transatlantic think tank for toxicology

t4 Report*

A Roadmap for Hazard Monitoring and Risk Assessment of Marine Biotoxins on the Basis of Chemical and Biological Test Systems

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Summary

Aquatic food accounts for over 40% of global animal food products, and the potential contamination with toxins of algal origin – marine biotoxins – poses a health threat for consumers. The gold standards to assess toxins in aquatic food have traditionally been in vivo methods, i.e., the mouse as well as the rat bioassay. Besides ethical concerns, there is also a need for more reliable test methods because of low inter-species comparability, high intra-species variability, the high number of false positive and negative results as well as questionable extrapolation of quantitative risk to humans. For this reason, a transatlantic group of experts in the field of marine biotoxins was convened from academia and regulatory safety authorities to discuss future approaches to marine biotoxin testing. In this report they provide a background on the toxin classes, on their chemical characterization, the epidemiology, on risk assessment and management, as well as on their assumed mode of action. Most importantly, physiological functional

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assays such as in vitro bioassays and also analytical techniques, e.g., liquid chromatography coupled mass spectrometry (LC-MS), as substitutes for the rodent bioassay are reviewed. This forms the basis for recommendations on methodologies for hazard monitoring and risk assessment, establishment of causality of intoxications in human cases, a roadmap for research and development of human-relevant functional assays, as well as new approaches for a consumer directed safety concept.

Keywords: marine biotoxins, risk assessment, consumer protection, regulatory toxicology

1 Introduction

Phytoplankton – planktonic algae – is at the basis of the marine food chain, i.e., it is the direct or indirect source of food for many higher level marine organisms. It is an essential source of nutrition for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) as well as for crustaceans and finfish in the marine environment, including marine aquacultures. About 300 marine algal species are described as producers of complex molecules that can be toxic to other organisms within the marine food web and are therefore designated as marine biotoxins. Exposure to these natural compounds can lead to adverse health effects including death in humans (Van Dolah, 2000). Although the function of marine biotoxins is not established, it is postulated that these compounds are produced to compete for space, hinder predation, or prevent the overgrowth by other organisms (Botana et al., 1996).

Whereas biotoxin producing algae, diatom, cyanobacteria, and dinoflagellate species (Chondria, Alexandria, Pyrodinia, Gymnodinia, Gambierdisci, Dinophyses, Kareniae, Karlodinia, Lyngbyae, Prorocentra, Azadinia, Protoperidinia, Pseudo-Nitzschiae, Protogonyaulaxes, Hydrocolea, Lyngbyae) are normal inhabitants of marine environments, their numbers can increase drastically in certain areas, thus leading to so called "harmful algal blooms" (HAB; see http://www.whoi.edu/redtide/). This phenomenon occurs under favorable conditions of high nitrogen, phosphorus, and CO_2 concentrations, high temperature, but also specific climatic conditions, e.g., in the aftermath of typhoons and hurricanes, etc.

The human influence, e.g., eutrophication in the marine environment due to farming, sewage processing and oil spills; changing biotic factors, e.g., El Niño-Southern Oscillation (ENSO) and decadal oscillations, and the effects of climate change, e.g., altered temperatures (water, surface and air) and CO_2 concentrations, have made the composition, frequency, magnitude, and also the spots of HAB occurrence less predictable (Hutchins et al., 2007; Anderson et al., 2002; Glibert et al., 2001; Colin and Dam, 2002; Purcell, 2005; Condon and Steinberg, 2008; Lomas et al., 2002).

Therefore, risk assessment of marine biotoxins must proceed on several levels: hazard monitoring regarding the occurrence of higher concentrations of harmful algal species in the marine environment, risk assessment and management of marine biotoxins in marine foods and non-foods, and finally meticulous registration of analytically verified intoxications in humans (Goater et al., 2011). The analytic verification of incidences will allow better evaluation of quantitative exposure to single marine biotoxins or combinations of marine biotoxins and intoxication symptomatology as well as monitoring the "success" of controls implemented during fishery and aquaculture production, processing, and retailing. For this reason, a workshop was organized in Ermatingen, Switzerland by the Center for Alternatives to Animal Testing – Europe (CAAT-Europe) within the framework of the Transatlantic Think Tank for Toxicology (t^4).

In contrast to other food products where hygiene and potential contamination with microbes or mold toxins is the prime issue, the emphasis in aquatic products (shellfish and finfish) is, beyond viral and bacterial contaminations, primarily placed on potential contamination with marine biotoxins owing to the numerous and recurring human intoxications.

The gold standards to assess toxins in aquatic food have traditionally been *in vivo* methods, i.e., the mouse and the rat bioassays. Besides the ethical issues of *in vivo* bioassays there are specific difficulties, e.g., different exposure route (i.p. versus oral), low inter-species comparability, high intra-species variability, and questionable extrapolation of quantitative risk to humans, thus highlighting the need for the development of more reliable detection and quantification methods. Based on this reasoning, the European Food Safety Authority (EFSA) advocates the use of an analytical method, i.e., LC-MS (Liquid Chromatography coupled Mass Spectrometry), as a substitute for *in vivo* bioassays for almost all classes of marine toxins. However, although LC-MS is a very sensitive method that has the advantages of being able to detect multiple toxins in a single analysis and being good for confirming the identity of toxins, the quality of quantitative analysis is dependent on the availability of calibration standards. While many new toxin structural analogues have been detected and identified using LC-MS, this technique – as with most other methods – is not good at detecting new toxin types. When new toxin analogues are detected but accurate standards are not yet available, only an approximate quantitation is possible using estimated response factors.

For the purpose of risk assessment of food, however, it is imperative to focus on the possible adverse effects, independent of whether they stem from one toxin or a combination of toxins. As toxicity is defined by functional and structural changes of biological systems, the development of a human-relevant *in vitro* system for appropriate risk assessment of marine biotoxins in seafood stands to reason. Moreover, poor correlation of human intoxications, of acute symptomatology and long-term adverse effects, and of predictive *in vitro*, *in vivo*, and analytical detection methodologies of marine biotoxins stems not least from a lack of appropriate documentation of human intoxica-

tions in a central registry accompanied by verification of intoxication via analytical techniques.

As highlighted by the *Escherichia coli* infections that spread in Germany and France from contaminated fresh salad (EFSA, 2011) and the horse meat scandal within Europe (Wise, 2013), global distribution of foods demands a rigorous control over production, processing, and quality. Moreover, controls should not remain primarily in the hands of the producers but a rigorous system involving all nations trading with foods or consuming the end-product is needed to ensure safety of aquatic products and to avoid entry of contaminated and incorrectly labeled food into global trade markets as has been recently the case with horse meat. Thus, in light of the growing global significance of fisheries and aquacultures and the global distribution of these products, the development of globally harmonized quality and safety assurance and regulations for consumer health protection worldwide is of central importance.

1.1 Global importance of marine food and its safety for consumers

Today already 40% of the globally distributed animal food products are of aquatic origin (USDA, 2013; FAO, 2012). Marine food (seafood) refers to any marine life form used as a source of nutrition by humans, including finfish (including whales and dolphins) and shellfish (mollusks, crustaceans, and echinoderms) from open seas as well as from aquacultures. According to the FAO report on "The State of World Fisheries and Aquaculture" from 2012 the global production of fisheries and aquaculture increased more than sevenfold from 1950 (20 million tons) to 2011 (over 154 million tons, providing 18.8 kg fishery and aquaculture products per capita and year respectively) and this trend seems likely to continue in the future with at a growth rate of approximately 3.6% per year (FAO, 2010; WHO, 2002) and will exceed that of beef, pork or poultry within the next decade. In comparison, the ratio of global population growth from 1950 to 2008 shows a 2.7-fold increase. This shows that fishery and aquaculture have gained significant importance as a source of world food. Notably, approximately 70% of nutrition from aquatic sources is consumed as "food" (fresh, frozen, cured, and canned); the remaining 30% finds its way into the food chain of consumers as "non-food", i.e., fishmeal, fish oil, pharmaceuticals, and supplements.

Economically, over 200 million persons are currently employed in this sector, and both the world total export and import markets passed the 100 billion US$ mark in 2008 (FAO, 2010, 2012). This increase in international fish trade parallels the globalization phenomenon, causing changes in the fisheries' supply chains and distribution channels, favoring few large retailers, inducing outsourcing of processing and also leading to introduction of advanced technologies. Regarding the advanced technologies, e.g., the development of long-distance refrigerated transportation vehicles and fast large-scale shipments of huge amounts of marine organisms – originating from ill-defined locations – e.g., from long-line catching in the South Pacific and along the coast of Africa – has played a significant role in achieving global distribution of marine catches. In other words, marine organisms can be caught or produced in one region, processed in another part of the world, and consumed in a variety of further regions. Moreover due to overfishing – primarily in Europe – there is an increasing need to import fish from other regions (FAO, 2012).

1.2 A primer on marine biotoxins

Twelve classes of marine biotoxins have been described and over 197 analogues and congeners are currently known (Tab. 1). Marine biotoxins are relatively heat-stable, i.e., resistant to cooking temperatures. The route of uptake of these toxins in humans is mainly via ingestion (oral) but can also be absorbed due to dermal and respiratory exposure. The latter route may have importance for fishermen and coastal populations, possibly affecting the development and progression of airway and lung diseases (Backer et al., 2003; Baden et al., 1995; Cheng et al., 2005; Fleming et al., 2001b, 2005; Morris et al., 1991; Music et al., 1973; Pierce et al., 2003; Kirkpatrick et al., 2004; Asai et al., 1982; Baden, 1983; Singer et al., 1998).

However, little is known about the relative composition of toxin analogues and congeners of different marine biotoxins in the different stages of the food chain. Indeed, as most studies to date were performed with extracted toxins, current knowledge is mainly limited to the performance of the extraction methods, although in most cases "documented" purified toxins have been used. Further, it is assumed that the most prevalent marine biotoxins are also the most toxic. These assumptions are based on the behavior and systemic response of rodents, which are the basis for toxic potency ranking (toxicity equivalent factors), usually evaluated after intraperitoneal injection (i.p.) of purified toxins or shellfish extracts. The endpoints of rodent assays, e.g., the mouse bioassay (MBA) (Kimura et al., 1982), are acute and sub-acute effects that depend on the toxins present but include death, piloerection, food uptake refusal, and diarrhea. The MBA for regulatory toxicological risk assessment of marine food and products is estimated to consume at least 300,000 mice per year in Europe (http://bit.ly/1cnFqiC). Beside ethical issues regarding stress and pain severity level, there are significant scientific issues demonstrating that the rodent system is irrelevant if not misleading as a surrogate for the detection of marine biotoxins in fishery and aquaculture products. These are: the route of application of the toxins (i.p. injection) is not comparable to the most likely routes of exposure in humans (oral, dermal, inhalation), the questionable correlation of the responsiveness of rodents with humans (inter-species variability with regard to the specific toxin interaction, species specific anatomical/physiological issues, e.g., rodents, which cannot vomit, are poor surrogates for the description of emesis induced by marine biotoxins, e.g., brevetoxin, ciguatoxins, maitotoxins, etc. in humans.), the poor sensitivity, significant intra-strain variations in sensitivity, high incidence of false positive results mainly due to the administration route (i.p.), lack of detection of false-negatives, subjective onset of toxic symptoms, and impracticality as field method *inter alia*. As pure analytical methods are restricted to identifying and possibly quantifying known marine biotoxins, there is a major gap of knowledge that could describe the effects of small quantities of single marine biotoxins or combinations thereof as a single or following multiple repeated exposures and

the onset of adverse effects in humans. In other words, there is a lack of validated human-relevant functional assays for the purpose of adequate risk assessment of marine biotoxins and also for hazard evaluation addressing the relative potencies of the 12 recognized classes of relevant marine biotoxins including the analogues and congeners described so far.

2 Occurrence, source, and chemical structure of marine biotoxins

2.1 Amnesic shellfish poisoning (ASP) toxins

ASP is caused by domoic acid (DA) and a number of toxic DA isomers (Clayden et al., 2005), primarily produced by benthic marcophytes (red algae) of the genera Chondria, Alsidium, Amansia, Digenea, and Vidalia (Wright et al., 1989; Lefebvre and Robertson, 2010) as well as diatoms of the genera Pseudo-Nitzschia, Nitzschia, and Amphora (FAO/IOC/WHO, 2004; Lefebvre and Robertson, 2010). In recent years shellfish containing DA have been reported in the USA, Canada, France, UK, Spain, Ireland, Portugal, and Italy (Perl et al., 1990; Bill et al., 2006; Campbell et al., 2001; Blanco et al., 2006; James et al., 2005; Vale and Sampayo, 2001). ASP toxins are water-soluble cyclic amino acids of low molecular weight (DA: 311 Da), whereby three carboxylic acid groups are responsible for water solubility and polarity (Quilliam, 2001). Due to their cyclic structure, ASP toxins are fairly heat stable, i.e., cooking procedures do not destroy them (McCarron et al., 2007). Around 10 isomers and analogues of DA have been described (Holland et al., 2005; Maeda et al., 1986; Walter et al., 1994; Wright and Quilliam, 1995; Zaman et al., 1997) and each can exist in different charged states, depending on pH (Jeffery et al., 2004; Pineiro et al., 1999). Storage, ultraviolet light, and heat cause DA to epimerize to epi-DA and iso-DA (Djaoued et al., 2008; Quilliam, 2003; Quilliam et al., 1989; Wright et al., 1990; Wright and Quilliam, 1995). Although limited data are available, current understanding suggests that some analogues may occur in a higher concentration in shellfish than in others

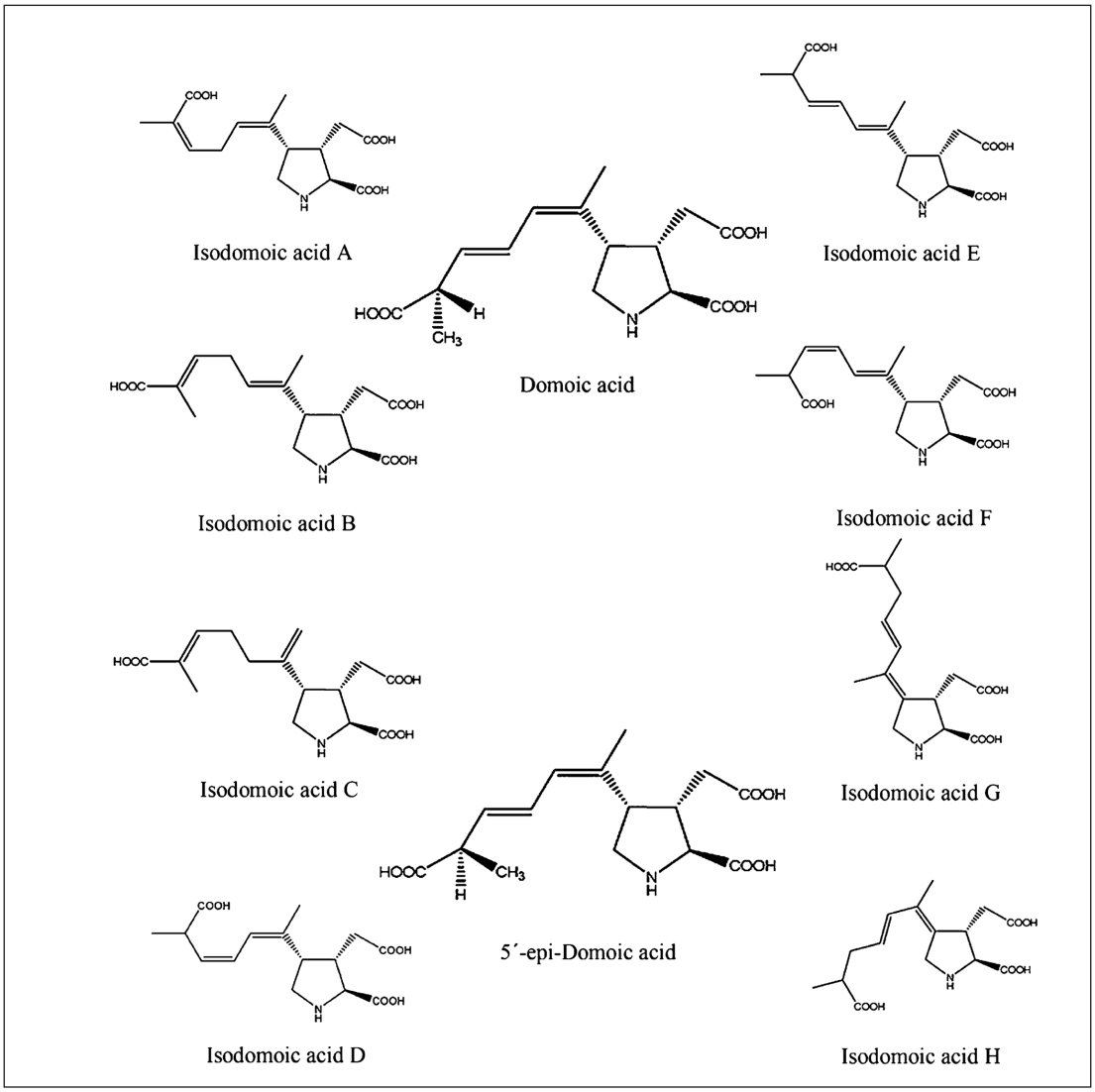

Fig. 1: Chemical structures of some ASP toxins
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(Jeffery et al., 2004; Zhao et al., 1997). Of the nine DA isomers (isodomoic acid A to H and the C5' diastereomer, see Fig. 1), isomers A, B, and C appear to have a lower toxicity in humans (Munday, 2008).

2.2 Azaspiracid shellfish poisoning (AZP) toxins

AZP is caused by azaspiracid (AZA) and its analogues, and so far 32 AZA analogues have been described (Satake et al., 1999; Ofuji et al., 2001, 1999; Brombacher et al., 2002; James et al., 2003; Rehmann et al., 2008; Krock et al., 2012). The structural formulae for AZA1 - AZA5 are displayed in Figure 2. The first reported incidents of human intoxication by AZA occurred in 1995 in the Netherlands after ingestion of contaminated mussels coming from Ireland, but AZA also was detected along the western coastline of Europe, especially along the Norwegian coast and also Chile (James et al., 2004; Magdalena et al., 2003; Vale, 2004; Lopez-Rivera et al., 2010). Notably, dinoflagellates of Azadium species could be shown as a source of azaspirazid (Jauffrais et al., 2012a,b, 2013; Potvin et al., 2012). AZP toxins show a spiral ring assembly baring a piperidine ring (heterocyclic amine) and an aliphatic carboxylic acid moiety (FAO, 2004). AZP toxins belong to the group of nitrogen-containing polyether toxins. In an acidic environment (methanol) AZA1 and AZA2 are unstable at temperatures above 70°C (Alfonso et al., 2008). In tissue samples at temperatures above 100°C AZP toxins are prone to degradation (McCarron et al., 2007), thus suggesting that cooking at higher temperatures could possibly degrade the AZP toxins. Whether or not the AZP toxin degradation products are also toxic has not yet been established.

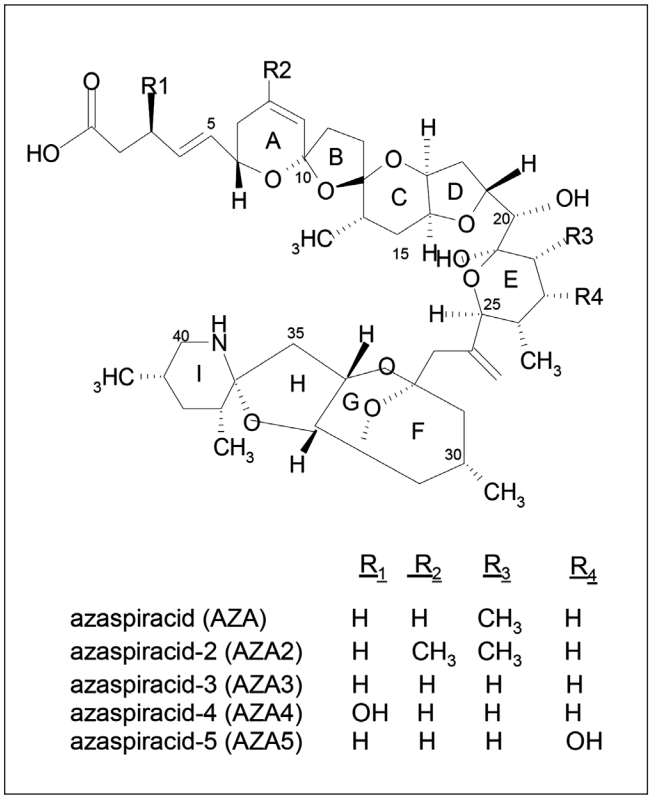

Fig. 2: Chemical structures of some AZP toxins
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2.3 Ciguatera fish poisoning (CFP) toxins

CTX are produced in fish (Barbotin and Bagnis, 1968) and mollusks, e.g., the turban snail (*Turbo picta* synonymous with *Lunella cinerea*) and possibly in giant clams (*Tridacna gigas*) by biotransformation from gambiertoxins originating from the dinoflagellates of the genus Gambierdiscus (Lehane and Lewis, 2000). Ciguatera toxins are found in the tropical and sub-tropical regions of the Pacific, Atlantic (Caribbean region, coast of Cameroon, Canary Islands, Madeira), and Indian Ocean. As the CTX from these regions differ with regard to their chemical structures as well as their toxicity/symptomatology and potency when ingested by humans, CTX are distinguished accordingly, i.e., Pacific (P-CTX), Caribbean (C-CTX) and Indian Ocean CTX (I-CTX) (Lehane and Lewis, 2000; Lewis, 2001; Lewis et al., 1991, 1998; Pottier et al., 2002; Vernoux and Lewis, 1997). More recently evidence has surfaced that *Gambierdiscus* spp. can be found in the Mediterranean (Aligizaki and Nikolaidis, 2008; Aligizaki et al., 2008; Bentur and Spanier, 2007). CTXs also have been identified in the Atlantic islands west of Africa (Boada et al., 2010; Gouveia et al., 2009; Nunez et al., 2012; Otero et al., 2010b; Perez-Arellano et al., 2005).

More than 30 CFP toxins have been described so far (Lehane, 2000; Lehane and Lewis, 2000; Lewis et al., 1991; Murata et al., 1990; Satake et al., 1993a,b, 1997, 1998). The structural formulas for some CFP toxins are displayed in Figure 3.

The primary sources of CFP toxins (CTX) are dinoflagellates of the genera Gambierdiscus, Prorocentrum, Gymnodinium, and Gonyaulax (Aseada, 2001). CFP toxins (CTX) occur in finfish, mussels, clams, and conches, whereas CTX accumulate along the trophic levels, thus occurring at highest concentrations in top predatory fish, e.g., barracuda (Sphyraenidae), amberjack (Seriola), grouper (Family: Serranidae), snapper (Family: Lutjanidae), po'ou (Chelinus spp.), jack (Family: Carangidae), and travelly (Caranx spp.) (FDA, 2011). The CFP toxin producers, as well as the concentrations of CTX concentrations in mollusks and finfish, can vary significantly, most likely as a result of weather and water conditions, as suggested for Australasia and the South Pacific Islands (Derne et al., 2010).

CTX toxins are lipophilic polyether compounds with 13-14 rings and as such are heat-stable, frost resistant, and stable in mild acidic and basic environments (FAO, 2004; Lange, 1994). Structural changes appear primarily due to modifications at the termini of the toxins, making them more hydrophilic (and toxic) as the toxins/toxin metabolites move from the herbivorous reef fish through the trophic levels to the carnivorous top predatory fish (Naoki et al., 2001; Yasumoto et al., 2000; Dechraoui et al., 1999). Following uptake and transfer through trophic levels, CFPs may undergo substantial conjugation and oxidation reactions in the respective species, thus resulting in a number of CTX "structural variants".

Gambiertoxin and maitotoxin have also been isolated from *Gambierdiscus toxicus*. Gambiertoxins are actually ciguatoxins and some of the CTX found in the dinoflagellate also are found in the fish (e.g., P-CTX-3C). Maitotoxin (MTX) occurs similar

to CTX also primarily in predatory fish consumed by humans (snapper, barracuda, amber jack and moray eel), and more investigations should be conducted on the role on MTX in human intoxication.

2.4 Cyclic imine poisoning (CIP) toxins

Gymnodimines (GYM), pinnatoxins (PnTX), pteriatoxins (PtTX), and spirolides (SPX) belong to the toxin group of cyclic imines (CI). The dinoflagellates *Alexandrium ostenfeldii* and *Alexandrium peruvianum* are the main producers of SPX, some strains of *A. ostenfeldii* also can produce saxitoxins – STX (Cembella, 1998; Gribble et al., 2005; Kremp et al., 2009; MacKenzie et al., 1996), while *A. peruvianum* produces not only SPX (Borkman et al., 2012; Tomas et al., 2012; Touzet et al., 2008, 2011) but also 12-methyl gymnodimine, STX and gonyautoxins (Tatters et al., 2012; Van Wagoner et al., 2011). SPXs were found in the early 1990s in Canada (Hu et al., 1995). SPX profiles produced by *A. ostenfeldii* can differ significantly due to localization and environmental conditions (Cembella et al., 2000; Otero et al., 2010a). GYM are produced by the dinoflagellate *Karenia selliformis* and were first described from occurrences in New Zealand (Haywood et al., 2004; MacKenzie et al., 1996; Miles et al., 2000, 2003; Seki et al., 1995, 1996). GYM may persist in shellfish even years after *K. selliformis* blooms. (MacKenzie et al., 2002).

PnTX (A-G) and PtTX (A-C) were initially found in Japan and New Zealand (Chou et al., 1996; Takada et al., 2001a; Uemura et al., 1995). The source of PnTX and PtTX currently is thought to be the dinoflagellate *Vulcanodinium rugosum* (Rhodes et al., 2010; Selwood et al., 2010; Nézan and Chomérat, 2011; Rhodes et al., 2011; Smith et al., 2011), and PtTX may be biotransformation products of PnTX (Selwood et al., 2010).

