 2004).

The presence of toxicity in shellfish is the ultimate result of a complex cascade of environmental, biological, physiological, and biochemical interactions. Protection of human consumers of shellfish has focused research and management on all aspects of this cascade of interactions. Research has presented the management community with options for consumer protection, and these options often are context-specific; i.e., specific for a given shellfish species or a specific biotoxin. Thus, a review of biotoxin contamination and shellfish safety requires description of general principles and then application of these principles to specific instances.

There are approximately 4000 described species of marine microalgae, or phytoplankton, but only a few (70–80 species, about 2%) produce toxic compounds (Scoging, 1998). These harmful algae are natural and have been part of the ecosystem for millennia. As human activities have increased, incidence and severity of harmful algal blooms (HABs) have also increased and so have risks for human exposure (Hallegraeff, 2004).

Generically, a phytoplankton 'bloom' refers to the development of a microalgal population in a given water mass (Smayda, 1997). The maximal population density of a toxic bloom can vary from 200 cells/ml to more than 50 000 cells/ml, depending on the species of toxic algae and the conditions of the bloom. Certain species of toxic phytoplankton contain red or brown pigmentation; if the concentration of the bloom is high enough, a bloom can become visible and thus described as a 'red tide' or a 'brown tide' (Shumway, 1990). Proliferation of harmful algae does not necessarily impart color to the water and, conversely, harmless algae can also discolor water masses.

Chemical compounds produced by phytoplankton, collectively termed phycotoxins, potentially are responsible for poisoning incidents worldwide. These incidents can trigger disease and death of marine life (Landsberg, 2002) and are also a threat for human intoxication or death if contaminated seafoods are ingested or aerosols are inhaled. The types of intoxication or disease associated with specific harmful algae are diverse, and several seafood-poisoning syndromes have been described, depending upon the type of phycotoxin to which humans are exposed (Table 2.1). Although these toxic syndromes can result from the consumption of finfish or shellfish, this review will focus only on the phycotoxins present in shellfish.

The main shellfish-mediated, phycotoxin-associated, seafood poisoning syndromes are: paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), diarrheic shellfish poisoning (DSP) and azaspirazid shellfish poisoning (AZP). A few other phytoplankton species can cause harmful effects in humans, but are not included in the aforementioned, symptom-based syndromes, e.g. phytoplankton species including *Pfiesteria* spp. and *Alexandrium monilatum*.

Bivalves are filter feeders that, as they consume phytoplankton, can

Table 2.1 Origins of phycotoxins

Disease	Causative organisms	Major toxins	Bioactive mechanism	Acute symptoms	Chronic symptoms	Diagnostic
Paralytic shellfish poisoning (PSP)	Pelagic dinoflagellates *Alexandrium* spp. *Gymnodinium catenatum* *Pyrodinium bahamense*	Saxitoxins Gonyautoxins C-Toxins	Na^+ Channel Blocker: neurotoxins bind to site 1 on the voltage-dependent sodium channel, blocking the influx of sodium into excitable cells and restricting signal transmission between neurons	Tingling and numbness in mouth, ataxia, dizziness, headache, respiratory distress and muscular paralysis Nausea, vomiting, diarrhea, paraesthesia, respiratory depression	None known	Clinical, mouse bioassay of food, HPLC
Neurotoxic shellfish poisoning (NSP)	Pelagic dinoflagellates and raphidophytes *Karenia* spp.	Brevetoxins and brevetoxin-like compounds	Na^+ channel blocker: binds to site 5 on the sodium voltage-sensitive channels and alters properties of excitable cells by shifting activation to more negative potentials, triggering membrane depolarization	Nausea, vomiting, diarrhea, bronchoconstriction, reversal of temperature sensations, paraesthesia	None known	Clinical, mouse bioassay, HPLC, ELISA
Amnesic shellfish poisoning (ASP)	Pelagic diatoms *Pseudo-nitzschia* spp.	Domoic acid	Glutamate receptor agonist: binds to kainite-type glutamate receptors in the brain, functioning as an excitatory neurotransmitter causing depolarisation of neurons, followed by calcium ion influx, neuronal swelling, and cell death	Nausea, vomiting, diarrhea, amnesia, paraesthesia, respiratory depression	Amnesia	Clinical, mouse bioassay of food, HPLC

Table 2.1 Origins of phycotoxins

Disease	Causative organisms	Major toxins	Bioactive mechanism	Acute symptoms	Chronic symptoms	Diagnostic
Diarrheic shellfish poisoning (DSP)	Pelagic or benthic dinoflagellates *Dinophysis* spp., *Prorocentrum lima*	Okadaic acid Dinophysis-toxins Pectenotoxins and yessotoxins	Protein phosphatase type 1 and 2A inhibitor: increases protein phosphorylation Unknown mechanisms for PTX and YTX	Gastrointestinal distress, nausea, vomiting, diarrhea	None known	Clinical, mouse bioassay, HPLC, ELISA
Azaspirazid shellfish poisoning (AZP)	Pelagic dinoflagellate *Protoperidinium* sp.	Azaspiracid	Unknown mechanism	Nausea, vomiting, severe diarrhea (i.e., similar symptoms to DSP) and stomach cramps		
Additional taxa implicated in human poisoning	*Pfiesteria* spp.	Radical forming toxic organic-ligated metal complex	Newly documented carbon-sulfur-metal-based radical production			
	Prorocentrum minimum	Uncharac-terized	Human poisoning not confirmed			
	Prorocentrum micans	Uncharac-terized	Human poisoning not confirmed			
	Alexandrium monilatum	Goniodomin A	Can change its conformation which might alter the actomyosin ATPase activity			

HPLC, high-performance liquid chromatography.
ELISA, enzyme-linked immunosorbent assay.
Details compiled from Shumway (1990), Landsberg (2002), and Hallegraeff (2004).

accumulate toxins within tissues, along with nutritional compounds associated with the phytoplankton. As little as 6 hours of filtration of toxic microalgae by bivalves can be enough for the shellfish to become toxic to human consumers (Scoging, 1998). Shellfish accumulate microalgal biotoxins generally in the digestive gland (hepatopancreas), but also in other tissues. Toxin retention time varies according to the group of toxins, the tissues in which the toxins are located, and also the shellfish species (Schantz, 1984).

Very effective and protective monitoring and regulatory mechanisms have been established to limit human risk of exposure to toxic shellfish in many countries (Shumway *et al.*, 1988; Smayda, 2004; Andersen *et al.*, 2004; Todd, 2004; Fernández and Shumway, 2004; Backer *et al.*, 2004). Thus, public health relies on biotoxin monitoring programs, which generally close shellfish-harvesting areas when shellfish are likely to be contaminated. In non-industrialized countries, the monitoring programs are usually not as well developed, and more cases of intoxication can or do occur. Monitoring programs can be based upon one of two fundamental approaches: either by monitoring the water for presence of harmful algal taxa, or monitoring shellfish for toxicity. (See Chapter 5, this volume for a more detailed discussion on monitoring programs and regulations.)

2.2 Origins of phycotoxins

There are several types of biotoxins produced by various phytoplankton species. Phycotoxins are accumulated and can sometimes be metabolized by shellfish. These compounds can be noxious or lethal to humans. The toxins are listed in Table 2.1; included are compounds in the saxitoxin (STX) family (20 different toxins, including STX, NeoSTX, GTX) responsible for PSPs. Okadaic acid (OA), the dinophysistoxins (DTX), the pectenotoxins (PTX), and yessotoxins (YTX) are responsible for DSP; NSP is attributed to the exposure of shellfish to a group of polyethers called brevetoxins. ASP is caused by an amino acid, domoic acid, as the contaminant in shellfish. Finally, azaspiracid toxins are responsible for AZA. Some algal species also produce toxins that have not yet been identified or fully characterized chemically, such as *Prorocentrum minimum, Prorocentrum micans*, *Pfiesteria* spp., and *Alexandrium monilatum*. Human sensitivity to microalgal biotoxins varies among individuals; recorded intoxication levels vary between 144 and 1660 μg per person, and lethal quantities can reach 300 to 12 400 μg pr person (Van Egmond *et al.*, 1993).

