Saxitoxin and Other Paralytic Toxins: Toxicological Profile

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Contents

Introduction 24
The Saxitoxins 25
Producing Organisms and Vector of Poisoning 27
Epidemiological Data 28
  Human Studies 29
Toxicokinetics 29
  Human Studies 29
  Animal Studies 30
Toxicity 31
  In Vivo Toxicity After Repeated Administration 31
  Genotoxicity 31
Limitations of Current Assays and Analytical Methods 32
Limitations of Monitoring and Management Procedures 34
Conclusions 35
Cross-References 36
References 36

Abstract

Toxic microalgal blooms that produce paralytic shellfish poison (PSP) have increased worldwide in frequency, duration, and extension of affected areas, with severe impacts on human health, local economies, and exports. PSP is a variable mixture of tetrahydropurine marine biotoxins collectively named as saxitoxins (STXs) that are produced by dinoflagellates from the genera *Alexandrium, Pyrodinium,* and *Gymnodinium.* Harmful algal blooms of these species (named "red tides") produce accumulation of saxitoxins in shellfish,

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fishes, and other organisms. Ingestion of contaminated tissues may lead to paralytic shellfish poison intoxications in humans and deaths by muscle paralysis and cardiorespiratory failure. Tetrodotoxins (TTXs) are compounds chemically different from STXs, synthesized by cyanobacteria present in freshwater ecosystems that produce a similar paralytic syndrome in cattle, birds, and fishes. TTXs (and STXs) are also present in puffer fishes and cause also human intoxications. In addition, saxitoxins and tetrodotoxins are produced by a number of other species that may represent additional health risks. The severe public health and economic impacts of PSP intoxications brought the attention of researchers almost 80 years ago. Since then a large body of scientific studies on PSP shellfish toxins has accumulated. In recent years an international regulatory effort has been developed to assess, manage, and control health risks caused by PSP toxins and other marine toxins. However, in many countries of the developing world, monitoring and management programs for marine biotoxins in products destined for domestic consumption are limited in scope, geographical extension, frequency, and methodologies. Evidence from physiological, toxicological, and risk management studies is examined that may indicate that current approaches to manage risk and to protect consumers may be still insufficient, especially for underdeveloped countries. Strategies for future work are suggested.

Introduction

The molecular target of saxitoxins (STXs) and tetrodotoxins (TTXs) is the voltage-dependent sodium channel, a transmembrane protein that undergoes subtle conformational changes upon activation by voltage. These molecular movements open a pore within the protein that conducts sodium ions across the cell membrane generating action potentials in excitable neuronal, endocrine, and muscle cells (Catterall 2014). Saxitoxin and tetrodotoxin and their derivatives block ion conduction causing inhibition of action potentials, inducing respiratory paralysis and death (Hall et al. 1990; Nagashima and Arakawa, ▶ Pufferfish Poisoning and Tetrodotoxin, this volume). Saxitoxin is the most toxic parent compound of a group of over 50 known tetrahydropurines (Thottumkara et al. 2014; Wiese et al. 2010). Just 1–4 mg can cause death in humans, while other derivatives display a range of toxicities that range from 100 % to 0.05 % maximal value that are caused by structural molecular modifications (Oshima 1995). The potent toxicological effect is due to the extremely high affinity of saxitoxin for its receptor site located in the outer amino acidic structure of the sodium channel (Suarez-Isla 2008). Bioaccumulation of saxitoxins in filter-feeding bivalves and other marine species may reach concentrations that can cause human illnesses and death upon consumption. This is the cause of thousands of human cases of paralytic shellfish poisoning occurring every year, affecting mostly poor coastal communities worldwide (Callejas et al. 2015; Hernandez-Orozco and Garate 2006; Rodrigue et al. 1990; Yen et al. 2006). Monitoring programs have been developed in several countries to assess,

manage, and prevent the severe health and economic risks associated with the international trade of contaminated shellfish and fish (Anderson et al. 2001; EFSA 2009a; EFSA: European Food Safety Authority). In many countries, monitoring and management programs for marine biotoxins in products destined for domestic consumption are limited in scope, geographical extension, and methodologies.