There is evidence that CIP toxins and their producers also are found in Europe, the USA, and North Africa (Aasen et al., 2005a; Bire et al., 2002; Cembella and Krock, 2008; Ciminiello et al., 2006; John et al., 2003; MacKinnon et al., 2006; Marrouchi et al., 2010; Rundberget et al., 2011; Touzet et al., 2008; Villar Gonzalez et al., 2006), and their presence, along with PSP

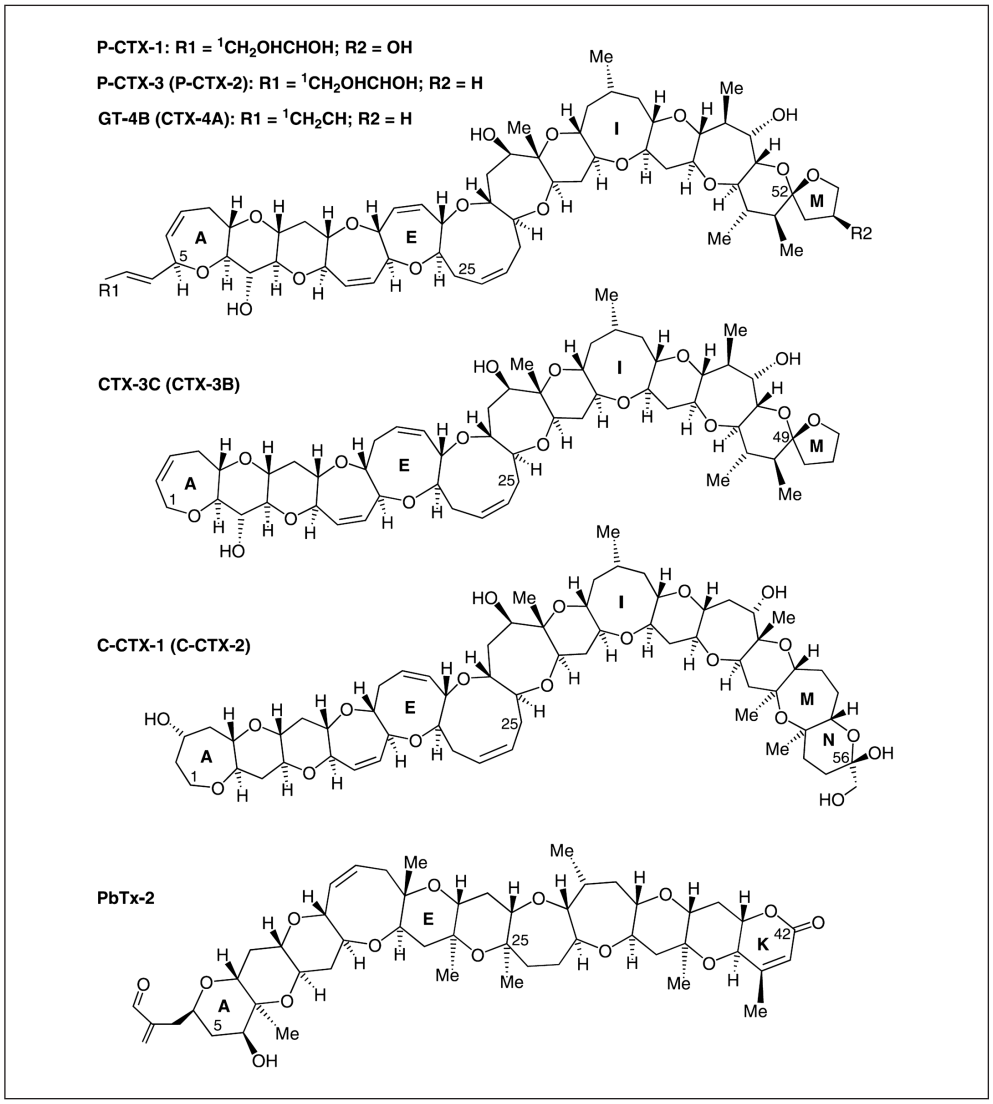


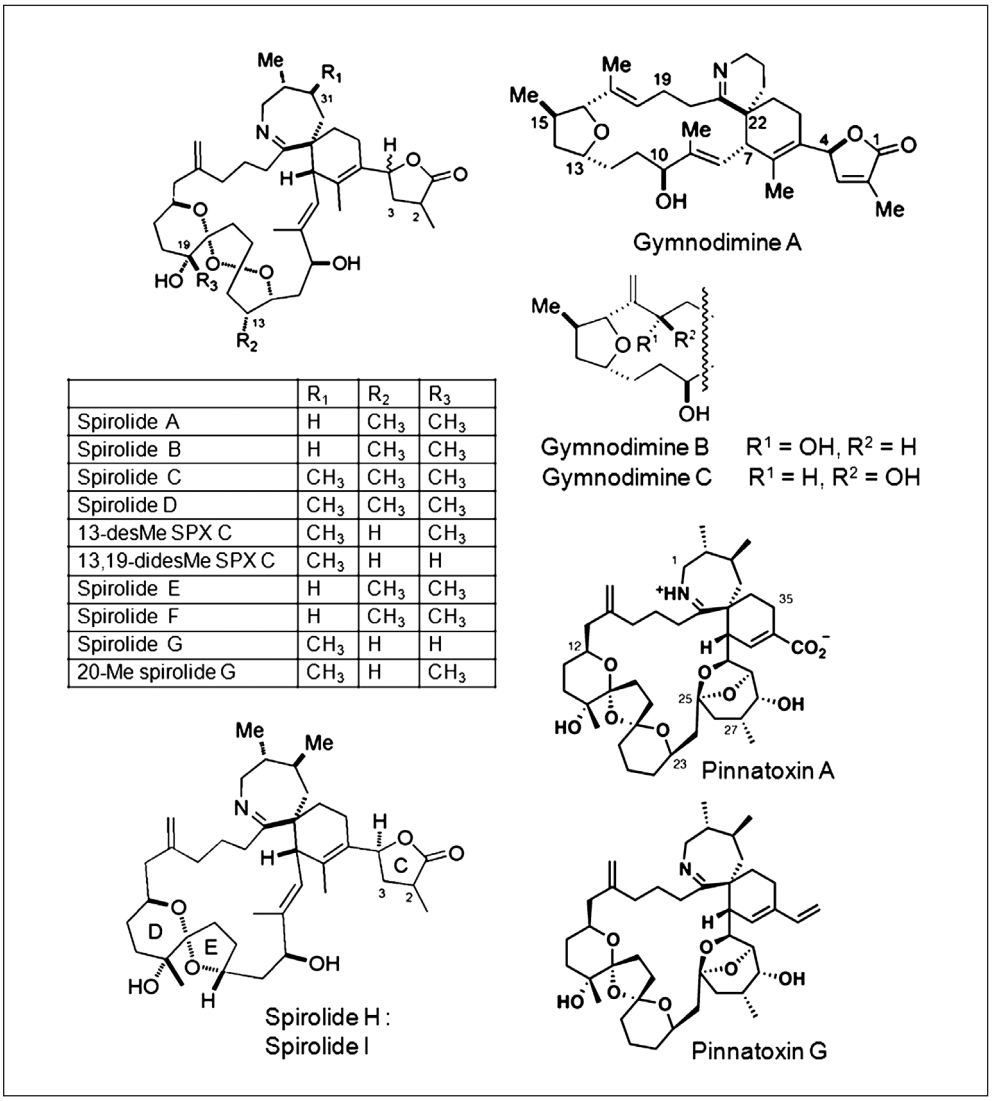
Fig. 3: Chemical structures of some ciguatoxins and brevetoxin PbTx-2
Reproduced with permission from (Lewis, 2006).

and DSP, has been reported in new invertebrate vectors (Silva et al., 2013).

Chemically, CI are amphiphilic macrocyclic compounds consisting of imine- and spiro-linked ether moieties of which more than 24 structural analogues have been described. Structures of spirolides and gymnodimines are displayed in Figure 4. In general, CI are structurally very similar, e.g., having 70% structural homology among PnTX and SPX (Cembella and Krock, 2008). To date, 3 classes of SPX (A-D, E-F, and G, H, I) and 34 GYM analogues (A, B, C) and 12-methyl gymnodimine have been identified. SPX-C and SPX-D appear to be resistant to acidic and enzymatic degradation in shellfish, while SPX-E and SPX-F could be metabolites of SPX-A and SPX-B (Christian et al., 2008; Hu et al., 1996). The characteristic of CI is the presence of an imine moiety as part of a bicyclic ring system (Fig. 4). The majority of these toxins contain a cyclic spiroimine motif, present in gymnodimines, spirolides, pinnatoxins, and pteriatoxins. The spiroimine ring system can be either 6:6 (gymnodimines) or 6:7 (spirolides, pinnatoxins, pteriatoxins). Another feature of this toxin family is the presence of a trispiroketal ring system, with either a 5:5:6 arrangement, specific for spirolides, or 6:5:6 arrangement, specific for pinnatoxins. There are a few exceptions to this rule, e.g., a 5:6:6 arrangement for spirolide G and bispiroketal 5:6 ring system for spirolides H and I. The spiroketal fragment, in the latter case, is replaced by a tetrahydrofuran ring in the gymnodimine family.

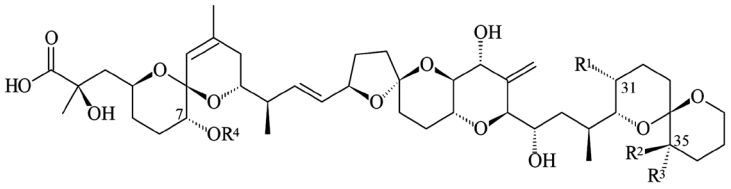
2.5 Diarrheic shellfish poisoning (DSP) toxins

Since the first documented human intoxication case in 1961, DSP toxins (DTX) were frequently reported in shellfish or algae along the coasts of Europe, Canada, South America (Chile), Japan, Australia, and North Africa (Elgarch et al., 2008; Garcia et al., 2005; Kat, 1979; Scoging and Bahl, 1998; Korringa and J., 1961). The causative agents of DSP are okadaic acid (OA) and its analogues dinophysistoxins (DTXs), which are produced by the dinoflagellate genera Dinophysis and Prorocentrum. The OA toxin producers, as well as the concentrations of these DSP toxins in shellfish, can vary significantly, most likely as a result



	R_1	R_2	R_3
Spirolide A	H	CH_3	CH_3
Spirolide B	H	CH_3	CH_3
Spirolide C	CH_3	CH_3	CH_3
Spirolide D	CH_3	CH_3	CH_3
13-desMe SPX C	CH_3	H	CH_3
13,19-didesMe SPX C	CH_3	H	H
Spirolide E	H	CH_3	CH_3
Spirolide F	H	CH_3	CH_3
Spirolide G	CH_3	H	H
20-Me spirolide G	CH_3	H	CH_3

Fig. 4: Chemical structures of spirolides and gymnodimines



	R^1	R^2	R^3	R^4
OA	CH_3	H	H	H
DTX1	CH_3	CH_3	H	H
DTX2	H	H	CH_3	H
DTX3 (acylated forms of OA, DTX1 and DTX2)	H/CH_3	H/CH_3	H/CH_3	fatty acid

Fig. 5: Chemical structures of some DSP toxins Reproduced with permission from (EFSA, 2008c).

of weather and water conditions (FAO, 2004). DSP toxins are heat-stable, frost-resistant, lipophilic polyethers (FAO, 2004; McCarron et al., 2007; Yasumoto and Murata, 1990), which can also occur in acylated forms (see Fig. 5) (Hallegraeff et al., 1995; Wright, 1995; EU/SANCO, 2001).

2.6 Neurotoxic shellfish poisoning (NSP) toxins

The causative agents of NSP are brevetoxins (BTX), primarily produced by the dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve* or *Ptychodiscus breve*) (Gunter et al., 1947; Davis, 1948), which appears to be expanding in global presence as is has been observed for other dinoflagellates (Grovel et al., 2003; Kerzaon et al., 2008; Otero et al., 2010a; Poirier et al., 2007; Rodriguez et al., 2008), either due to warming ocean temperatures, enhanced distribution with ballast water, increased nutritional loading of coast-line waters or a combination thereof (FAO/IOC/WHO, 2004). It is also produced by species of the genera Chattonella, Fibrocapsa, and Heterosigma (FAO, 2004). To date NSP intoxications have occurred in the USA (Gulf of Mexico and Florida) and New Zealand (Heil, 2009; Ishida et al., 2004c). The mass occurrences of bloom of NSP toxin producing dinoflagellates often are referred to as "red tides" (Music et al., 1973; Steidinger, 1975; Steidinger and Baden, 1984). Fifteen different BTX analogues are cur-

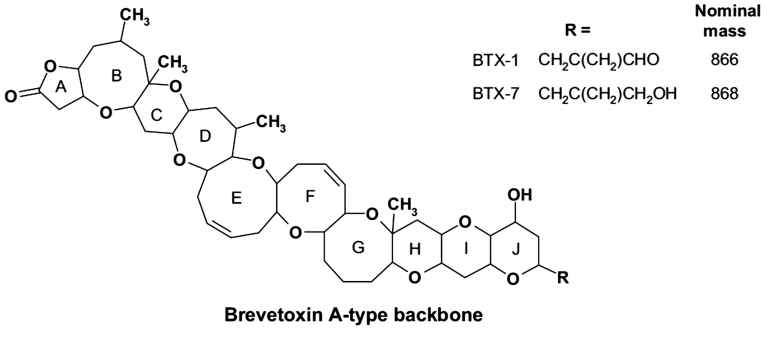


Fig. 6: Chemical structures of some brevetoxins representing the two backbone types
Reproduced with permission from (Abraham et al., 2008).

rently known that can be grouped into two classes (type A and B) according to differences in the backbone of the chemical structure. Structurally, BTX consist of 10-11 connected rings (Fig. 6) that are heat and acid stable. BTX-1 belongs to type A and appears to be a precursor of other NSP toxins (Baden et al., 2005). BTX-2 (type B) currently is assumed to be the most abundant in algae (Landsberg, 2002; Plakas and Dickey, 2010). It currently is assumed that human exposure occurs via consumption of shellfish, mollusks, and finfish, and that humans are primarily exposed to BTX-metabolites formed by shellfish, mollusks, and finfish rather than to the parent toxin molecules (Abraham et al., 2008, 2006; Dickey et al., 1999; Fire et al., 2008; Ishida et al., 1996, 2004a,c, 1995; Morohashi et al., 1995, 1999; Murata et al., 1998; Plakas and Dickey, 2010; Plakas et al., 2002; Shimizu et al., 1986; Wang et al., 2004).

2.7 Palytoxin poisoning (PaP) toxins

PaP results from exposure to palytoxins (PlTX), 42-OH-PlTX, mascarenotoxins, ostreocins, and ovatoxins which were first detected in Hawaii and Japan but have been observed in the European Mediterranean countries as well as in other temperate areas worldwide (Moore and Scheuer, 1971; Moore and Bartolini, 1981; Moore, 1985; Ciminiello et al., 2009; Shears and Ross, 2009; Deeds and Schwartz, 2010). To date, more than 15 analogues of PlTX have been described. The main producers of PlTX are dinoflagellate species of the genera Ostreopsis, as well as soft corals (zoanthids) of the genera Palythoa (Deeds et al., 2011) and marine cyanobacteria of the genus Trichodesmium (Kerbrat et al., 2011).

PlTX are amphiphilic, complex poly-hydroxylated alkaline compounds with an aliphatic backbone containing a cyclic ether residue (Fig. 7) having high resistance to heat. The structure of PlTX analogues appears largely to depend on the PlTX producing organism (Moore, 1985; Moore and Bartolini, 1981), such as the palytoxin congener ovatoxin produced by *Ostreopsis ovata* (Ciminiello et al., 2012b,a) and palytoxin analogues produced by *Scarus ovifrons* (Suzuki et al., 2013).

2.8 Paralytic shellfish poisoning (PSP) toxins

PSP intoxications are a result of exposure to saxitoxins (STX), gonyautoxins (GTX) and C-toxins. More than 57 different analogues of STX have been described (Dell'Aversano et al., 2008; Wiese et al., 2010). PSP toxins have been detected in European, North American, and Japanese waters, as well as in Chile, South Africa, Australia, and other countries (Pitcher et al., 2007; Krock et al., 2007; Robertson et al., 2004). The predominant producers of STX are species of the genus Alexandrium. Other genera, however, e.g., Gymnodinium and Pyrodinium, also have been known to produce STX (FAO, 2004).

Chemically, PSP toxins are tetrahydropurines grouped in 7 divisions. They share similar structures (Fig. 8) and they are water-soluble as well as heat stable substances. However, under acidic conditions (mineral acids) C-toxins convert to GTXs, and the conversion was shown to occur at a greater rate at increased temperatures (Shimizu, 2000).

2.9 Pectenotoxin poisoning (PeP) toxins

Pectenotoxins (PTX) are lipophilic contaminants of shellfish detected in Australia, Japan, New Zealand, and in some European countries, where they often co-occur with okadaic acid (OA) (FAO, 2004). PTX are produced by dinoflagellates of the genus Dinophysis, which encompasses >200 species. PTX belong to the chemical group of macrolides (FAO, 2004; Miles, 2007), and they are heat-stable but can be destroyed under alkaline conditions (Yasumoto et al., 2005). To date, 15 analogues have been described, all containing spiroketal, bicyclic ketal, cyclic hemiketals, and oxolanes (Allingham et al., 2007). Examples of structural formulae of some pectinotoxins are displayed in Figure 9. PeP toxins are assumed to represent a mixture of parent compounds and biotransformation products

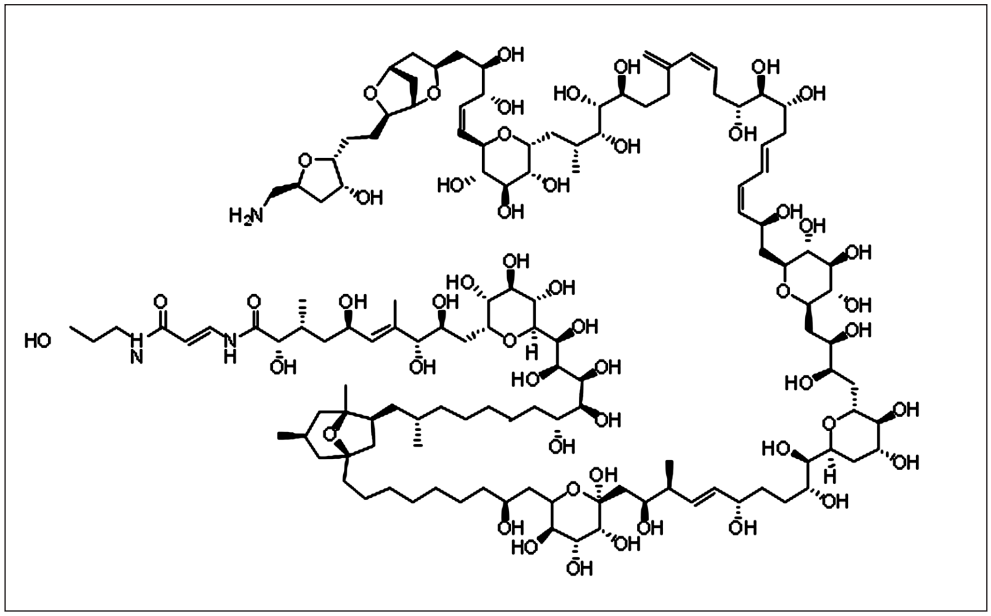


Fig. 7: Chemical structure of PlTX
Reproduced with permission from (Riobo and Franco, 2011).

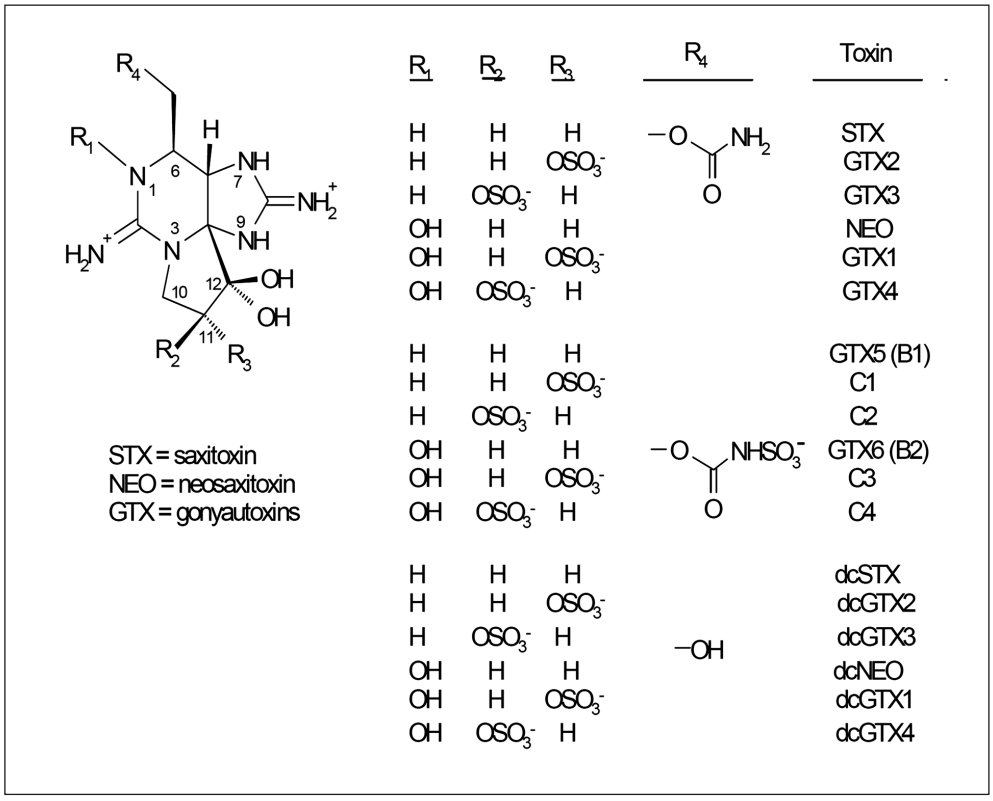


Fig. 8: Chemical structures of STX and GTX
Reproduced with permission from (EFSA, 2009d).

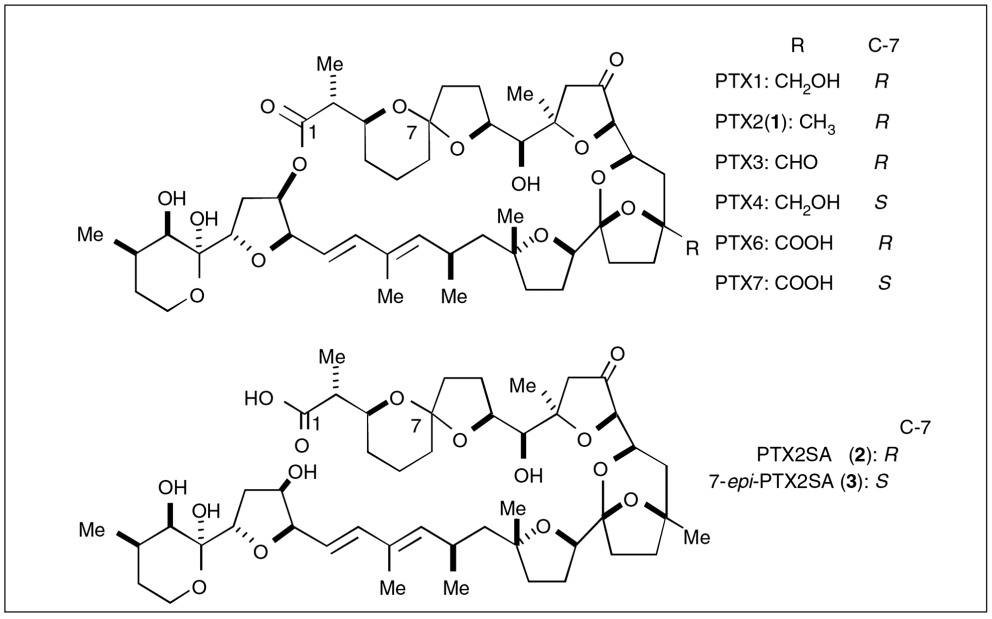


Fig. 9: Chemical structures of some pectenotoxins
Reproduced with permission from (Luisa Fernandez et al., 2006).

of PTX2 (Draisci et al., 1996; Yasumoto et al., 2001). PTX2 was shown to be metabolized to PTX2 seco acid (PTX2 SA) and its epimer 7-epi-PTX2 seco acid (7-epi-PTX2 SA) in the scallop *Patinopecten yessoensis*. PTX4 and PTX7 were shown to be isomers of PTX1 and PTX2 (FAO, 2004). Under acidic conditions PTX are labile and can transform into seco acid analogues. PTX2 SA and 7-epi-PTX2 SA can be metabolized in shellfish to the corresponding more lipophilic fatty acid esters and thus be present at 20-fold higher concentrations than the parent seco acids (Wilkins et al., 2006).

2.10 Yessotoxin shellfish poisoning (YSP) toxins

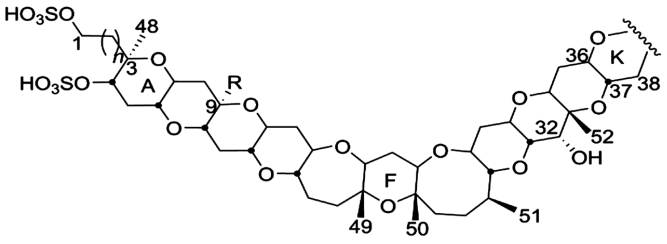
The etiological agents of YSP are yessotoxins (YTX), produced by dinoflagellates of the genus Protoceratium, Lingulodinium, and Gonyaulax (Aasen et al., 2005b; Ciminiello et al., 2007; Dominguez et al., 2010; EFSA, 2008b; Miles et al., 2004a;

Rhodes et al., 2004; Samdal, 2005; Satake et al., 1997; Suzuki et al., 2007; Yasumoto, 2001; Aune et al., 2002; Draisci et al., 1999; Tubaro et al., 2003; Rhodes et al., 2004).