PSTs are produced by dinoflagellate species, such as *Alexandrium* species, *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum,* and by freshwater cyanobacteria such as *Anabeana circinalis*, *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii*, *Lymgbya wollei*, and *Planktothrix* sp. These species produce a group of toxins referred to as saxitoxins and saxitoxin derivatives. Saxitoxin was first isolated from toxic Washington butterclams, *Saxidomus gigantea* (Schantz *et al.*, 1957; Schantz, 1960) harvested in the state of Washington (western North America), hence the name. *Alexandrium*

(formerly *Gonyaulax* and *Protogonyaulax*) *catenella* (Whedon et Foid) Balech, was the first dinoflagellate to be associated with PSP toxins. Indeed, 102 people were sickened and six people were killed after ingestion of shellfish exposed to *A. catenella* near San Francisco in 1927 (Sommer and Meyer, 1937). Since then, Scoging (1998) reported about 1600 annual cases of PSP worldwide, of which about 300 can be attributed to *Alexandrium*.

The two major species of dinoflagellates responsible for PSP exposure in humans are *Alexandrium catenella* and *A. tamarense* (Shumway *et al.*, 1988; Landsberg, 2002). These species are present in different regions and in different concentrations. Additionally, toxin composition can vary drastically from one species or strain to the next, according to geographic origin, environmental conditions, or culture conditions (Cembella *et al.*, 1988; Anderson, 1990; Anderson *et al.*, 1990, 1994). As toxin compositions and concentrations in the various toxin-producing dinoflagellates differ according to all these criteria, the amount of toxin to which any animal is exposed will differ as well (Landsberg, 1996). Blooms of toxic algae producing PSTs occur worldwide; they have been recorded throughout Europe, on coasts of South and North American continents, and in coastal Africa, as well as throughout the Asian continent and in the Pacific Ocean.

The different types of PSTs are STXs and derivatives, carbamate toxins (C toxins), gonyautoxins (GTX), neosaxitoxins (neoSTX), deoxydecarbamoylsaxitoxins (doSTX), and decarbomoylsaxitoxins (dcSTX), associated with several strains of dinoflagellates. Twenty-six derivatives of saxitoxins have been identified in shellfish or in the dinoflagellates responsible for PSP (Lagos and Andrinolo, 2000). PSTs are potent neurotoxins that bind to site 1 on voltage-dependent sodium channels, blocking the influx of sodium into excitable cells (Kao, 1966), thus limiting signal transmission between neurons.

PSPs in humans have thus far been caused exclusively by toxic dinoflagellates (Shumway, 1990, Hallegraeff, 2004); human PSP intoxication linked to saxitoxins from cyanobacteria has not been reported. Indeed, most cases of PSP in humans have been observed following ingestion of bivalves (Shumway, 1990), but also a few gastropods and crustaceans (Shumway, 1995), and, very rarely, toxic fish (Maclean, 1979; Adnan, 1984). PSTs can accumulate throughout the food chain, and experiments with copepods have highlighted the mechanisms controlling the availability of toxins to higher trophic levels (White, 1979, 1981; Turriff *et al.*, 1995; Teegarden and Cembella, 1996; Turner *et al.*, 2000).

NSP results from consumption of shellfish contaminated with brevetoxins. Neurotoxic shellfish poisoning following bivalve consumption has been known in Florida since the late 1800s (Walker, 1884), but the cause of these poisonings was not identified until the 1960s (McFarren *et al.*, 1965; Steidinger, 1993). Cases of NSP in the Gulf of Mexico have been mostly associated with the consumption of filter feeders, such as eastern oysters, *Crassostrea virginica*, quahogs, *Mercenaria mercenaria*, and *M. campechiensis*, surfclams, *Spisula solidissima raveneli*; sunray venus, *Macrocallista maculata*; coquinas, *Donax variabilis*; cross-barred venus, *Chione cancellata*; and a few other species.

Brevetoxins are produced by the dinoflagellate *Karenia brevis,* and by the raphidophyte *Chattonella* cf. *verruculosa*. Brevetoxin-like compounds have also been identified in other algal species, such as *Karenia* spp. and several raphidophytes, *Chattonella antiqua, C. marina, Heterosigma akashiwo*, and *Fibrocapsa japonica* (reviewed in Landsberg, 2002). Nine brevetoxins have been isolated from *K. brevis* (Baden, 1989; Schulman *et al.*, 1990): PbTx-1 to PbTx-3 and PbTx-5 to PbTx-10. *Karenia* spp. blooms generally produce more toxins than raphidophyte blooms and are therefore much more toxic to aquatic organisms; they are the only blooms reported to have effects on human health. Blooms of *Karenia* sp. triggering NSP originally occurred in the Gulf of Mexico and were observed only there until 1987 (Steidinger, 1993). In 1987 to 1988, the Atlantic coast of Florida was closed for shellfish harvesting because of NSP, and the Gulf Stream transported the Florida Atlantic coast *Karenia brevis* red tide to the coast of North Carolina. NSP was also observed in New Zealand for the first time in 1992 (Bates *et al.*, 1993; Chang *et al.*, 1995; Satake *et al.*, 1996). Brevetoxins can cause massive fish kills, but thusfar, no human deaths have been associated with NSP.

DSP cases have been observed worldwide; however, most cases have been reported in Europe, North and South America, Japan, and Southeast Asia (Sechet *et al.*, 1990). Diarrheic shellfish toxins (DSTs) are lipophilic toxins produced by various species of phytoplankton within the genera *Dinophysis* and *Prorocentrum.* Toxin production varies appreciably between the different species of dinoflagellates, but also according to regional and seasonal parameters (Vale and Sampayo, 2003). The first group of toxin-producing species, common in Europe, *D. acuta* and *D. acuminate*, produce primarily OA; whereas, in Japan, *Dinophysis fortii* is the principal source of DTX-1 (Landsberg, 2002). OA and DTX mainly accumulate in the hepatopancreas of shellfish (Edebo *et al.*, 1988; Alvito *et al.*, 1990; Aune and Yndestad, 1993). The second group of toxins responsible for DSP are PTXs, neutral toxins consisting of polyether-lactones. PTXS are produced by various *Dinophysis* species. Ten PTXs have been isolated thus far, six of which have been chemically identified: PTX1, -2, -3, -4, -6 and -7. The third group of toxins associated with DSP events includes a sulfated polyether, YTX, and its derivatives. YTX was first isolated from the digestive organs of scallops (*Patinopecten yessoensis*) in Japan (Ciminiello *et al.*, 1999). DSP in humans has exclusively been observed following consumption of shellfish.

ASP was first observed in Prince Edward Island, Canada, in 1987, after 107 people became ill from eating contaminated blue mussels (Todd, 1993). ASP cases have been mainly observed in Canada and in the USA. A few occurrences of ASP have been recorded in Europe, Australia, New Zealand, and Japan (reviewed in Hallegraeff, 2004). The toxin responsible for ASP, domoic acid (DA), was originally discovered as a product of a red macroalga, *Chondria armata,* and was later isolated from several other red macroalgae. Subsequently, DA was demonstrated to be produced by diatoms as well: *Pseudo-nitzschia* spp. and *Nitzschia* spp., but not by dinoflagellates. The ASP observed in humans

following the consumption of shellfish is exclusively attributable to DA produced by diatoms – nine species of *Pseudo-nitzschia* and one species of *Nitzschia* (reviewed in Landsberg, 2002).

AZP was first observed in November 1995 when several people from the Netherlands became ill after eating mussels (*Mytilus edulis*) cultivated in Killary Harbour, Ireland (McMahon and Silke, 1996; Satake *et al.*, 1998a). A few differences between symptoms of AZA poisoning and DSP, such as a slowly progressing paralysis, were observed in the mouse assay using mussel extracts. Moreover, no trace of any DSP-producing, toxic organism was detected in the waters. Thus, azaspiracid (formerly called Killary Toxin-3 or KT3) was identified as a new toxic syndrome. Azaspirazids (AZA) represent a new group of phytotoxins, first identified on the Irish coast, but now known to occur over the entire western coast of Europe. Several analogues of AZAs have been identified: AZ-1-11. AZAs are polyether toxins (Furey *et al.*, 2003); they accumulate in tissues of bivalves, which were previously exposed to harmful algae in the genus *Protoperidinium*, considered non-toxic previously. AZA has been associated specifically with the microalga *Protoperidinium crassipes* (Gribble, 2002). AZAs can be accumulated in several bivalve species, not only mussels. The presence of AZA has been reported in many different species of bivalves, including mussels (*M. edulis*), oysters (*C. gigas*), scallops (*P. maximus*), cockles (*C. edule*), and clams (*T. philippinarium*), and can reach levels that exceed the EU regulatory limit (0.16 μg total AZAs/g).