This review examines current knowledge on toxicology and toxicodynamics of saxitoxins. Recent advances on risk analysis are compared with previous comprehensive assessments on the saxitoxin group (EFSA 2009a; Paredes et al. 2011) to identify specific gaps in toxicological knowledge, analytical uncertainties, and limitations of monitoring and risk management policies that may still pose a significant public health risk.

Several comprehensive reviews are available on saxitoxins (EFSA 2009a; Lawrence et al. 2011; Otero 2014; Vale 2014) and tetrodotoxins including their chemistry (Ashihara et al. 2013; Choi et al. 2012; Ciminiello et al. 2012; Thottumkara et al. 2014; Wiese et al. 2010), pharmacology and toxicology (Daneshian et al. 2014; Llewellyn et al. 2006), detection methods (Humpage et al. 2010; Suarez-Isla 2008), and distribution and dynamics of toxic phytoplankton species (Weinstein 2013).

The Saxitoxins

Saxitoxin (STX) is an alkaloid that belongs to a large group of marine natural products that contain guanidino groups as their main structural components. The presence of two guanidino groups in STX explains the high polarity of this compound. The saxitoxin group includes over 50 analogues (Wiese et al. 2010) that differ in the functional side group R1, R2, R3, and R4 (Fig. 1). The best studied ones are hydrophilic and were isolated from contaminated shellfish and/or

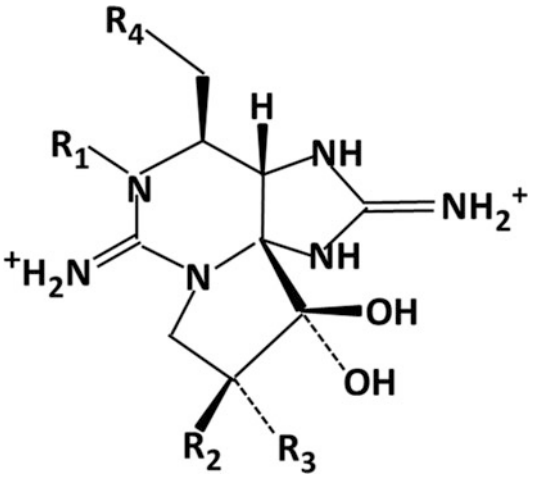
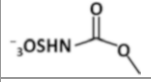
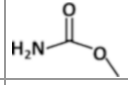


Fig. 1 The general structure of saxitoxins (Adapted from Oshima 1995)

Table 1 The relative toxicities of the main saxitoxin congeners

R1	R2	R3	R4		R4		R4	
			N-sulfocarbamoyl		carbamate		decarbamoyl	
								
				MU/μmol		MU/μmol		MU/μmol
H	H	OSO_3^-	C1	15	GTX2	892	dcGTX2	382
H	OSO_3^-	H	C2	239	GTX3	1,584	dcGTX3	935
H	H	H	B1		STX	2,483	dcSTX	1,274
OH	H	OSO_3^-	C3	33	GTX1	2,468	dcGTX1	
OH	OSO_3^-	H	C4	143	GTX4	1,803	dcGTX4	
OH	H	H	B2		NeoSTX	2,295	dcNeoSTX	
H	H	H	GTX5 (B1)	160				
OH	H	H	GTX6 (B2)	180				

Adapted from Oshima 1995 and EFSA 2009a. MU: mouse unit

cultured dinoflagellates. According to the functional group on the R4, there are carbamoyl, decarbamoyl, and N-sulfocarbamoyl saxitoxins, and toxicities measured by the mouse bioassay decrease in the order carbamoyl > decarbamoyl > N-sulfocarbamoyl (Table 1) (Oshima 1995). The carbamoylated analogues comprise the most toxic group members, such as STX, neoSTX, and gonyautoxins 1- and 4 (GTX1, GTX4). Loss of the carbamoyl moiety reduces toxicity significantly as is the case for the resulting analogues GTX2 and GTX3, respectively. The presence of a sulfamate group in position R4 generates the less toxic subgroup of B1 and B2 toxins (alternative designations GTX5 and GTX6, respectively). Metabolic transformations of the original mixture of saxitoxins present in the toxic dinoflagellate can take place in the shellfish (or other primary vectors) and other members of the trophic chain, including humans. These biochemical reactions can increase the total toxic burden and, rarely, decrease it. The most common modifications include N-oxidations and carbamoyl hydrolysis. Knowledge of the original composition and ensuing specific changes are essential for a correct risk assessment (Munday and Reeve 2013).

