Innovative mode of action based in vitro assays for detection of marine neurotoxins

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Contents

CHAPTER 1

General introduction

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1. General background

Marine biotoxins are naturally occurring chemicals produced by particular phytoplankton species. Harmful algal blooms (HABs), i.e. rapid increases of the population of phytoplankton, occur at certain environmental conditions. HABs can be harmful not only through the production of marine biotoxins, but HABs can also deplete oxygen from the water, block light to organisms living deeper in the water and can even clog fish gills. Because of the global warming, HABs are expected to take place more frequently and therefore represent a growing threat for public and environmental health. The majority of algae producing marine biotoxins are the dinoflagellates, although only a minority of them produces toxins affecting other organisms. The main producers of marine biotoxins are presented in Table 1.1.

Marine biotoxins are known to accumulate in seafood products such as fish, crabs and filter feeding bivalves. According to the Food and Agriculture Organization, the consumption of seafood in Europe records a constant increase from one year to another (see chapter 2). Marine biotoxins accumulate in the digestive gland of shellfish and do not harm the shellfish itself. However, marine biotoxins represent a threat for consumers and monitoring seafood for their presence is important (Fig. 1.1).

Table 1.1: Major groups of marine biotoxins and their main producers.

Biotoxin	Syndrome	Producer	Reference
Azaspiracids	Azaspiracid poisoning (AZP)	*Azadinium spinosum*	[58]
Brevetoxins	Neurologic shellfish poisoning (NSP)	*Karenia bicuneiformis, brevis, brevisulcata, concordia, cristata, mikimotoi, papilionacea, selliformis Chantonella cf. verruculosa*	[2, 24]
Domoic acid	Amnesic shellfish poisoning (ASP)	*Pseudo-nitzschia australis, calliantha, cuspidata, delicatissima, fraudulenta, galaxiae, multiseries, multistriata, pseudodelicatissima, punges, seriata, turgidula*	[59]
Gymnodimines	-	*Karenia selliforme* *Gymnodinium mikimotoi*	[60, 61]
Okadaic acids	Diarrhetic shellfish poisoning (DSP)	*Phalacroma rotundatum* *Prorocentrum arenarium, lima*	[62, 63]
Saxitoxins	Paralytic shellfish poisoning (PSP)	*Alexandrium spp.* *Gymnodinium catenatum* *Pyrodinium bahamense*	[64-66]
Spirolides	-	*Alexandrium ostenfeldii, peruvianum*	[67, 68]
Yessotoxins	Diarrhetic shellfish poisoning (DSP)	*Protoceratium reticulatum* *Lingulodinium polyedrum* *Gonyaulax polyhedra*	[24, 69]

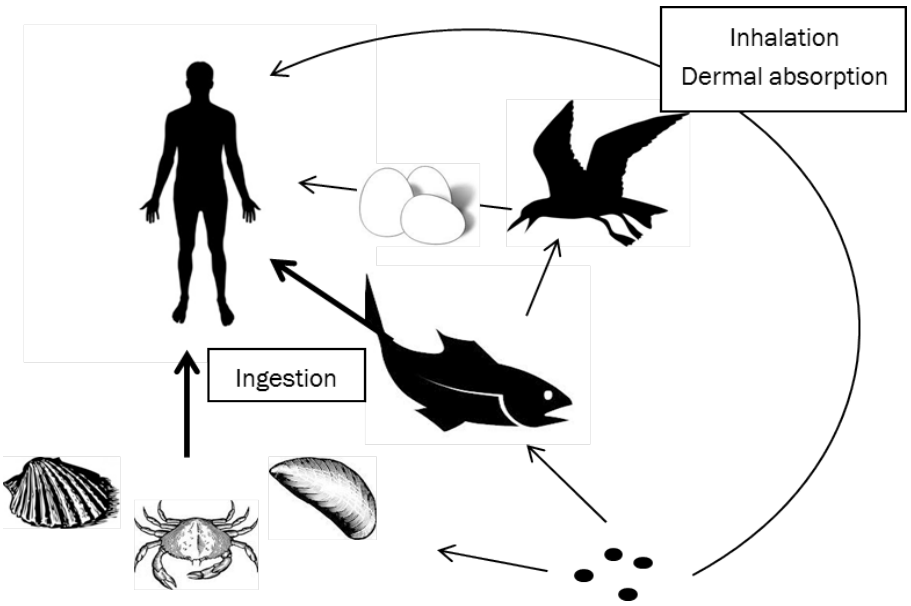

Figure 1.1. Different routes of human exposure to marine biotoxins. Humans are exposed to marine biotoxins mainly through consumption of contaminated seafood.

The main syndromes that can occur following consumption of seafood contaminated with marine biotoxins are Amnesic Shellfish Poisoning (ASP), Azaspiracid Shellfish Poisoning (AZP), Ciguatera Fish Poisoning (CFP), Diarrhetic Shellfish Poisoning (DSP), Neurologic Shellfish Poisoning (NSP) and Paralytic Shellfish Poisoning (PSP). The symptoms associated with the consumption of seafood contaminated with marine biotoxins vary from tingling or numbness around the lips to gastrointestinal disturbances, paralysis and in severe cases death [1, 2].