Tetrodotoxin (TTX) represents another type of potential human poisoning. TTX poisoning has been observed in humans following consumption of finfish but it has also been observed in bivalve molluscs, which can represent a source of human shellfish poisoning. TTX has been associated with bacteria, *Vibrio*, and *Aeromonas* (Noguchi *et al.*, 1986; Tamplin, 1990), and more recently with *Alexandrium tamarense* or the bacteria associated with it (Kodama *et al.*, 1993, 1996). Direct association between dinoflagellates and human cases of TTX poisoning has not been demonstrated (Landsberg, 2002). TTX produces similar symptoms in humans to PSP, as it blocks site 1 of the voltage-dependent sodium channel in nerve and muscle membranes (Kao, 1993).

Spirolides have been observed in shellfish from Nova Scotia, Canada, Northern Europe: Norway, Denmark, and from the Gulf of Maine, USA (Aasen *et al.*, 2005; Gribble *et al.*, 2005; MacKinnon *et al.*, 2006). These neurotoxins are produced by *Alexandrium ostenfeldii* (Cembella *et al.*, 2000; Hu *et al.*, 2001) and can trigger rapid death of mice injected with extracted spirulides (Landsberg, 2002). Thus far, even though it remains a potential toxic agent, no instance of human poisoning has been recorded.

Other algae

Alexandrium monilatum produces hemolysins, which have demonstrated neurotoxic effects on mice experimentally (Clemons *et al.*, 1980; Erker *et al.*, 1985). These hemolysins are different from STX or GTX. Moreover, recent analyses from Hsia *et al.* (2006) reported that *A. monilatum* also produces the toxin they

termed goniodomin A, a toxin also produced by the Japanese rockpool dinoflagellate, *Alexandrium pseudogoniaulax*.

Alexandrium monilatum causes sedation, abdominal constriction, fecal clumping in the perianal area, ataxia, tremors, cyanosis, loss of reflexes, convulsions, and death of adult mice. This dinoflagellate has been associated with fish kills in the last few years. Thus, entire cells as well as extracts of *A. monilatum* have been tested on mice, rats and fishes and triggered death of these species (Gates and Wilson, 1960; Aldrich *et al.*, 1967; Clemons, *et al.*, 1980; Erker *et al.*, 1985). So far, no poisoning has been observed in humans attributable to *A. monilatum*.

Prorocentrum micans has sometimes been suspected to be associated with shellfish poisonings, even though most studies have shown *P. micans* to be a non-toxic algal species (Landsberg, 2002). In Portugal, in 1955, several human poisonings and one death followed the consumption of toxic cockles, exposed to a bloom of *P. micans* (Pinto and Silva, 1956). The toxins of *P. micans* have not been identified, but the poisoning involved various neurological symptoms, including loss of sensitivity in the lips and chin, numbness and paraplegia, tremors, ataxia, and a feeling of floating. As these observations have not been repeated at other times in other places experiencing *P. micans* blooms, it appears possible that the one reported incident of poisoning may have been caused by a co-occurring, undetected microalgal species.

Prorocentrum minimum has also been suspected to be responsible of venerupin shellfish poisoning (VSP) (Denardou-Queneherve *et al.*, 1999). Putative toxin from this species is uncharacterized so far, but a B-diketone, or some uncharacterized venerupin or prorocentrin have been suspected to be responsible for the toxicity (see tables in Lansberg, 2002). Venerupin, which has been reported several times as the causative agent of hepatotoxicity following ingestion of poisonous clams and oysters in Japan, however, also has never been characterized (Hashimoto, 1979). Williams *et al.* (1997) observed tumor-promoting and hepatotoxic microcystin in mussels. These results, associated with the observations that liver damage found in all venerupin-related events, suggest that 'venerupin' toxicity could also have been caused by microcystins. This suggests that microcystins could potentially be responsible for a new type of shellfish intoxication, hepatotoxic shellfish poisoning (HSP). Microcystins are produced by cyanobacteria, which occur mainly in fresh or brackish waters, but have also been observed in marine waters.

The effects of *Pfiesteria* spp. have been observed on human health. A hydrophilic *Pfiesteria* toxin (*Pf*Tx) was isolated from *Pfiesteria piscicida* in 1997 (Fairey *et al.*, 1999), consisting of a metal–organic complex. This toxin was shown to affect fish and mammals; indeed, *Pf*Tx was demonstrated to be lethal to fish and toxic to mammals (Fairey *et al.*, 1999; Burkholder *et al.*, 2001, 2005; Moeller *et al.*, 2001; Levin *et al.*, 2003). Melo *et al.* (2001) purified *Pf*Tx and described a pharmacological mode of action. According to Moeller *et al.* (2007) the toxicity of *Pfiesteria* spp. is mediated by metal-containing, organic compounds which produce toxic free radicals based on carbon and sulfur. These

radicals have a very short lifetime, which explains the difficulty scientists encountered to isolate and characterize the toxins from *Pfiesteria* spp. *Pfiesteria* spp. also affects bivalve shellfish health (Springer *et al.*, 2002; Shumway *et al.*, 2006); therefore, the consumption of bivalves exposed to *Pfiesteria* spp. by humans could trigger a risk of poisoning.

2.3 Trophic dynamics of phycotoxins in molluscan shellfish

The intensity and the occurrence of phytoplankton blooms leading to toxic shellfish have increased drastically in the past few decades. Hallegraeff (2004) proposed four major reasons to explain this rise in outbreaks of toxic phytoplankton: the increase of scientific awareness of toxic species; enhanced utilization of coastal waters for aquaculture; stimulation of harmful algal blooms attributed to eutrophication or unusual climatic conditions; and the transportation of cells or cysts of harmful algae through ballast water or via transportation of shellfish from one body of water to another.

Toxin content in shellfish is a function of uptake, metabolism, and depuration. Rates of these processes are different for various toxins and shellfish species, complicating prediction of exposure risk for human consumers of the shellfish. Indeed, toxin accumulation and retention times vary among different bivalve species and depend upon intrinsic and extrinsic factors, such as the concentration of the bloom as well the toxicity of the algal cells and by the filtration and elimination rates of the shellfish (Shumway, 1990).

2.3.1 Toxin uptake

There are different vectors by which shellfish are exposed to microalgal toxins. The exposure can be direct, with toxin transfer directly from the algal cells, whether they are intact or lysed, or indirectly, by consumption of other grazers already contaminated, as toxins accumulate through the food chain.

Harmful microalgae are generally suspended in the water column and accordingly are encountered by filter feeders. Toxic cells, or particle-associated toxins, can sometimes sink to the bottom as well. Some harmful microalgae can also be benthic organisms, and some, especially dinoflagellates, have sedimentary cysts or resting stages as part of their life cycles. Sometimes these stages are even more toxic than the free-swimming stages; cysts of *Alexandrium* can for example be 100 times more toxic that the vegetative cells after they have undergone several months of dormancy (Dale and Yentsch, 1978). These toxins, cells, or cysts present in the sediment are consumed by benthic organisms, thereby exposing the deposit-feeders to biotoxins. Nevertheless, filtration of planktonic, vegetative cells represents the most direct means of exposure in bivalve molluscs.

Shellfish can also be affected by the extracellular toxins (exotoxins) exuded into the surrounding water by many microalgae. Another direct exposure of

shellfish to microalgal toxins consists of direct contact with the cells, either because the microalgal toxins are present at the surface of the cells or through mechanical damage caused by the microalgae interacting with the gills or epithelial tissues of the bivalve (Landsberg, 2002). These two processes do not generally result in accumulation of toxins within the shellfish and therefore are not often responsible for human intoxication.

Some shellfish, such as some gastropods and crabs, feed on bivalves or other organisms and can thereby be exposed to toxins indirectly from prey that have already accumulated phycotoxins. Toxins bioaccumulated can, in many cases, have been bioconverted and biomagnified and are now available to animals not feeding on microalgae. Humans are examples of predators only exposed to biotoxins following indirect exposures, as we may eat shellfish previously exposed to microalgal toxins.

2.3.2 Biotoxin accumulation

As described in the previous section, bivalve shellfish can be filter feeders or benthic, deposit feeders. Thus, if any harmful algae are present in the water or in the sediment, the bivalves encounter them. The algae then can be ingested and pass through the stomach and digestive system, or be rejected in the form of pseudofeces. When the cells are ingested, they can be assimilated or rejected as intact cells in the feces. As the harmful algal cells pass through the gut and the digestive tract, some are digested and the toxins are accumulated in the tissues of the bivalves, which affects the accumulation rate of toxins in the shellfish (Morono *et al.*, 2001) as well as the gut passage time.