Occurring globally YTX have been isolated from various species of shellfish. To date over 100 YTX analogues have been described of which the structures of about 40 were elucidated (Ciminiello et al., 2007; FAO/IOC/WHO, 2004; Miles et al., 2006). The high number of YTX structural analogues may be the result of metabolic conversion within shellfish. YTX are heat-stable (Alfonso et al., 2007) polyether compounds, consisting of 11 contiguously transfused ether rings, an unsaturated side chain, and two sulfate esters (Fig. 10) (Samdal, 2005; EFSA, 2008b).

3 Epidemiology of marine biotoxins

Epidemiology is the science of the study of causes and effects of disease and health conditions in defined populations – etiology, prevalence, and incidence. It identifies risk factors for human health and targets for preventive action. Epidemiological studies, including case studies of intoxication incidences resulting from marine biotoxin exposure, are an undisputable requirement for a proper evaluation of the health risk associated with the exposure to marine biotoxins in fishery and aquaculture products.



		R	n	m/z
1	YTX	H	1	1141
2	45-hydroxyYTX	H	1	1157
3	carboxyYTX	H	1	1173
4	1a-homoYTX	H	2	1155
5	45,46,47-trinorYTX	H	1	1101
6	ketoYTX	H	1	1047
7	40-epi-ketoYTX	H	1	1047
8	41a-homoYTX	H	1	1155
9	9-Me-41a-homoYTX	CH_3	1	1169
10	44,55-dihydroxyYTX	H	1	1175
11	45-hydroxy-1a-homoYTX	H	2	1171
12	carboxy-1a-homoYTX	H	2	1187

n=number of methyl groups in the molecule m/z=mass to charge ratio (molecular mass of the analogue diminished by 1 dalton ([M-H]$^-$ ions))

Fig. 10: Chemical structures of some yessotoxins
Reproduced with permission from (EFSA, 2008b).

3.1 Amnesic shellfish poisoning (ASP)

One of the first ASP outbreaks occurred in 1984 in Calgary, Canada and was thought to be related to exposure to DA. Here, 12 persons had consumed Prince Edward Island mussels and developed vomiting, diarrhea and impaired vision. Symptoms occurred one to three hours after the consumption of the mussels and lasted from one to seven days. However, the etiologic agent could not be clearly identified (Todd, 1993), thus prohibiting the establishment of causality between toxin exposure (form, exposure repetition and quantity per repetition) and type, onset, severity, and duration of adverse health effects. Towards the end of 1987, 153 acute cases of intoxication were registered that were retraceable to consumption of cultured blue mussels (*Mytilus edulis*) from Prince Edward Island (Quilliam and Wright, 1989), and DA was identified as the causative agent (Wright et al., 1989). Symptoms included vomiting, diarrhea, disorientation, confusion, memory loss, and coma, whereby 3 elderly patients died as a consequence of exposure (Wright et al., 1989). In some of the most severely affected patients, the neurological symptoms appeared to persist years after the intoxication. Based on the analyses of cultured blue mussels (*Mytilus edulis*) from Prince Edward Island (Quilliam and Wright, 1989) an estimated 0.9 DA mg/g fresh weight of edible mussel tissue was found. Based on an average consumption of 200-400 grams of mussels in a meal by a single person, one can calculate that the patients were exposed to an estimated 180-360 mg DA, i.e. about 2.57-5.14 mg DA/kg body weight (b.w.) when using an average b.w. of 70 kg (Quilliam and Wright, 1989; Wright et al., 1989). Using the latter calculations and the clinical symptoms observed, a lowest adverse effect level (LOAEL) for severe symptoms of 4.0 mg DA/kg b.w. and a LOAEL for mild symptoms ranging between 0.9-2.0 mg DA/kg b.w. was estimated.

Similarly, a series of ASP outbreaks – about 24 cases – was reported from the coasts of Oregon and Washington State, USA in 1991. 21 incidences could be traced to the consumption of razor clams (*Siliqua patula* Dixon) and Dungeness crabs (*Cancer magister* Dana) with no severe symptoms, though the involvement of ASP toxins in these cases appears controversial (Horner et al., 1997; Todd, 1993).

3.2 Azaspiracid shellfish poisoning (AZP)

There is little well-documented data available on azaspiracid intoxications and outbreaks. The first outbreak that could be associated with a new toxin (AZA was formerly named Killary toxin) other than DSP – as the symptoms are similar – occurred in 1995 in the Netherlands. The contaminated shellfish were from Killary Harbour, Ireland (Satake et al., 1998). Since AZA and some of its analogues could be purified and the chemical structure determined, it was possible to associate a series of outbreaks with this class of marine biotoxins: 8 cases in the Netherlands in 1995, approximately 24 in Ireland in 1997, 10 in Italy in 1998, 30 in France in 1998, 16 in the UK in 2000, and 219 in France in 2008 (EFSA, 2008a; FSAI, 2006; McMahon and Silke, 1996).

In 2001 the Food Safety Authority of Ireland (FSAI) performed the first AZA risk assessment in shellfish utilizing LC-MS. Mussels were collected from the Arranmore (Ireland) incident site frequently and their hepatopancreases were analyzed. Probabilistic modeling resulted in an estimate of 5.7-10.7 µg AZA/g hepatopancreas as the putative concentration that had led to the symptoms (FSAI, 2001). In 2006, FSAI revised the calculation made in 2001 by adding corrections resulting from the stability of AZA during the cooking procedure (Hess et al., 2005). Due to the largely differing AZA analogue compositions observed in the mussel samples, this revision also questioned the use of AZA analogue composition within the hepatopancreas as a surrogate analysis for the whole shellfish and thus as a means for extrapolation to the whole mussel meat. Indeed, up to 30 AZA analogues are known to date, of which many have not been thoroughly characterized and only a very few have been toxicologically tested in an acute setting, while some data on chronic toxicity are available for AZA1 (Ito et al., 2002).

3.3 Ciguatera fish poisoning (CFP)

CTX are known to have a rather high toxicity in humans due to the opening of the non-selective, non-voltage-activated ion channels, which elevates intracellular calcium concentration and leads to cellular toxicities (Lu et al., 2013) and thus to the symptoms, including numbness of the perioral area and extremities, reversal of temperature sensation, muscle and joint aches, headache, itching tachycardia, hypertension, blurred vision, and paralysis.

CFP is the most commonly reported marine toxin disease affecting 10,000-50,000 people per year in the world (Botana, 2008; Terao, 2000; De Fouw et al., 2001; Lehane, 2000) as a result of:

– The global trade and sale of fish originating from all areas in the world where CFP can occur.
– The lack of routine CTX analysis, i.e., single fish are rarely analyzed.
– Lack of standardized CTX material for LC-MS/MS confirmation, i.e., C-CTX, P-CTX and I-CTX, as well as the lack of rapid detection methodologies.

As testimony of this global distribution and trade of finfish, the presence of C-CTX-1, as well as P-CTX-1 was confirmed as follow-up analytical confirmations after CFP incidents in Europe (Gouveia et al., 2009; Otero et al., 2010b; Perez-Arellano et al., 2005) or as was recently the case in New York, where intoxications took place following consumption of C-CTX contaminated barracuda (Graber et al., 2013). Also, in Germany, the first outbreak of ciguatoxin poisoning after eating fish was confirmed recently by analytical methods (BfR, 2013). Notably, for detection and quantification of P-CTX-1, a non-certified method with a non-certified standard for P-CTX-3C is typically employed. Beyond the shipping of contaminated fish to Europe from various parts of the world, it is currently assumed that the Mediterranean levels of CTX toxins also are sufficiently high to cause outbreaks in Europe (EFSA, 2010a).

Though the number of reported cases is significant for epidemiological (statistical) analysis, there are only few cases in which the concentrations of CTX toxins were determined and the toxicity estimated. For example, in the New York barracuda

consumption case, meal remnants were analyzed with the *in vitro* neuroblastoma cell assays for sodium channel toxins to provide a qualitative estimate, whereby positive samples were subsequently analyzed via LC-MS/MS. The latter analyses demonstrated at least in one of the barracuda meal remnants the presence of 1.1 μg C-CTX-$1_{equiv.}$/kg fish, thus surpassing the currently established FDA guidance level of 0.1 μg C-CTX-$1_{equiv.}$/kg fish flesh by a factor 10. In a similar case, whereby grouper was consumed, a contamination level of 1.9 μg C-CTX-$1_{equiv.}$/kg fish was confirmed (Graber et al., 2013).

The original reference system for testing the toxicity of CTX was the MBA, whereby an i.p. bolus dose of 35 μg P-CTX-$1_{equiv.}$/kg b.w. was found to be lethal. Accordingly, 1 mouse unit (MU) corresponds to approximately 7 μg P-CTX-1 (Oshiro et al., 2010; Yasumoto, 2001). Analysis of contaminated material associated with symptoms of CFP in humans in the past suggested a threshold of 0.1-5 µg P-CTX-$1_{equiv.}$/kg fish (Hokama et al., 1998a; Lehane, 2000; Lehane and Lewis, 2000; Lewis, 1994; Lewis et al., 1999; Lewis and Sellin, 1992, 1993). More recent evaluations narrowed the range from 0.1-5 μg to 0.3-5.6 μg P-CTX-$1_{equiv.}$/kg fish, whereas the lower margin was assessed with a sodium channel-specific mouse neuroblastoma assay and the upper margin with the MBA (Arnett and Lim, 2007; Oshiro et al., 2010). Notably, P-CTX-1 was thought to be more toxic than C-CTX-1 (FAO, 2004). However, recent C-CTX induced CFP cases corresponding to 0.6, 1.0, 1.1, 1.9, and 20 μg C-CTX-$1_{equiv.}$/kg fish, suggested that CFP by C-CTX can be induced within a similar concentration range as P-CTX-1 (CDC, 2009; Perez-Arellano et al., 2005; Poli et al., 1997). However, the above values where determined by various analytical and biochemical assays, thus making determination of a more reliable concentration range and thus threshold for the onset of CFP symptoms difficult.

Above datasets all were gained from adult or juvenile intoxication cases, whereas there are only a very few limited descriptions of cases that have been associated with pregnancy. From the latter cases it was estimated that a contamination of approximately 1.0 µg P-CTX-$1_{equiv.}$/kg fish in conjunction with the consumption of 500g fish by the mother will lead to symptoms in the mother and to tumultuous movements of the unborn child. As neither these mothers nor their newborns were subjected to routine observation after birth, there are no additional data that could provide insight as to the potential long-term effects of prenatal exposure to CTX, e.g., on the ability to learn (Fenner et al., 1997; Lehane, 1999; Pearn et al., 1982; Senecal and Osterloh, 1991).

3.4 Cyclic imine poisoning (CIP)

To date there are no records of human intoxications due to SPX, GYM, PnTX or PtTX. Despite the presence of CI in local waters and marine organisms (e.g., up to 200 μg CI /kg oyster tissue in Rangaunu Harbour, New Zealand in 1994), consumption of local marine products in the same period did not lead to adverse effects in humans (McCoubrey, 2009; Munday et al., 2004; Richard et al., 2001). Based on the latter observation, there are no current regulatory limits for CI in shellfish and fish, despite the global distribution and thus the presence of CI in shellfish and products thereof in Europe, Asia-Pacific, Japan and the USA.

Nevertheless, there is a high degree of uneasiness of regulatory authorities with regard to the risk assessment of CI (EFSA, 2010c), especially as the MBA for some CIs demonstrate large differences between oral and i.p. toxicity. SPX and GYM interact with both muscle and neuronal nicotinic ACh receptors (nAChR) at subnanomolar concentrations (Bourne et al., 2010; Kharrat et al., 2008), and this interaction leads to respiratory paralysis in rats and mice treated with GYM (Kharrat et al., 2008). The question that needs to be answered is whether or not this mechanism also applies to humans and thus whether the data from the MBA are suitable for risk assessment for humans. The EFSA panel of contaminants in the food chain, CONTAM panel, considered the MBA as being inappropriate due to poor specificity and for ethical reasons (EFSA, 2010c). However, no alternative to the *in vivo* assays for estimating the potential health risk to humans is currently available.

In a recent investigation, Wandscheer and colleagues (Wandscheer et al., 2010) demonstrated the irreversible competitive antagonism of SPX 13-desmethyl C spirolide at muscarinic ACh receptors (mAChR) in human neuroblastoma cells, thereby emphasizing that CI's are likely to induce adverse effects in humans upon sufficient exposure. Thus, the lack of reported human adverse effects clearly associated with the consumption of CI contaminated shellfish may be primarily the result of poor recognition and reporting of minor to moderate adverse health effects, e.g., gastric distress and tachycardia, in humans (FAO/IOC/WHO, 2004) within the European, US, Japanese, Chinese and the Asia-Pacific health systems rather than the absence of toxicity. Moreover, due to the potential lack of recognition of acute intoxications, possible chronic intoxications would not be recognized either, therefore regulatory authorities are concerned about the very likely health risks associated with chronic CI exposure. Indeed, CI are rapidly taken up via the GI tract following oral exposure (Otero et al., 2012) and reach the central nervous system of exposed mice (Alonso et al., 2013a,b), thus demonstrating the capacity to cross the blood brain barrier and to potentially interact with muscarinic ACh receptors *in vivo*.

3.5 Diarrheic shellfish poisoning (DSP)

The first incidents in Japan occurred in the period from 1976 to 1977, where over 164 individuals developed diarrhea, nausea, vomiting, and abdominal pain after eating scallops contaminated with dinophysistoxin-1, DTX1 (Murata et al., 1982; Yasumoto et al., 1978a). The analysis of the meal leftovers originally consumed by eight of the individuals was the basis for estimating cut-off values of 48 µg okadaic acid (OA) equivalents/person for mild symptoms and 80-280 µg OA equivalents/person for severe symptoms. In the following decades DSP incidents were reported also in other countries: Belgium (De Schrijver et al., 2002), Canada (Quilliam et al., 1993a), Chile (Garcia et al., 2005; Lembeye et al., 1993), France (Van Egmond et al., 1993). New Zealand (Fernandez and Cembella, 1995), the Netherlands (Kat, 1979, 1983), Norway (Torgersen et al., 2005; Underdal et al., 1985), Portugal (Vale and Sampayo, 1999, 2002), Sweden

(Krogh et al., 1985), the UK (COT, 2006; Scoging and Bahl, 1998), and Italy in 2010 (Aurelia Tubaro, personal communication). Of these incidents several hundred cases with mild symptomatology corroborated the LOAEL of 50 µg OA equivalents/ person (EFSA, 2008c).

An incident in 2006 in the UK involved 159 individuals who developed symptoms of intoxication after eating mussels in different restaurants. Three samples from the suppliers were examined with the MBA and LC-MS/MS. The concentration of OA in these samples ranged between 258 and 302 μg OA equivalent/ kg shellfish, whereas the MBA could only give positive results for two of the three samples. Notably, the samples were also contaminated with a concentration of 513 µg/kg shellfish pectenotoxins (PTX), but the toxicological significance of this was not examined further and thus remained unclear (COT, 2006).

Additionally, long-term exposure to low concentrations of DSP toxins OA and DTX1 is postulated to be linked to a significantly higher incidence of digestive cancers in populations of coastal areas in France (Cordier et al., 2000). A similar report from Spain supports the association between digestive cancer and consumption of mussels: a two-fold higher risk of colorectal cancer was associated with a 7-fold higher consumption of bivalve mussels. Notably, none of the authors were able to substantiate that this increased risk of colorectal cancer was also incurred via exposure to marine biotoxin(s) in the mussels consumed or in the mussels from that region (Lopez-Rodas et al., 2006).

3.6 Neurotoxic shellfish poisoning (NSP)

Overall the epidemiology of NSP, caused by brevetoxins (BTX), lacks proper documentation. The few published case reports and overviews do not allow judgment whether NSP illness is simply not recognized/identified (misjudgment) or rather if this intoxication is a rare event (Backer et al., 2003, 2005; Fleming et al., 2001a,b). However, in most incidences of NSP that have occurred along the Gulf of Mexico, east coast of the United States, and in New Zealand, affected patients presented within minutes to hours with nausea, emesis, diarrhea, paresthesia, cramps, bronchoconstriction, seizures, paralysis, and coma (Plakas and Dickey, 2010). The reviews of the largest outbreak of NSP recorded in New Zealand in 1992/1993 suggested that different toxin producing organisms were found in the responsible bloom including the BTX producer *Karenia mikimotoi* (Ishida et al., 1996; Ishida et al., 1995; Morohashi et al., 1995, 1999; Todd, 2002). Of the few accounts of intoxication with BTX that have been studied, the best characterized is the outbreak in North Carolina 1987, where 48 of 85 persons that consumed BTX contaminated oysters displayed symptoms of adverse health effects (Morris et al., 1991). In the latter case, the number of symptoms increased with the amount of contaminated oyster meat consumed. Based on the degree of BTX contamination determined in the leftovers of the meals consumed and oysters harvested from the same general areas, a contamination of 35-170 mouse units (MU) BTX-group toxins was estimated. The latter would make a low toxic dose of 42-72 MUs per person, when assuming a total consumption of 100 g oyster meat, corresponding to approximately 168-288 µg BTX-2, i.e., for a 60 kg person to 2.8-4.8 µg/kg b.w. (Gessner, 2000). Indeed, Florida has reported BTX contamination levels ranging from 880 to 49,000 µg BTX-2 equivalents/ kg shellfish meat (Naar et al., 2007; Pierce et al., 2004; Steidinger et al., 1998; Watkins et al., 2008), which would exceed the current permissible levels of 0.8 mg BTX-$2_{equiv.}$/kg shellfish meat (20 MU/100 g) currently applied in the US (FDA, 2012), New Zealand and Mexico (Rodriguez-Velasco. Similar analyses of contaminated fish (Naar et al., 2007), albeit using a different analytical technique, provided values ranging from 580 to 6000 µg BTX-3 equivalents/kg fish meat. Thus inadvertent distribution of BTX contaminated shellfish and fish could occur where no BTX regulation or routine detection of shellfish and fish products is in place, e.g., in the EU, and thus could lead to human intoxications. Despite the lack of a European regulation limit for BTX, the EFSA CONTAM Panel considered that an acute reference dose (ARfD) should be established (EFSA, 2010b). However, EFSA considered that the presently available data were too scant to allow for derivation of an ARfD. The mere fact that no NSP intoxication cases have been registered in the EU appears more a matter of lack of recognition and corresponding reporting rather than a lack of actual incidences. The latter however does suggest that NSP incidences to date do occur rarely, as more frequent intoxications would have gained attention and thus have become more public.

In a more atypical setting, human adverse effects from BTX can also be incurred via the inhalation of aerosolized dinoflagellates containing BTX as can occur during red tides as reported from the Florida and Texas coasts (Backer et al., 2003; Cheng et al., 2005; Fleming et al., 2005; Pierce et al., 2005, 2003).

3.7 Palytoxin poisoning (PaP)

All in all, the possible PlTX exposure routes apart from oral intake of contaminated seafood are via inhalation and cutaneous exposure after direct contact with aerosolized seawater during Ostreopsis blooms and/or from home aquaria containing Zoanthids (Cnidaria) (Tubaro et al., 2011b). The epidemiology of PlTX associated incidences overall lacks proper reporting, such that the data available are insufficiently robust to allow for a proper hazard characterization.

After oral exposure, the uncertainty in definition of case reports arises from the difficulty in performing analysis for the confirmation/quantification of the toxins both directly in leftover food and/or in clinical specimens. In fact, only in four case reports referring to 15 patients, direct evidence of PlTX contamination is reported, although the PlTX identification was sometimes problematic (Tubaro et al., 2011b). Most of the patients report a metallic taste immediately after testing the seafood, then developed general malaise, nausea, vomiting, diarrhea, myalgia, dyspnea and, sometimes, abnormalities in cardiac function (Tubaro et al., 2011b). Other case reports, related to about 135 people, are ascribed to PlTX either on the basis of analysis carried out on the same seafood species (but not directly on the leftovers) or on the basis of the symptoms and anamnesis (Tubaro et al., 2011b). For instance, Noguchi and colleagues described an incident involving two individuals which appeared to suffer from PaP; but neither the palytoxin as the causative agent nor the concentration of the predominant agent was determined (Noguchi et al., 1988). In a much

more detailed case, a 49-year old patient in the Philippines died 15 hours after consuming parts of a hairy crab (*Demania reynaudii*). The symptomatology presented as dizziness, nausea, fatigue and cold sweat within a few minutes of crab consumption, which was followed by diarrhea, paresthesia, restlessness, muscle cramps, vomiting, bradycardia, respiratory problems, and renal failure prior to death. Based on the analytical method used (HPLC), tetrodotoxin and saxitoxins could be excluded, while the presence of PlTX or PlTX-like toxin was assumed via co-chromatography with purified PlTX (Alcala et al., 1988) and appears to be corroborated by comparison of the chromatographic properties published much later (Riobo et al., 2006). Using the MBA as reference, the remaining crab leg flesh (10.4 g) was analyzed and found to contain 800 MU of PlTX in the methanol extract, which calculates to approximatedly 77 MU/g. The MU was defined as the amount of toxin that would kill a 17 g mouse in 24 h by i.p. injection. When employing the reported mouse LD_{50} values ranging between 0.15 –0.72 µg/kg b.w. (Munday, 2006; Rhodes et al., 2002; Riobo et al., 2008), the MU as observed with the 17 g mice employed by (Alcala et al., 1988) would be equal to 2,55 – 12,24 ng PlTX/mouse. Using this range and the approximate 77 MU/g crab tissue, the PlTX concentration in the crab would have been approximately 196-942 ng/kg crab tissue. Although many parameters decisive for proper hazard calculations were missing (e.g., amount of crab flesh eaten, weight of the victim, storage of remaining crab until analysis, etc.), when assuming 60 kg body weight of the patient and a similar sensitivity of mice and humans, the lethal dose of 9-43.2 μg PlTX would have been reached by consuming 10-220 g crab flesh. In Madagascar a 49-year old woman experienced malaise, then uncontrollable vomiting and diarrhea within the first two hours, followed by tingling of the extremities, delirium, and death within 17 hours. This fatality occurred following the consumption of sardines (*Herklotsichthys quadrimaculatus*) that were most likely contaminated with PlTX or PlTX-like toxins, as suggested by concomitant analysis of the extracts from the frozen remainders of the sardines using the MBA, the mouse blood haemolysis assay, the neuroblastoma cell cytotoxicity assay and MALDI-TOF-MS (Onuma et al., 1999). However, more detailed information as to the amount of fish consumed, amount of fish flesh analyzed subsequently and how the 2-20 ng PlTX-like toxins would refer back to the whole amount of food consumed are missing from the report.

The recent identification of Ostreopsis ssp. in marine aerosols (Casabianca et al., 2013) suggests that the occurrence of respiratory irritation and malaise related to aerosol exposure during *Ostreopsis ovata* blooms could be due to both the algal fragments and/or to the presence of PlTXs. Several cases of inhalation toxicity related to Ostreopsis proliferation occurred mainly in temperate climates, e.g., in the Mediterranean and Portuguese costs (Barroso García et al., 2008; Durando et al., 2007; Gallitelli et al., 2005; Sansoni et al., 2003; Tichadou et al., 2010; Tubaro et al., 2011b; Kermarec et al., 2008). Dermal toxicity has been associated with skin contact by PlTX-containing zoanthid corals or seawater containing Ostreopsis cells. In the former, intoxications occurred primarily in aquarium hobbyists who came into contact with Palythoa while cleaning aquaria (Deeds and Schwartz, 2010; Durando et al., 2007; Hoffmann et al., 2008; Nordt et al., 2011; Sud et al., 2013).

3.8 Paralytic shellfish poisoning (PSP)

Though there are hundreds of recorded PSP cases and outbreaks reported from around the world since the 1940's, there is significant variation in the estimate of STX toxin doses associated with the symptoms. This variation is a reflection of the unreliability and/or lack of comparability of the toxin extraction and detection methodologies employed as well as – more often – of missing information that would be necessary to calculate the concentration of toxin per b.w., such as the amount of flesh consumed, the amount emitted due to vomiting directly after consumption, the patient's body weight and potential predisposing factors of the specific patient. Analyses of existing data indicate that the mean value for the lowest observed adverse effect level (LOAEL) of PSP toxins is approximately 1.5 μg $STX_{equiv.}$/kg b.w. (range between 0.7-70 μg $STX_{equiv.}$/kg b.w.), moderate intoxication symptoms occur at a range between 1.5-150 μg $STX_{equiv.}$/kg b.w., whereas the dose associated with severe symptoms ranged between 5.6-2058 μg $STX_{equiv.}$/kg b.w. (EFSA, 2009d). Notably, a fatal outcome of PSP was reported for an individual in East Timor consuming parts of a crab (Zosimus aeneus) which resulted in an approximate intake of only 1-2 μg $STX_{equiv.}$/kg b.w. (Llewellyn et al., 2002). However, there are also reports of individuals who were exposed to up to 90 μg $STX_{equiv.}$/kg b.w. without developing any notable symptoms (Gessner and Middaugh, 1995).

Another example is the report of a PSP incident in Norway, in which meal leftovers from 5 patients (out of 8) were subjected to the MBA. The authors postulated a dose range for slight symptoms from 1.8 μg to 13.5 μg $STX_{equiv.}$/kg b.w., for moderate symptoms from 6.3 μg to 18 μg $STX_{equiv.}$/kg b.w., and for severe symptoms from 15.3 μg to 18 μg $STX_{equiv.}$/kg b.w.; however, two individuals who did not develop symptoms were subjected to 3.6 μg $STX_{equiv.}$/kg b.w.(Langeland et al., 1984).