Besides the impact on health that may arise from the consumption of contaminated shellfish, it is estimated that there is a loss to the tourism and shellfish industry of about 900 million euros per year due to HABs [3]. It is therefore important to develop effective strategy plans to limit their impact. To do so, monitoring programs have been put in place and regulatory limits have been established in order to protect seafood consumers (see chapter 2).

2. Legislation

Different regulations and surveillance approaches are being applied in countries, mainly depending on the type of toxins present in their coastal waters. However, due to globalization, i.e. increase in imports and exports, countries have to adapt to the legislations established in other and new markets. For example, while CFP was mostly reported in the Pacific Islands in the twentieth century, recent intoxications were reported in France and Germany, due to import of seafood products (see chapter 3). Regulation 853/2004 is another example, where production areas in Europe should be closed as long as necessary when toxic phytoplankton is detected to ensure consumer safety, and seafood can be transferred to a toxic phytoplankton-free area to allow detoxification prior to market release. International trading therefore requires the establishment of specialized structures, e.g. national reference laboratories for the detection of a wide range of marine biotoxins that may end up on consumers plates. Requirements before commercializing seafood are briefly summarized in Figure 1.2.

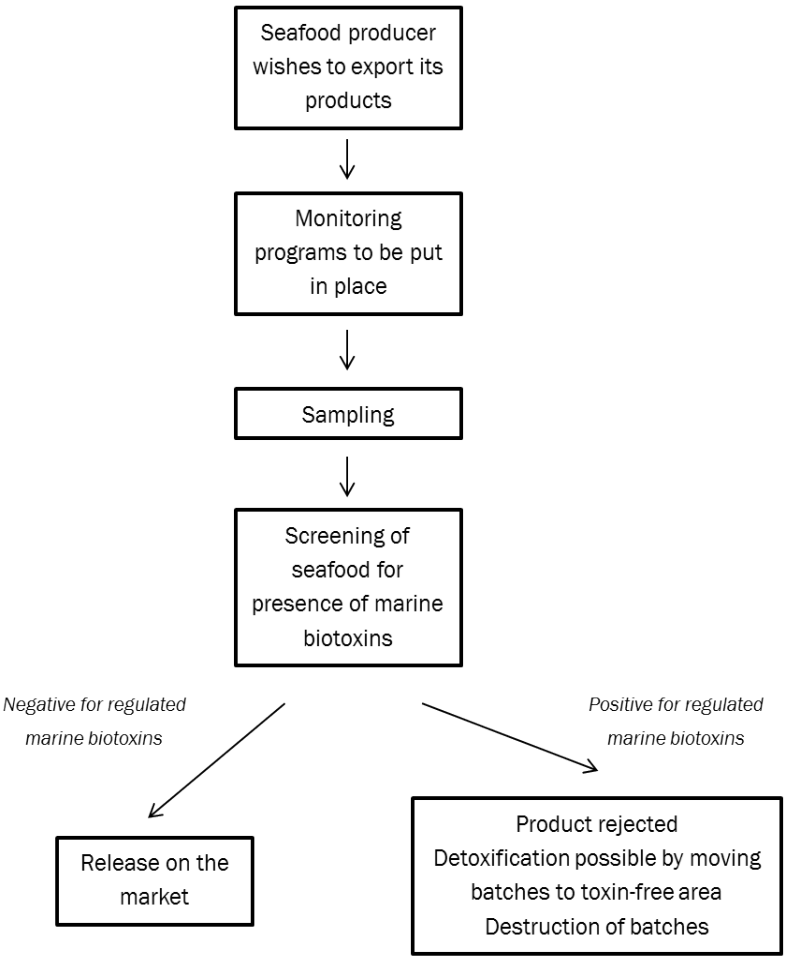

Figure 1.2. Steps required prior to commercializing seafood: example for the European market. As the food business operator is responsible for the products that reach the market it is common that these businesses perform their own checks before the commercialization of their products.

3. Methods of detection

The detection of marine biotoxins in seafood is currently performed through either in vivo assays or chemical methods, including HPLC-UV, LC-FLD and LC-MS/MS [4, 5]. Antibody-based assays such as enzyme-linked immunosorbent assay (ELISA) kits are also available, e.g. for okadaic acid, dinophysistoxins and domoic acid. A receptor binding assay has been validated to be used as an official method for screening of PSP toxins in the US [6]. Due to the lack of faith