Producing Organisms and Vector of Poisoning

Saxitoxins are produced by marine dinoflagellates from the genera *Alexandrium*, *Pyrodinium*, and *Gymnodinium*, eukaryotes that are distributed worldwide (Anderson et al. 2012a, b). Dinoflagellate species such as *Alexandrium catenella*, *Alexandrium tamarense*, and *Gymnodinium catenatum* generate harmful algal blooms that can reach up to 1×10^4 cells/L or higher. These blooms that rarely produce water discolorations (red tides) display complex distribution patterns in the water column (McGillicuddy et al. 2014). Blooms of these species may lead to PSP toxin accumulation in shellfish and fishes and PSP intoxication and human deaths by cardiorespiratory failure upon ingestion of contaminated tissues. The most common toxin vectors for human intoxications are filtering bivalves, but other species such as gastropods, echinoderms, tunicates, and ascidians can accumulate toxic levels of saxitoxins (Bricelj and Shumway 1998; Deeds et al. 2008; García et al. 2015; Zamorano et al. 2013) and other marine biotoxins. Accumulation of PSP toxins in sardines (Costa et al. 2010) and salmon (Cembella et al. 2002; Sephton et al. 2007) has been established although at sub-toxic levels. In addition, saxitoxins and tetrodotoxins are produced by a number of species that represent an additional health risk. Fugu fish poisoning is caused by TTX and derivatives present in the fish tissues (Arakawa and Nagashima, ▶ Pufferfish Poisoning and Tetrodotoxin, this volume). These toxins are also produced by prokaryotes from freshwater environments, such as filamentous cyanobacteria that include *Anabaena circinalis*, *Aphanizomenon gracile*, *Cylindrospermopsis raciborskii*, and *Lyngbya wollei* (Kellmann et al. 2013). Cyanobacteria blooms have caused livestock intoxications and contamination of freshwater reservoirs (Fitzgerald et al. 1999). Monitoring programs in drinking water in Australia, New Zealand, and Brazil (Costa et al. 2010; Humpage et al. 2010) have been implemented to address these hazards.

Precise knowledge of dinoflagellate bloom distribution and dynamics and possible triggering factors is limited to a few coastal systems (Crespo et al. 2011; Kleindienst et al. 2013; McGillicuddy et al. 2014; Vale et al. 2008). Causal relationships between cyst abundance and distribution with bloom initiation and toxicity levels are complex (Cox et al. 2008), and in spite of the quality of field information in specific well-studied ecosystems (Anderson et al. 2014), current results still have limited applicability for resource management decisions (Boesch et al. 1997).

Correlations between PSP accumulation and changes in global or local weather parameters are very complex, and key initiation factors for harmful algal blooms are still elusive. Moore et al. (2009) examined short term and changes in weather parameters and concluded that PSP accumulation in mussels seemed to be favored by a combination of high air and water temperatures and low stream flow, conditions that favor water column stability and stratification. McGillicuddy et al. (2014) have proposed a prediction model for HAB (harmful algal bloom) occurrence and severity in the Gulf of Maine that is based on extensive sets of environmental observations. However, predictors are rather general and do not provide sufficient spatial resolution for short-term management decisions. As a result of these difficulties, monitoring programs for PSP toxins have relied almost exclusively on toxin analyses in shellfish tissues and, in some instances, also on phytoplankton monitoring (Anderson et al. 2001).

Epidemiological Data

PSP intoxication symptoms include gastrointestinal disorders and several manifestations of neurological nature that result from sensory, cerebellar, and motor function impairment after sodium channel blockade by saxitoxins.