Kuiper-Goodman and Todd reviewed outbreaks and cases in Canada from 1944 to 1970 as well as in Guatemala in 1987. Their calculation estimates a dose range for mild symptoms from 0.7 μg to 70 μg $STX_{equiv.}$/kg b.w., for moderate symptoms from 1.5 μg to 150 μg $STX_{equiv.}$/kg b.w., and for severe and extremely severe symptoms from 5.6 μg to 300 μg $STX_{equiv.}$/kg b.w., whereas one patient with severe symptoms was exposed to 0.3 µg STX equivalents/kg b.w. (Kuiper-Goodman and Todd, 1991). Lastly, Gessner and Middaugh analyzed PSP cases from 1973 to 1992 that occurred in Alaska and estimated a LOAEL of 0.2 µg STX equivalents/kg b.w. (Gessner and Middaugh, 1995).

3.9 Pectenotoxin poisoning (PeP)

To date there are no reported human incidents that can be clearly correlated to PTX exposure. This may be associated with the fact that the producers of PeP toxins – dinoflagellates of the genus Dinophysis spp. – also produce DSP, thus PTX and DSP usually co-occur (FAO/IOC/WHO, 2004; Dominguez et al., 2010) and therefore may produce adverse effects in humans that are indistinguishable or superseded by the fulminant effects of co-occurring OA exposure (Burgess and Shaw, 2001; FAO/IOC/

WHO, 2004). PeP is currently still treated as if belonging to the class of lipophilic shellfish toxins in the EU (EFSA, 2009b), Canada (http://www.pac.dfo-mpo.gc.ca) and elsewhere. Miles and colleagues (Miles et al., 2004b) however demonstrated that PTX do not have a diarrheic effect in mice and have little if any toxicity when given orally. However, whether this data can be directly extrapolated to humans is unclear (EFSA, 2009b).

PTX2 was shown to be cytotoxic, most likely as a result of the interaction with actin filaments (Allingham et al., 2007; Ares et al., 2007; Butler et al., 2012; Zhou et al., 1994).

3.10 Yessotoxin shellfish poisoning (YSP)

Human YTX exposure occurs primarily from the consumption of shellfish (oysters, clams, scallops, and mussels), however, currently there are no epidemiological data on human YSP incidences (EFSA, 2009e, 2008b)

3.11 Summary of uncertainties with regard to epidemiological analysis

For evaluation of epidemiological data the presence of sufficient information regarding the causative (etiological) agent is necessary; this includes also information on the exposure (concentration, amount of contaminated tissue consumed, amount emitted via emesis or diarrhea, etc.), chemical properties (stability), and the metabolic fate of the activated and/or the parent form of the causative agent (half-life, excretion). In the following, sources of uncertainties and hence inappropriate epidemiological data, also affecting the descriptive toxicological estimates (e.g., NOAEL and LOAEL), are summarized:

Symptomatology: Obviously intoxications must be recognized as such by the emergency medical staff, house physician, or regulatory physicians, such that appropriate treatment as well as the corresponding exposure assessment including sample preservation can be carried out. This implies that physicians can recognize marine biotoxin intoxications and differentiates these types of intoxication from other types of food poisoning. The dearth in well-documented case studies and robust epidemiological assessments is also a result of a lack of reporting by treating physicians and primary care providers to a central reporting authority, either due to lacking recognition of the type of intoxication or a lack of a centralized reporting unit.

Body weight: The patient's body weight is necessary for any relevant calculations on the amount of toxins consumed and thus the concentrations of toxins that led to the symptoms observed. Absence of body weight information, as is the case for most epidemiological data, renders the reported data nearly useless for the purpose of toxicological risk assessment and consumer protection.

Intoxication timetable: In many cases a detailed description of when the meal was consumed, the timing of the onset of first symptoms and a detailed description of the clinical progression and intensity of symptoms and adverse effects is missing. A detailed description of the latter would help to understand differences in patients as to onset and progression of adverse effects.

Patient description: The present epidemiological and case datasets do not provide for a detailed individual patient description, e.g., with regard to prior or existing illnesses, potential medical/disease predispositions (e.g., existing disease or metabolic polymorphisms) and lower or higher likelihood for a more serious or light onset and/or progression/recurrence of adverse effects and symptoms. Information on the latter would promote our understanding of high sensitivity sub-cohorts and thus allow for an improved risk calculation affording protection also to the high-risk consumers.

Toxin content: The information on the amount of identified toxin incorporated (amount consumed – minus amount excreted via diarrhea and/or emesis) together with the information of the patient's body weight enable the estimation of the range of toxin concentrations that induce adverse effects in patients. In the current database, the amount consumed is either unknown or had to be estimated. The actual amount consumed however, can vary dramatically as in many moderate to severe intoxications patients react with immediate diarrhea or emesis and thus a major reduction of the meal consumed. As toxin concentrations, especially in crustaceans and fish, may differ substantially between different body parts/organs consumed (Lin et al., 2004), the analysis of meal leftovers can seriously underestimate/overestimate the amount consumed and thus the amount of toxins the patient was originally exposed to. Moreover, transport and storage conditions (frozen, cooled, uncooled) to the laboratory and type and time of storage until analysis of the meal upon preservation, the method of toxin extraction as well as the method of analysis will have major influence on the amount of toxins detected in the leftovers, leading to a poor estimate of the actual amount ingested. The use of analogous samples, i.e., fresh samples from the same area and possibly the same harvest, can serve as a good estimate of the range of toxin contamination observed, but do not provide an appropriate and robust basis for analysis of the intoxication. Additionally, post-mortem analyses of stomach contents for estimation of the toxin amount involve uncertainties and often result in underestimation of the actual toxin dose, as acidification of the stomach contents as well as diarrhea and emesis will greatly influence the amount of toxin recovered.

Toxin profile: Knowing the identity of the toxin, and also the profile of analogues (also co-occurrence with other contaminants) is not only imperative for risk assessment, epidemiology, and hazard monitoring, but also for the purpose of medical treatment. The MBA, the method routinely used in the past, does not deliver any information on the profile/identity of the toxin(s) present. For profiling and quantification of toxins in a given sample, the availability of a complete set of certified reference materials (toxins) as well as the knowledge of metabolites formed in the shellfish, fish, and crustaceans consumed as well as those formed in the patient, is imperative and a prerequisite for appropriate analyses.

Methodical issues: Marine biotoxins are heat stable and for most toxins the concentrations in cooked tissue is higher (EFSA, 2008c) than in the living organisms (toxin enrichment via cooking can range between 125% to 180% of the concentration in the fresh sample). Despite this, there is a dearth of studies describing the effects of cooking on the different toxin analogues (including the toxin metabolites formed in the species

consumed) of the same toxin group. Further, the procedure of toxin extraction from the tissue is often not well described and also the effect of the extraction method on the method used to analyze toxin content is not taken into account (e.g., boiling tissue at low pH for MBA analysis).

Comparability: For systematic analysis of cases and outbreaks, it is important that the methods and techniques of sample preservation and analysis, the source (finfish and shellfish, processed food or algae), and routes of exposure (oral, dermal, inhalatory) are comparable, and that documentation is sufficiently detailed to understand even small differences in procedure. The value of estimates for toxicological action levels remains questionable when the sources – finfish and shellfish species – differ, even when the etiological agent is evidently the same. In many cases toxicological estimates are derived from different analytical and biochemical assays. Notably, for significant evaluation for any of the methods in use, the availability of certified reference material is essential. Moreover, for most documented cases, non-validated methods were combined with non-certified reference material, or the assessors used a reference material to refer to another toxin or analogue. Unfortunately, a number of these toxins currently are not commercially available. It is per se questionable to extrapolate results from the MBA acute toxicity assays to the human, especially as the use of uncertainty factors used lack a scientific basis, only being an artificial construct to aid risk assessment.

Occurrence: Due to lack of harmonized processes there is a lack of data on the correlation of the occurrence of toxins in the water and the presence of benthic and pelagic dinoflagellates, toxic algae /diatoms with the respective toxin contamination of crustaceans, finfish and shellfish. In addition, there is only limited information on regions of occurrence and dynamics of marine species and also little information on seasonal aspects, water depth, and mixing behavior, and temperature related to the time and location of the sample collection.

Consumption: There are some attempts to collect data on consumption habits involving surveys and questionnaires, but still the amount and quality of this type of information is insufficient. The uniformity and accuracy of questionnaires and surveys are important qualifiers in the establishment of robust datasets. Moreover, some parameters of current questionnaires and surveys have to be reconsidered, e.g., a survey per meal or per day would make more sense than the consumption patterns over several days, as the initial signs and symptoms of marine biotoxin poisoning occur within minutes to hours, when the concentration of toxin(s) is sufficiently high. Notably, most of the surveys and questionnaires do not aim to gain information on the meal size, the species consumed, or the processing status of the organisms used to make the meal. More importantly, as the consumption frequency of finfish, shellfish, and crustaceans may vary largely, questionnaires must be adapted to include information that may be of regional importance and relevance or may be used to identify high or low risk groups.

Chronic exposure: Beyond some studies with DA, there have been only few attempts to gather information on the consequences of low-dose repeated or moderate intermittent-exposure to marine biotoxins in mammals and specifically in humans. As a consequence, none of the regulatory authorities in Asia-Pacific, USA, Canada, or Europe declare themselves capable of estimating a tolerable daily intake (TDI) or a provisional tolerable weekly intake (PTWI). However, in view of the strong recurrent adverse reactions as reported (e.g., by ciguatoxin patients, or the DA exposure associated temporal lobe epilepsy in a patient nearly a year after poisoning and after all symptoms had disappeared) the establishment of systematic long-term studies, preferably employing *in vitro* systems with human cells or artificial organs would be advisable.

Public outreach: The basis for epidemiological data ascertainment for consumed food products is an alert (also for slight and mild symptoms) and educated medical personnel. With the exception of regional areas with regular exposure to marine biotoxins, e.g., Florida, USA, there are no efficient attempts recorded at the global or supraregional level to build up an adequate awareness in the public or to educate and train medical personnel in the recognition and treatment of intoxications by marine biotoxins. No standard operating procedures (SOP) are in place at primary care providers that secure meals consumed and possibly vomit or stool for later analysis by a certified reference laboratory. Such a SOP must also maintain a chain of custody of the samples such that all processes are transparent and reproducible and thus avoid any form of adulteration or changes that could affect the toxin content or form. Such a chain of custody was recently exemplified by a ciguatoxin intoxication of Germans exposed to contaminated fish from India (German Federal Institute for Risk Assessment, 2013). Simultaneously, medical primary care providers should be trained and provided with a national intoxication center where they can report intoxications as well as receive advice on treatment options.

Database: From an epidemiological point of view and for risk assessors the establishment of a detailed database on toxicity of different marine biotoxins and their analogues is a prerequisite for any reasonable hazard identification and risk assessment. This database should provide information on the range of adverse effects, on immediate and long-term effects, on treatment and on dose-effect relationships as well as on patient specific traits (body weight, ethnicity, predispositions, etc.) that could influence the progression of the intoxication (e.g., DA) and or the likelihood of much more severe recurrence upon re-exposure (e.g., ciguatoxin).

4 Hazard monitoring, risk assessment and risk management

Hazard monitoring: Due to the different chemical and physical properties of hazards relevant to consumer health, monitoring of marine biotoxins requires a wide range of actions. Hazard monitoring involves routine measurements with robust and sensitive methods to detect changes in the environment. Therefore, defined acceptable levels of exposure and thresholds of toxicological concern are the basis for evaluation of monitored hazards. Considerations guiding towards relevant methods for monitoring include identification, detection and quantification of critical agents. However, threshold identification is largely

dependent on the relevant toxicological database, which in the case of marine biotoxins is primarily related to acute intoxications of a very few well documented human cases and a high number of surrogate animal tests with yet questionable relevance to humans (see above).

Risk assessment: The process of evaluating risks to health from recognized hazards is risk assessment. This includes systematic examination of the potential sources of exposure and adequate sampling strategies proving a representative base for assertions. The evaluation of risk is a combination of information on magnitude and frequency of human exposure, on the inherent toxicity of the hazardous compound and on the nature and extent of the contamination.

Risk management: Risk management refers to the regulatory safety science procedure aiming to protect public health. Therefore, thresholds for contaminations with hazardous compounds are set. Whereas risk assessment provides information on potential burden, risk management is the regulatory preventative action based on the information provided by risk assessment. Thus it is essential for the purpose of risk management to have relevant information regarding the scientific background (toxicology, chemistry, epidemiology, ecology, and statistics *inter alia*) as well as information regarding technological possibilities.

Monitoring, assessment, and management of marine biotoxins: As marine sourced nutrition is a significant component of world food and as marine food and products are subjected to global distribution, it is important to have reliable, robust, and sensitive methods for continuous routine *in situ* monitoring of toxin occurrence in both producing and vector organisms as well as the free toxin concentrations in the environment. Information from risk assessment, which is the basis for risk management, should be relevant to human physiology for the purpose of human-relevant risk management. This includes also information on the relative potencies of marine biotoxins, preferably in humans, to judge the potential burden of exposure to these hazards and thus to define relevant thresholds, the acceptable levels of exposure and thresholds of toxicological concern (Botana et al., 2010).

Although the globalization of distribution of finfish, shellfish and crustacean products as well as an increased distribution of marine toxin producers is recognized in science, attempts to address these pressing issues at the regulatory level have been unsatisfactory so far. Most efforts have been directed at risk management via emplacement of regulatory levels of marine biotoxins to be controlled at the level of the producer. Currently, the most comprehensive approaches have been implemented by the Canadian and Japanese Health Authorities, the European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA); all of which support a maximum allowance level for specific marine biotoxins. The scientific basis for these allowance levels was primarily derived from acute human intoxication incidents, i.e., case studies. However, due to the limitations associated with these case studies (see above), the toxin concentrations ingested that subsequently led to the presentation of adverse effects in the patients are more often than not mere estimates of the truly effective dose. Thus, the presently employed allowance factors heavily rely on the safety factors employed for calculating the allowable concentration in the final products. Indeed, for most if not all marine biotoxins, there is a dearth of human-relevant methods aiming to address all relevant physiological endpoints in the intoxication cases. For example, although the human neuroblastoma assay (see below) is an excellent biological and human relevant method for assessing the sodium channel-specific interaction of ciguatoxins, brevetoxins, tetrodotoxins or saxitoxins (Manger et al., 1993, 1994, 1995), subchronic, chronic, or recurring exposure toxicity cannot be tested. In addition, and despite the fact that strenuous efforts are continuously being put forth, there is a lack of reference materials for toxin analysis. The current regulatory allowance levels – and their background – for the toxins discussed in this workshop are briefly summarized. Most of the data are reported as presented in the original documents, revealing a lack of harmonization in the units and in the calculation of the safe accepted level.

4.1 Amnesic shellfish poisoning (ASP)

In the USA, the FDA revised guide for the control of shellfish from 2007 sets an official limit of 20 mg DA/kg fish weight (f.w.), except in the viscera of Dungeness crab, where the action level is 30 mg DA/kg f.w. (FDA, 2011). FDA considers the HPLC method for detection of DA as "totally satisfactory", as the detection limit of the HPLC method for DA corresponds to 0.75 μg DA/kg f.w. (FDA, 2012). Similarly, the legal framework within the EU regulates that producers of live bivalve mollusks marketed for human consumption cannot exceed the limit of 20 mg DA/kg f.w. (determined in the whole body or any edible parts thereof), see Regulation EC No. 853/2004, chapter V (2) b of section VII Annex III (EC, 2004).

The strategy of EFSA aims to establish an acute reference dose (ARfD) for exposure to ASP toxins. It is noteworthy that based on the available data from oral dose toxicity studies, humans appear to be several fold more sensitive to the DA excitotoxic effects than rodent model species (Lefebvre and Robertson, 2010). It is primarily for this reason that an incidence of ASP outbreak in Canada in 1987 was employed for risk calculations by EFSA (EFSA, 2009c). The dose of DA leading to the symptoms was estimated from 9 poisoning cases (6 with mild gastrointestinal symptoms, 4 with dizziness, 1 with memory loss). The range of exposure was estimated from 0.9 to 4.2 mg DA/kg b.w., therefore the EFSA CONTAM panel suggested 0.9 mg DA/kg b.w. as LOAEL for ASP toxin exposure (EFSA, 2009c). A safety factor of 3 to derive a NOAEL from the reported LOAEL was employed, plus an additional factor of 10 to account for human variability, thereby establishing an ARfD of 30 μg DA/kg b.w. Because DA can be converted to epi-DA during storage, the ARfD applies to the sum of DA and epi-DA. EFSA recommends a safety threshold of 4.5 μg DA/g shellfish in order not to exceed the acute reference dose (ARfD), which is lower than the maximum tolerated residue level of 20 μg DA/g f.w. (20 mg DA/kg f.w.) effective in Canada, USA and the EU. The EFSA recommendation is based on an assumed consumption of a 400 g portion of shellfish, the ARfD of 30 μg DA/kg b.w, and a 60 kg adult person. Obviously, the calculation of the ARfD as well as the calculations based on the con-

sumption of a 400 g shellfish meal by a 60 kg adult would not protect embryos or neonates, albeit the safety factors employed should be protective of individuals with an inherently higher susceptibility (Giordano et al., 2013). Experimental studies in rodents have shown that DA readily passes across the placenta and enters the fetus and also to a small extent DA transfers into milk, but in this case is not absorbed by the neonate (Maucher Fuquay et al., 2012a,b,c; Maucher et al., 2007). The risk of maternal fetal transfer has been shown in multiple rodent studies reporting adverse outcomes after birth (Dakshinamurti et al., 1993; Levin et al., 2005). Consequently, the inclusion of an additional safety factor of 10 would add a necessary additional layer of protection for embryos and neonates with regard to late onset of neurological deficiencies due to subchronic or repeated exposures to very low concentrations of DA via food.

Notably, in all current risk calculations, subchronic, repeated intermittent or chronic exposure scenarios have not been sufficiently considered when calculating the maximum tolerated residue levels in shellfish and crustaceans considered safe for distribution, sale and thus consumption. Indeed the EFSA panel (EFSA, 2009c) did not calculate a TDI (tolerable daily intake) due to absence of "chronic data" (supported by rodent and primate studies that have failed to provide evidence for increased acute or long term toxicity to repeated DA exposure), although current *in vitro* results strongly suggest that repeated or subchronic exposure could lead to late onset as well as propagation of neurological defects (Lefebvre et al., 2012).

To monitor maximum residue levels in shellfish and crustaceans, EFSA recommends AOAC validated high-performance liquid chromatography (HPLC), referred to as the Quilliam-method (Quilliam et al., 1995), as well as ELISA methods to screen for DA and analogues in shellfish and crustaceans cultivated for European market (EFSA, 2009c). Both methods have sufficient robustness and sensitivity to detect DA and analogues at tissue levels below the recommended safe level of 4.5 mg/kg f.w. The use of LC/MS with electrospray ionization in tandem mode (Furey et al., 2001) was additionally considered a rapid and valuable tool for the detection of DA and its isomers in crude extract of shellfish. Using the additional arbitrary safety factor of 10 to protect embryos and neonates would result in 0.45 DA mg/kg f.w. of tissue as maximum tolerated residue level considered safe for consumption. The latter however, would be right at the limit of detection (LOD) of the Quilliam-HPLC method (Quilliam et al., 1995), thus suggesting that shellfish and crustacean products considered safe for consumption possibly could not be safe for pregnant women or breast-feeding mothers.

4.2 Azaspiracid shellfish poisoning (AZP)

Currently only attempts of regulatory bodies of Ireland (FSAI, 2001, 2006), the European Commission (EC) and US FDA to evaluate and manage the risk of exposure to AZP toxins have been reported.

FDA sets the action level for AZP toxins at 0.16 ppm azaspiracid equivalents (FDA, 2011), but does not make recommendations regarding detection methods.

In Europe, the EC working group considered the attempts of FSAI, EU Community Reference Laboratory for Marine Biotoxins (CRL-MB) working group and FAO/IOC/WHO to estimate an ARfD for AZP toxins (CRLMB, 2005; FAO/IOC/WHO, 2004; FSAI, 2001, 2006): FSAI 0.63 µg/kg b.w. applying an uncertainty factor of 3, CRL-MB 0.127 µg/kg b.w. applying an uncertainty factor of 3, FAO/IOC/WHO 0.04 µg/kg b.w. applying a safety factor of 10. This revision of the initial FSAI assessment (FSAI, 2001) led to an estimate of 113.4 µg AZA per 60 kg person that would result in symptoms (FSAI, 2006). As the latter risk estimate assumed equal potencies for all AZA analogues, the EFSA risk assessment on AZA (EFSA, 2008a) employed toxicity equivalent factors (TEF) that were based on very few mouse experiments using i.p. injection, suggesting TEF as AZA1 = 1.0, AZA2 = 1.8, AZA3 = 1.4, AZA4 = 0.4, AZA5 = 0.2 (see Fig. 1 for structures), whereby AZA1, 2, and 3 were considered to be the most prevalent AZAs found in mussels. The latter estimation was carried out as a provisional measure only, thus emphasizing that a better toxicity database is required in order to provide a more reliable risk assessment. Similarly, lack or extremely limited data on the chronic toxicity of AZA prevented the establishment of a TDI, again emphasizing the need for an improved and validated database for risk assessment. Using the revised FSAI level from 2006 and the reported range of human effects, EFSA used the 5th to the 95th percentile range, i.e., 50.1 μg-253.3 μg and thus the median of 113.4 μg per person as a point of departure for calculating an ARfD. Using 113 μg AZA1$_{equiv.}$ per person (i.e., 1.9 μg/kg b.w. in a 60 kg person) an uncertainty factor of 3 was employed to deduce a NOAEL from the LOAEL. A factor of 3 was used to account for human variability, assuming that the persons affected during the Arranmore (Ireland) incident were most likely more susceptible to AZA effects than the more general population. Based on the use of an uncertainty factor of 9, EFSA established an acute reference dose (ARfD) of 0.2 µg AZA1$_{equiv.}$/kg b.w., corresponding to 12 μg AZA1$_{equiv.}$/60 kg person (EFSA, 2008a). However, it must be noted that when a consumption of 400 g mussel meat is used as an average meal for a 60 kg person and applying this to the current EU limit of 160 μg AZA1$_{equiv}$/kg shellfish meat, the calculated ARfD would be exceeded by a factor of 5, thus emphasizing that lower contamination limits, e.g., 30 μg AZA1$_{equiv}$/kg shellfish meat would need to be set to ensure protection from intoxications with AZA when consuming mussels.

For detection of AZA the EC suggests the EU-RL LC-MS/MS method as the reference method for detection of AZA1, AZA2, and AZA3. For other toxins from the AZA group the MBA or the rat bioassay may be used until December 31, 2014. After this period, the MBA can be used only during the periodic monitoring of production areas and relaying areas for detecting new or unknown marine toxins on the basis of the national control programs elaborated by the Member States (EC, 2011; EFSA, 2008a).

4.3 Ciguatera fish poisoning (CFP)

There are few specific regulations for CFP toxins and also no officially accepted NOAEL or LOAEL values. Notably, there are recommendations for thresholds of CFP-toxins (Lehane, 2000; Lehane and Lewis, 2000; Dickey and Plakas, 2010).

The FDA has recommended action levels for P-CTX and C-CTX at 0.01 ppb P-CTX-1 equivalents and 0.1 ppb C-CTX-1 equivalent (FDA, 2011). FDA recommends the use of the MBA as the "generally accepted method of establishing toxicity of suspect fish" caused by CFP toxin contamination (FDA, 2012).

In the EU, legislation covering fishery products states that "fishery products containing biotoxins such as ciguatera toxins" cannot be placed on the market, however neither a threshold nor suggestions regarding the method(s) of detection are provided. In view of the latter, the EC has prepared the groundwork for regulatory allowance levels for CFP toxins (EFSA, 2010a). Interestingly, while France, as part of the EC, does not list action levels for P-CTX and C-CTX, it does regulate the sale of endemic fish species well known to be involved in ciguatera in its tropical and sub-tropical overseas territories. The EFSA CONTAM panel considered 1 µg/kg of P-CTX-1 as the dose causing "clear toxic symptoms" with regard to case reports in humans. Moreover, the panel extrapolated the lowest P-CTX-1 concentration in fish associated with mild toxicity in humans as 0.1 µg/kg. In the case of CFP toxins EFSA waives the establishment of an ARfD threshold, and established a concentration of 0.01 µg P-CTX-$1_{equiv.}$/kg of fish flesh, which is "expected not to exert effects in sensitive individuals" – by applying an uncertainty factor of 10 to the lowest concentration 0.1 µg P-CTX-$1_{equiv.}$/kg of fish flesh associated with mild symptoms.

Concomitantly, EFSA recommends the validation and implementation of LC-MS/MS as the method of choice for the detection of P-CTX and C-CTX (EFSA, 2010a).

4.4 Cyclic imine poisoning (CIP)

Currently there are no regulatory limits for CIs in shellfish in Europe or in other regions of the world.

The EU Community Reference Laboratory for marine biotoxins (CRL-MB) has proposed a guidance level of 400 µg of CIP SPXs/kg shellfish meat (CRLMB, 2005; Pigozzi et al., 2006). The CONTAM panel of EFSA has decided to establish a margin of exposure (MOE) for CIP SPX instead of an ARfD. The panel calculated data on SPX occurrence in shellfish with a 95th percentile of exposure of 0.06 µg/kg b.w., involving deterministic and probabilistic approaches. On the basis of this value and the LD_{50} values of SPX in mice (50 µg/kg b.w. and 500 µg/kg b.w. administered by gavage or in feed, respectively) the panel considers that exposure to SPXs does not raise concern for the health of the European consumer (EFSA, 2010c). However, it must be noted that the MOE is based on acute data only and thus bears no weight for the protection of consumers against sub-chronic or chronic toxicity.

4.5 Diarrhetic shellfish poisoning (DSP)

While DSP toxins are subject to regulation in different parts of the world, all regulatory levels are primarily based on toxicity data derived from toxicity studies in mice. However, the analysis of hundreds of human intoxication cases with mild symptomatology corroborated the LOAEL of 50 µg OA equivalents/person (EFSA, 2008c).

The US FDA has established an action level for OA at 0.16 ppm, and it recommends "a commercially available immunoassay (detection limit for okadaic acid = 1 fg/100 g of meats; 0.01 ppm) for DSP" as replacement for the MBA (FDA, 2011, 2012).