in the completeness of these alternatives to cover the whole spectrum of marine biotoxins, in vivo assays such as the mouse bioassay (MBA) are still used in some countries for the screening of marine biotoxins in seafood. This assay is regarded as unethical, as mice are intraperitoneally injected with extracts from suspected seafood samples and the endpoint is lethality. Moreover, these in vivo assays give high rates of false positive and false negative results. Extracts from seafood with safe levels for oral human consumption of spirolides for example have been shown to cause death of mice within minutes [7]. From 2015 onwards, the MBA is forbidden, except for the periodic control of production areas [5]. More precisely, regulation 15/2011 states that the MBA "should be used not as a matter of routine and only during the periodic monitoring of production areas for detecting new or unknown marine toxins". Besides in vivo assays, chemical techniques have been developed, including the European official HPLC-UV method for detection of amnesic shellfish poisons (ASPs), the EU official LC-FLD method for screening of paralytic shellfish poisons (PSPs) and a LC-MS/MS method that can be used as a validated alternative routine method for the MBA in Europe to detect lipophilic marine biotoxins (diarrhetic shellfish poisons (DSPs) and azaspiracid poisons (AZPs)) [4, 5]. However, such chemical analyses are expensive and do not allow for the detection of all or presently unknown marine biotoxins, e.g. the LC-MS/MS technique developed by Gerssen et al. [8] allows for the detection of pacific ciguatoxin-1 (P-CTX-1) with a limit of detection of 0.2 µg P-CTX-1-eq)/kg seafood while the FDA regulatory limit is 0.01 µg P-CTX-1-eq/kg seafood. Thus there is an urgent need to develop in vitro alternatives that enable a sensitive detection of both known and currently unknown marine biotoxins in seafood products. Mode of action-based approaches represent an interesting way to follow, as theoretically they will allow the detection of a wide range of compounds through the measurement of functional endpoints.

The objective of this thesis was to develop mode of action-based assays for the screening of marine neurotoxins in seafood, replacing the current in vivo assays. Given the fact that a large number of marine neurotoxins target ion channels or receptors situated on the cell membrane, models that have been developed during this 4-year period were directed at a wide range of channels and receptors targeted by marine neurotoxins. While developing such mode of action-based assays one should bear in mind that to be implemented as routine techniques for the detection of marine neurotoxins in seafood, screening assays should be predictive and reproducible and should be performed at low costs,

with high throughput and high sensitivity.

In the following sections of this chapter, a brief overview of the origin of the major marine neurotoxins and their associated modes of action will be given to set the framework for the development of mode of action-based assays for the screening of marine neurotoxins in seafood, followed by a short introduction to the models that have been developed in this thesis.

4. Modes of action

4.1. Marine neurotoxins

Most marine neurotoxins are known to target ion channels/pumps or neuronal receptors. Here a brief summary is given. More details and structural formulas of the marine neurotoxins are presented in chapter 3.

Ion channel modulators

Saxitoxins (STXs) are produced by several species from the genus Alexandrium (Tab. 1.1). These toxins are responsible for PSP upon consumption of seafood. Tetrodotoxins (TTXs), which have a similar mode of action as STXs, can be ingested accidentally or intentionally through the consumption of puffer fish [9]. Few intoxications to TTXs occurred and in patients acutely exposed to TTXs neuronal excitability was reduced. This was due to the blockade of voltage-gated sodium channels (VGSCs). Under normal conditions VGSCs undergo a conformational change when detecting a change in ion concentration inside the cell, resulting in the opening of the channel and a sodium (Na^+) influx leading to membrane depolarization and regulation of cell excitability. Both STXs and TTXs bind to site 1 of this channel. The binding of these toxins to the VGSC blocks its ion conductance. This loss of Na^+ conductance in excitable cells prevents membrane depolarization and the transmission of the action potential. STXs and TTXs can be lethal to humans.

Brevetoxins (PbTxs), produced by Karenia spp. (Tab. 1.1), induce NSPs without being fatal to humans. However, these toxins induce a widespread killing of fish, birds and marine mammals [10]. PbTxs bind to the VGSC on site 5, leading to changes in the gating properties of these channels [11, 12]. The activation of the VGSC is thus enhanced and the Na^+ entry into the cells is raised. This Na^+ entry is responsible for excitatory cellular responses, but the cells may eventually not be excitable anymore, leading to paralysis [13]. Jeglitsch et al. (1998) [14]

showed that PbTx-3 specifically inhibits inactivation of the VGSC in rat sensory neurons. While exposure to most marine biotoxins occurs through consumption of contaminated seafood, inhalation of red tide (algal bloom of red or brown colour) vapours represents an important route of exposure to PbTxs.

Palytoxins (PlTxs) are produced by different genera of algae as well as corals [15] (Tab. 1.1). It is certainly one of the most potent marine biotoxins discovered up to now. In the past PlTxs were not of high concern in Europe, but since a few years these toxins are detected in temperate areas such as the Mediterranean sea [16, 17, 18]. Therefore, the number of studies on the evaluation of the toxicological effects induced by these toxins has increased and their mode of action is now well-known, i.e. PlTxs affect the Na^+/K^+-ATPase pump receptor. Few models have been investigated (guinea-pig papillary muscle, frog spinal cord, cockroach axons) and as an example Muramatsu et al. (1984) [19] showed, using a voltage-clamp technique, that PlTx concentrations ranging from 10^{-8} to 10^{-6} M induced membrane depolarization in giant squid axons.