Depending on the severity of intoxication, first symptoms can appear within 0.5 and 2 h after ingestion, followed by a variable development phase of up 12 h (Murphy 1936; Gessner et al. 1997a; Gibbard and Naubert 1948; Montebruno 1993; Tenant et al. 1955). Patients may display oral and facial paresthesias, dysphagia, weakness of lower and upper limbs, abdominal pain, and dyspnea due to respiratory muscle paralysis. This may result in respiratory arrest, cardiovascular failure, coma, and death if untreated (Batoreu et al. 2005; Long et al. 1990; Montebruno 1993). Alert patients may report swallowing difficulties, double vision, headache, and dizziness (Hurley et al. 2014). A survival after 12 h has a good prognosis of recovery without long-term effects. The lethal average dose (LD_{50}) of saxitoxin is dependent on the animal species, age, and the administration route. In humans LD_{50} ranges from 1 to 4 mg depending upon age and physical condition of the patient (Lawrence et al. 2011). Intraperitoneal (i.p.) injection of STX in rats causes a LD_{50} of 10–12 μg STX equivalents/kg and 9–10.6 μg STX equivalents/kg in mice. However, orally administered STX has an $LD_{50}$of 260–263 μg STX equivalents/kg. These values have been critically reviewed by Munday and Reeve (2013) that advocate a complete revision to obtain a better estimation of specific toxicities in experimental animals employing oral administration.

Human Studies

Garcia et al. (2005) examined four intoxicated patients that were successfully treated during a major PSP outbreak in the Island of Chiloe in southern Chile (47°S) (March–May 2002). Patients had eaten a few ribbed mussels (*Aulacomya ater*) that accumulated 8,575 μg STX equivalents/kg. Gonyautoxins GTX2 and GTX3 were detected in shellfish tissue, serum, and urine. Pathological signs such as hypoxia, low arterial O_2 pressure concomitant with respiratory acidosis, and bradycardia as a consequence of respiratory failure were observed. Treatment included respiratory support, vasoactive drugs, and diuretics. In this case patients were stabilized within 8 h and recovered completely after 48 h. As reported previously (Hurley et al. 2014; Price et al. 1991), patients did not report subsequent long-lasting effects, confirming previous studies (Stafford and Hines 1995).

It is important to notice that there is no specific treatment for PSP intoxication and there are no antidotes approved for human treatment. First care symptomatic treatment during the initial phase is crucial for later recovery as PSP intoxication is a life-threatening emergency characterized by several signs and symptoms (Garcia et al. 2005; Hurley et al. 2014). There is no substitute for rapid recognition of the specific symptoms and of the circumstances of the accident by the medical team. Early diagnosis may attribute symptoms to other causes, such as intoxication with drugs of abuse, psychiatric disorders, or cerebrovascular accidents. Patients may need intubation, evacuation of stomach content, and ventilator support. The use of diuretics and close monitoring of cardiovascular parameters are important, as cases of hypertension and tachycardia have been reported (Gessner et al. 1997b; Hurley et al. 2014). Severely affected patients may become ataxic and will not respond to usual tests, but maintenance of emergency care is essential as severe cases that may resemble coma or brain death symptoms have been reported to recover. Thus, special focused training on emergency procedures and availability of respiratory aids are essential.

Toxicokinetics

Human Studies

Due to the life-threatening nature of PSP intoxications, clinical reports on human intoxications and findings about toxicokinetic parameters of saxitoxins are infrequent, as sampling and measurements have to be made in an emergency room setting. In spite of these constraints, it could be determined that saxitoxins were rapidly eliminated by urination, the main route of toxin excretion (Gessner et al. 1997a; Montebruno 1993). Initial studies suggested that elimination took place apparently without modification. However, later studies provided the first evidence of specific metabolic and detoxification routes for saxitoxins in humans that could occur in the gastrointestinal tract and liver. In fact, composition of saxitoxins found in shellfish was different from that found in blood and urine samples of patients during a toxic outbreak in Kodiak, Alaska (Gessner et al. 1997b).

Postmortem analysis of tissues of a fisherman after ingestion of highly toxic crab meat indicated the presence of saxitoxin in urine, liver, and blood (Llewellyn et al. 2002). The additional detection in urine of neoSTX that is produced by N-oxidation from STX and of decarbamoyl STX via STX hydrolysis of the carbamoyl moiety confirmed metabolic modifications of saxitoxins.