The ability of shellfish to retain and accumulate toxins varies greatly according to the shellfish species (Bricelj and Shumway, 1998; Fernandez and Shumway, 2004). In Maine coastal waters, PSTs were detected 12 days earlier in mussels *Mytilus edulis* than in clams *Mya arenaria*; *M. edulis* accumulate two to four times more PST than the clams (Hurst and Gilfillan, 1977; White, 1982; Larocque and Cembella, 1991; Bricelj and Shumway, 1998). Hence, mussels appear more appropriate for monitoring purposes as they accumulate more toxin, and more rapidly, than most of the other bivalve species, thereby allowing earlier detection. Thus, the toxicity of shellfish species can vary according to intrinsic parameters, such as previous exposure to HABs, uptake dynamics and detoxification capabilities, tissues affected, feeding rate, and food retention. Extrinsic factors associated with the phytoplankton species and the bloom characteristics, amounts of toxins present in the algal species, or the environmental conditions also affect toxin accumulation in shellfish. Some species of shellfish, when exposed to a harmful alga, reduce or suppress completely filtration, moderating or eliminating the accumulation of toxins internally (Fernandez and Shumway, 2004). Filtration rate can be reduced as a shellfish species avoids the specific harmful alga, or because the harmful algae affect the mechanism for food capture. Fernandez and Shumway (2004) reported the retraction of the siphon of quahogs, *Mercenaria mercenaria*, when exposed

to *Alexandrium tamarense*, followed by a complete isolation of the clams from the environment by shell-valve closure. Efforts to induce toxicity in these clams appeared to be unsuccessful. In contrast, blue mussels, *Mytilus edulis,* are known to accumulate large concentrations of PST when exposed to toxic dinoflagellates (Shumway, 1990). Certain bivalve shellfish can accumulate more toxin than others. Mussels, *M. edulis,* feed actively on toxic cells (Bricelj *et al.*, 1990), as they possess nerves insensitive to PSP toxins; this species therefore accumulates high toxin levels (Bricelj and Shumway, 1998). In contrast, oysters, *Crassostrea virginica*, reach only a very low level of toxicity; they are very sensitive to PSP toxins (Bricelj and Shumway, 1998) and display behavioral and physiological mechanisms to eliminate or diminish exposure to the toxic algae.

DSP-producing species have, to date, not been demonstrated to affect the filtration rate of shellfish, neither in the field, nor in laboratory experiments (Bauder *et al.*, 2001). Similarly, Whyte al al. (1995) showed no effect of the ASP-producing species *Pseudo-nitzshia multiseries,* on feeding by mussels, *Mytilus edulis*. Conversely, DSP-containing dinoflagellates seemed to induce valve closure in *Crassostrea gigas* (Jones *et al.*, 1995). *Karenia brevis*, responsible for the production of the brevetoxins triggering NSP, affected the valve closure of oysters *C. gigas* and mussels *Brachiodontes recurvis* (Sievers, 1969).

Shellfish toxicity also depends upon the biomass of the organisms coupled with the amount of biomass into which the toxins are distributed. Thus, as the toxin content remains constant, if an organism, after toxin contamination, changes its weight, toxicity will be adjusted as well.

Moreover, bivalves accumulate different concentrations of toxins in different tissues. Overall, the visceral mass of bivalves seems to concentrate toxins more than the rest of the body (review in Bricelj and Shumway, 1998). Clams also accumulate most of the toxins primarily in their siphon, as it is the first organ in contact with the algal cells. Similarly, brevetoxins accumulate in the gut and hepatopancreas in most species (McFarren *et al.*, 1965; Steidinger *et al.*, 1973, 1993; Hemmert, 1975; Roberts *et al.*, 1979; Baden *et al.*, 1982; Tester and Fowler, 1990; Steidinger *et al.*, 1998).

The accumulation of PSP toxins has been demonstrated to vary among individuals in a bivalve population according to extrinsic, environmental factors, including: temperature, microhabitat differences, especially availability of the toxic dinoflagellates, concentration of the toxic dinoflagellates, or tidal immersion time of the shellfish population (Quayle, 1969; Bricelj and Shumway, 1998). The difference can also be the result of intrinsic factors, such as feeding rate of the shellfish or variation in body mass (Bricelj and Shumway, 1998). Bricelj *et al.* (2005) demonstrated also a genetic basis for differences in biotoxin accumulation in individual shellfish. They suggested the existence of genetic selection for resistance to PST in areas subjected to PSP outbreaks of sufficient intensity and occurrence. Interspecific differences in the accumulation of PST have been associated with differences in nerve resistance to toxins, as measured by the concentration of saxitoxin necessary to block the conduction of the nerve action potential in *in vitro* trials (Twarog *et al.*, 1972).

Bivalves can also accumulate different concentrations of DST depending on many factors (Alvito *et al.*, 1990; Suzuki and Mitsuya, 2001), and can remain toxic for variable periods of time. OA and DTX mainly accumulate in the hepatopancreas of shellfish (Edebo *et al.*, 1988; Alvito *et al.*, 1990; Aune and Yndestad, 1993).

Domoic acid (DA) has also been observed in various species of bivalves (reviewed in Landsberg, 2002). The responses of each bivalve to the diatoms and to the DA itself vary according to the bivalve and algal species and are very species-specific. Moreover, DA accumulates in various tissues in different concentrations. In mussels collected from a naturally contaminated site, for example, 93.4 ± 1.9% of the DA contained in the whole animal was found in the hepatopancreas, which represents only 30% of the total biomass of the animal (Grimmelt *et al.*, 1990). Thus, the amounts of toxins in the body of animals depend upon the nature of the tissues. Additionally, detoxification rates are different in each tissue. As DA is a hydrophilic toxin, a large portion of it is excreted instead of being bioaccumulated (Novaczek *et al.*, 1992). When accumulated, DA can also be biotransformed; for example, DA present in the digestive gland of mussels can be converted into isodomoic acid isomers (Wright *et al.*, 1990).

The balance in the organisms of the accumulation of toxins is regulated by the intake, mostly from direct filtration or consumption of organisms already toxic, the loss to the environment, and by biotransformation into other toxins (Fernandez and Shumway, 2004).

2.3.3 Biotransformation

Toxins accumulated within the tissues of bivalve molluscs may undergo metabolic reactions by which they are changed chemically, sometimes into more or less toxic forms. These biotransformations may be a consequence of digestive or active detoxification processes, or may simply result from participation of toxins in general metabolic processes intra- or extracellularly. Metabolic reactions involving phycotoxins can be quite important in determining the safety of human consumers of shellfish, particularly in terms of estimating risk of illness when toxin-producing phytoplankton are observed in the water and determining a safe moratorium on harvest after toxin accumulation by shellfish.

Specific instances of biotransformation of phycotoxins within bivalve tissues are mainly apparent from mis-matches between toxin profiles of toxic phytoplankton ingested and toxin profiles within the animal over time. For example, some PTXs, such as PTX-2, have been observed exclusively in dinoflagellates and not in shellfish, suggesting that an oxidation reaction takes place in the hepatopancreas of shellfish producing other PTXs. Indeed, oxidation of PTX2 to PTX6 in scallops (*Patinopecten yessoensis*) was demonstrated by Suzuki *et al.* (1998). Evidence of bioconversion of brevetoxin PbTx-2 in PbTx-3 in various shellfish species has also been demonstrated in Florida following a NSP outbreak, as PbTx-2 and 3 were present in the water, but only PbTx-3 was found in shellfish (Poli *et al.*, 2000).

Issues related to biotransformation of microalgal biotoxins within the tissues of edible bivalves may become more important to seafood-safety monitoring if and when chemical toxin-detection technologies replace bioassays currently employed. The chemical specificity inherent in chromatographic or immunologically based (e.g., ELISA) methods carries the risk that small, structural changes in toxin molecules resulting from biotransformation may render still-toxic metabolites undetectable. Clearly, chemical toxin-detection methods will need to be informed by knowledge of biotransformation of microalgal toxins within the tissues of bivalves sampled for testing.