Ciguatera is a seafood poisoning occurring in tropical areas. Ciguatoxins are accumulating in carnivorous fish nourishing on herbivorous fish and in invertebrates feeding on microalgae [20]. Despite recent advances in terms of detection methods, this marine biotoxin is a great risk, as it is toxic at very low levels, and at the same time difficult to detect at such low concentrations with analytical methods. Lehane and Lewis (2000) [21] evaluated the risk associated with Ciguatera poisoning and according to the FAO, not less than 50000 cases of intoxication following ciguatoxin food poisoning are reported each year. In addition, one should keep in mind that the number of intoxications is most likely under-reported. The majority of the reports reveals a persistence of the neurological symptoms following exposure to ciguatoxin-contaminated food [22, 23]. Some CTXs bind the VGSCs on site 5, leading to changes in the gating properties of these channels. This enhances the sodium entry into the cells leading to adverse effects similar to those of PbTxs [24]. Ciguatera is also partly caused by maitotoxins (MTXs). MTXs are produced by the marine dinoflagellate Gambierdiscus toxicus [30]. It accumulates in the flesh of fish. MTXs increase sodium and calcium (Ca^{2+}) entry into both excitable and non-excitable cells [31]. This supports the idea that MTXs target non-selective cation channels (NSCCs) [32]. Wang et al. (2009) [33] investigated how the NSCCs are activated and identified the activation of sodium hydrogen exchangers (NHE) as playing an important role in MTX-induced toxicity in rat cortical neurons. NHEs

are membrane proteins involved in regulating different cell processes and their over-activation in the central nervous system leads to ischemic injuries, stroke and other excitotoxic events [33].

Gambierol is a polycyclic ether isolated from the algae Gambierdiscus toxicus [25]. This marine biotoxin is thought to affect two types of voltage-gated ion channels: Na^+ [26, 27] and potassium (K^+) channels [28, 29]. Inoue et al. (2003) [26] observed that Gambierol inhibited the binding of [^{3}H]PbTx-3 to the sodium channels situated on synaptosomes prepared from rat brains. This result shows that Gambierol could either bind to site 5 or allosterically modulate the VGSCs to displace brevetoxin from its binding site. In another study, a specific fluorescent dye (bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (DiBAC4(3), bis-oxonol)) was applied to to monitor depolarization in human neuroblastoma cells. The authors showed that 30 μM gambierol induced a depolarizing sodium current.

Neuronal receptor modulators

Macrocyclic imines are fast-acting lipophilic marine biotoxins causing respiratory distress. This group of marine biotoxins comprises spirolides, gymnodimines, pinnatoxins and pteriatoxins. No human intoxication has been reported following exposure to any of these toxins and they are therefore considered “safe for human consumption” according to EFSA [44]. The micro-organism that produces spirolides is Alexandrium ostenfeldii. Information on the neurological mode of action of spirolides is limited, essentially because of the poor availability of pure standards or even toxic materials. What is known is that the toxins from the cyclic imines group are inhibiting the nicotinic and muscarinic receptors at the central and peripherous nervous system level as well as at the neuromuscular junctions. When administered intraperitoneally, as in the MBA, cyclic imines induce a cascade of neurological symptoms within a few minutes. Spirolides are capable of weakly activating L-type Ca^{2+} channels [46]. Gymnodimine is a metabolite of the planktonic dinoflagellate Karenia selliformis [45]. Pinnatoxin is one of the best characterized neurotoxin from the cyclic imine group. This toxin is produced by marine dinoflagellates and was identified after a food poisoning in China in 1990. While it was hypothesized that the VGCC was its target [47], Araoz et al. (2011) [48] suggested another mechanism of action. The authors described pinnatoxin A as selectively interacting with the human neuronal α7 subtype of nicotinic receptors.

Domoic acid (DA) is produced by the red algae Chondria armata and some

marine diatom species of the genus Pseudo-nitzschia. This marine toxin induces amnesic shellfish poisoning (ASP). One of the most important intoxications occurred in Canada in 1987 [49]. DA has been shown to primarily affect the hippocampal regions of the brain [50]. DA is structurally related to kainic acid (KA) which is an analogue of the neurotransmitter and excitatory amino acid L-glutamate. DA is known to be an agonist of the kainate receptor, a non-NMDA ionotropic glutamate receptor [51, 52]. The affinities of isodomoic acids to kainate receptors compared to DA are much lower, indicating that the structures of isodomoic acids result in different receptor binding affinities [51]. The binding of DA to glutamate receptors induced an increase in neuronal firing when the toxin was administered in the hippocampus of rats [53, 54]. Addition of DA to rat hippocampal neurons in vitro resulted in the same observation, suggesting that DA induces an excitatory response in neuronal cells both in vivo and in vitro. DA exerts its toxicity through the activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/KA subtype of glutamate receptors [55]. Berman et al. [56] monitored the intracellular Ca^{2+} accumulation in cerebellar granule neurons following DA exposure. The authors observed a rapid and concentration-dependent elevation of $[Ca^{2+}]i$, partly responsible for neuronal degeneration following DA exposure. This elevation of $[Ca^{2+}]i$ causes glutamate release that subsequently activates NMDA receptors, leading to cell death [57].