Analysis of tissues and body fluids from two fishermen that died 2–4 h after ingestion of highly toxic ribbed mussels (*Aulacomya ater*; 8,575 μg STX equivalents/kg as measured by mouse bioassay) detected PSP toxins such as GTX1, GTX4, GTX5, GTX2, and GTX3 in the gastric content and neoSTX and GTX4/GTX1 epimers in urine and bile (Garcia et al. 2004). As neoSTX was not detected previously in gastric fluids, it was suggested that it was an N-oxidation product from the original STX. Similarly, GTX1/GTX4 epimers should have been produced by an oxidizing metabolic step from GTX2/GTX3 during the time between toxin ingestion and death. Later in vitro studies using fresh human hepatic extracts have confirmed that saxitoxins can be N-oxidized and glucuronidated as many other xenobiotics, using a common catabolic pathway that would be expected to contribute to PSP detoxification in humans (García et al. 2009, 2010). The glucuronidated saxitoxins may have gone undetected in previous studies.

Analysis of fluid samples from a surviving intoxicated patient that ate a large portion of cockles contaminated with saxitoxins during a bloom of *Gymnodinium catenatum* in the Portuguese northwest coast, indicated also a significant modification of the original shellfish profile (Rodrigues et al. 2012). Urine samples showed an increase in the molar percentages of dcSTX, neoSTX, and GTX5. In contrast congeners with an O-sulfate at C11 that were abundant in bivalves could not be detected in urine.

Recent studies report that total PSP toxicity is eliminated with a half-life between 10.4 and 12.6 h (DeGrasse et al. 2014). These are values consistent with previous observations of patient recovery times after intoxication. These authors could also demonstrate that the sulfated derivatives of STX and neoSTX, namely, GTX1/GT4 and GTX2/GTX2 epimers, had a shorter half-life of elimination.

Animal Studies

A large fraction of saxitoxin was eliminated intact in the urine of rats after intravenous injection during the first 24 h (Stafford and Hines 1995) as determined by pre-oxidation high-performance chromatography. A slower phase that lasted another 36 h could be detected, pointing to a biphasic process. Intravenously injected STX in anesthetized cats (Andrinolo et al. 1999) was mainly eliminated via glomerular filtration without accumulation in body fluids. Similar findings were observed in cats after oral administration of sublethal doses of gonyautoxins GTX2/GTX3 epimers (Andrinolo et al. 2002a). Interestingly, STX has been detected in cat (Andrinolo et al. 1999) and rat brain tissues (Cervantes Cianca et al. 2011), a finding that has not been observed in humans. Metabolic modification of saxitoxins and involvement of xenobiotic mechanisms have been well established in rodents

(Hines et al. 1993; Hong et al. 2003; Naseem 1996) and fishes (Bakke and Horsberg 2010; Gubbins et al. 2000). In vitro transport experiments of intestinal absorption in rat and human cell lines using GTX2/GTX3 epimers have provided evidence for an active transport system for saxitoxins (Andrinolo et al. 2002b).

Toxicity

In Vivo Toxicity After Repeated Administration

Several marine biotoxins are slowly eliminated, and shellfish can maintain detectable toxin levels below the regulatory limit during a detoxification phase that may last months or years (Deeds et al. 2008). Some shellfish species can retain saxitoxins for considerable periods of time (Shumway et al. 1994). In order to address this long-term exposure risk, the determination of tolerable daily intake (TDI) values is needed. TDI is defined as "the daily intake of a chemical in food that, in the light of present knowledge, can be consumed every day for a lifetime with no appreciable harmful effects" (Lawrence et al. 2011). TDI values have to be determined by repeated dosing animal studies following approved protocols (OECD 2008, 2009a). This type of normalized studies is practically absent in the marine biotoxin literature, and this has precluded EFSA (2009a) to define TDIs for this group of toxins.

Other studies using repeated doses are infrequent and contradictory. Early findings by Sommer and Meyer (1937) described that an i.p. injection of mice with a toxic shellfish extract made them more susceptible than control animals to a second dose. In contrast, many years later McFarren et al. (1961) reported that an initial sublethal dose of STX made the animals more resistant to a later administration. Recently, in a search for nontoxic levels of neoSTX to be used in experimental local anesthesia, doses of subcutaneous administration of neoSTX to rats during 12 weeks (1, 3 and 6 μg/kg) proved to be non-lethal. Under these special conditions, no signs of adaptation to previous doses were observed (Zepeda et al. 2014).