In order to estimate an ARfD value, EFSA employed the human LOAEL of 50 μg $OA_{equiv.}$/person (60 kg) which corresponds to 0.8 μg $OA_{equiv.}$/kg b.w. for adults. The CONTAM panel of EFSA chose an uncertainty factor of 3 for calculation of NOAEL (0.27 μg $OA_{equiv.}$/kg b.w.), because the symptoms considered were relatively mild and reversible. In comparison, the rodent toxicity studies indicated a NOAEL of 50 μg $OA_{equiv.}$/kg b.w. Applying an uncertainty factor of 100 for intra- and inter-species variations, an ARfD of 0.5 μg $OA_{equiv.}$/kg b.w. for humans would be established, which is slightly higher than the NOAEL, based on the evidence collected from human intoxication cases (EFSA, 2008c).

The EC refers to the LC-MS/MS method as the reference method for detection of DSP toxins and also recommends other internationally validated methods to be used until 31 December 2014 (EC, 2011).

In order to account for the DTX variants, Garibo and colleagues (Garibo et al., 2013) established inhibition equivalency factors for OA of 1.1 and 0.9 for DTX-1, and 0.4 and 0.6 for DTX-2, for recombinant and wild human PP2A, respectively. The latter values were applied to the determination of OA equivalent contents in spiked and naturally contaminated shellfish samples and demonstrated good agreement with those obtained in LC-MS/MS analysis.

4.6 Neurotoxic shellfish poisoning (NSP)

There are currently no NOEL/LOAEL or ARfD values established for this toxin class, though toxicity occurs in the nanomolar concentration range (Baden, 1989; Toyofuku, 2006).

The FDA, established an action level of 0.8 ppm of BTX-2 equivalent analyzed by the MBA (FDA, 2011, 2012). Similarly, New Zealand employs an acceptable BTX-2 level of 20 MU/100 g f.w. mussel tissue (Van Apeldoorn, 2001). Notably the underlying information is based on studies from the 1960s in which death of mice injected by crude extracts from shellfish were compared with human symptoms (Van Apeldoorn, 2001). Additionally, shellfish bed closures in the USA are based on the density of the causative dinoflagellate, *Karenia brevis*, and reopened based on MBA analysis of shellfish.

Despite the lack of a European regulation limit for BTX, the EFSA CONTAM panel considered that an acute reference dose (ARfD) should be established (EFSA, 2010b). However, EFSA considered that the presently available data were too scant to allow for derivation of an ARfD.

The European authority recommends the LC-MS/MS approach and also further development of bio-molecular assays (EFSA, 2010b).

4.7 Palytoxin poisoning (PaP)

Currently there are no regulations for management of PaP toxins.

The experts of the EFSA CONTAM panel however decided to establish an ARfD value for PaP toxins for humans on the basis of oral and sublingual toxicity in experimental animals. The LD_{50} values for PlTX ranged from 510 to 767 μg/kg and

651 μg/kg, (Munday, 2006; Sosa et al., 2009; Tubaro et al., 2011a) in mice. Ostreocin-D exhibits effects similar to that of PlTX, but somewhat less severe at similar doses, after a single oral administration in mice (Ito and Yasumoto, 2009). A provisional NOAEL of 3 μg/kg/day was estimated after repeated oral exposure (7 days) of PlTX in mice. Higher PlTX doses provoke macroscopic alterations at the gastrointestinal level (gastric ulcers and intestinal fluid accumulation) in mice, while histological analysis highlighted severe inflammation, locally associated with necrosis, at pulmonary level, as well as hyper-eosinophilia and fiber separation in myocardium (Tubaro et al., 2011b). The PlTX induced cardiac pathology was further studied *in vitro* on cardiomyocytes, indicating a severe and irreversible impairment of their electrical properties (Del Favero et al., 2013). As the reference point for establishment of the ARfD, the LOAEL for oral toxicity (gavage) in mice 200 μg PlTX/kg b.w. (Ito and Yasumoto, 2009) was chosen as point of departure (EFSA, 2009a). As EFSA deemed mice less sensitive to PlTX toxins than other species, an extra safety factor of 10 to the routine factor 100 was applied resulting in an ARfD of 0.2 μg/kg b.w. for the sum of PlTX and its analogue ostreocin-D. Based on the assumption of a 60 kg person and a consumption of 400 g crab, shellfish or fish flesh, the maximum contamination of consumable flesh should not exceed 30 μg PlTX and ostreocin-D analogues/kg flesh.

The EC does not recommend any particular method of analysis of PlTX toxins, yet recommends to develop and optimize methods other than the MBA for lipophilic toxins (EFSA, 2009a).

4.8 Paralytic shellfish poisoning (PSP)

Based on the acute i.p. MBAs and TEF calculations proposed by Oshima et al. (Oshima, 1995), the National Reference Laboratories for marine biotoxins including the FAO/WHO supported a TEF calculation for the different STX and GTX analogues in finfish, shellfish, and crustacean products (EFSA, 2009d; Van de Riet et al., 2009, 2011; Watanabe et al., 2013): STX = 1, NeoSTX =1, GTX1 = 1, GTX2 = 0.4, GTX3 = 0.6, GTX4 = 0.7, GTX5 = 0.1, GTX6 = 0.1, C2 = 0.1, C4 = 0.1, dc-STX = 1, dc-NeoSTX = 0.4, dc GTX2 = 0.2, GTX3 = 0.4, and 11-hydroxy-STX = 0.3 (see Fig. 8 for some of the structures). Based on a LOAEL of 1.5 μg $STX_{equiv.}$/kg b.w. and applying a safety factor of 3, a NOEL and thus an acute reference dose (ARfD) of 0.5 μg $STX_{equiv.}$/kg b.w. was calculated. When assuming the consumption of 400 g mussel flesh per meal per person and applying the current FDA and EU limit of 800 μg $STX_{equiv.}$/kg shellfish meat, the consumption of such a meal would result in the exposure to 4.6 μg $STX_{equiv.}$/kg b.w. in a 70 kg person and thus exceed the ARfD by a factor of 9-10. Thus reducing the allowable level in shellfish meat to approximately 80 μg $STX_{equiv.}$/kg shellfish meat would be a matter of prudence, irrespective of the fact that the LOAEL may have been overestimated.

EFSA recommends the optimization of LC-MS/MS approaches, while declaring the MBA and the AOAC-HPLC method as the official methods for PSP detection (EFSA, 2009d). EFSA suggested the inter-laboratory testing of biomolecular methods, e.g., receptor binding assays, to be validated as alternative assays. An international validation exercise of the receptor binding assay was carried out by van Dolah and coworkers (Van Dolah et al., 2012) and demonstrated an acceptable correlation with the MBA (slope of 1.64 and an $r^2 = 0.84$), and the HPLC detection method using precolumn oxidation (slope of 1.20 and an $r^2 = 0.92$).

Similarly, the US-FDA recommends the HPLC method "… to identify individual PSP toxins (with a) detection limit for saxitoxin = 20 fg/100 g of meat" (FDA, 2011, 2012).

4.9 Pectenotoxin Poisoning (PeP)

There are currently no regulatory attempts for risk management of PeP toxins except for Europe and the USA.

The EFSA CONTAM panel aimed to establish an ARfD value for exposure to PeP toxins. Based on the rodent toxicity studies and the corresponding LOAEL of 250 μg PTX2/kg b.w. i.p. (Ishige et al., 1988), the EFSA extrapolated a NOAEL using a risk factor of 3 and then a default risk factor of 100 to establish an ARfD of 0.8 μg $PTX2_{equiv.}$/kg b.w. (EFSA, 2009b). For safety reasons, EFSA employed a provisional TEF value of 1 for PTX1, PTX2, PTX3, PTX4, PTX6, and PTX11, while no TEF values were assigned to PTX7, PTX 8, PTX 9, PTX2 SA, and 7-epi-PTX2 SA due to the limited information available and the much lower i.p. toxicity of these analogues. However, due to its current regulation, a total of 160 μg lipophilic toxins / kg shellfish meat (primarily associated with the limit of 160 μg $OA_{equiv.}$/kg shellfish meat) would be permissible in both the EU and the USA (FDA, 2011). Upon consumption of 400 g shellfish meat per 60 kg person, the latter regulatory limit would result in an intake of 64 μg $PTX2_{equiv.}$ i.e., 1.06 μg $PTX2_{equiv.}$/kg b.w. and thus exceed the ARfD by approximately 33%. Although the latter was not considered to represent an excessive additional health risk, especially considering the currently reported contamination levels of shellfish on the European markets, EFSA proposed lowering of the permissible $PTX2_{equiv.}$ level to 120.0 μg $PTX2_{equiv.}$/kg shellfish meat (EFSA, 2009b).

The European Commission refers to the LC-MS/MS method as the reference method for detection of PTX toxins (EC, 2011; EFSA, 2009b).

4.10 Yessotoxin shellfish poisoning (YSP)

Currently there are no regulatory limits for YSP toxins outside of Europe. The EFSA CONTAM panel considered for definition of the corresponding ARfD for human exposure data from a small number of acute toxicity studies using single and repeated oral administration of YTX and resulting in LD_{50} values ranging between 100 to 5000 μg YTX/kg b.w. (Aune et al., 2002; Dell'Ovo et al., 2008; Tubaro et al., 2008, 2004, 2003; Espenes et al., 2006). A NOAEL of 5 mg YTX/kg b.w. for acute cardiotoxicity was observed following oral administration of YTX and served EFSA as point of departure. In view of the >90 structural analogues and the dearth of information as to their individual toxicities and thus lack of reliable TEF values, EFSA employed a safety factor of 2 to allow for analogue differences, in addition to a safety factor of 100 to account for intra- and inter-species differences, finally arriving at an ARfD of 25 μg $YTX_{equiv.}$/kg b.w. When employing the 400 g shellfish meat portion size and the currently permissible levels of YTX of 1 mg $YTX_{equiv.}$/kg shellfish meat, the resulting

exposure of a 60 kg person would approximate to 6.7 μg YTX-$_{equiv.}$/kg b.w., thus being well below the ARfD extrapolated.

The EC refers to the LC-MS/MS method for lipophilic toxins as the reference method for detection of YTX toxins and recommends the MBA (EFSA, 2009e).

4.11 Some shortcomings regarding risk assessment and management

Chronic exposure: To date there are no data on the effects of long-term low-dose or repeated human exposures to marine biotoxins. Available data refer to acute situations, as statistically sufficient amounts of epidemiologically appropriate data are lacking. More problematic is the fact that currently there are no concerted efforts for well-designed epidemiological studies that would enable the derivation of a tolerable daily intake (TDI). Thus marine biotoxin consumer-centric risk assessment and management remains scientifically not well founded.

ARfD: The bases for the most robust ARfD values are animal experiments, primarily acute toxicity studies after oral administration of the toxins, generally in a small number of animals, due to the small amount of toxins and the primary interest in defining an acutely toxic effect. To be more effective proper experimental designs would need to be developed that allow derivation of chronic effects and consequently a TDI. However, determination of chronic effects would demand a high number of animals, high concentrations of toxins to test with and consequently would mean a huge financial investment. More importantly however, the acute as well as the chronic rodent biassays lack the necessary scientific basis for the transferability of these data to the human situation. Indeed, for some marine biotoxins the mice appear less susceptible, e.g., to PlTX toxins, than humans or more susceptible, e.g. to yessotoxins. In cases where data from human incidences could be recruited the number of intoxication cases employable for the estimation of NOAEL's or LOAELs is inappropriately small and therefore statistically insufficiently robust.

Analytical methods: Although a number of laboratories, foremost the National Research Council Canada (http://www.nrc.ca/crm), are constantly working on developing and producing internationally available certified reference material for many marine toxin analogues, there are still insufficient certified reference materials for many toxins. Although the latter is understandable in view of the difficulties of the task of preparing material and the huge number of toxin analogues, the incompleteness of certified reference toxins represents a major obstacle to the use of analytical methods for hazard assessment or use of new methodologies for hazard and risk assessment of whole toxin classes. Since a European provider of certified reference material and standards, Cifga laboratories (http://www.cifga.com), commercially develops toxin standards, this will partially ameliorate the problems with certified reference materials but not the issue of the high cost and the huge diversity of toxin analogues.

Co-occurrence: It is well-recognized that marine biotoxins of different classes can co-occur, also in the same vector organism, e.g., more-than-additive or possibly synergistic toxicity of AZA and YTX (Tubaro et al., 2004), co-occurrence of CTX and PlTX (Kodama et al., 1989), PTX and OA (FAO/IOC/WHO, 2004) and OA, PTX and YTX (EU/SANCO, 2001). Though there are rigorous attempts for proper risk assessment of mixture effects in pesticides (FSA-UK, 2003; ILSI, 1999; Meek et al., 2011; US-EPA, 2001), no such evaluations and thus risk assessments are currently being carried out for marine biotoxins.

Transparency: The current decision finding processes for setting action and allowance levels for marine biotoxins are not sufficiently transparent to allow understanding and comprehension of these decisions. For example in most cases cited, although human intoxication cases have been used, the analytical confirmation for toxin levels is lacking or questionable, safety factors were employed for the derivation of NOAELs or LOAELs. Concomitantly, the scientific explanation for the absolute values of uncertainty and safety factors used are generally lacking (no weight of evidence studies published or discussed). Finally, detailed guidance, characterization, and reasoning for the deterministic and probabilistic approaches used are often completely missing.

Harmonization: Currently there are no international efforts at harmonizing epidemiological, hazard monitoring, and risk management approaches. However, it is crucial that responsible safety authorities find agreements on semantics (e.g., action level vs. allowance level), risk calculations, and reference systems (ppm/ppb vs. g/60-kg person vs. g/kg fish vs. g/fish portion vs. g/kg body weight), the portion of sample finfish or shellfish (e.g., GI tract, muscle tissue) to be analyzed and being considered as representative for the potential meal to be consumed, etc. Finally, there is a need for an enhanced harmonized risk assessment, e.g., the expansion to toddlers, children, pregnant women and the elderly and in consequence for a specific risk management regarding labeling of produce that could be of risk to specific cohorts in the consumer population (see "population" below).

Analytical shortcomings: The use of LC-MS as a monitoring method is creating an important uncertainty related to the fact the LC-MS is a target methodology, it seeks the desired compounds, but it does not cover unexpected toxins. Therefore, this will mean a breach in safety monitoring. On the other hand, there seems to be a large variation in interlaboratory results if the parameters in the method being used are not well controlled (Otero et al., 2011). Notably, the uncertainties regarding the use of LC-MS as well as the position of Otero and colleagues are still a matter of debate.

Population: Throughout all biosciences population differences in reactivity, responsiveness, and sensitivity due to age (developing organisms, infancy) and maternal conditions (pregnancy, lactation) are taken into account. These considerations urgently should also be applied to the field of toxicological risk and safety assessment of marine biotoxins.

5 *In vivo* assays for toxicological risk assessment of marine biotoxins

Traditionally mice and also rats are most commonly used in toxicological risk assessment of pharmaceuticals, pesticides,

chemicals, personal care products, food and food-components, and consequently also of marine food products.

The mouse bioassay (MBA) and rat bioassay (RBA) are critical parts of regulatory compliance, e.g., European Commission Regulation (EC) No 2074/20054 Annex III, Chapter III, until December 31, 2014. After this period, the MBA shall be used only during the periodic monitoring of production areas and relaying areas for detecting new or unknown marine toxins on the basis of the national control programs elaborated by the EU Member States. The MBA is employed for the assessment of toxins responsible for ASP, AZP, CFP, DSP, NSP, PeP, PaP, CIP, and PSP, while additionally the RBA is employed for toxins responsible for AZP and DSP. Approximately 300,000 mice are used per year for this regulatory purpose in Europe alone (http://bit.ly/1cnFqiC), whereas the additional mice and rats employed for investigative toxicology with marine biotoxins are not included in this number.

The MBA for PSP was first suggested in 1937 (Sommer and Meyer, 1937), the method then described in detail in 1960 (Banner et al., 1960). A DSP MBA was established as an internationally acknowledged assay by Yasumoto and colleagues between 1971 and 1984 (Yasumoto et al., 1971, 1984; Yasumoto et al., 1978b) followed by additional further adaptations and optimizations. The DSP MBA has been expanded over time to include additional lipophilic toxins that are detected by death in mice used in the assay, although over and under sensitivity for some of these toxins occurs, depending on the specific toxin and extraction method. Toxin analyses using the MBA consist of a toxin extraction from whole flesh of finfish, shellfish, or crustaceans by boiling in water miscible solvents, e.g., acetone, methanol for some but not all lipophilic compounds (exceptions are DSPs and similar lipophilic compounds), or in acids, e.g., hydrochloric acid for PSP (Aune et al., 2007). Following evaporation and resuspension, e.g., in a 1% solution of Tween 60, resuspended extracts are injected into mice i.p. and their survival is monitored. The lipophilic assay is run over a period of up to 24 h, with the death of two out of three animals considered to indicate a positive sample, whilst that for PSP is run for up to one hour, with the median time of death of 5 or more animals used to give an indicative toxin level.

Toxin testing using the RBA for those lipophilic toxins that are capable of causing diarrhea (Kat, 1983) consists of mixing whole flesh of finfish, shellfish, or crustaceans, or the hepatopancreas of shellfish with the food of pre-starved rats. Following exposure of the rats to this "toxin contaminated" food, the rats are observed for 16 hours for signs of diarrhea, consistency of the feces and food refusal. These parameters are correlated to symptoms of diarrhea and nausea in humans (FAO/IOC/WHO, 2004).

Although expert panels repeatedly raised significant doubts regarding use and relevance of the rodent-based assays to humans, there are no systematic reviews or studies highlighting the shortcomings and pitfalls of these methods. EFSA (EFSA, 2008a,c, 2009c,b,e,d,a, 2010a,c,b), raised the following methodical shortcomings of the mouse and rat bioassays:

- the outcome depends on the choice of solvents used for toxin extraction and i.p. injection;
- extraction solvents show different extraction efficiencies. Furthermore, results may differ several fold if different extraction pHs are used for extraction of some toxin classes;
- the boiling step in acidic solutions may result in overestimation of the toxicity, depending on the toxin profile, e.g., when analyzing for PSP;
- inherently high variability of results amongst laboratories primarily resulting from various factors, e.g., strain, sex, age, weight, general state of health, diet, stress, as well as experience of the animal facility, in handling the animals, and in carrying out the *in vivo* assay;
- the potential for false positive results;
- the potential for false negative results;
- for most toxins not quantitative;
- in the MBA, the injection volume of 1 ml exceeds good laboratory practice guidelines (less <0.5 ml), thus producing stress in the mice and therefore confounding or obscuring the potential specific effects;
- not sufficiently sensitive to detect relevant levels of some toxins;
- have never been subjected to a proper validation for the detection of most of the toxins, thereby emphasizing the lack of safety in extrapolation provided by these assays when considering the huge variability in the endpoints determined amongst laboratories;
- the i.p. route is not appropriate for the detection of toxins that require hydrolysis in the gastrointestinal tract;
- are prone to interferences by other factors present, e.g., metal salts.
- in many countries the use of the MBA and RBA are considered unacceptable for ethical reasons;
- labor intensive;
- cannot be readily automated;
- require specialized animal facilities and expertise.

6 Symptoms, modes of action, relevant endpoints, and functional assays

Each of the individual marine biotoxins in finfish, shellfish, and crustaceans provide for an array of symptoms in exposed humans, some of which are restricted to acute intoxications, others may appear at a later stage, e.g., upon recurrent or chronic exposure or progression to disease states. As already stated, most current knowledge is primarily based on a rather low number of case studies of acute intoxications, whereas subchronic or chronic exposures have not been well investigated or documented, mainly because of the lack of sufficient amounts of toxins, and the enormous cost of the studies involved. Based on the latter, the current modes of action (MOA) of marine biotoxins refer primarily to the molecular interaction and subsequent apical endpoints of the acute intoxication, whereas the MOA due to subchronic or chronic exposures may differ dramatically. While we know that some marine biotoxin intoxications can lead to lethal outcomes in humans, as detected by mouse and rat bioassays, little information can be gleaned from these *in vivo* assays as models regarding the physiological interactions and the relevance of the symptomatology in rodents for the human.

Specific *in vitro* assays addressing the relevant and specific MOA of each individual marine biotoxin may lead to complementary physiologically relevant assays with high selectivity, robustness, high reproducibility, low variability and high sensitivity as well as a qualitative and quantitative capability. However, the latter pertains only to toxins for which the MOA is known and does not address the MOA of unknown toxins or unknown compounds for which there is only scant evidence for their toxicity. These *in vitro* assays, called "functional assays", are usually related to the actual toxicity that can be carried out with human cells, thus providing a higher relevance for risk extrapolations to humans while adding the potential for high-throughput applicability and thus affordability. These however only reflect a very specific MOA in humans, whereas the broader symptomatology of a given marine toxin intoxication cannot be simulated. Thus the question that remains to be answered is whether or not all relevant MOA of an acute intoxication with a specific marine biotoxin can be addressed with simple *in vitro* systems. If not, undetermined relevant MOA could lead to possibly non-lethal intoxications that would remain unrecognized as marine biotoxin related, but possibly affecting a larger number of consumers than otherwise predicted. In the following section the known MOA of individual marine biotoxin groups will be reviewed.

6.1 Amnesic shellfish poisoning (ASP)

Symptoms

Adverse effects include gastrointestinal disorders, nausea, vomiting, abdominal cramps and diarrhea within 24 h. Headaches, dizziness, memory loss, confusion and hallucinations can occur within 48 h (Kumar et al., 2009; Jeffery et al., 2004; Grant et al., 2010). However, "non-neurological" symptoms also include vomiting, cramping, coma and death with or without the typical symptoms associated with amnesia (Grant et al., 2010).

Mode of action

DA has two primary targets in the central nervous system: the hippocampal formation and its associated regions, which are involved in memory processing, and the brain stem region of the area postrema and nucleus of the solitary tract associated with visceral function (Adams et al., 2009). DA can cause extensive damage to the central nervous system (Colman et al., 2005). The olfactory bulb and hippocampus are most susceptible to damage and each may play a role of transmitting damage to the other brain regions (Peng et al., 1994; Pulido, 2008; Tryphonas and Iverson, 1990).

The neurotoxic mechanism of DA is mediated via activation of N-methyl-D-aspartate (NMDA) receptors (Berman and Murray, 1997; Novelli et al., 1992). DA interacts with subclasses of glutamatergic ionotropic receptors comprised of KA and GluR5-7 subunits through a partial agonistic interaction that induces partial ion conductivity and prevents normal inactivation of channel opening resulting in ion conduction well beyond that achieved by natural glutamatergic transmitters (reviewed by Ramsdell, 2007). Excitotoxicity results from integrative action on both sides of the synapse. DA depolarizes the presynaptic membrane to release glutamate into the synapse. DA depolarizes the postsynaptic membrane and promotes activation of NMDA ionotropic receptors. DA targets the high concentration of glutamatergic ionotropic receptors in the granule cell layer of the olfactory bulb and CA3 region of the hippocampus, leading to seizure-prone circuitry in the medial temporal lobe of the brain. Excitotoxicity leads to cellular and ultrastructural damage to pathways responsible for the learning and recall of sequences underlying spatial memory and the restraint of seizure circuitry.

The mechanism of DA acute toxicity is the high affinity binding of DA, its isomers and analogues to excitatory non-N-methyl-D-aspartate (non-NMDA) glutamate receptors (GluR), similar to the neurotoxin kainic acid. Due to its structural analogy to glutamate kainic acid, it activates both the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptor and kainate subtypes of glutamate receptors. Upon DA binding to AMPA receptors, Na^+ channels are opened thus resulting in a Na^+-influx into neuronal cells and in a membrane depolarization. In contrast, DA binding to kainate receptors results in an influx of extracellular Ca^{2+} (EFSA, 2009c). It is currently recognized that NMDA receptors as well as glutamate released from the synapses as a consequence of DA binding to pre-synaptic AMPA/kainate receptors, also participate in the cascade of events observed following DA receptor binding (Fig. 11).

The result of DA binding, largely depending on the DA concentration *in situ*, is an increased intracellular Ca^{2+} and loss of intracellular Ca^{2+} homeostasis in neurons with the consequent loss of neuron viability via necrotic or apoptotic cell death (Giordano et al., 2008, 2009, 2006, 2007; Ozawa et al., 1998; Lerma et al., 2001; Ramsdell, 2007). Upon acute DA excitotoxic insults, neurons of the hippocampus undergo rapid swelling in both the soma and dendrites, whereby dendrites appear to be more susceptible to DA. In consequence, neuronal pathology is observed primarily in the olfactory bulb and hippocampus and the amygdala upon i.p. or i.v. exposure, while little is known regarding the doses or concentrations required for acute effects upon oral exposure.