4.2. Other marine biotoxins

Yessotoxin and its analogues are produced by the microalgae Proteceratium reticulatum and Lingulodinium polyedrum. These toxins were first isolated from the scallop Patinopecten yessoensis [34]. According to EFSA (2008) [35], yessotoxins (YTXs) could be excluded from legislation, as it has been assessed that they do not pose a risk in the European Union for its population at the levels at which they occur. A large number of studies focused on the specific mode of action of YTXs, but many problems arose, e.g. the number of analogues produced by the algae is very large, the metabolism of the toxins in shellfish leads to the production of new analogues and toxin standards were not yet available. As a result, the molecular target of YTXs is still unknown. Moreover, studies to identify the YTX molecular target showed contradictory results. However, contrarily to other lipophilic marine biotoxins such as OA, YTXs do not inhibit protein phosphatases PP1 and PP2A [36]. What is known is that YTX exposure triggers a small but significant Ca^{2+} increase within minutes. This increase is limited

(less than double) and is induced by concentrations of 10^{-8} M in neuronal cells and 10^{-7} M in other cell systems. The consequences of this limited increase are still unknown. It is expected to lead to arrhythmia in cardiac cells. However, the principal way YTXs are thought to exert their toxicity is through the involvement of phosphodiesterases (PDEs) [37, 38]. This activation leads to a decrease in the intracellular levels of adenosine 3′,5′-cyclic monophosphate (cAMP), a second messenger important for a large number of biological processes, depending on the presence of extracellular Ca^{2+}. By modulating cAMP levels, YTXs induce an extracellular entry of Ca^{2+} through nifedipine-sensitive Ca^{2+} channels. On the other hand, YTXs also inhibit the capacitive entry of Ca^{2+} in human lymphocytes [39]. Despite the fact that YTX has a chemical structure close to that of PbTx and CTX, this toxin does not interact with the VGSC.

Azaspiracids (AZAs) have been identified by Satake et al. (1998) [40] following consumption of AZA contaminated shellfish in the Netherlands in 1995. The marine algae responsible for the production of AZAs are still not clearly established, although it is thought that the genus Azadinium is the major producer of AZAs [41, 42]. AZA-1, AZA-2 and AZA-3 are the most prevalent AZAs found in mussels. AZAs elicit the same symptoms as observed for Diarrheic Shellfish Poisons (DSPs). However, these marine biotoxins are not included in the DSP group, as neurological symptoms, including effects on synaptic transmission, have been observed as well following exposure to AZAs. Only a few studies have been performed to elucidate the mode of action of AZAs and their molecular target is still unknown. Some authors suggest that AZAs could act on voltage-gated ion channels. However, Kulagina et al. [43] showed, using a whole-cell patch clamp technique, that AZA-1 did not act on the voltage-gated Na^+, K^+ and Ca^{2+} currents in cultured spinal cord neurons from primary cultures. It was concluded that the observed effect on the synaptic transmission did not involve voltage-gated channels.

As described above, many marine neurotoxins target ion channels or neuronal receptors. Therefore, when developing mode of action-based assays, one should look for models presenting such channels and receptors.

5. Outline of the thesis

Marine neurotoxins represent a threat for consumers. Therefore monitoring programs should be put in place and simple, reliable tools for high throughput

detection of marine biotoxins in seafood should be available.

The MBA is forbidden from 2015 onwards, except for the control of production areas [5], and chemical analyses do not allow for the detection of all known and unknown marine biotoxins that threaten human health. Therefore, effort has been put on developing alternative methods based either on the chemical properties of the different marine biotoxins or on their specific modes of action, resulting in respectively biochemical assays and cell-based assays. Especially the cell-based assays represent a promising tool for the screening of marine biotoxins, as such assays will theoretically detect unknown toxins with a similar effect (mode of action) too. Cell lines or primary cell cultures can be used, allowing high throughput analysis of samples. The most promising cell-based assay for in vitro detection of marine biotoxins in seafood is the neuroblastoma neuro-2a assay, where cytotoxic effects of marine biotoxins are measured as an endpoint. However, additional functional endpoints can be investigated.

The aim of the research described in this thesis was to develop mode of action based alternatives to the current in vivo assays for the screening of marine neurotoxins in seafood. At the end, an integrated in vitro testing strategy was set up, contributing to the reduction, refinement and replacement of animal experiments.

This chapter, **chapter 1**, presents the aim of the studies, a short summary of the different marine neurotoxins and their mode of action and the outline of the thesis.

In **chapter 2**, an overview of the occurrence of outbreaks following consumption of seafood contaminated with marine biotoxins is given in order to underline the need for more effective and better monitoring programs and screening tools in order to further prevent such outbreaks and anticipate to effects of climate changes on HABs.

Chapter 3 depicts the modes of action of different marine neurotoxins and the different methods developed up to date to detect these toxins in seafood products. In addition, data gaps and bottlenecks are identified for the further development of new mode of action based in vitro bioassays.

Because neurons and cardiomyocytes share similar ion channels, the embryonic stem cell-derived cardiomyocytes were chosen as a model in **chapter 4**. Results obtained for different neurotoxic model compounds as well as two pure marine neurotoxins were compared to those obtained in the neuroblastoma neuro-2a assay. The endpoint measured in the neuro-2a assay is cytotoxicity,

which is not specific as it may be affected by external factors, including handling procedures, temperature and changes in pH. Thus, **chapter 5** focused on the development of new specific/functional endpoints using the neuro-2a cells. Changes in membrane potential as well as ion flux measurements were recorded using fluorescent probes in order to screen a wide range of neurotoxins targeting ion channels or pumps. In addition, microarray analysis of exposed cells were performed to identify potential genes up- or down-regulated by specific marine neurotoxins that could be suitable biomarkers for the detection of these toxins in seafood extracts.