Genotoxicity

To date few studies have been reported on mutagenicity effects of marine biotoxins, and no studies on carcinogenicity employing approved standard methods using animal models or cell lines have been carried out (Munday and Reeve 2013; OECD 2009b, c). Tetrodotoxin gave negative genotoxicity results in the mouse bone-marrow micronucleus test (Guzmán et al. 2007). However, effects of saxitoxins have been detected in fish and shellfish species frequently exposed to these toxins in their natural habitats. Saxitoxin produced cytogenetic damage in white seabream with significant increase of nuclear abnormalities after 2 and 6 days i.c. (intracutaneous) injection as assessed by the ENA assay (erythrocytic nuclear abnormality assay) (Costa et al. 2012).

Limitations of Current Assays and Analytical Methods

Management of toxic PSP outbreaks and TTX intoxications has been based worldwide on the mouse bioassay for the last 65 years (AOAC 2000; AOAC, Association of Official Analytical Chemists; Schantz 1986; Sommer and Meyer 1937). The mouse bioassay is a semiquantitative assay that provides sufficient reproducibility and detection limit for regulatory purposes. Its major advantage is that it measures biological toxicity directly and as such should be maintained as a reference method. While reliable for regulatory purposes, this assay is costly and time-consuming and cannot be automatized, and its detection limit (320–380 μg STX equivalents/kg edible parts) is very near to the maximum permitted level of 800 μg STX equivalents/kg (AOAC 2000; Wekell et al. 2004). Some countries have decreased that level, as children and elderly people have shown signs of intoxication at lower levels (Callejas et al. 2015; Campàs et al. 2007).

Sodium channel toxins pose a significant public health threat and an enormous economic challenge to the shellfish industry worldwide. Fatal PSP intoxications represent the most serious threat of marine origin worldwide (Batoreu et al. 2005; Zhang et al. 2013) with severe public health and economic impacts in Asia, Europe, North America (Anderson et al. 2001), and South America (Sar et al. 2002). As a consequence, the large majority of coastal countries and all seafood-exporting economies have established mandatory PSP toxin screening programs.

Risk assessment analyses (EFSA 2009a, b; Paredes et al. 2011) have determined an acute reference dose (ARfD) of 0.5 μg STX equivalents/kg bw. (bw, body weight). This implies if a 70 kg person eats 400 g of mussels contaminated at the current permitted level of 800 μg STX equivalents/kg, the exposure would be 4.6 μg STX equivalents/kg bw. This is equivalent to 9–10 times the ARfD. As a consequence, EFSA (2009a) has suggested a permitted level 10 times lower at 80 μg STX equivalents/kg. Thus, future methods should have a robust limit of quantification (LoQ) lower than 80 μg STX equivalents/kg. This may be a problem as current HPLC-FD versions (HPLC, high-performance liquid chromatography; FD, fluorescent detection) of the official AOAC method (AOAC 2000) have LoQs higher (or very near to) than that value for some derivatives (STX in particular; EFSA 2009a). According to EFSA (2009b) this critical analytical limitation means that it is not possible to perform a correct exposure assessment, as many samples containing saxitoxins in the range of the newly proposed permitted level will go undetected.

Certainly, the mouse bioassay with a detection limit of 320–380 μg STX equivalents/kg cannot meet a requirement of a new maximum permitted level of 80 μg STX equivalents/kg. However, the mouse bioassay is currently used worldwide, and countries from the developing world will continue to do so due to the lack of sufficient funding to implement new methodologies in all necessary locations. These countries are facing a new difficulty. For many years standard saxitoxin dihydrochloride was used as a calibrant of the mouse bioassay, and it was provided free of charge to all accredited laboratories in the world by the US Food and Drug Administration, Office of Seafood. Since 2012 this invaluable service has ceased to

exist, and laboratories that need to continue using of the mouse bioassay have to rely on a sustainable production of standard saxitoxin from the National Research Council Canada.

As it is the case with other critical supplies for regulatory work, such as assay kits and analytical standards, their continued production depends on market variables that are beyond the control of laboratories from health or fisheries services. This is a mayor risk for a sustained monitoring and management effort on these lethal marine biotoxins. Uncertainties of provision of certified analytical standards are a major risk for the sustainability of all analytical methods currently in use for marine biotoxins.