2.3.4 Natural shellfish detoxification

The rate of detoxification in shellfish depends upon toxin and shellfish species (Shumway, 1990; Bricelj and Shumway, 1998; Fernandez and Shumway, 2004). Data are mostly available for commercially important shellfish species. Fernandez and Shumway (2004) reviewed the approximate retention times of DA for various species of bivalve molluscs, by recording the time it took for the bivalve species to reach a level of toxin below quarantine or detection level. The retention time varies considerably according to the species, from a few hours to a few days for DA in the mussels *Mytilus edulis* and *M. galloprovincialis* (Novaczek *et al.*, 1992, Blanco *et al.*, 2002a) to several months or years for DA in the scallop *Pecten maximus* (Blanco *et al.*, 2002b). Toxin elimination can occur fairly rapidly in some cases. Fletcher *et al.* (1998) reported that oysters, *Crassostrea gigas*, which reached a NSP level of 25 to 100 mouse units (MU) per 100 g of drained oyster meat, following a 24 hour exposure to *K. brevis* cells at a concentration of 10 to 25 millions per oyster, reduced their level of toxicity considerably and were almost at the acceptable regulatory limit for human consumption, after only 3 days of depuration. Toxin depuration is also highly dependent upon the tissues in which the toxins are accumulated. Toxins present in the digestive glands are usually eliminated more rapidly than toxins present in the other organs. DSP toxins in *Argopecten irradians* (Bauder *et al.*, 2001) and DA in *Pecten maximus* (Blanco *et al.*, 2002b) appear to be exceptions.

The factors regulating depuration are not fully understood; for example, relative importance of season (Prakash *et al.*, 1971), or water temperature (Shumway and Cembella, 1993) are difficult to differentiate. Elevated temperature has been reported to retard DSP toxin loss in *Mytilus galloprovincialis* (Blanco *et al.*, 1999), to advance ASP toxin loss in *Mytilus edulis* (Silvert and Subba Rao, 1992; Novaczek *et al.*, 1992) and *Pecten maximus* (Blanco *et al.*, 2006), and to have no effect on the PSP toxin loss of *Saxidomus giganteus* (Madenwald, 1985). The amount of non-toxic phytoplankton ingested appears to be another factor enhancing slightly the detoxification rate in shellfish exposed to PSP and DSP toxins (Sampayo *et al.*, 1990; Marcaillou-Le Baut *et al.*, 1993; Blanco *et al.*, 1997, 1999). Metabolic processes have also been cited as affecting detoxification rate of DA in *Pecten maximus* (Fernandez and Shumway, 2004).

2.4 Human health impacts

As humans consume shellfish, they can be exposed to several types of poisoning syndromes from the phycotoxins accumulated in shellfish. The five major types of shellfish poisoning defined above are, after all, based upon clinical symptoms in human victims: PSP, NSP, ASP, DSP, and AZA. The symptoms associated with each type of poisoning present general and specific characteristics.

2.4.1 Effect on humans, toxins involved and their biochemical mechanisms

PSP begins with paresthesia and a sensation of tingling and numbness around the lips and the mouth, then the face and the neck. A sensation of muscular weakness, of lightness and feeling of floating develops, followed by uncoordinated gestures, dizziness, ataxia, incoherence, and progressive respiratory depression. In high concentration, PSP can lead to respiratory paralysis and can be fatal to humans (Catteral, 1985; Kao, 1993).

NSP in humans is characterized by paresthesia, reversal of temperature sensations, fever, myalgia, dizziness, vertigo, ataxia, muscle or abdominal pain, chills, nausea, diarrhea, burning pain in the rectum, headache, bradycardia, and dilated pupils (Steidinger *et al.*, 1973; Hemmert, 1975; Baden, 1983, 1988; Morris *et al.*, 1991). Despite the intense symptoms, to date, no human deaths have been reported following NSP exposure. NSP cases have been recorded in the west coast of Florida in the US, on the southern Atlantic coast, in the Caribbean Sea and in New Zealand. Toxins responsible for NSP are potent neurotoxins and hemolysins called brevetoxins. Brevetoxins are complex, polycyclic ethers, which bind to sodium channel site 5 on the voltage-sensitive channels on neurons and alter the properties of the excitable cells, shifting activation to more negative potentials, thereby triggering membrane depolarization (Huang *et al.*, 1984; Catterall, 1985; Poli *et al.*, 1986; Lombet *et al.*, 1987). Brevetoxin pathogenic dose for human is very low, about 42–72 MU, and oral LD50 value in rats varies between 520 to 6600 μg/kg (Llewellyn, 2001).

Acute symptoms associated with DSP in humans involve gastrointestinal pain, diarrhea, nausea, and vomiting (Aune and Yndestad, 1993; Quilliam and Wright, 1995). Symptoms can appear as soon as 30 minutes after ingestion of the shellfish and last for 3 or 4 days; however, DSP has never been reported to generate human mortalities. DSP in humans is attributable to consumption of shellfish containing diarrheic shellfish toxins (DSTs), including three groups of toxins: (1) OA and DTX1-4, which are OA derivatives (Aune and Yndestad, 1993); (2) PTX (Yasumoto *et al.*, 1985; Murata *et al.*, 1982, 1986); and (3) YTX (Yasumoto and Satake, 1998).

OA and DTXs are produced by *Dinophysis* spp. and *Prorocentrum lima*. Biochemical mechanisms by which these toxins affect humans exposed have been well described. OA inhibits protein phosphatases types 1 and 2A, which increases protein phosphorylation. Consequently, intracellular processes, such as metabolism, cellular division, contractility, gene transcription, maintenance of cytoskeletal structure, membrane transport and secretion, and receptor-mediated

signal transduction are affected. Moreover, OA stimulates expression of certain proto-oncogenes and activation of H1 kinase *in vitro*. Finally, OA induces several mitosis-specific events (Bialojan and Takai, 1988; Fujiki *et al.*, 1989; Haystead *et al.*, 1989; Herschmann *et al.*, 1989; Yamashita *et al.*, 1990; Sakai and Fujiki, 1991; Fujiki and Suganuma, 1993; Rossini, 2000). Mice were exposed experimentally to OA, DTX-1, and DTX-3 to assess short-term effects and estimate exposure levels. After less than 15 minutes, severe diarrhea associated with destruction of the absorptive epithelium of the ileum villi occurred (Terao *et al.*, 1993). OA triggered rapid changes the large and small intestines of rats, leading to hypertension, and accumulation of goblet cells (Edebo *et al.*, 1988; Lange *et al.*, 1990). DTX-1 was responsible for excessive fluid accumulation in the intestines of suckling mice (Hamano *et al.*, 1985). In adult mice, the level of toxins triggering diarrhoea is equal to or above 40 μg for OA and equal to or above 35 μg for DTX1–4 (Scoging, 1998).

PTXs are neutral toxins, consisting of polyether-lactones, which originate from *Dinophysis* species also (reviewed in Landsberg, 2002) and are present in various species of bivalves. Possible impacts of PTXs on human health are not yet clearly defined, and neither is the mechanism of action. Similarly, the mechanisms of action, as well as the impact on human health, of YTX and its derivatives are poorly understood. These toxins are known to be sulfated polyethers, but molecular structures have not been clearly defined. YTXs appear to interact with calcium channels (de la Rosa *et al.*, 2001). YTXs are produced by the dinoflagellate *Gonyaulax grindleyi* (= *Protoceratium reticulatum*) (Satake *et al.*, 1997; Draisci *et al.*, 1999).

ASP is the result of DA intoxication. The acute symptoms are nausea, vomiting, abdominal pain, diarrhea, and neurological effects such as memory and consciousness loss, seizures, dizziness, disorientation, and confusion (Debonnel *et al.*, 1989; Wright *et al.*, 1989; Perl *et al.*, 1990; Teitelbaum *et al.*, 1990; Todd, 1990, 1993; Nijjar and Nijjar, 2000). In cases of severe intoxication, these symptoms may be followed by coma or death in human victims. Chronic symptoms of permanent amnesia have been reported, including loss of short-term memory (Todd, 1993), but the extent of the chronic effects of ASP are still unclear.

DA is part of the group of amino acids called kainoids, which are exocitotoxins or neuroexcitants that affect the mechanisms of neurotransmission in the brain (Quilliam, 2004). It is a crystalline, water-soluble, heat-stable toxin with properties of a typical amino acid; it is an analogue of glutamate, and acts as a glutamate receptor agonist. DA binds to the kainite type of glutamate receptor in the brain, especially in the hippocampus, acting as an excitatory neurotransmitter (Debonnel *et al.*, 1989; Sutherland *et al.*, 1990). DA causes depolarization of neurons, followed by an influx of cellular calcium ions, neuronal swelling, and cell death (Novelli *et al.*, 1990; Bates, 1998). The neurons located in the hippocampus are responsible for memory retention, which explains the loss of memory associated with ASP (Bates, 1998).