While the neuro-2a assay is a promising assay for the broad screening of marine biotoxins (chapter 7), it does not allow for the detection of DA. An in vitro screening strategy that includes the neuro-2a assay thus requires an additional assay for DA. **Chapter 6** investigated whether the multielectrode array (MEA) platform could be of added value, i.e. for the screening of a wide range of marine biotoxins, including DA, through the monitoring of neuronal network activity. Rat cortical neurons were isolated and placed on 48-well plates containing 16 electrodes, i.e. a total of 768 electrodes, to monitor electrical changes directly correlated to neuronal activity. Both pure marine neurotoxins and marine neurotoxins present in fish/shellfish extract were tested.

Finally **chapter 7** assesses the suitability of the neuro-2a assay for the screening of a wide range of pure marine biotoxins and marine biotoxins present in complex food matrices (including in addition to marine neurotoxins also marine biotoxins inducing gastrointestinal disturbances). In addition to a wide range of pure marine biotoxins, real samples of fish and shellfish (including natural contaminated samples) were tested.

At the end, given the data available in the literature and the results obtained in this thesis, a screening strategy for the detection of marine biotoxins is proposed in chapter 8. Future perspectives regarding the improvement of monitoring programs and implementation of in vitro alternatives for the MBA in a routine set-up to ensure food safety are also discussed in chapter 8.

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CHAPTER 2

Marine biotoxins and associated outbreaks following seafood consumption: prevention and surveillance in the 21st century

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Abstract

Marine biotoxins are produced by particular types of microalgae. Depending on environmental conditions, including temperature, pH and salinity of the water and current patterns, proliferation of algae producing marine biotoxins, also known as harmful algal bloom (HAB), occurs worldwide. Marine biotoxins can accumulate in seafood products and as such present a threat for consumers. This paper reviews and compiles up-to-date literature on reported outbreaks that occurred worldwide in humans following exposure to marine biotoxins through seafood consumption. The review includes a discussion about prevention of such outbreaks and surveillance programs in order to identify possible limitations and solutions for limiting the impact of HABs on human health. It is concluded that marine biotoxins represent a threat for human health associated with the food chain as thousands of poisonings following consumption of seafood contaminated with marine biotoxins were reported in the 21st century, emphasizing the need for carrying on/developing surveillance programs to detect the presence of HABs and for the development, validation and implementation of sensitive high throughput methods for the screening of these biotoxins in seafood to protect consumers. Regarding the possible presence of unknown toxins and the general lack of standards for many known toxins, in vitro effect-based bioassays may play an important role in the monitoring for biotoxins.

1. Introduction

Marine biotoxins are naturally occurring chemicals produced by phytoplankton. Worldwide occurrence of marine biotoxins in seafood is correlated with proliferation of algae, i.e. harmful algal blooms (HABs), the occurrence of which depends on different environmental factors. Despite the fact that less than 0.02% of the existing species of phytoplankton are capable of producing marine biotoxins [1], HABs present a serious threat to wildlife and humans. Due to global warming and therefore changes in weather conditions and current patterns, specific HAB taxa tend to occur more often and at unexpected places. As a result marine biotoxins are considered a growing concern for public health [2, 3].

Marine biotoxins can accumulate in fish and shellfish products. Consumption of contaminated seafood can lead to various types of poisoning. Four major groups of syndromes are described: amnesic shellfish poisoning (ASP), diarrhetic shellfish poisoning (DSP), neurologic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). Besides these additional syndromes exist: azaspiracid poisoning (AZP), ciguatera fish poisoning (CFP), palytoxin poisoning and tetrodotoxin poisoning [4]. Each type of poisoning is associated with a specific group of marine biotoxins, all covered in this review.

According to the Food and Agriculture Organization (FAO), it is estimated that from 2000 to 2011, the fish and seafood supply quantity increased from 15.8 to 18.9 kg/capita/year, i.e. an increase of about 20% (Tab. 2.1), certainly due to reported beneficial health effects [5].

Maximum limits as established for a number of marine toxins in the EU and the US are shown in table 2.2. The European Food Safety Authority (EFSA) recently reviewed the toxicity of the various classes of marine biotoxins and established for a number of them an acute reference dose (ARfD), in most cases based on doses reported to cause adverse effects in humans. Subsequently, it was established which amount of toxin could be safely consumed via one portion of seafood. Regarding the acute nature of the adverse effects EFSA used a relatively high but not unrealistic portion size of 400 grams shellfish meat. The calculated levels corresponding to the ARfD are also shown in table 2.2 and are in some cases lower than the existing limits. It is clear that besides regulatory limits, established to ensure food safety, suitable monitoring programs, including animal friendly high throughput detection methods, should be developed and put into practice for preventing outbreaks related to marine biotoxins.

Table 2.1: Fish and seafood supply quantity in the world in 2000 and 2011 according to the Food and Agriculture Organization of the United Nations.