The major limitation of the mouse bioassay, however, is the controversial use of live animals. The assay measures the time to death after intraperitoneal injection of seafood extract, a procedure that has received such ethical criticism that it can no longer be carried out in some European countries. In addition, high salts and metals such as zinc, manganese, and cadmium interfere severely with bioassay determinations, suppressing the apparent PSP toxicity (Suarez-Isla 2008; Turner et al. 2012). These disadvantages stimulated the development of several alternative methods and assays (Reverté et al. 2014; Suarez-Isla 2008) that are beyond the scope of this review. Suffice it to say that in today's regulatory environment, any new method has to be validated following strict internationally agreed requirements to demonstrate that its performance is at least equivalent to the current reference method. In this context, liquid chromatographic-based methods with fluorescent detection have been most successful at validation (European Commission Decision 2002/657/EC 2002; Kruve et al. 2015).

Current analytical methods for saxitoxins have to perform within limits of detection and quantitation that are fit for their purpose. There are two AOAC-validated HPLC-FD methods for PSP toxins (DeGrasse et al. 2011), a pre-column oxidation method (AOAC 2005.06; Anon 2005) and a post-column oxidation method (AOAC 2011.02; Anon 2011). Both require oxidation of STX analogues for fluorescent detection.

The official methods display detection and quantitation limits that differ for each derivative available as a certified standard (see Table 4; EFSA 2009a) and also depend on specific implementation protocols and instruments used in each laboratory. As stated before, for some derivatives, these performance parameters may be still insufficient, especially if the proposal from EFSA (2009b) to reduce the regulatory limit 10-fold, from 800 μg STX equivalents/kg to 80 μg STX equivalents/kg, is finally accepted.

However, recent work on an improved version of the AOAC 2005.06 pre-column oxidation method (Harwood et al. 2013) will help to avoid the interference from matrix components and from fluorescent STX derivatives display similar or identical retention times that can lead to overestimation of concentrations. Recent advances in LC-MS/MS methods are also very promising and may provide the needed performance characteristic sensitivity to solve these limitations (Turner et al. 2015).

Analytical HPLC methods require analytical standards to quantitate toxins in a mixture. Availability of certified reference materials and analytical standards is

critical. As mentioned before, the composition of acidic extracts from naturally contaminated PSP shellfish samples is highly variable and may contain over 18 different analogues of STX in variable proportions (García et al. 2015; Oshima 1995). To date only 12 STX analytical standards and a reference material are provided by the laudable efforts of the National Research Country Canada (www.nrc.ca).

But there is another problem. Analytical methods provide the molar composition of toxic extracts and a total toxin concentration, a quantity that has to be transformed into toxicity values. This calculation relies on toxicity factors obtained by the mouse bioassay carried out with intraperitoneal injection of pure STX analogues (EFSA 2009a; Hall et al. 1990; Oshima 1995). These STX analogues need to be certified standards in order to correlate acute toxicity in mice with a known concentration. Currently, countries in the European Union apply a set of equivalent toxicity factors (TEFs) which are based on a set of relative toxicities derived from specific toxicities determined previously by mouse bioassay (Oshima 1995) (see Table 4; EFSA 2009a). However, these agreed TEF values have poor correlation with acute toxicities measured as median lethal doses of saxitoxin, neosaxitoxin, decarbamoylsaxitoxin, and mixtures of gonyautoxins 1/4 and gonyautoxins 2/3, using several routes of administration (Munday et al. 2013). This situation should prompt a revision of current TEF values and a renewed effort to obtain a revised set of these critical values.

In summary, results of toxicities derived from analytical measurements by HPLC-FD methods rely on a biological method that has been replaced in several countries. The lack of a complete set of analytical standards in sufficient amounts hampers the determination of equivalent toxicity factors for saxitoxin derivatives and limits the capabilities of analytical methods.