AZP has been recognized recently as a serious risk for human health (Ofuji *et*

al., 1999a) and is attributed to the toxin azaspiracid (Satake *et al.*, 1998a,b; Ofuji *et al.*, 1999a,b; Draisci *et al.*, 2000; James *et al.*, 2000). The symptoms – nausea, vomiting, severe diarrhea, and stomach cramps – are very similar to those of DSP (Satake *et al.*, 1998a). Azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3) have been identified specifically as 8-methylazaspiracid and 22-demethyl-azaspiracid, respectively (Ofuji *et al.*, 1999a). Two analogs, azaspiracid-4 (AZA-4) and azaspiracid-5 (AZA-5), were identified as 3-hydroxy-22-demethylazaspiracid and 23-hydroxy-22-demethylazaspiracid (thus hydroxylated analogs of AZA-3) (Ofuji *et al.*, 2001). James *et al.* (2003) recently isolated AZA-6–11 five new hydroxyl analogs of azaspiracids. Despite importance in human health, the mechanisms of action of azaspiracid are still largely unknown. Several studies are ongoing to understand the mechanisms involved in azaspiracid poisonings. Twiner *et al.* (2005) highlighted cytotoxic and cytoskeletal effects of azaspiracid-1 (AZA-1) on mammalian cell lines. Alfonso *et al.* (2005) also demonstrated that azaspiracid-4 (AZA-4) inhibits plasma membrane Ca^{+2} entry by stored, operated channels in Ca signaling within human T lymphocytes.

TTX can be responsible for fatal human poisoning; it has been recorded in numerous species of finfish, but has also been found in gastropods (Shumway, 1995; Lin and Hwang, 2001) and in molluscs such as the Japanese scallop, *Patinopecten yessoensis* (Kodama *et al.*, 1993). TTX produces similar types of symptoms as STX; these toxins are chemically different, but their mechanisms of action are fairly similar. TTX blocks the voltage-dependent sodium channels in nerve and muscle membranes (Kao, 1993). The effects of TTX on aquatic organisms are currently unknown.

Other algae

Prorocentrum micans has also sporadically been associated with human intoxications, but no toxins have been identified. Indeed, in 1955, *P. micans* was present in Portuguese waters when humans ingested toxic cockles. One death occurred, as well as various symptoms, such as neurological abnormalities, loss of sensitivity in the lips and the chin, lack of feeling in the arms and hands, paraplegia of the legs, ataxic walking, and floating sensations were reported (review Landsberg, 2002).

2.5 Management responses

Programs and guidelines have been developed to minimize risk of human exposure to phycotoxin-contaminated shellfish. These programs are based upon intervening at several points in the sequence of activities leading to consumption of shellfish: (1) stopping harvest when potentially toxic phytoplankton species are present, (2) sampling shellfish populations before harvest and analyzing toxicity in the shellfish tissues, (3) post-harvest removal of specific tissues containing phycotoxins, and (4) issuing advisories to cook harvested shellfish

thoroughly in cases wherein phycotoxins are heat-labile. These programs can involve, therefore, highly developed monitoring programs as well as safe depuration techniques, both discussed in the following section.

2.5.1 Monitoring programs

Biotoxin accumulation in shellfish makes them toxic for human consumption. Thus, monitoring programs and regulations need to be established to limit the risk of intoxication of humans by toxic shellfish. To be efficient, the monitoring programs must allow rapid detection of any toxic algae or presence of toxic shellfish, to be able to limit the harvest and avoid contamination, as well as to prevent unnecessary disposal of toxic shellfish already harvested. Thus, many countries have monitoring programs screening the phytoplankton present in the water, but also the flesh of the shellfish on a regular basis. Moreover, a good network needs to be developed to be able to transfer the information very rapidly and efficiently to seafood harvesters, distributors, and consumers, as well as public health and medical professionals.

Thus, the major points of monitoring and management programs preventing biotoxin intoxication often include the following elements (Anderson *et al.*, 2001): (1) environmental observations of the plankton, fish kills, and abnormal animal behaviors, (2) regular sampling of plankton, and shellfish, (3) analysis of samples of water and animals for presence and quantification of harmful algae and toxicity of shellfish, (4) evaluation of the results, (5) dissemination of information and implementation of regulatory action, and finally (6) action plan or mitigation measures.

Each country develops its own monitoring program, identifying one or several agencies as responsible, and may also include industry, fishermen, or private consultants. Developing efficient monitoring programs, based upon phytoplankton observations and shellfish toxicity rather than relying only upon post-harvest toxin analyses, allows a very early response to protect the public and to minimize product losses for the harvesters and costs associated with shellfish poisoning.

2.5.2 Detection methods and analysis of toxins

As mentioned previously, the first method for detection of harmful algae is visual monitoring of the water, both on a macro-scale and with the microscope. Observation of the phytoplankton present in the water gives early information about the potential occurrence of HABs and subsequent contamination of shellfish with phycotoxins. The second method involved in monitoring is to quantify toxins present in marine organisms. Each toxin has a detection level at which shellfish harvesting should be closed, and different toxins may be detected with different methods.

The mouse bioassay is the most classical test for analysis of most of the biotoxins transferred by shellfish (Schantz *et al.*, 1958). This method needs to be

standardized, with a known strain, size and condition of the mouse. Mice are then challenged by injection of shellfish extract, and responses are compared with the responses of animals injected with different known concentrations of toxins. The mouse bioassay is a rapid, inexpensive method, but it can lack specificity. Thus, other, more accurate detection methods are also used to identify the presence and quantities of toxins in animal tissues.

Antibody-based immunoassays can be used for detection of certain marine toxins (Lewis, 2001), but there are only few available because of the difficulty to obtain sufficient quantities of pure toxins. These assays can be available in rapid test kits, such as 'DSP Check Elisa Kit' (Camacho *et al.*, 2007).

Other methods used for the toxin determination include HPLC, which allows definition of the toxin profile in both shellfish and toxic algae (Oshima *et al.*, 1984; Sullivan *et al.*, 1985). Moreover, analytical chemistry techniques that combine the physical separation capabilities of liquid chromatography (aka HPLC) with the mass analysis capabilities of mass spectrometry (LC-MS) are also used for determination of toxins, such as ASP (Holland *et al.*, 2003) and lipophilic marine algal toxins such as OA/DTXs, PTXs, YTXs, AZAs are detected with LC/MS (Aasen *et al.* 2003). The mouse bioassay detects the presence of domoic acid in the tissues only when the concentration of domoic acid is higher than 40 μg per gram of shellfish meat. As this concentration is much higher than the regulatory level, an alternate method was developed to monitor the concentration of toxin present in shellfish. HPLC with ultraviolet detection (HPLC-UV) was the first method used to detect the presence of domoic acid in shellfish samples (Wright *et al.*, 1989) and is the principal analytical method used at present. Other methods have also been developed, including thin layer chromatography (TLC), for semi-quantitative detection (Quilliam *et al.*, 1998), capillary electrophoresis (CE), a promising new technique for detection of domoic acid in bivalves (Zhao *et al.*, 1997), and LC-MS.

Recently a series of *in vitro* assays have been developed for rapid detection of toxins in shellfish (Cembella *et al.*, 2004). These assays generally are expensive to develop, but economies of scale can lower the cost to where these assays can become economically competitive. A receptor-binding assay for ASP toxins has been developed to detect DA and its analogues. Alternative assays have also been developed recently, such as the evaluation of a protein-phosphatase inhibition assay for monitoring OA and derivatives (Botelho *et al.*, 2003) or caspase-8 activation associated with OA-induced apoptosis (Cabado *et al.*, 2003, 2004).

The two screening strategies included in most monitoring programs – water and shellfish monitoring – are both necessary under different circumstances, but each carries advantages as well as disadvantages (Todd, 2004). Analyses of phytoplankton are usually more cost-effective than shellfish testing, and results are faster, permitting quicker decisions concerning harvest of resources in the monitored area. Moreover, phytoplankton monitoring allows observation of all species of phytoplankton present in the water, not only the species producing toxins, providing additional information on trophic conditions. Unfortunately,

determination of species of phytoplankton in water samples requires a very highly trained phytoplankton taxonomist. Further, local hydrographic conditions must be known so that areas sampled are chosen to represent surrounding areas (Todd, 2004). Similarly, the monitoring of the shellfish flesh presents advantages as it provides a straightforward answer on the question of possible health effects of eating the shellfish. Also, monitoring shellfish tissues for toxicity gives a cumulative picture of what may have occurred in the water previously as shellfish were accumulating and possibly metabolizing the toxins. Conversely, shellfish-tissue analyses are more expensive, require specialized chemists, and take longer than microscopic observation of phytoplankton samples, which can delay decisions on the management of fisheries (Todd, 2004).