Subchronic, repeated exposure to DA also results in brain pathology, i.e., cytoplasmic vacuolization of neurons and astrocytes, as well as injury to the mitochondria in the pyramidal cells at high doses only. These high doses compare to assumed doses that have been suggested to result in no clinical symptoms, e.g., seizures or overt neurotoxicity (Pulido, 2008). Decreased levels of neuronal glutathione (GSH) enhance susceptibility to DA neurotoxicity, suggesting that repeated exposure to non-toxic doses of DA could result in overt neurotoxicity in individuals with polymorphic glutamate cysteine ligase and thus low GSH (Giordano et al., 2013). Experimental studies in rodents have shown that DA readily passes across the placenta and enters the fetus and also to a small extent transfers to milk, but in this case is not absorbed by the neonate (Maucher Fuquay et al., 2012b,c; Maucher and Ramsdell, 2005; Lefebvre and Robertson, 2010). Moreover, the epidemiologic data not only suggests that males are more susceptible than females but rather that developing embryos as well as neonates are more prone to DA mediated late neurological persistent adverse effects (Lefebvre and Robertson, 2010; Pulido, 2008). Devel-

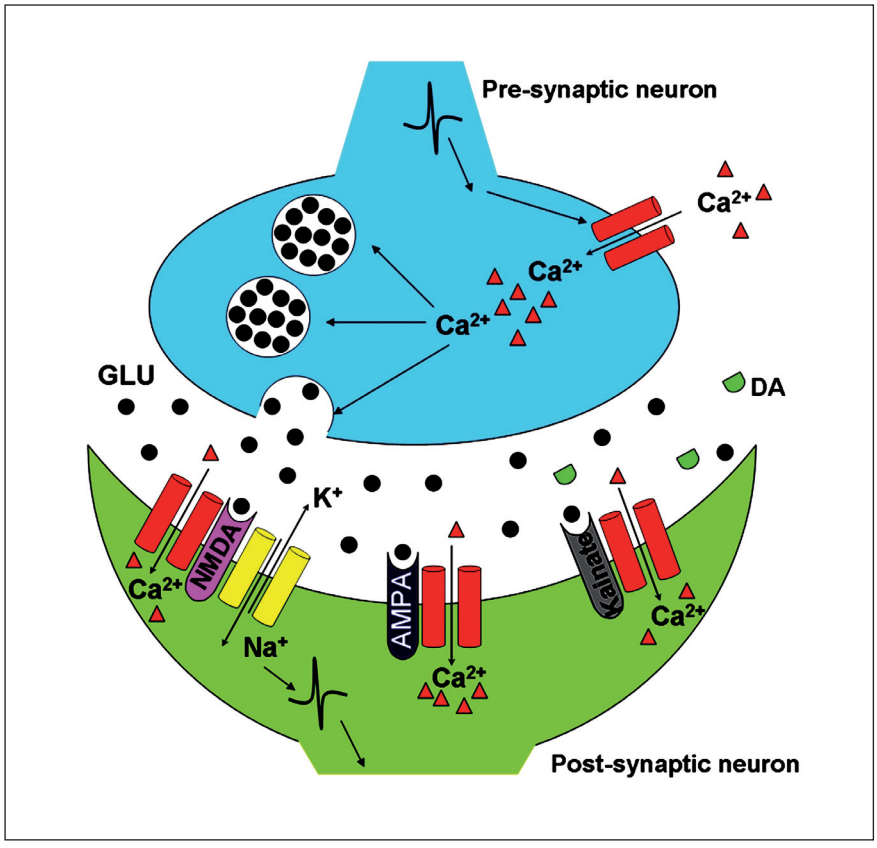


Fig. 11: Glutamate (GLU) and domoic acid (DA) induced interaction with neuronal signaling
Endogenous Ca^{2+} is released upon depolarization of the pre-synaptic cell which leads to mobilization of vesicles containing GLU to the membrane surface. GLU is then exocitotically released into the synaptic cleft, interacting with cell surface receptors. DA can interact within the synaptic cleft with the three ionotropic receptor subtypes, i.e. the kainate, AMPA, and NMDA receptors, on cell membranes. Kainate and AMPA receptor activation results in release of Ca^{2+} into the post-synaptic cell. The influx of Na^+ and Ca^{2+} can also be triggered by the binding of DA to NMDA receptors, which are linked to both Ca^{2+} and Na^+/K^+ ion channels. DA binding induces, unlike GLU binding, prolonged receptor activation, which leads to a constant influx of cations into the cells inhibiting desensitization processes. The excessive intracellular Ca^{2+} concentration causes swelling of the cells, functional disruption and cell death (Reproduced with permission from (Lefebvre and Robertson, 2010)).

oping and aging brains, having discrete windows of exposure and a different spectrum of effects during neurogenesis, synaptogenesis and maturation of dendritic spines, appear to be more susceptible to DA than brains of the typical 70 kg (or 60 kg as EFSA deploys) standard human routinely used for risk assessment purposes (Grant et al., 2010; Ramsdell and Zabka, 2008). The latter has implications with regard to risk assessment and tolerable residue levels in edible tissue of shellfish and crustaceans (see above).

Functional, biochemical and biomolecular assays

There are several analytical approaches regarding detection and quantification of ASP group toxins. The most robust and sensitive (LOD <20 µg DA/kg shellfish tissue and the LOQ 40 µg DA/kg shellfish tissue) tandem mass spectrometry (LC/MS/MS) approach was subjected to single-laboratory validation and inter-laboratory studies (McNabb et al., 2005) and optimized methods documented for various biological fluids and tissues.

As a biochemical method an ELISA (antibody based) is available for detection of DA, iso-DA A, D, E, F, and epi-DA with a limit of detection (LOD) of 0.003 mg/kg and a limit of quantification (LOQ) of 0.01 mg/kg. This method is accepted as the AOAC (Association of Official Analytical Chemists) method 2006.02 (Kleivdal et al., 2007). With development of a complete set of certified ASP toxin reference materials, antibodies against other DA analogues can be developed. Hence this approach seems promising for the detection of ASP toxins. Antibodies against DA have also been used in the development of optical immunosensors based on surface plasmon resonance (Stevens et al., 2007; Yu et al., 2005).

A receptor-binding assay was developed on the basis of binding competition of DA with [^{3}H]-kainic acid for the kainate/quisqualate glutamate receptor, involving rat GluR6 glutamate receptor (Quilliam et al., 1995; Van Dolah et al., 1997, 1994). Further adaptations by involvement of human receptors and optimization of the sensitivity could qualify this approach as a high-throughput competent functional assay for evaluation of the risk and potency of some of the ASP toxins, although it would be important to identify whether or not the "non-neurological" symptoms, e.g., vomiting, cramping, coma, and death can also be explained by the interaction with the excitatory non-NMDA glutamate (GluR) and AMPA receptors.

Recent findings in the field also suggest a significant role of ASP toxins on Na^+/K^+-ATPase activity, which is postulated to determine neuronal vulnerability. Na^+/K^+-ATPase assays in relevant cell types for detection and evaluation of toxic potencies of ASP toxins may also lead to promising approaches (Watanabe et al., 2011).

Currently, there are no test systems evaluated to quantify the increase of intracellular Na^+ and Ca^{2+} in relevant cells resulting from the exposure to ASP toxins. As systems for detection of a rapid intracellular increase of Na^+ and Ca^{2+} (also for neurons) are commercially available, the recruitment of these methods for assessment of ASP toxins should be enhanced. Notably, the lat-

ter methods can also be applied to calcium mediated cell death scenarios, which are relevant to the action of ASP toxins.

Standardized reference compounds
Certified reference calibrant DA solution and a mussel tissue certified reference material are provided by the National Research Council Canada, NRCC, Halifax and Cifga. The NRCC materials also contain defined amounts of epi-DA.

6.2 Azaspiracid shellfish poisoning (AZP)

Symptoms
Acute intoxications of humans results in nausea, vomiting, diarrhea and stomach cramps (Ito et al., 2000; Paredes et al., 2011). Mouse studies suggest that chronic exposure to AZP toxins could lead to a higher likelihood of the development of lung tumors (Furey et al., 2010), albeit it remains a matter of discussion whether the latter is of any relevance for humans.

Mode of action
It has been postulated that some AZP toxins can inhibit protein phosphatases (PP), yet other scientists question this (Flanagan et al., 2001; Twiner et al., 2005, 2008). *In vitro* experiments with mammalian cell lines showed alterations in the cytoskeletal structure (Roman et al., 2002; Twiner et al., 2005; Ronzitti et al., 2007; Vilarino et al., 2006; Paredes et al., 2011). AZP toxins increase intracellular Ca^{2+} concentrations and cAMP levels in human lymphocytes and primary cultures of cerebellar granule cells (Roman et al., 2002; Vale et al., 2007b). In addition these toxins induce increased phosphorylated Jun-kinases in primary cultured neurons (Vale et al., 2007a). New findings in the field of AZP toxin research reveal also the ability of AZA1 to inhibit endocytosis by inhibition of maturation of cathepsin D in MCF-7 cells, (Sala et al., 2013). Other findings point to the ability of AZP toxins to block the K^+ conductance of hERG1 channels in HEK-293 cells (Twiner et al., 2012a). It could also be shown that AZP toxins induce apoptosis pathways in Jurkat T lymphocytes, Caco-2 intestinal cells, and BE(2)-M17 neuroblastoma cells in the nM range (Twiner et al., 2012b).

Functional, biochemical and biomolecular assays
Currently there is one protocol for analytical quantification of AZA1 available which was subjected to inter-laboratory validation (McNabb et al., 2005). The LOQ for this method is 0.05 mg/kg.

Two antibodies were developed, i.e., mAbs 6B11 and 9E8, both of which bind azaspiracid-1 with a relative binding affinity of 0.2 μM (Frederick et al., 2009). These could be used to develop a sandwich-ELISA which could be suitable for a high-throughput application.

For AZP toxins there is a lack of further biochemical and biomolecular assays, most likely due to the conflicting theories as to the MOA of AZPs. Possible relevant targets could be the increase of cytosolic Ca^{2+} and cAMP in human lymphocytes, the alteration of cytoskeletal structure (actin, E-cadherin) in human cell lines, the blockage of potassium channels, the impact on endocytosis, but also the pro-apoptotic activity of AZP toxins.

Standardized reference compounds
Standardized reference compounds (CRM, certified reference material) of AZA1, AZA2, and AZA3 are available from NRCC. A mussel tissue certified reference material for azaspiracids is also available from the NRCC. This material contains most of the azaspiracid analogues. Quality controlled standards for AZA1, AZA2, AZA3, AZA4, and AZA5 are supplied by Cifga.

6.3 Ciguatera Fish Poisoning (CFP)

Symptoms
The CFP toxins induce about 175 ciguateric symptoms, classified into four categories: gastrointestinal, neurological, cardiovascular, and general symptoms (Terao, 2000; Lewis et al., 2000). The symptoms of CFP toxins differ dependent on local occurrence: neurological symptoms predominate in the Pacific Ocean, gastrointestinal symptoms in the Caribbean Sea, and both symptom categories are reported in the West Indies.

Symptoms may recur during periods of stress, such as exercise, weight loss, or excessive alcohol consumption (Lehane and Lewis, 2000). Individuals who have previously suffered from CFP may become sensitized and experience a recurrence of symptoms after eating fish that does not cause symptoms in other persons (De Fouw et al., 2001). It has also been reported that the toxin can transfer between partners during sexual intercourse, resulting in localized pain and other symptoms in the partner who had not consumed the affected fish (De Fouw et al., 2001).

The nature, duration and severity of symptoms vary between ethnic groups and between the sexes, although it is not clear whether this is due to genetic predispositions, different eating preferences, or different toxin profiles in different regions or types of fish consumed.

In the Pacific, men are more likely to experience diarrhea and abdominal pain, whereas women more often report arthralgia and myalgia. Whilst cases have spanned all ages, there appear to be more males than females affected, and more patients within the 30-49 year age group (FAO, 2004; Lehane and Lewis, 2000).

Mode of action
The voltage-gated sodium channel (NaV) is the primary target of CFP toxins in the nM range. Receptor binding opens the ion pore, leading to Na^+ influx (Cameron et al., 1991; Mattei et al., 1999). This causes membrane depolarization and functional impairment of excitable cells (Boyarsky and Rayner, 1970; Setliff et al., 1971; Bidard et al., 1984; Lombet et al., 1987; Catterall et al., 2007). Secondary responses observed in cells exposed to CTX-group toxins include Ca^{2+} entry into the cell by reverse action of Na^+/Ca^{2+} exchangers (Lewis and Endean, 1986; Molgo et al., 1993; Terao, 2000), which leads to muscular contraction and neurotransmitter release in a variety of experimental systems (Kakizaki et al., 2006). However, as noted by Dickey and Plakas, not all symptoms observed in exposed humans can be explained by the interaction of CTX with the NaV, thereby emphasizing that a better understanding of the symptomatology observed in patients and the respective CTX concentrations to which the patient was exposed is required in order to identify

new MOA and thus relevant endpoints for development of functional assays (Dickey and Plakas, 2010).

Maitotoxin appears to exert its effects on endogenous membrane calcium channels inducing Ca^{2+} entry in different mammalian cells (Estacion, 2000).

Functional, biochemical and biomolecular assays

An analytical method developed for detection and quantification of CFP toxins is an LC-MS/MS method with a LOD for P-CTX-1 of 0.03 µg/kg fish (Lewis et al., 2009; Stewart et al., 2010). However, analytical approaches suffer from the lack of knowledge and reference materials on the structural variants of which the impact on CFP is unknown.

Since the 1970s various immunoassays have been developed for sensitive detection of CFP toxins, e.g., a radioimmunoassay (Hokama et al., 1977, 1998a), an enzyme immunoassay, available also as stick test (Hokama et al., 1983, 1989), and a membrane immunobead assay with a LOD of 32 ng P-CTX-1/kg fish flesh (Empey Campora et al., 2008; Hokama et al., 1977, 1998a,b; Manger et al., 1995). As the antibodies are not commercially available and as the antibodies address only P-CTX toxins, these approaches were not subjected to further optimization or validation. Nevertheless, there are on-going attempts to develop immunoassays for sensitive detection of CFP toxins (Caillaud et al., 2010b; Tsumuraya et al., 2010, 2012).

Another approach with the potency for detection of CTX and evaluation of their risk for human health are the neuroblastoma (Neuro-2a cells) cell-based assays addressing the cytotoxic action of the toxins. This test shows high sensitivity and specificity for screening purposes (Caillaud et al., 2012; Pawlowiez et al., 2013; Dechraoui et al., 1999, 2005), and has proven useful for monitoring toxin levels in blood in laboratory rodents and wildlife.

As a functional assay for detection and quantification of CFP toxins, a competitive receptor binding assay was developed and evaluated (Dechraoui et al., 1999, 2005; Legrand and Lotte, 1994; Lombet et al., 1987; Perez et al., 2011). This assay is based on the competitive binding of radio-labeled [^{3}H]-brevetoxin-3 to sodium-channels in rat brain synaptosomes in the presence of CTX-group toxins. The limit of quantification (LOQ) for this assay was reported at 0.16 μg P-CTX-3C$_{equiv}$/kg fish (Darius et al., 2007). The approach of receptor binding assays refers to the MOA of CFP toxins and has the potential not only to detect and quantify CTX contamination but also to provide the basis for evaluation of human responsiveness to these toxins. Notably, findings from the field of pharmacology may provide further competitive binding partner for this assay (Meraj et al., 2012). The introduction of chromophore-linked competitive binding partners instead of radioactively labeled ones would also be advantageous in terms of safety and feasibility and also increase throughput.

Standardized reference compounds

Currently certified reference compounds for CFP toxins are not available, and only very limited amounts of purified materials are available, although standards are now being developed by Cifga.

6.4 Cyclic imine poisoning (CIP)

Symptoms

Cyclic imines are fast acting toxins when injected intraperitoneally into mice. There are no reports of human intoxication from exposure to CI toxins (Munday et al., 2012; Richard et al., 2001) although pinnatoxins may have been involved in a shellfish mediated food poisoning in China (Zheng et al., 1990).

Mode of action

The toxicity of the CIs to rodents appears to be mediated by blocking of muscarinic and nicotinic acetylcholine receptors (AChR) in the central and peripheral nervous system (Munday, 2008). The interaction with the receptors may differ between the different groups of CIs as both, reversible and irreversible binding, have been observed (Molgo et al., 2008, 2007; Fonfria et al., 2010; Vilarino et al., 2009).

Functional, biochemical and biomolecular assays

There are currently no biochemical methods available for the detection of CIP toxins. However, LC-MS/MS methods are presently refined that allow highly sensitive detection of GYM, PnTX, PtTX, and SPX (Aasen et al., 2005a; Alvarez et al., 2010; Bire et al., 2002; Cembella et al., 1999; Ciminiello et al., 2006; Fux et al., 2007; Gerssen et al., 2009a,b; MacKenzie et al., 2002; Quilliam et al., 2001; Stirling, 2001; Takada et al., 2001a,b; Villar Gonzalez et al., 2006; Miles et al., 2010).

The interaction of CIP toxins with AChR allowed the development of a functional assay (Vilarino et al., 2009). The latter method is based on the competition of GYM A and 13-desmethyl SPX C with fluorescent-labeled alpha-bungarotoxin for nicotinic AChR of the electric organ of the *Torpedo marmorata*. The LOQ is 80 μg GYM A/kg and 85 μg 13-desmethyl SPX C/kg. This functional assay is a high throughput method for rapid detection of CI directly in environmental samples with minimal sample handling, high sensitivity, reduced matrix effect and low cross-reactivity (Araoz et al., 2012). The microplate-receptor binding assay was shown to be highly sensitive and specific for the detection of six CI toxins. However, this functional method cannot identify a given CI in a shellfish sample. To tackle this drawback it was coupled to mass spectrometry. The cyclic imine toxins tightly bound to the coated Torpedo-nAChRs were further eluted from the wells and analyzed by mass spectrometry, shortening the time between screening and CI toxin characterization (Araoz et al., 2012). The latter approach was developed further by immobilizing the AChR using a microsphere-flow cytometry system with a range of detection of 10-6000 μg 13-desmethyl SPX C/kg of shellfish (Rodriguez et al., 2013).

Standardized reference compounds

Currently NRCC provides certified toxin reference standards (CRM) for 13-desmethyl-spirolide C and gymnodimine. A reference standard for pinnatoxin-G will soon be available from NRCC. Certified reference material for 20-methyl spirolide G and quality controlled standards for 13,19-didesmethyl spirolides C and 13-desmethyl spirolide C are available at Cifga.

6.5 Diarrheic shellfish poisoning (DSP)

Symptoms

Symptoms of intoxication with DSP toxins are diarrhea, nausea, vomiting and abdominal pain (Garcia et al., 2005), starting within 30 minutes to a few hours following ingestion. Complete recovery occurs approximately within three days. Some DSP toxins have been shown to promote tumors in animal models (Manerio et al., 2008) at the skin and gastric level, although only at very high concentrations.

Mode of action

Inhibition of type 1 and, mainly, 2A serine-threonine phosphoprotein phosphatases (PP) appears to be the primary mode of action of this toxin group (Bialojan and Takai, 1988; Haystead et al., 1989; Nishiwaki et al., 1990), albeit it was demonstrated that OA can also inhibit PP 3 and 4 (McNabb, 2008). Diarrhea is most likely induced by cytoskeletal phosphorylation of enterocytes with the ensuing loss of function and thus loss of ion and fluid homeostasis within the GI tract similar to the effects of *Vibrio cholera* (Cohen et al., 1990).

Functional, biochemical and biomolecular assays

Beyond analytical methods, e.g., HP-LC with a LOD and LOQ of 40 μg OA/DTX1/kg and 100 μg OA/DTX1/kg shellfish meat (McNabb et al., 2005; McNabb, 2008; Stobo et al., 2005) or LC-MS/MS (e.g., ESI triple quadrupole) (Suzuki and Quilliam, 2011) with a LOD and LOQ of 10 μg OA/DTX1/kg and 25 μg OA/DTX1/kg shellfish meat (Stobo et al., 2005), there are several antibody-based approaches for detection and quantification of DSP toxins in free concentrations and in shellfish tissue. A recent multiplex immunoassay for free concentrations could be shown to detect OA with a LOQ of 20 ng/l (Desmet et al., 2012). Electrochemical immunosensors have also been developed for the detection of OA (Campas et al., 2008). In this case, the use of an enzyme recycling system to amplify the amperometric signals provided a LOD of 30 ng/l. The immunosensor was applied to the analysis of oysters and mussels, providing results similar to those obtained by LC-MS/MS. Another attempt in this regard involving a flow-through amperometric immunosensor could detect free concentrations of OA at 0.15 μg/l with a linear range of 0.19-25 μg/l (Dominguez et al., 2012). Furthermore an enzyme-linked immunosensor for OA with paramagnetic nanobeads was introduced which provided for a slightly higher LOD than other methods, i.e., 0.38 μg/ml (Hayat et al., 2011). Stewart and colleagues introduced a biosensor immunoassay with a working range of 31 to 174 μg OA/kg shellfish tissue, which could be correlated with LC-MS/MS findings using certified reference material ($R^2 = 0.99$), laboratory reference material, and naturally contaminated mussel samples (Stewart et al., 2009). Apparently, the research and development in the field of antibody-based detection of DSP toxins is advancing rapidly. It is to be expected that in near future some promising setups in this regard will be subjected to inter-laboratory validations.

To evaluate the risk of DSP toxins for human health several protein phosphatase inhibition assays were developed. A colorimetric phosphatase assay was introduced using recombinant human PP2A with a LOD of 2 μg $OA_{equiv.}$/kg shellfish hepatopancreas (Tubaro et al., 1996). PP2A inhibition fluorometric assays were also developed with a LOD of 12.8 μg $OA_{equiv.}$/kg shellfish hepatopancreas (Vieytes et al., 1997), followed by further optimizations and adaptations, e.g., competitive displacement (Doskeland et al., 2000; Serres et al., 2000). Recent developments on the basis of phosphatase inhibition (single laboratory validated) suggested a LOD and a LOQ of 44 μg $OA_{equiv.}$/kg and 56 μg $OAe_{equiv.}$/kg shellfish meat, which could be confirmed with chemical analytical methods (Smienk et al., 2012). Shellfish matrix has been shown to affect the applicability of the PP2A inhibition assay when used at high concentrations. Consequently, a careful evaluation of this effect is needed when analyzing contaminated samples (Garibo et al., 2012a). Strategies based on a chromatographic separation of the shellfish sample components may help to eliminate undesirable matrix effects, making the toxin determination more reliable (Canete et al., 2010). The same strategy can be used in the determination of trace amounts of OA in phytoplankton samples (Caillaud et al., 2010a). The determination of inhibitory equivalency factors for DTX1 and DTX2 with respect to OA may also help to better correlate the results provided by the enzyme inhibition assay with those obtained by LC-MS/MS analysis in the analysis of samples with multi-DSP toxin profiles (Garibo et al., 2013). The PP inhibition-based assay has also been the basis for the development of electrochemical biosensors, using entrapment of the enzyme into polymers (Campas and Marty, 2007). Strategies to improve the enzyme immobilization, such as the use of magnetic particles as supports, could improve the limits of detection and also pave the way towards the development of portable and compact analysis devices (Garibo et al., 2012b).

As PP inhibition is the primary effect that leads to dysregulated protein-phosphorylation homeostasis, downstream endpoints of the initial PP inhibition could become more important as functional endpoints, e.g., inhibition of autophagy (Magnaudeix et al., 2013), alteration of gene expression, tau hyper-phosphorylation in neuroblastoma cells (Chen et al., 2012; Valdiglesias et al., 2012), blockage of CaBP4, and SNAP-25 dephosphorylation (Haeseleer et al., 2013; Iida et al., 2013).

In addition, protein phosphatase independent effects of DSP toxins are suggested that could possibly be employed to describe human symptomatology that is more likely to be present as a result of sub-acute toxic or at subchronic exposure levels: The latter could also be addressed via functional assays, e.g., cytotoxicity of OA in T lymphocytes (Martin-Lopez et al., 2012), caspase-3 independent apoptosis (Hanana et al., 2012; Jayaraj et al., 2009), OA induced neuro-inflammation (Kamat et al., 2012), and granulation of tubulin in cytoplasma of hepatocytes (Rubiolo et al., 2012).

Standardized reference compounds

Certified reference standards (CRMs) for OA, DTX1, and DTX2 can be obtained from the NRCC, as well as from the Japan Food Research Laboratories (Suzuki et al., 2009) and Cifga. A certified mussel tissue reference material for the OA group of toxins is also available from the NRCC.

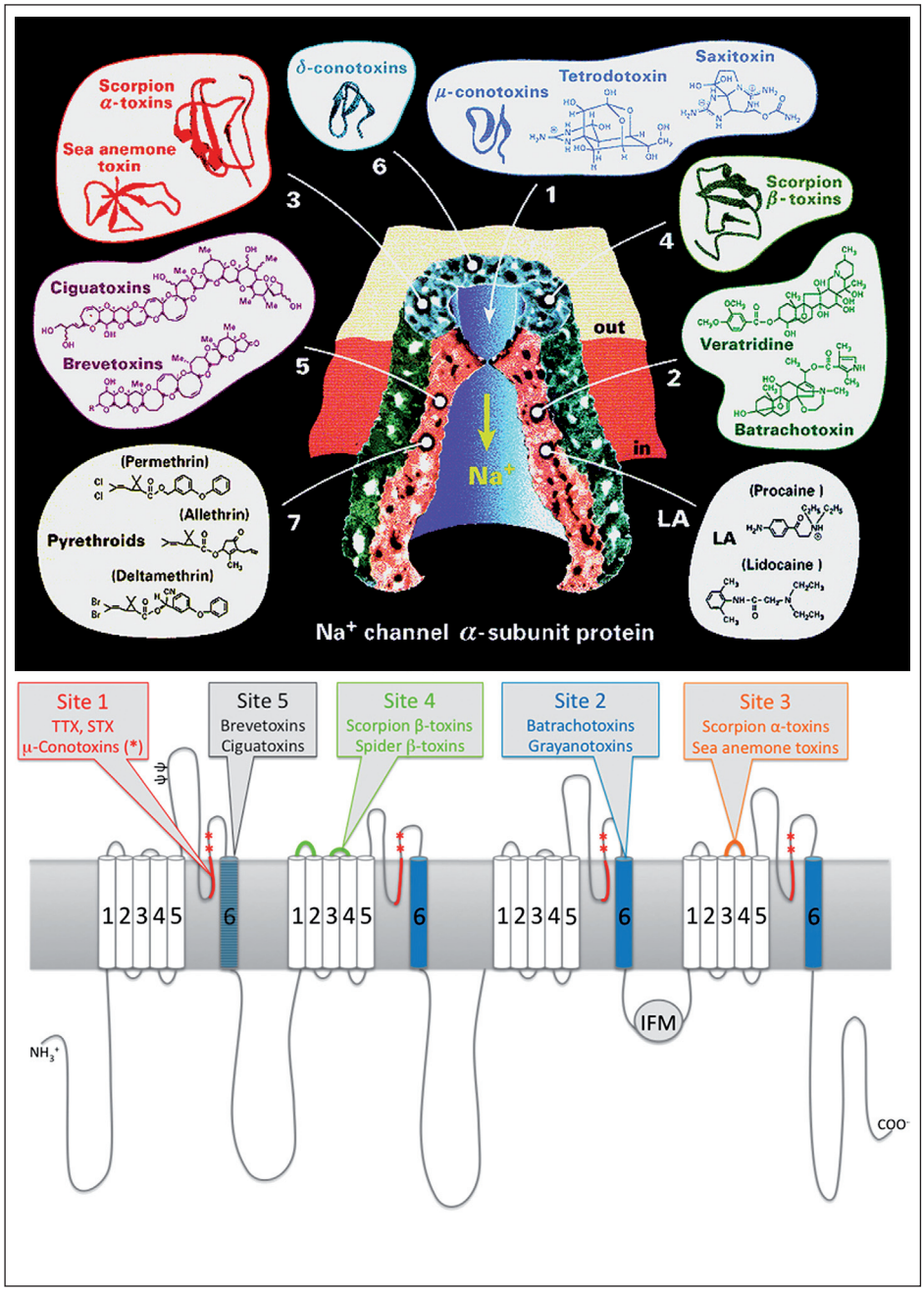


Fig 12: Inhibitory binding sites of voltage-gated sodium channel for different toxins including CTX, BTX, TTX, and STX

Upper figure adapted with permission from (Gordon et al., 2007), lower figure adapted with permission from (Stevens et al., 2011). Receptor binding sites for neurotoxins are denoted with numbers. Receptors of the lipid-soluble sodium channel modifiers, such as alkaloid toxins (e.g., veratridine, grayanotoxin, batrachotoxin – receptor site-2), marine cyclic polyether toxins (e.g., brevetoxins and ciguatoxins receptor site-5), synthetic pyrethroids (e.g., permethrin, deltamethrin – receptor site-7), and local anesthetics (LA, e.g., lidocaine), are located within the hydrophobic protein core. Receptor binding sites of water-soluble polypeptide toxins, such as scorpion α- and β-toxins (receptor sites 3 and 4), and δ-conotoxins (receptor site-6), are located at the extracellular side of the channel protein. The external vestibule of the ion conducting pore, at the centre of the protein, contains receptor site-1, which binds the sodium channel blockers μ-conotoxins, tetrodotoxin (TTX) and saxitoxin (STX). The part of the channel responsible for fast inactivation is the short, highly conserved intracellular linker that connects domains III and IV (lower part of the figure). The three hydrophobic amino acids Ile, Phe, and Met (IFM motif) are the key sequence necessary for fast inactivation.