Limitations of Monitoring and Management Procedures

Monitoring and management for harmful algal blooms and marine biotoxins is a complex task (Anderson et al. 2001). Countries that export shellfish that may be potentially affected by marine biotoxins, such as New Zealand and Chile, have implemented management programs based on EU directives that are focused on monitoring of growing areas and end product testing. These procedures follow well-established international regulations (see EFSA 2009a). However, due to limitations of sampling geographical density and frequency, these programs may underestimate spatial and intrapopulational variability in live samples from growing areas. As has been reported before, high spatial variability in PSP levels in the same species has been found in samples collected from sites distant 1–10 km (Nishitani and Chew 1984; Prakash et al. 1971). In the absence of concomitant information on phytoplankton dynamics and distribution, it is very difficult to explain the mechanisms underlying intrapopulational variability.

As reported by García et al. (2015) and Zamorano et al. (2013), the distribution of PSP toxin levels may also show large interindividual variability as a function of species, specific location, and depths in natural shellfish beds or in cultures

(Turner et al. 2014). Factors affecting variability have been discussed by Bricelj and Shumway (1998) and more recently by García et al. (2015). Significant factors include among other variables the degree of exposure to toxic phytoplankton, assimilation capacity of toxins by shellfish, toxin elimination rates, developmental stage of the shellfish species, and species-specific biotransformation pathways.

Large variability of PSP toxin profiles and lack of predictability of PSP events have been reported by Turner et al. (2014) after a 5-year period of study in Great Britain using official HPLC-FD methods (Turner et al. 2009). Examination of a large number of samples from 12 marine species in over 50 sampling sites indicated some regularities of PSP toxin profiles for particular species. However, the occurrence or absence of toxic events during a specific year could not be correlated with successive occurrences. It was observed that only 0.267 % of all tested samples (41/15640) showed levels above the regulatory limit (RL) of 800 μg/kg, while the number of samples above RL and another group over a reporting limit of 160 μg/kg were 249 (1.62 %). This is consistent with observations in other countries using the mouse bioassay with percentages of samples above RL below 0.5 %. Several reasons could explain the very large number of nontoxic samples found in long-term monitoring programs in several parts of the world that use the mouse bioassay. The simplest explanation is related to the high limit of detection of the mouse bioassay (ca 320–280 μg STX equivalents/kg) that would leave most samples go undetected.

Interestingly, other factors may be at play to explain the large variability of the toxicity levels found in shellfish as a function of species and geographic distribution, such as genetic forcing mechanisms affecting the primary structure of sodium channel proteins in specific amino acids that reduce the binding affinity for STX and TTXs (Soon and Venkatesh 2008). These factors have been suggested to explain the differential behavior of species that have been exposed to PSP toxins for many generations as compared to other populations that grow in areas without a previous history of toxic blooms. This is the case of the soft-shell clam *Mya arenaria* (Bricelj et al. 2005) and of razor clams (Navarro et al. 2014). Relatively PSP-insensitive shellfish species such as mussels and clams can accumulate and sustain exceedingly high toxic levels. In contrast, PSP-sensitive shellfish species such as oysters that display inhibition of feeding mechanisms for saxitoxins during blooms, as is the case for the Pacific oyster *Crassostrea gigas* (Moore et al. 2009), may lead to accumulation of low levels of the toxins that may be undetected by the mouse bioassay. This potential genetically determined variability may add to the uncertainties of any risk analysis and forecasting effort. Therefore, it is advisable to sample PSP-sensitive and PSP-insensitive species to reduce exposure risk.

Conclusions

After almost 80 years of sustained research effort, there are still significant gaps that have to be addressed to improve public health protection with better tools for monitoring and management to protect international trade (Anderson et al. 2001). While it is essential to prioritize the implementation of analytical techniques for

efficient monitoring and early warning in developing countries, it will be necessary to preserve capacities for mouse bioassays as screening tool for saxitoxins and new toxins from uncommon sources. Analytical techniques have to be validated with local matrices from potential toxin vectors that are consumed locally following long established traditions. The critical issue of availability of analytical standards should be tackled and supported by a public effort to generate low-cost reference materials. In the long term, the intriguing impact of sodium channel adaptive evolution should be addressed in those coastal areas with endemic PSP presence.

As previously stated by Boesch et al. (1997), as government support for monitoring has declined worldwide, new reliable and more sensitive methods of detection are urgently needed to insure early warning decisions. Together with a better-trained public health workforce and information technologies applied for early warning and dissemination of risks, it should be possible to mitigate impacts on public health and local economies.

Cross-References

► Pufferfish Poisoning and Tetrodotoxin

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