After the results of the sampling have been determined and evaluated by the several institutions responsible for making decisions regarding the management of the fisheries, dissemination of information followed by regulatory actions or mitigation measures take place. For example, Todd (1993) reported the toxicity level of DA for humans to be 1 to 5 mg/kg in the Prince Edward Island outbreak in 1987. At present, shellfish contaminated with a concentration of DA higher than 20 μg per gram of shellfish meat is considered to be excessive for human consumption, and this became the regulatory level set by the Canadian regulatory authorities after the outbreak of ASP in Canada in 1987.

Similarly, monitoring programs in Europe and in the US regularly detect high concentrations of PSTs (Van Egmond *et al.*, 1993). As there are no known antidotes to PSP shellfish poisoning, monitoring programs are very well developed and need to be implemented very carefully. Shellfish are monitored in many states and countries to control shellfish harvest by commercial and recreational fishermen. Recognition of the potential lethality of PSP has led to increases in monitoring programs limiting the risk of human intoxication. Most human intoxication with PSP followed consumption of toxic bivalves (Shumway, 1990), but a few reports also record intoxication attributed to consumption of toxic gastropods and crustaceans (Shumway, 1995). Saxitoxin, and its numerous derivatives, accumulated in shellfish eaten by human consumers can cause PSP, but risk of exposure can be mitigated through monitoring and control programs.

Blooms of PST-producing algae occur mainly from April to October in the US and in Europe and can occur several times a year. The blooms of toxic algae are dependent on variable factors, such as light, temperature and salinity of the waters, nutrient availability, niche availability, as well as other environmental factors. Blooms producing PSTs generally occur in 'cold waters', but the waters have to be above 5–8 °C for blooms to occur. As dinoflagellates can also form cysts, if the temperature decreases drastically or if environmental conditions change and become inhospitable, the dinoflagellates may form cysts and survive in surface sediments. PSP occurrence is not predictable, and shellfish can remain toxic for various periods of time afterward. Closure of shellfisheries must be determined based upon persistence of the toxins in the shellfish; therefore, some areas can be closed for short or very long periods of time.

Commercial harvesting is regulated and stopped for various periods of time, but as recreational fishing is also important in many regions, a very large effort is also made to advise the public with harvest warnings through the media and signs posted on public beaches.

2.5.3 Processing for detoxification

Processes for active detoxification have been developed to artificially eliminate toxins from shellfish after harvest and before sale, using two different groups of techniques. The first group of techniques includes temperature or salinity stresses, ozonation, chlorination, transplantation, and other methods that accelerate the rate of detoxification of shellfish while maintaining them alive. The second group of techniques aims to select products free of toxins after applying specific processing procedures.

Relay has been used as a method to facilitate detoxification with limited success, but it is effective in preventing reintoxication (Blanco *et al.*, 1997, 1999). Methods involving temperature, salinity (Gilfillan *et al.*, 1976, Blogoslawski *et al.*, 1979), electric shock (Kodama *et al.*, 1989), pH (Neal, 1967), or chlorination have shown positive results in shellfish detoxification efforts. Numerous studies have been conducted to assess the potential of ozonation to enhance detoxification of shellfish. The results appear different according to the toxins and the shellfish species; Fernandez and Shumway (2004) highlighted the cost-ineffectiveness, as well as the limited safety of using these methods in the majority of the cases. Thus, the best way to obtain shellfish free of toxin is to monitor the water and the shellfish before harvesting and to only harvest non-toxic shellfish.

The second group of techniques includes a very wide range of methods, from selective evisceration or selection of certain tissues to more highly developed industrial processes. Some species accumulate toxins in particular organs and not in every tissue, thus, selective removal of the toxic tissue, if the animal is large enough, can be an effective process. For example, bay scallops accumulate most brevetoxins in the hepatopancreas, but as most people eat only the adductor muscle, the scallops are usually safe to eat. These techniques are very tightly controlled, but have the advantage of allowing the sale of shellfish harvested from HAB-affected areas, which helps mitigate the economic losses associated with a toxic outbreak. Cooking the shellfish is another method used to decrease the levels of some toxins in the meats. As many toxins are water-soluble and very heat stable; however, they generally survive regular cooking. For example, cooking food containing PSP toxin for 5 minutes will reduce the toxicity by denaturation by only 30%; 20 minutes cooking by only 40% (Scoging, 1998). Similarly DSP toxins and brevetoxins are also heat-stable and overcome standard cooking, and can survive temperatures up to 300 °C for brevetoxins. As the denaturation of toxins with heat is only slight, consumption of shellfish is allowed only when initial levels of toxins are very low. Depuration and ozonation are not effective in reducing PSP toxin level in shellfish either, and thus are not used (Anderson *et al.*, 2001).

Table 2.2 Monitoring of toxins and regulatory tolerance established by FDA (1998)

Toxin	Toxicity	Regulatory tolerance	Method of analysis
PSP	PD: 0.1–2 mg; LD: ≥0.3–12 mg	80 μg/100 g tissue	Mouse assay
DSP	35–40 μg	0–60 μg/100 g tissue	Mouse assay
NSP	PD: 42–72 MU	0.8 ppm (20 MU/100 g)	Mouse assay
ASP	PD: 1–5 mg/kg	20 ppm domoic acid	HPLC

PD = Pathogenic dose for humans.
LD = Lethal dose for humans.

Regulations, especially for the canning industry, now accept very low legal levels of toxins in shellfish (Table 2.2), but slightly contaminated shellfish must undergo a series of sequential procedures. Shellfish must be cleaned with freshwater for a minimum of 2 minutes at 20 ± 2 °C, followed by pre-cooking for 3 minutes at 95 ± 5 °C in freshwater. The flesh and the shell are then separated, and a second cleaning with freshwater for at least 30 seconds at 20 ± 2 °C precedes cooking for a minimum of 9 minutes at 98 ± 3 °C in freshwater. The shellfish flesh then must be cooled for 90 seconds under running, cold, freshwater before mechanical separation of the edible and non-edible parts with water pressure. The shellfish meat is then conditioned in hermetic containers in a non-acidified liquid medium and sterilized in an autoclave at 116 ± 5 °C for more than 15 minutes.

In the previous paragraph, approved detoxification methods were presented, but as it is almost impossible to reliably reduce the amounts of biotoxins present in shellfish, the best technique to avoid human consumption of toxins in molluscan shellfish is to measure the presence of toxins and limit the harvest of highly toxic shellfish by controlling and classifying shellfish harvesting areas through monitoring programs. Additionally, the development of a system verifying the type and quantity of the shellfish after harvest, the harvester, the date and location of harvest and developed by the US FDA (1998) is necessary to verify that shellfish sold for human consumption were harvested from areas not impacted by HABs.

2.5.4 Regulation of shellfish safety and quality: international policies and harmonization

Harmonization of regulations is needed as shellfish can be traded internationally. The General Agreement on Tariffs and Trade (GATT), included in the World Trade Organization (WTO) composed of 125 countries, represents an agreement on sanitary and phytosanitary regulations to protect health, which does not discriminate among all the countries' members and where the decisions are based on scientific evidence and risk assessment.

The EU allows free trade among the different countries, and over the past

decade, numerous Directives concerning food-safety legislation have been developed to ensure high-quality products, consumer protection, and fair competition. In the case of molluscan shellfish, directives are being developed to advance the process of harmonization, involving traceability of the products, and controlling shellfish production areas, harvesting methods, transport, depuration, storage, processing, and marketing, but also chemical and microbiological parameters as well as toxin content. Monitoring of water and bivalve molluscs in production areas occurs periodically in numerous areas to detect the presence of toxins in the shellfish. However, much effort still needs to be made to develop harmonization of the sampling, methods of measurement, and the levels of toxins accepted.

APEC (Asia Pacific Economic Cooperation) includes 18 coastal and archipelago economies very much involved in marine resources. One of the goals is to eliminate barriers between the countries and develop a free trade area; APEC is therefore working toward standardization of sanitary controls. HABs occur on a regular basis in these countries and have resulted in major economic losses. The need for monitoring programs is recognized in these countries, but they differ according to region. Some countries have very highly developed monitoring programs and are very well equipped for analyses; whereas, others have not developed programs to control the presence of toxins in marketed shellfish. The need for harmonization in monitoring methods for toxins is urgent; therefore, APEC created a Red Tide/Toxic Algae Project, developed by the Working Group on Marine Resources Conservation, to establish standards and legislation for all countries belonging to APEC.