6.6 Neurotoxic shellfish poisoning (NSP)

Symptoms

Symptoms and signs of NSP include nausea, vomiting, diarrhea, chills, sweats, reversal of temperature sensation, hypotension, arrhythmia, paresthesia of the lips, face and extremities, cramps, bronchoconstriction, paralysis, seizures, coma, and death. Symptoms typically occur within 30 minutes to 3 hours of consumption of contaminated shellfish and can last for a number of days (Watkins et al., 2008). The inhalation of brevetoxin aerosols may result in respiratory difficulties and eye and nasal membrane irritation (Pierce et al., 2005; Kirkpatrick et al., 2006).

Mode of action

NSP toxins have multiple actions on the transition states of voltage-gated sodium channels (NaV) leading to a shift in voltage dependence, inhibition of inactivation, increase in mean open times and subconductance states (Jeglitsch et al., 1998; Schreibmayer and Jeglitsch, 1992). This leads to uncontrolled Na^+ influx and depolarization of neurons followed by a persistent inactivated state and blockade of nerve conduction (Huang et al., 1984; Plakas and Dickey, 2010), similar to DA (see Fig. 11) and ciguatoxins (see Fig. 12) and an increase in intracellular Ca^{2+} (Baden et al., 2005; Watkins et al., 2008; LePage et al.,

2003). Brevetoxin has differential effects on striated, cardiac, and smooth muscle and discrete actions on diaphragm are predominated by a block of nerve conduction (reviewed in (Ramsdell, 2008)). Systemically BTX targets the autonomic nervous system at the ganglionic level and midbrain resulting in its depressant effects on the cardiovascular system (Borison et al., 1980). NSP toxin activation of NaV has been shown in immune cells, inducing cell proliferation, gene transcription, cytokine production and apoptosis (Murrell and Gibson, 2011).

BTX have a high first pass effect, i.e. they are rapidly absorbed and metabolized in the liver (Cattet and Geraci, 1993; Poli et al., 1990a,b) and then removed primarily via the bile within 48 h of exposure. However, some metabolites reach the blood stream and are distributed systematically. Due to their lipophilicity, BTX can pass more readily through cell membranes including the blood-brain barrier, thus also explaining the onset of neurological symptoms observed upon intoxications. Interestingly, following oral administration of non-lethal doses of BTX to rats, a wide distribution to all organs was observed, while the highest concentrations were found in the liver up to 8 days subsequent to exposure (Cattet and Geraci, 1993; Toyofuku, 2006). The latter strongly suggests that although BTX may appear to have a short half-life in serum, total body clearance may take days (Poli et al., 1990a,b), thus providing some evidence that recurring or intermittent exposure to BTX may result in relevant body concentrations in exposed patients with potential health consequences.

BTX-1 and BTX-2 are noted for chemical reactivity and yield multiple reactive intermediates. The formation of adducts to nucleic acids in lung is associated with DNA damage; however, tests have not shown evidence of mutagenicity. Formation of adducts with cysteine, histidine, and lysine yield adducts to protein and the formation of adducts to glutathione and cysteine (BTX-B2) are common in shellfish, fish, and mammals. Adduction of fatty acids to cysteine BTX (BTX-B4) yields a diversity of potent brevetoxin intermediates. These conjugated toxins have different potencies and elimination kinetics in shellfish and mammals and require consideration for human toxicity assessment.

Functional, biochemical and biomolecular assays

LC-MS/MS methodologies have been developed, evaluated and have been used for detection of BTX since the mid 1990s (Dickey et al., 1999, 2004; Fire et al., 2008; Hua and Cole, 2000; Hua et al., 1995; Ishida et al., 2004a,b,c; Nozawa et al., 2003; Pierce et al., 2006; Plakas et al., 2002, 2008, 2004; Wang and Cole, 2009; Wang et al., 2004; McNabb et al., 2012). The reported LOQ are 2, 0.2, 0.4, and 0.4 μg/kg shellfish meat for BTX-2, BTX-3, BTX-B5 and BTX-B1, respectively.

There are also a variety of antibody-based methods (Baden et al., 1995, 1988; Fire et al., 2008; Levine and Shimizu, 1992; Naar et al., 2002, 1998; Plakas et al., 2008; Poli et al., 1995, 2007; Trainer and Poli, 2000; Zhou et al., 2010). For example, a competitive ELISA was established with a LOD of 25 μg BTX/kg oyster (Naar et al., 2002). Further adaptation to chemiluminescence lowered the LOD to 1 μg BTX/kg (Poli et al., 2007). The cross-reactivity of the cysteine conjugates of BTX-2 (S-desoxy-BTX-B2 and BTX-B2) antibody to other BTX was reported by several groups (Dechraoui et al., 2007; Maucher et al., 2007; Plakas et al., 2004), thereby raising some doubt as to the specificity of this antibody. Due to recruitment of a magneto-controlled immunosensing platform a LOD of 1 pg BTX-2/ml extract with a range of 1 pg/ml to 10 ng/ml could be reached (Tang et al., 2012). Another approach with a multiplexed immunoassay achieved a LOD of 1.8 pg BTX-2/ml and a LOQ of 6 pg BTX-2/ml extract (Zhang et al., 2012). The development of an electrochemical immunoassay revealed a LOD of 10 pg BTX-2/ml extract with a linear range of 0.03 ng to 8 ng BTX-2/ml (Tang et al., 2012). The progress in the field of antibody-based detection of NSP toxins appears to be advancing rapidly. However, validation of these methodologies across different laboratories is key to any true advancement.

Functional receptor binding assays for NSP toxins were developed based on the affinity of BTX toxins for the sodium channel receptor. Here BTX toxins compete with radioactive BTX toxins (^{3}H-BTX-3) binding the receptor (site 5 of Nav) using membrane fragments of excitable tissues or cell preparations (Dechraoui et al., 2007; Fire et al., 2008; Trainer and Poli, 2000; Van Dolah et al., 1994). The LOQ of the receptor binding assay is around 30 μg BTX-3$_{equiv.}$/kg oyster homogenate (FAO/IOC/WHO, 2004). Notably, a fluorophore-labeled brevetoxin was recently introduced to replace the current radioligand in the receptor binding assays (McCall et al., 2012). Further progress in the field of voltage gated sodium channels suggests that the BTX-2 effect on intracellular sodium and calcium concentrations can also be mediated by the N-methyl-D-aspartate (NMDA) receptors, thus adding other functional endpoints that can be exploited for a better understanding and quantification of the physiologic effects of BTX (George et al., 2012).

Other findings and developments on the impact of NSP toxins may also lead to the identification of endpoints for the development of functional assays, e.g., proteomics approaches analyzing the molecular range of action of NSP toxins in several tissues (Tian et al., 2011), gene alterations (Murrell and Gibson, 2011), mast cell activation and cytokine release (Hilderbrand et al., 2011), induction of DNA damage and apoptosis (Murrell and Gibson, 2009), and induction of inflammatory response in immune cells (Sas and Baatz, 2010).

The cytotoxicity of NSP toxins is generally recognized, and several cytotoxicity assays have been developed (Bottein et al., 2010; Dechraoui et al., 2007; Dickey et al., 1999; Fairey et al., 1997; Louzao et al., 2004; Manger et al., 1995, 1993, 1994; Trainer et al., 1995). The cytotoxicity of BTX is assumed to result from the interaction with the voltage-gated sodium channels. A protocol involving neuroblastoma cells aims to quantify the mitochondrial reduction of MTT tetrazolium dye as a measure of the viability of veratridine pretreated cells. The LOD for this assay is reported at 250 μg BTX-1/kg shellfish (Manger et al., 1993, 1994; Plakas and Dickey, 2010). Cytotoxicity may occur at higher concentrations than the typical neurotoxic effects.

Standardized reference compounds

Currently certified reference material for NSP toxins are not available.

6.7 Palytoxin Poisoning (PaP)

Symptoms

The symptoms of intoxication with PlTX toxins include general malaise, nausea, vomiting, diarrhea, myalgia, dyspnoe and, sometimes, abnormalities in cardiac functions (Tubaro et al., 2011b). In severe cases, rhabdomyolysis of skeletal muscles is followed by renal failure, which may be lethal (Deeds and Schwartz, 2010; Taniyama et al., 2002).

Mode of action

PlTX toxins interact with Na^+/K^+-ATPases, whereby the ATPase is converted to a non-specific cation channel which leads to membrane depolarization in both excitable and non-excitable cells and uncontrolled contraction of muscle cells (Habermann, 1989; Habermann and Chhatwal, 1982; Redondo et al., 1996; Scheiner-Bobis et al., 1994). The Na^+/K^+-ATPase is a transmembrane pump belonging to the family of P-type ATPases, which are essential for maintaining cellular homeostasis by transferring three Na^+ ions out of the cell in trade for two K^+ ions in a cyclic process that uses ATP. Many studies report the ability of the cardio-active glycoside ouabain to inhibit PlTX effects *in vitro* (Habermann and Chhatwal, 1982; Pelin et al., 2012, 2011; Schilling et al., 2006; Vale-Gonzalez et al., 2007), and thus support this mechanism of action. However, incomplete abolishment of PlTX biological activity by ouabain suggests that ouabain does not directly compete with PlTX on the binding site. Indeed, Artigas and Gadsby demonstrated that PlTX and ouabain can simultaneously bind to Na^+/K^+-ATPase (Artigas and Gadsby, 2004), suggesting the possibility of two different binding sites on the pump, as also confirmed by Pelin and colleagues (Pelin et al., 2013a). PlTX toxins were also shown to affect other membrane ion transport components (Frelin and Van Renterghem, 1995; Ikeda et al., 1988; Muramatsu et al., 1984; Sauviat, 1989; Kockskamper et al., 2004): the toxin could also interfere with other P-type ATPases such as the sarcoplasmic endoplasmatic reticulum Ca^{2+} pump (SERCA) (Coca et al., 2008; Kockskamper et al., 2004) and the non-gastric Na^+/K^+ ATPase pump (Qiu et al., 2006; Scheiner-Bobis et al., 2002).

Intracellular Na^+ accumulation induces the transport of Ca^{2+} into the cells via Na^+/Ca^{2+} exchangers (Frelin and Van Renterghem, 1995; Ikeda et al., 1988; Kockskamper et al., 2004; Vale et al., 2006). The latter increased intracellular Ca^{2+} concentration affects skeletal, heart and smooth muscle cells (Frelin and Van Renterghem, 1995; Ito et al., 1977; Karaki et al., 1988; Kockskamper et al., 2004; Nagase and Karaki, 1987; Vale et al., 2006).

Functional, biochemical and biomolecular assays

The LOD of LC-MS/MS methods for PlTX in shellfish were reported to be 2 and 36 μg/kg and the LOQ were reported as 9 and 120 μg/kg (EFSA, 2009a). However, recent analyses suggest that the LOD and LOQ are higher, largely depend on the tissue extraction method as well as the instrument employed (Ciminiello et al., 2011). A novel screen approach has been developed that uses LC-MS/MS analysis of substructures generated by oxidative cleavage of vicinal diol groups present in the intact toxin. Oxidation of palytoxins, ovatoxins or ostreocins using periodic acid generates two nitrogen-containing aldehyde fragments: an aminoaldehyde common to these toxins, and an amide aldehyde that may vary depending on toxin type. The method's LOD was determined to be approximately 1 ng/ml and the LOQ 4 ng/ml extract, which corresponds to 10 µg/kg in fish or shellfish flesh (Selwood et al., 2012).

There are antibody-based methods for detection and quantification of PlTX since the end of 1980s (Bignami et al., 1992; Levine et al., 1988). Recent reviews on marine biotoxin antibodies provide some insight on availability of antibodies for PaP toxins but also for other marine biotoxins (Garet et al., 2010a,b). A novel sandwich ELISA for detection of PlTX was introduced and also evaluated, providing a LOQ of 11 ng PlTX/ml mussel extract, 9.6 ng PlTX/ml algae sample and 2.4 ng PlTX/ml for seawater samples (Boscolo et al., 2013). Via introduction of an electrochemiluminescence-based sensor a LOQ of 2.2 μg PlTX kg of shellfish tissue was achieved, which is in the area – if not lower – than the LOQ of some of the LC-MS/MS approaches (Zamolo et al., 2012). Recently also a novel surface plasmon resonance immunoassay was introduced with an instrumental limit of detection for PlTX in sub-ng/ml sample range (Yakes et al., 2011).

Functional assays focus on the inhibition of Na^+/K^+-ATPases by PlTX. On this basis, an erythrocyte haemolysis and a haemolysis neutralization assay was developed (Bignami, 1993; Lenoir et al., 2004; Riobo et al., 2006, 2008; Taniyama et al., 2003, 2002). The erythrocyte haemolysis assay reached a LOD of 1.6 ng PlTX/kg shellfish tissue, but is quite unspecific due to hemolytic substances present in the shellfish extracts.

Also neuroblastoma cytotoxicity assays could be adapted for evaluation of PlTX action (Canete and Diogene, 2008; Espina et al., 2009; Ledreux et al., 2009). A LOD of 50 μg PlTX/kg shellfish tissue was reported. A cytotoxicity assay on MCF-7 breast cancer cells detecting lactate dehydrogenase was reported to have a LOD of 10 ng PlTX/kg shellfish tissue (Bellocci et al., 2008).

Notably, fluorophore-labeled Na^+/K^+-ATPase and its interaction with PlTX could be analyzed by a fluorescence polarization technique leading to an instrumental LOQ of 10 and a LOD of 2 nM PlTX (Alfonso et al., 2012).

Other findings in the field of PaP toxin research may open new possibilities to define endpoints for development of functional assays, e.g., the role of oxidative stress in exposure of keratinocytes to PlTX (Pelin et al., 2013b) or non-oxidative necrosis induced by PlTX in PC12 cells (Sagara et al., 2013).

Standardized reference compounds

There are no certified standards or certified reference materials for PlTX-group toxins.

6.8 Paralytic shellfish poisoning (PSP)

Symptoms

Adverse effects from PSP start with numbness around the lips that spreads to the neck and face and may progress to include prickly sensations of the fingertips and extremities (arms and legs), headache, dizziness, stiffness and lack of limb coordination, general weakness, slight respiratory difficulty, rapid pulse and backache. Nausea, vomiting, and diarrhea can also occur and temporary

blindness has been reported. When high concentrations of PSP toxins are consumed, voltage-gated sodium channels are blocked resulting in blockage of nervous transmission followed by nerve dysfunction and muscular paralysis, resulting finally in respiratory arrest and death if supportive care is not administered (EFSA, 2009d; FAO/IOC/WHO, 2004; de Carvalho et al., 1998; Azanza, 2006; Catterall et al., 2007; Paredes et al., 2011).

Mode of action

PSP toxins block voltage-gated sodium channels (Hille, 1966, 1968; Kao, 1966; Ritchie, 1975), which leads to loss of neuromuscular function (Hartshorne and Catterall, 1984; Hille, 1968, 1975; Catterall et al., 2007; Paredes et al., 2011). Nine isoforms of the relevant subunit of this channel are described, thus variations in sensitivity to PSP toxins can be expected (Goldin et al., 2000).

Functional, biochemical and biomolecular assays

Currently there are several analytical methods available for detection and quantification of PSP toxins with the most sensitive LOD of 0.08 μg $STX_{equiv.}$/kg shellfish (Cefas, 2010; Botana et al., 2007; Lawrence et al., 1995; Quilliam et al., 1993b).

There are also several antibody-based methods for detection and quantification of PSP toxins, which include ELISA (Chu and Fan, 1985; Usleber et al., 1995), radioimmunoassay (Carlson et al., 1984), lateral immunoflow assay (Jellett et al., 2002), and surface plasmon resonance biosensor technology (Campbell et al., 2007; Fonfria et al., 2007). A comparative analysis of different ELISA methods revealed that PSP toxins could be detected at 50 μg $STX_{equiv.}$/kg shellfish without matrix interference (Garet et al., 2010b). Recently, a solid-phase inhibition immunoassay was introduced with a detection range of 2.2 to 19.7 ng $STX_{equiv.}$/ml (Fraga et al., 2012). A multiplex biotoxin surface plasmon resonance method was introduced for detection of marine biotoxins in algae and water, for which a LOD of 0.82 ng $STX_{equiv.}$/ml has been reported (McNamee et al., 2012). Another approach for a rapid competitive assay – a fluorescence planar waveguide biosensor – for PSP toxins in marine algae revealed a LOD of 12 pg $STX_{equiv.}$/ml and a CCβ of 20 pg $STX_{equiv.}$/ml (Meneely et al., 2013).

A novel approach for detection and quantification of STX addresses the concentration-dependent, selective binding of STX to a DNA aptamer (Handy et al., 2013).

Several functional assays were developed employing the inhibitory properties of PSP toxins with regard to voltage-gated sodium channels. The latter assays include radioactive methods with rat brain homogenates (Davio and Fontelo, 1984; Doucette et al., 2000; Vieytes et al., 1993), fluorescent meth-

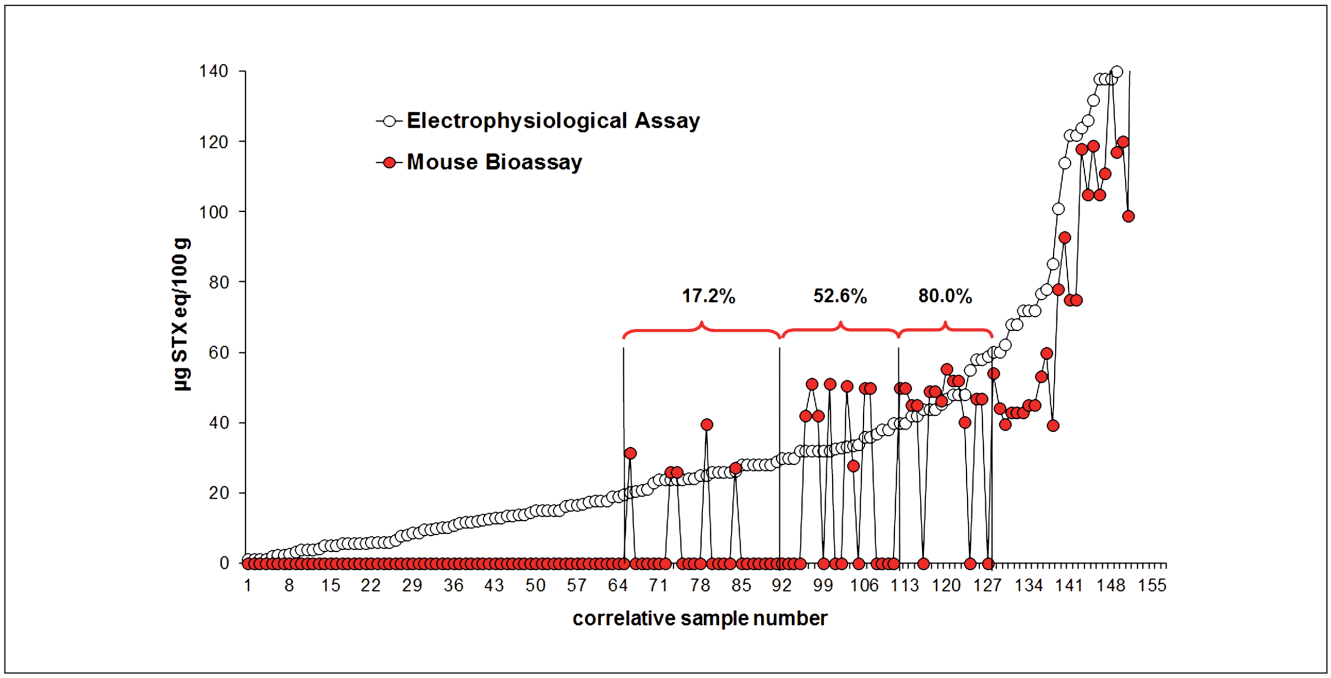


Fig. 13: Comparison of the MBA with the electrophysiological assay for STX

A set of 157 samples was analyzed by the electrophysiological assay and the AOAC mouse bioassay; levels of toxicity determined by the electrophysiological assay range from 1.1 to 1744 μg STXeq/100g oysters; the data demonstrate the inability of MBA to detect lower concentrations than 20μg STX in 100g oysters; there is a 82.8% likelihood of false negative results involving MBA for detection of STX concentrations between 20-30 μg/100g oyters (17.2% positive recovery rate), a 47.4% likelihood of false negative results involving MBA for detection of STX concentrations between 30-40 μg/100g oyters (52.6% positive recovery rate), and 20% likelihood of false negative results involving MBA for detection of STX concentrations between 40-60 μg/100g oysters (80% positive recovery rate); only 26 out of 128 samples (20.3%) under 60 μg STXeq/100g could be found to contain STX by mouse bioassay; data were generated during the studies leading to the publication (Turner et al., 2011).

ods involving neuroblastoma cells (Louzao et al., 2003, 2001, 2004; Vale et al., 2008; Manger et al., 2003), an approach with rhodamine and synaptoneurosomes (Nicholson et al., 2002), and a patch clamp method (Velez et al., 2001). The radioactive method for saxitoxin analysis utilizing brain homogenates was recently accepted as an AOAC validated method (Van Dolah et al., 2012) and may be used for high-throughput screening of environmental samples (Doucette et al., 1997; Ruberu et al., 2003). The LOD of the latter method is reported at 2 μg STX-$_{equiv.}$/kg shellfish tissue. The modified method was recently subjected to a validation procedure (Van Dolah et al., 2012). An action potential voltage-clamp technique was introduced which could be the next generation tool for development of functional assays also for marine biotoxins acting via ion channels (Szentandrassy et al., 2011). Another electrophysiological assay for saxitoxins was developed at the laboratory for Marine Toxins in Chile and is based on the determination of percental inhibition of maximal sodium current elicited at -10 mV on rat skeletal muscle (Turner et al., 2011). Notably, the direct comparison of this assay with the MBA demonstrated an incredible inaccuracy of the MBA at values below 60 μg STX per 100 g of shellfish (Fig. 13). Even though very promising, the electrophysiological assays still require cross-laboratory validation and regulatory acceptance.

The cytotoxicity of PSP toxins is presumed to result from their interaction with voltage-gated sodium channels. A protocol involving neuroblastoma cells aims to quantify the mitochondrial reduction of tetrazolium dye as a measure of the viability of veratridine pretreated cells (Gallacher and Birkbeck, 1992; Kogure et al., 1988; Manger et al., 1993). This commonly used assay quantifies cells with normal morphology following treatment with PSP toxins with a LOD of 20 μg PSP$_{equiv.}$/kg shellfish (Jellett et al., 1992).

Standardized reference compounds

Certified reference materials (CRMs) are provided for the following PSP toxin analogues and mixtures by NRCC: saxitoxin dihydrochloride, decarbamoylsaxitoxin, neosaxitoxin, decarbamoylneosaxitoxin, gonyautoxin-1 and -4, gonyautoxin-2 and -3, gonyautoxin-5, decarbamoylgonyautoxin-2 and -3, N-sulfocarbamoylgonyautoxin-2 and -3. CRMs for gonyautoxin-2 and -3 and N-sulfocarbamoylgonyautoxin-2 and -3 are also provided by Cifga.

6.9 Pectenotoxin Poisoning (PeP)

Symptoms

Unclear, as reported symptoms may be related to the co-occurrence with OA toxins.

Mode of action

Isolated morphological alterations of hepatocytes from the organs of mice can be detected upon exposure to PTX1. This implies that PTX-group toxins affect the cellular cytoskeleton and cause apoptosis (Terao et al., 1986; Aune et al., 1991; Zhou et al., 1994; Hori et al., 1999; Leira et al., 2002b; Ares et al., 2007; Chae et al., 2005). PTX2 forms a 1:1 complex with actin (Allingham et al., 2007).