Region	Fish/seafood supply quantity (kg/capita/year) in 2000	Fish/seafood supply quantity (kg/capita/year) in 2011	% increase
Africa	7.8	10.8	38.5
America	13.5	14.2	5.2
Asia	17.3	21.2	22.5
Europe	19.1	21.8	14.1
Oceania	22.8	26.5	16.2
Worldwide	15.8	18.9	19.6

The detection of marine biotoxins in seafood products is currently primarily performed by in vivo assays and chemical analysis [7, 8]. Besides this, ELISA kits are available for okadaic acid, dinophysistoxins and domoic acid and for the screening of PSP toxins a receptor binding assay has been validated according to the "Association Of Analytical Communities" (AOAC) guidelines [9]. The main in vivo assay is the mouse bioassay (MBA), where mice are intraperitoneally injected with a seafood extract and lethality is the final readout, making the assay being considered as highly unethical. For PSP toxins the MBA has proven to be suitable and still is applied worldwide. For the lipophilic marine biotoxins (including DSP and AZP) the MBA is unspecific. Low levels of spirolides for example, can cause the death of mice within minutes at levels not considered relevant for human consumers [10]. In Europe, the MBA for routinely testing for the presence of lipophilic toxins is banned from 2015 onwards, except for the control of production areas for the presence of unknown toxins [8]. The current EU official method for the routine analysis of DSPs and AZPs is LC-MS/MS [11]. ASP is analysed by the EU official HPLC-UV method, while the official methods for PSPs in Europe are based on either HPLC-FLD [7, 8] or the MBA. The chemical methods that are in place in the EU legislation have gone through internationally recognized intra-laboratory validation studies. For example, the LC-MS/MS method for DSPs (DTXs, OAs, PTXs, YTXs) and AZPs, developed by Gerssen et al. [12], and the HPLC-FLD, developed by Lawrence et al. (2005) for PSPs have been

validated according to international guidelines as alternative routine methods to the in vivo assays. The HPLC-FLD method for saxitoxins is however very laborious and not really an alternative for routine monitoring but merely for confirmation.

Table 2.2: Main marine neurotoxins in seafood, EU and US regulatory limits.

Toxin	Current EU limits	Current US limits	European Food Safety Authority opinion (µg/kg)
Azaspiracids	160 µg AZA eq/kg SM	160 µg AZA eq/kg SM	30
Palytoxin	NR	NR	30
Ciguatoxins	a	0.01 µg P-CTX-1 eq/kg meat or 0.1 µg C-CTX-1eq/kg meat	More research needed
Brevetoxins	NR	0.8 mg PbTx-2eq/kg SM	-
Saxitoxin	800 µg STX/kg SM	800 µg STX eq/kg SM	75
Tetrodotoxin	b	Importation of puffer fish products restricted	-
Domoic Acid	20000 µg DA/kg SM	20000 µg DA/kg SM except in viscera of dungeness crab (30 mg DA/kg SM)	4500
Okadaic acid and analogues	160 µg OA eq./kg SM	160 µg OA eq./kg SM	45

SM: shellfish meat. NR: Not regulated. a: Fishery products containing biotoxins such as ciguatoxin or muscle-paralysing toxins must not be placed on the market. However, fishery products derived from bivalve molluscs, echinoderms, tunicates and marine gastropods may be placed on the market if they have been produced in accordance with Section VII and comply with the standards laid down in Chapter V, point 2, of that Section (for more information see [6]). b: Fishery products derived from poisonous fish of the following families must not be placed on the market: Tetraodontidae, Molidae, Diodontidae and Canthigasteridae [6].

At present, no chemical analytical method is routinely applicable for the broad detection of all lipophilic and hydrophilic marine biotoxins, primarily due to the

rather different chemical properties of the toxins. As such, the MBA still seems to have the broadest application, despite its problems with sensitivity and specificity.

The objective of this review is to give an overview of reported outbreaks, i.e. periodic human poisonings, that occurred worldwide in the 21st century and affected human health following consumption of resulting contaminated seafood products. The different types of poisoning and their associated reported outbreaks are highlighted first, followed by a short description and strength analysis of actual methods for surveillance and prevention of such outbreaks, and ending with the development of new techniques to cope with the potential increase of HABs and their possible impact on seafood consumers, as it is often concluded that improvements are needed to better protect seafood consumers.

2. Types of poisoning and associated reported outbreaks

This section focuses on the different types of poisoning associated with exposure to marine biotoxins: ASP, DSP, NSP, PSP, and other types of poisoning, i.e. AZA poisoning, CFP, cyclic imines poisoning, palytoxin and tetrodotoxin poisoning. Most marine biotoxins are odorless, cannot be destroyed by freezing or cooking and do not present any particular taste. There are thus no clear warning signals for consumers of contaminated seafood products. An overview of the worldwide reported human outbreaks following oral exposure to marine biotoxins is depicted per area (Europe, America, Asia and Oceania) in a chronological order, including the levels of toxins detected when available.

2.1. ASP: domoic acid (DA)

Amnesic shellfish poisoning (ASP) is characterized by symptoms varying from nausea, vomiting, short-term memory loss to coma and eventually death. Domoic acid (DA) is produced by the red algae Chondria armata and some marine diatom species of the genus Pseudo-nitzschia. DA is a heterocyclic amino acid and a kainic acid analog (Fig. 2.1). DA activates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, inducing Ca^{2+} influx therewith perturbing neuronal communication [13]. However, marine wild life is affected by DA as underlined by several reports and peer reviewed papers [14-17].