In the US, the Food and Drug Administration (FDA) is responsible for all shipping of food items interstate. In 1925, the FDA received authorization to enlist the efforts of each individual state, which led to the creation of the National Shellfish Sanitation Program (NSSP), In 1982, the Interstate Shellfish Sanitation Conference (ISSC), composed of members of the state and federal agencies, of shellfish industry and academic institutions, was created. It provides updated regulations for sanitation processes, harvesting, processing and shipping of shellfish.

After monitoring of microalgal blooms in the water and monitoring of toxin levels in shellfish exposed to toxic microalgae, the decision to close an area to harvesting is made. Indeed, based upon the level of toxin content of the shellfish, water areas are classified, and shellfish harvest can be closed.

Moreover, knowledge of the provenance, quality, date of harvest, etc., of the shellfish is also required. For example, FDA (1998) developed a control program, including a requirement for each container of in-shell, molluscan shellfish to bear a tag identifying the quality and type of shellfish, as well as the location and date of the harvest. Moreover, the harvesters also must be licenced. When the molluscan shellfish is shucked, it has to be certified and has to bear a label of the processor's name, address, and certification number.

2.6 Economic impacts of harmful algal blooms (HABs)

The economic impacts of HABs have been classified under four distinct categories: (1) public health impacts, (2) commercial fisheries impacts, (3) recreation and tourism impacts, and (4) monitoring and management costs. The economic costs associated with public health impacts are represented by the costs involved in medical treatment and investigation, but also attributable to loss of income and work days (Hoagland *et al.*, 2002). This cost can be minimized by better information-dissemination and effective shellfish closure. In countries with efficient monitoring programs, the cost of HABs on public health impacts remains fairly low. Todd (1995) estimated the cost of a PSP illness at $1400 per reported case and $1100 per unreported case, including the cost of medical treatment and transportation (for the reported cases), as well as the cost associated with investigation following illness and the costs of lost productivity during sick days. Following the guidelines developed by Todd (1989), Hoagland *et al.* (2002) estimated the annual cost of public health attributable to shellfish poisoning in the US at $400 000 per year.

The impacts of HABs on commercial fisheries are diverse. HABs can cause direct fish or shellfish mortalities, but can also lead to loss of habitat for certain species, can generate a forced closure of the fisheries, increase the cost of processing the contaminated harvested resources, and depress consumer demand (Hoagland *et al.*, 2002). In the US, annual impacts of HAB on commercial fisheries vary between $7 to $19 million, averaging about $12 million per year.

HABs have also demonstrated economic impacts on tourism and recreational activities (Hoagland *et al.*, 2002). Examples of the economic impacts of HABs on recreation are very diverse, including: accumulation of dead fish on the coast after a HAB event, noxious odors produced on the beach by the death of the algae or of the dead marine organisms, water discoloration, closure of recreational fishing, mortalities of protected marine species, and air-quality impacts caused by aerosols that can be produced by the harmful algae and affect human health. In the US, between 1987 and 1992, annual impacts of HABs on tourism and recreational activities varied between $0 and $29 million, averaging about $7 million per year (Hoagland *et al.*, 2002).

The last main economic impact of HABs is represented by the monitoring and management costs of HABs (Hoagland *et al.*, 2002). The cost involved in management and monitoring of the HAB varies considerably, according to country or even areas within one country. Many countries do not have a regular monitoring program; others, such as the US, include variation among the different states. Moreover, monitoring programs and regulations are different according to the countries and regions of the world (see previous section on monitoring and management). The costs are represented by the daily, weekly, or monthly monitoring, but also surveys or investigations taking place after a one-time HAB event. The real costs associated with HABs, are, however, very difficult to estimate properly, and more effort to quantify economic effects and to standardize the collection of data will be necessary to be able to better evaluate these costs (Hoagland *et al.*, 2002).

2.7 Conclusions

2.7.1 Other marine poisonings

Even though not covered in this chapter, it is important to note that other species of animals, such as horseshoe crabs, snails, puffer fish, and other fishes can also be responsible for human poisonings. Indeed, other marine toxins have been identified, such as ciguatoxin (CTX), tetrodotoxin (TTX), or scombrotoxin. Human ciguatera poisoning, caused by CTX, scombroid poisoning (also called histamine poisoning), caused by scombrotoxin, and poisoning with TTX have occurred in humans only following ingestion of finfish. To date, no occurrences of these toxins have been recorded in shellfish; therefore, these toxins and their effects are not covered in this chapter.

Moreover, this chapter was limited to shellfish poisoning, but another pathway for biotoxin effects upon humans can be through aerosols produced by the phytoplankton or by means of direct contact with the skin or human tissues following drinking or swimming. Cylindrospermopsin was, for example, responsible for human hepatoenteritis after drinking of contaminated, domestic water (Bourke *et al.*, 1983; Hawkins *et al.*, 1985). *Karenia brevis* blooms producing brevetoxins can also affect human health through inhalation of toxic aerosols (Music *et al.*, 1975).

2.7.2 Impact of HAB on bivalves

This review presents the effect of the major biotoxins on human health, and does not report the effect of these toxins on marine organisms. Effects of toxic phytoplankton and biotoxins have been reported on marine organisms (reviews by Shumway, 1990, and Landsberg, 2002), but these studies are very sporadic, and the impacts of biotoxin accumulation on the shellfish themselves are very poorly documented. Indeed, as the shellfish are toxic, they are not harvested, and thus not observed. Therefore, even though harmful algae producing toxins can also have deleterious effects on the shellfish themselves, effects upon commercial quality, usually the level of toxin at which these effects occur is higher than the level required for closure of the fishery. Shellfish toxins are predominantly a human health problem rather than a shellfish quality issue.

2.8 Future trends

The research community has been pursuing innovations in toxin detection, although most new methods have not been implemented in established regulatory programs. Three basic technological approaches have been pursued: chromatographic separation and detection of toxins, molecular-biological identification of toxin-producing microalgae, and 'functional assays' for detection of toxins (Rossini, 2005; Hess *et al.*, 2006). Comparisons of traditional and experimental toxin-detection protocols have yielded enough variability between methods to indicate caution in application of new methods (Llewellyn

et al., 2001). HPLC has long been the method of choice for quantifying many microalgal toxins in research settings (Quilliam, 2004). The potential, however, for interfering compounds to result in false positive results and unnecessary closures of shellfishing, or the worse scenario of ineffective extraction or analysis allowing sale of contaminated shellfish, has limited HPLC methods to follow-up analyses after positive bioassay results. Molecular detection of toxin-producing microalgae in water samples (Tengs *et al.*, 2001) can lessen the need for highly trained taxonomists in monitoring programs and can aid in quantification of target algal species if coupled with quantitative fluorescence detection. Again, the possibility of not detecting an unexpected, toxic species limits the practical application of this technology to routine monitoring programs. Finally, perhaps the most promising technological advance in toxin detection is the 'functional assay,' or affinity binding approach to detection of biotoxins in shellfish meats. Surface-adsorbed compounds that selectively bind toxin molecules, producing a color reaction can be packaged in commercial units that resemble home pregnancy test kits (Turrell *et al.*, 2007). Such test units have been implemented in two ways. First, fisherman can use these units to test shellfish harvested at sea; if the first catch is contaminated, harvest is voluntarily halted. Nevertheless, any shellfish that are harvested still must be cleared for sale with bioassay monitoring. Second, in some very rural areas where monitoring methods are not implemented and artisanal harvest and consumption of shellfish occur, these test units can be distributed to consumers with the provision that consumption of shellfish still is not 'approved', but at least the health of the consumer is somewhat protected.

In addition to investigations into new methods for toxin detection, innovations have also been pursued in satellite imaging to forecast movements of water masses containing HABs (Schofield *et al.*, 1999). The ability to anticipate interactions between advection of blooms into areas with shellfish resources would enable more informed decisions about harvest, relay, and closure as management options.

Implementation of new technologies for HAB detection and forecasting may add another level of sophistication, along with complexity, to monitoring and control programs. Nevertheless, ultimately, protection of human consumers of shellfish from illnesses caused by microalgal biotoxins remains of paramount importance. Further, changes in, or additions to, legal documents defining responsibilities and approved methods for shellfish harvest and marketing in the face of expanding HAB effects will require thorough documentation of both technical and legal considerations. Accordingly, the process moving innovations from scientific discovery to practice will be methodical and thorough.

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