Functional, biochemical and biomolecular assays

A validated LC-MS/MS method for lipophilic toxins including PTX was validated (CRLMB, 2006), the limit of quantification (LOQ) appears to be around 1 μg PTX2/kg (Gerssen et al., 2009a; These et al., 2009).

At present there are no validated biochemical assays available for the detection of PTX. The few promising attempts in this regard have been abandoned (Briggs et al., 2005).

For the establishment of functional assays the inhibitory effects of PTX on polymerization of actin units is used. The first promising attempts in this regard revealed a dose-dependent decrease of skeletal muscle actin polymerization in the nM range (Butler et al., 2012).

Standardized reference compounds

The NRCC provides a certified calibration solution for PTX2. A mussel tissue certified reference material containing PTX2 has been prepared in collaboration with NRCC, Institute for Reference Materials and Measurements (IRMM), AgResearch (New Zealand) and Norwegian Veterinary Institute (NVI) and will be available in the third quarter of 2013. Cifga provides a quality control standard for PTX2.

6.10 Yessotoxin shellfish poisoning (YPS)

Symptoms

No human intoxication has been reported (Terao et al., 1990; Satake et al., 1997), thus the relevance of YTX for human risk assessment is currently an unresolved issue.

Mode of action

The observed mechanisms of action of YTX comprise the modulation of calcium movement into lymphocytes (de la Rosa et al., 2001; Malagoli and Ottaviani, 2004; Malagoli et al., 2006), modulation of cellular cAMP levels (Alfonso et al., 2003, 2004, 2005; Pazos et al., 2004, 2005, 2006), alteration of protein disposal (Pierotti et al., 2003), and apoptosis (Malaguti and Rossini, 2001; Malaguti et al., 2002; Leira et al., 2002a; Korsnes et al., 2006b,a). YTX inhibited the beating frequency in cardiomyocytes *in vitro* (Dell'Ovo et al., 2008). High cytotoxicity affecting a variety of cellular activities has been reported (Malaguti et al., 2002; Malaguti and Rossini, 2001; Ronzitti et al., 2004). YTX opens the permeability transition pore in rat liver mitochondria causing cytoskeletal disruption (Bianchi et al., 2004).

Functional, biochemical and biomolecular assays

The limit of quantification of LC-MS/MS is around 2 μg/kg shellfish (EFSA, 2009e).

A quantitative YTX direct competitive ELISA has been developed by AgResearch New Zealand, and has been used for the quantification of YTX in blood of rodents (Tubaro et al., 2008). This YTX-ELISA is presently in preparation for commercialization (following interlaboratory validation and optimization) by Biosense (http://www.biosense.com).

As there are no human symptoms clearly related to intake of YTX, it is difficult to address mechanisms underlying the MOA in order to establish functional assays. Nevertheless, there are several attempts to develop assays on the basis of putative phys-

iological effects of YTX. The modulation of cAMP is thought to be one of the key events in the toxicity of YSP toxins, leading to development of assays addressing phosphodiesterases. This method could establish a range of quantification of 0.1 to 10 μM (Alfonso et al., 2004, 2007, 2005; Fonfria et al., 2008; Pazos et al., 2005, 2004; Campas et al., 2010). A recently reported effect of YTX regarding cAMP modulation and mitochondrial A-kinase anchor proteins could build a basis for further assay development (Tobio et al., 2012). Another test aims at dose-dependent accumulation of E-cadherin fragments in MCF-7 cells upon YTX treatment giving a detection range of 0.2 to 1.8 mg $YTX_{equiv.}$/kg (Schirone et al., 2013). Furthermore, the apoptotic activity of YTX with regard to chromatin condensation, DNA laddering, caspase-3 activation, disruption of mitochondrial membrane potential and increase of cytosolic calcium levels could be revealed providing a potential for development of functional assays (Pang et al., 2012). Further findings pointing to inhibitory potency of YTX with regard to phagocytosis of macrophages (Orsi et al., 2010) or new binding proteins of YTX (Ujihara et al., 2010) may open the door for further research in the field of YSP.

Standardized reference compounds

Certified reference standards for yessotoxin (YTX) and homoyessotoxin (hYTX) are available from NRCC. A mussel tissue certified reference material containing YTX has been prepared in collaboration with NRCC, Institute for Reference Materials and Measurements (IRMM), AgResearch (New Zealand) and Norwegian Veterinary Institute (NVI) and will be available in the third quarter of 2013 (Michael Quilliam, personal communication). Certified standards for YTX and hYTX are also provided by Cifga.

7 Recommendations

7.1 Standard reference material for analytical methods

As there are both ethical and legal obligations to replace the MBA (EC, 2010) and as the MBA shows crucial inter-strain variations and, more importantly, the human response is not mirrored appropriately and adequately by this assay, the initiative to introduce alternative methods for detection of marine biotoxins by EFSA and the European Community is most appreciated and welcome. Consistent with this need, the European Commission has adopted liquid chromatography-mass spectrometry (LC-MS/MS) as a standard method to detect lipophilic toxins in bivalve mollusks, and mandated a phase out of the MBA by 31 December 2014.

As summarized in Table 1, there is a limited number of reference materials for marine biotoxins, their analogues and metabolites. LC/MS methods that are validated against reference materials for marine biotoxins can quantify the burden of these toxins, but they cannot provide information on other marine biotoxins potentially present within the same samples in quantitatively meaningful concentrations if no reference material for these is available.

Moreover, as LC/MS methods cannot be readily applied as routine in-field and on-board detection and quantification methods, the demand by the European Community to determine allowable market levels of finfish and shellfish via LC/MS methodology cannot fully protect consumers from exposure to the majority of marine biotoxins. The uncertainty of the institutions responsible for consumer protection, i.e., primarily the aquaculture industry and fishermen, may well lead to further use of the MBA in parallel to the chemical-analytical methods promoted by EFSA.

This consortium therefore recommends the establishment of international funding for existing institutions with expertise to prepare reference standard materials for known marine biotoxins, their analogues and variants including potential metabolites. Furthermore, research on the chemical synthesis of marine biotoxins for the purpose of producing reference materials should be promoted.

7.2 Human-relevant functional reference systems

Chemical-analytical assays are highly sensitive methods that can detect marine biotoxins for which reference materials are available. However, they do not reflect the physiological effects of the detected toxins or mixtures of toxins in humans. These are also only partially addressed with animal-models, as the systemic response of animals is not necessarily comparable to the human response to oral or inhalation exposure (see above).

Where we understand the MOA of marine biotoxins, *in vitro* or *ex vivo* methods that reflect these molecular mechanisms can be employed to detect marine biotoxins and replace *in vivo* assays.

This consortium recommends the further development and validation of physiological and functional assays, preferably using human cells or cell systems, to replace animal testing. As an example that this is feasible, the receptor-binding assay for PSP toxins has been validated via AOAC as an appropriate and suitable cell-free method. As the technical and methodical possibilities and the knowledge underlying the action of toxins also grows rapidly, the suggestions below are by no means meant to be exhaustive. The following recommendations refer to some verified and most likely MOA of toxins as the basis of further functional assays.

- Recommended targets for ASP toxins: Glutamate receptor binding, Na^+ influx respectively membrane depolarization and/or Ca^{2+} influx. The system could be based on *ex vivo* neuronal cells or iPS and stem cell derived neuronal cells or neurospheres.
- Recommended targets for AZP toxins: Increase of cytosolic Ca^{2+} and cAMP concentrations in lymphocytes and/or in Caco-2 cells, potassium channel blockage (in HEK cells), inhibition of endocytosis (inhibition of cathepsin D maturation) and induction of apoptosis in a variety of neuroblastoma or Caco-2 cells *inter alia* as well as c-Jun kinase activation (Vale et al., 2010).
- Recommended targets for CFP toxins: cellular increase of Ca^{2+} concentration, e.g., in Caco-2 cells. Decrease of beating of stem cell derived cardiomyocytes *in vitro* (bradycardia), cytotoxicity assays involving neuroblastoma assays, competitive NaV channel binding assays, human sensitization assays,

human peripheral neuron assay based on human embryonic stem cells.
- Recommended targets for CI toxins: Blocking action of muscarinic and nicotinic acetylcholine receptors in neurites, in neurospheres and/or in human retinal progenitor cells and/or in electric organ of the *Torpedo marmorata*, and/or with immobilized AChR.
- Recommended targets for DSP toxins: Protein phosphatase (PP) inhibition of a variety of PPs and the increase of cellular Ca^{2+} concentration. For example PP inhibition in Caco-2 cells and cardiomyocytes or any other suitable cell type, granulation of tubulin filaments in hepatocytes or decreased viability of T lymphocytes (the specificity of the latter still needs to be verified).
- Recommended targets for NSP toxins: Non-radioactive receptor binding assay, depolarization of neuronal and muscle cell membrane, increase of cellular Ca^{2+} and Na^{+} involving neurites, neurospheres and/or in HSMM respectively SkMC *in vitro* or/and in HSkMC cells *ex vivo*, cytotoxicity assay with human neuroblastoma cells, function inhibition of human peripheral neurons developed from human embryonic stem cells.
- Recommended targets for PaP toxins: Binding to sodium-potassium ATPase in neurites, neurospheres, and/or on relevant structures *in vitro* or *ex vivo* and subsequent membrane depolarization, inhibition of the function of human peripheral neurons derived from human embryonic stem cells.
- Recommended targets for PSP toxins: Binding to voltage gated sodium channels in neurites, neurospheres and/or on relevant structures *ex vivo* and subsequent membrane depolarization, growth or function inhibition of human peripheral neurons developed from human embryonic stem cells.
- Recommended targets for PeP toxins: Disturbance of cytoskeleton and cytotoxic potential. A variety of *in vitro* and *ex vivo* models can be recommended here, e.g., hepatic and kidney cells and cell lines (e.g. HepG2 cells, HepaRG cell line, WIF-B and WIF-B9 cells, HEK293 cell line, MDCK cells, podocytes) as well as neurites and/or macrophages *inter alia*.
- Recommended targets for YSP toxins: Decrease of cellular cAMP concentrations, e.g., in phosophodiesterase assays, cytoskeletal disruption and/or the modulation of permeability transition pores in mitochondria of hepatocytes in 2D and 3D cultures or engineered tissues; quantification of E-catherin fragments in epithelial cells, caspase-3 activation in a variety of cells and inhibition of phagocytosis in macrophages and AKAP activation in a variety of cells.

The recommended target structures may be combined to different batteries of methods employing high-content and high-throughput detection and quantification systems. Indeed, the development of aptamers, e.g., the one already existing for PSP toxins, would also be of interest. This may not be an easy task, but it is very promising.

7.3 Further research and development

As the marine food resource is gaining global importance, filling the gaps in our knowledge in the field of marine biotoxins is critical in order to protect consumers. The following recommendations from this consortium refer to the most urgent scientific gaps regarding relevant test methods and biotoxin evaluation. In particular, a roadmap for safety assurance with regard to target-oriented research and development urgently needs international financial cooperation and intense collaborative efforts.

Testing and risk assessment strategies:
- A considerable number of relevant cells, cell-lines (for 2D and 3D culturing), and also organotypic tissues are available. As the symptoms and, in some cases, the modes of action of marine biotoxins are quite well described, a number of relevant cells, cell-lines, and tissues should be subjected to systematic testing regarding their ability to reflect the physiological reaction involving the functional modes of action (MOA). The relevant testing batteries may differ in their composition depending on biotoxins and MoA addressed.
- The future of toxicity testing is heading towards the description of pathways of toxicity (PoT) on the gene, protein, and metabolite level. It is recommended to systematically catalogue the cellular toxicity pathways to identify patterns (targets) that need be addressed as adverse outcome pathways (AOP).
- It is highly recommended to implement modern high-throughput and high-content systems, i.e., automated -omics and imaging technologies for risk assessment of and research on marine biotoxins. These would, on the one hand, facilitate the search for suitable methods for different biotoxins and, on the other hand, accelerate the evaluation of samples due to the automation capacity of these techniques. A simplified methodology could be developed that would allow detection of mixtures of toxins present, increasing the sample numbers and the application to on-board detection of contaminated produce.
- It is recommended to investigate the respiratory and dermal route of exposure to marine biotoxins involving also biobarrier models with human cells. This is important for the coastal populations as well as for fishermen who are repeatedly exposed to these toxins.

Marine biotoxin research:

In the field of marine biotoxin research there are still uncertainties that need scientific clarification. These are integral for assessment and evaluation of the risk posed by exposure to marine biotoxins.
- Relative composition of analogues and congeners of certain toxin classes in vector organisms (shellfish and finfish). Due to metabolic and physico-chemical modifications vector organisms show a certain composition of analogues and congeners of incorporated toxin classes. Though potency rankings are assumed, the occurrence, abundance, and effective toxic potencies have to be subjected to scientific evaluation involving human-relevant methods and endpoints. Moreover, the assumption that the most toxic forms are the most abundant may be misleading for risk evaluation approaches and must be clarified scientifically.
- Hazard monitoring: The composition of phytoplankton, their seasonal occurrence and the localization of the hot spots of

their bloom are subject to alteration by biotic/physical (hurricanes, water temperature shift) and anthropogenic factors (nutritional overload, particle loading from erosion, ballast water distribution, oil spills, etc.). This consortium recommends establishing an internationally harmonized enterprise to develop rapid methods (e.g., dip-sticks) and additional analytical methodologies to monitor the shift of algal bloom hotspots and their composition. As the regulatory recommendations on on-boat proof of safety are applicable for very few toxins, it is recommended to focus also on the development of reliable methods for other relevant toxins.

– Distribution of marine biotoxins in the food chain: There are two levels of distribution, i.e., the biological and anthropogenic. The biological distribution of marine biotoxins refers to the natural food chain leading from plankton to shellfish and finfish in marine habitats that are incorporated by human consumers by eating the marine food. The anthropogenic distribution comes from the processing of marine products to non-food products. It is recommended to monitor the fate of non-food marine products, as consumers could be subjected to long-term or repeated low-dose exposure to marine biotoxins by this pathway.
– Hazard spike quantification: For the evaluation of exposure and risk it is essential to have a quantitative base for both the recovery rate of incorporated marine biotoxins in shellfish and finfish as well as for the ratio of free toxin concentrations in water and phytoplankton. As spike recovery studies do not deliver satisfactory results, it is recommended to introduce small-scale experiments to address these questions systematically. Therefore, the production of marine biotoxins in relevant phytoplankton strains (in correlation with different conditions), the incorporation rate of the toxins into shellfish and finfish in correlation with the recovery rate due to the extraction methods can be systematically evaluated (which is essential for adequate exposure and risk assessment). Moreover, the performance of the extraction methods from the vector organisms, as well as after initial processing and cooking should be optimized and validated, especially with regard to the parent compound and the respective metabolites present.
– *In silico* approaches: As the present data set does not provide any information as to the toxicokinetics of marine biotoxins in humans, experiments that could provide at least some initial insight are urgently needed. As *in silico* methodology has recently become an increasingly valuable tool for understanding the kinetic and dynamic portion of an intoxication, structure activity relationships (SAR) of given toxins with a specific interactant, e.g., PSP, TTX, BTX, etc. with the voltage gated channels could serve to better understand the dynamics, while PBPK (pharmacologically-based-pharmacokinetic) models could help in understanding the kinetic portion of the intoxication.

7.4 Recommendations for regulations of marine biotoxins

As summarized in Table 1, there are approximately 12 marine biotoxin groups that have been reported in finfish, shellfish, and other mollusks. The European Union has legal implementations that aim to assure that any fish or shellfish catch imported into or produced within the EU is routinely tested for marine biotoxins, but regulatory limits are only set for 5 of the 12 classes. For example, the EU regulation requires that no fish products containing ciguatoxins are placed on the market – thus excluding shellfish and mollusks – but does not specify the analytical method, nor does it regularly control fish or fish products for ciguatoxin contamination. It is thus not surprising that ciguatoxin intoxications by fish products are reported (German Federal Institute for Risk Assessment, 2013). Additionally, there are no regulatory requirements for monitoring cyclic imines in Europe despite the fact that these toxins show toxicity similar to other known neurotoxins, e.g., organophosphate pesticides or the chemical warfare agents' sarin, soman, and tabun. In addition, tetrodotoxin (TTX), which is not discussed in detail in this report, is not included in the European regulation, though this toxin class is found also in gastropods of several species (i.e., *Charonia lampas lampas*, *Gibbula umbilicalis*, *Monodonta lineata* (Silva et al., 2012)) and fish (*Lagocephalus sceleratus*) in the Mediterranean Sea and has recently resulted in severe human intoxications (Bentur et al., 2008; Rodriguez et al., 2012). In contrast, the EC mandates the routine analysis of yessotoxin (YTX) despite that fact that YTX was not demonstrated to be acutely toxic when given orally to mice, the putative mechanism of intraperitoneal toxicity in mice being unknown, and there being no reported cases of acute human intoxication due to YTX exposure.

This consortium of experts recommends in this regard that an internationally harmonized regulation for all marine biotoxin groups listed in Table 1 in marine nutrition products is developed, including hazard monitoring with analytical methods and risk assessment with human-relevant functional assays. This includes the regulatory recognition of TTX as well as CFP and CIP group toxins, and the revision of regulation for the YSP toxins by the EU, provided there is no additional evidence conclusively demonstrating potential toxicity of YTX.

Furthermore, this consortium recommends international harmonization regarding the monitoring and evaluation of risks of exposure to marine biotoxins. In this regard this consortium also raises a plea for a global designation of institutions responsible for the first on-line hazard monitoring and exposure evaluation. The current shift of responsibility to the fishermen and aquaculture enterprises automatically leads to doubtful data as the financial burden cannot be carried and only regulated toxins will be analyzed, thus again precluding the analysis of toxin analogues with relevance for human health. A routine assessment of toxin levels in aquaculture and fishery products sold in Europe and elsewhere would allow assessment whether the allowable levels of currently regulated marine biotoxins offer sufficient protection of the consumers and whether the methods employed by the producers are sufficiently advanced to avoid processing, shipment and sale of contaminated products.

7.5 International register for intoxication cases

This consortium emphasizes the need to establish an international register for human marine biotoxin intoxication cases. This also implies the demand for further education of medical

Tab 1: Marine biotoxin classes, regulatory levels in EU and USA, availability of reference standards, occurrence and intoxication incidents in Europe

Toxin Type	EC regulatory level (mg/kg shellfish or fish tissue)	US-FDA regulatory level (mg/kg shellfish or fish tissue)	Number of analogues	LC-MS/MS Certified Reference Standards from NRCC[a] and Cifga[b]	Presence in European marine waters	Possible presence in European imports	Reported human intoxications in Europe
Amnesic shellfish poisons (ASP; *domoic acid* (DA))	20.0 (DA equiv.)	20.0 (DA equiv.)	≈ 10	3	YES	YES	YES
Azaspiracid poisons (AZP, azaspiracid (AZA))	0.16 (AZA equiv.)	0.16 (AZA Acid equiv.)	≈ 20	3 (AZA1, AZA2, AZA3)	YES	YES	YES
Ciguatoxin fish poisons (CFP; ciguatoxins (P-CTX, I-CTX and C-CTX))	–	0.01 P-CTX (equiv.) 0.10 C-CTX1 (equiv.)	≈ 15	0	YES	YES	YES
Cyclic imine poisons: (CIP; spirolides (SPX), gymnodimines (GYM), pinnatoxins (PnTX), pteriatoxins (PtTX))	–	–	> 30 ≈ 3 ≈ 6 ≈ 3	2 0 1 0	YES Unknown YES Unknown	YES YES YES Unknown	Unknown, but possible[c]
Diarrheic shellfish poisons (DSP; okadaic acid (OA), dinophysistoxins (DTX))	0.16 (OA equiv.)	0.16 (OA equiv.)	3 (plus ester metabolites)	3 (OA, DTX1, DTX2)	YES	YES	YES
Neurotoxic shellfish poisons (NSP; brevetoxin (BTX))	–	0.80 (BTX equiv.)	≈ 15	0	YES	YES	YES
Palytoxin and ostreocins	–	–	> 15	0	YES	YES	Unknown
Paralytic shellfish poisons (PSP; saxitoxins (STX))	0.80 (STX equiv.)	0.80 (STX equiv.)	> 30	13	YES	YES	YES
Pectenotoxin poison (PTX)	–	–	≈ 13	1 (PTX2)	YES	YES	Unlikely[d]
Yessotoxin Poisons (YSP; yessotoxin (YTX))	1.00[a]	–	≈ 36	2 (YTX, hYTX)	YES	YES	Unlikely[d]
Maitotoxin	–	–	–	0	Unknown	YES	Unknown
Tetrodotoxins (TTX)	–	?	≈ 9	0	YES	YES	YES

[a] National Research Council Canada – Certified Reference Material, Halifax, Canada (http://www.nrc.ca/crm)

[b] Laboratorio Cifga S.A. – Lugo, Spain (http://www.cifga.com)

[c] Although acute human intoxications have not been reported, sub-chronic or chronic neurodegenerative effects in humans following subchronic or chronic consumption of contaminated marine food appears possible.

[d] Based on the presently available data, intoxication of humans even upon ingestion of high amounts of contaminated seafood is highly unlikely as the toxicological data from the MBA is extremely weak. Oral ingestion of YTX and PTX does not produce toxicity in mice.

personnel with regard to the recognition and treatment of intoxicated patients, establishment of the associated supporting analytical institutes for toxin level verification, long-term epidemiological studies, and education of the public toward awareness for the range of symptoms caused by exposure to marine biotoxins.

The establishment of a centralized human intoxication registry is highly recommended, as the compilation of all assumed marine biotoxin intoxications by medical personnel would mean that for each intoxication case the toxins in question and the exposure dose can be identified and verified using toxin standards and validated methodology. In addition, the source of the toxins, including suppliers and fishing areas, can be identified, which is crucial for hazard monitoring and predictive approaches. Without a centralized registry for human marine biotoxin intoxication cases the regulators, the responsible medical personnel, as well as consumers will remain unaware of the prevalence, the incidence, and the nature of intoxications caused by exposure to these marine toxin classes.

The acquisition of intoxication data fundamentally needs medical personnel trained in the recognition of the whole range of symptoms caused by exposure to marine biotoxins. Not only the patients' symptoms must be interpreted correctly and treated accurately, but also a follow-up of post-intoxication complications is made possible, e.g., for CTX.

Data collected in a patient intoxication registry would also help to set up epidemiological studies that allow determination of risk levels, frequently affected populations, repeatedly exposed populations, or risk groups, differences in response due to variations between populations, and putative long-term effects of exposure *inter alia*.

In such a registry not only information on intoxication cases would be evaluated, but also information on the type of exposure, the concentrations of toxins in human body fluids, data from *post mortem* analyses and also information on recurrence of symptoms in survivors of intoxications could be gathered as a basis for adequate risk assessment and management.

8 Conclusions

Marine biotoxins are a large, heterogeneous group of highly complex chemicals produced by unicellular and multicellular aquatic organisms. The purported use of this energy-consuming biosynthetic process is a survival advantage of the producers in locally limited chemical warfare involving other marine organisms. Human injury is, in this sense, an unintentional collateral damage that has been historically limited in the past to local incidents. With an increasingly globalized food economy this scenario is changing, and large populations across different locations may be affected. Thus, there is an urgent need for improved risk assessment and risk management, and the application of the most suitable methods and strategies for this purpose.

It appears that three key issues need more attention and work in the future:

1. The overall human adverse effects (and not exposure levels of few measurable compounds) need to be the major reference point for risk assessment of marine products; the judgment should be independent of whether the intoxication arises from one single compound or a broad mixture of different, potentially unknown toxins. This principle has far-reaching implications on future testing and research needs.
2. Improved analysis methods and their governance need to be adapted and optimized to the respective purpose, considering that there is no "one-size fits all" approach. Scenarios that need to be distinguished are on-board control, port control, general risk monitoring of sites, and analysis in poisoning episodes. Moreover, responsibilities for analysis of marine biotoxins cannot be predominantly with the producer in all areas. Stronger involvement of national and international agencies requires harmonization and enforcement of adequate and appropriate routine analysis.
3. To establish appropriate risk assessment models for marine biotoxins it is essential to learn more about human susceptibility and to make use of high quality epidemiological data. Currently, the gaps of knowledge regarding human intoxications are to a considerable extent due to insufficient documentation, ignorance of medical personnel, and lack of public awareness. Centralized institutions, such as a European registry for intoxications that interacts intensively with the European reference laboratory could remedy this issue.

Concerning the analytical approaches for marine biotoxin detection, currently three major strategies are being discussed: the mouse/rat bioassay, chemical analytical methods (typically LC-MS, but to a certain extent also immunoassays or HPLC), and *in vitro* bioassays. The animal bioassay appears to be a particularly poor option for all application scenarios. Its human predictivity is unknown, albeit it originally served to reduce PSP associated fatalities. The correlation of its outcome measures with human poisoning data and epidemiological findings remains unclear. Moreover, sensitivity, standardization and robustness of data generated are considered highly problematic. Also, the non-discriminant use of chemical analytical methods by themselves is unlikely to provide sufficient safety for the global consumer. Currently, standard reference materials are available for the most prominent representatives of most of the major regulated toxins, but there are many minor structural analogues for which there are no available standards. The lack of standard reference material is limiting the utility of this approach and since these methods are target-oriented, any unexpected or new toxin would not be detected. Moreover, there is a lack of knowledge regarding the concentration-response relationships as well as mixture effects of these toxins.

Functional bioassays reflecting human-relevant mechanisms of action promise to provide relevant data for safety and risk assessment of marine biotoxins.These can be recruited as micro- or macro-scale approaches. The application of these approaches and the development of further human-relevant *in vitro* systems for evaluation of adverse effects in biological

systems, i.e., for appropriate risk assessment of marine biotoxins in seafood, are an important way forward for ensuring future safety in the area of marine food products.

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