The only outbreak of ASP, i.e. periodic human poisoning, that has been well

documented occurred on the coasts of Prince Edward Island (Canada) in 1987 where 107 individuals suffered from gastrointestinal and neurological toxicity following ingestion of mussels (Mytilus edulis) [18]. After this outbreak, the toxin is well monitored in shellfish sanitary monitoring programs resulting in a significant reduction of toxic shellfish entering the market. Globally certain shellfish production sites are still frequently closed due to the presence of high levels of DA in various types of shellfish.

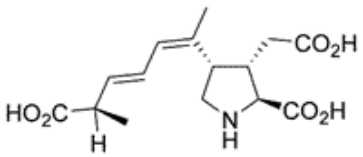

Figure 2.1. Chemical structure of domoic acid (DA)

2.2. DSP: dinophysistoxins (DTXs), okadaic acid (OA), pectenotoxins (PTXs)

Diarrhetic shellfish poisoning (DSP) is characterized by symptoms varying from diarrhoea to nausea, vomiting and abdominal cramps. The principal toxin responsible for DSP is okadaic acid (OA) (Fig. 2.2), produced by the dinoflagellates Prorocentrum and Dinophysis. DSPs exert their toxicity through inhibition of protein phosphatase-1 (PP1) and -2A (PP2A) except for pectenotoxins (PTXs) which exert their action through alteration of the actin-based cytoskeleton [19-21]. PTXs are historically included in the class of DSPs as they are produced by the same dinoflagellates although they do not induce diarrhoea. Human poisonings through shellfish contaminated with PTXs are not described in literature.

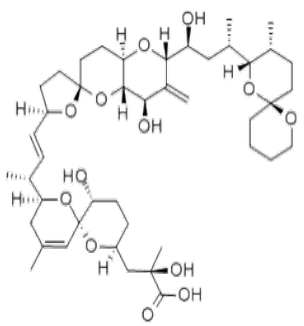

Figure 2.2. Chemical structure of okadaic acid (OA)

Europe

In 2000 in Thessaloniki (Greece), 120 persons presented acute gastrointestinal disturbances after consumption of mussels (Mytilus galloprovincialis) harvested from the Thermaikos Gulf [22]. Samples were analyzed using the MBA and were found positive for the presence of marine biotoxins. In a follow-up monitoring program, two more mussel samples collected from the Thermaikos Gulf were found positive for DSPs, but no outbreak has been reported during this period. In addition to the MBA, the protein phosphatase 2A inhibition assay as well as HPLC with fluorometric detection confirmed the presence of OA in these mussels [23].

In 2002, two outbreaks involving more than 70 victims following consumption of blue mussels (Mytilus edulis), razor clams (Solen marginatus) and green crabs (Carcinus maenas) from the Aveiro lagoon (Portugal) were reported by the Health Inspection Office of Aveiro's Retail Fish Market [24, 25]. The presence of OA and esterified OA in these seafoods was confirmed by LC-MS with selected single ion monitoring. The maximum concentration found in the remaining sample of the meal was 320 µg OA-eq/kg, twice the regulatory limit. Low levels of DA (30 µg/kg, far below the regulatory limits) were also measured, but were thought not to have contributed to the outbreak. Late 2002 in the region of Póvoa Varzim (Portugal) local health authorities reported 13 poisonings following consumption of wild mussels with levels of DSP as high as 18000 µg OA-eq/kg shellfish meat [26]. In the same year, 403 cases of DSP following ingestion of blue mussels imported from Denmark occurred in Antwerp (Belgium) [27]. The MBA was positive and the presence of OA, DTXs, yessotoxins (YTXs), PTXs and AZAs was confirmed by LC-MS. The responsible toxins OA and OA-esters were determined at a total level of 529 µg OA-eq/kg. During the summer of 2002, about 200 people were affected by DSP following consumption of self-harvested crabs (Cancer pagurus) feeding on blue mussels (Mytilus edulis) in the southern part of Norway [28]. LC-MS/MS analysis of crabs harvested in that specific area a week after the incident revealed levels of OA above regulatory limits (max. 1329 µg OA-eq/kg). Besides these outbreaks, contaminations occurred in different regions in Portugal between 2001 and 2005 (unpublished data, mentioned in [26]).

In 2009 in Vilaine Bay (France), 45 individuals suffered from illness due to consumption of mussels contaminated with OA and DTXs [29]. The levels found with LC-MS/MS were approximately eight times higher (1261 µg OA-eq/kg) than the current EU limits.

Recently, in 2012 and 2013 an advice was released by Food Safety Authorities

in Ireland and UK to avoid harvesting seafood products. Seventy people in South East England presented symptoms typical for DSP, following consumption of mussels harvested in Shetland (Scotland) while over ten people in Galway, Mayo and Sligo (Ireland) became ill after consuming suspected seafood [30, 31]. Frozen cooked Irish mussel meats were withdrawn from the market by the Food Safety Authority of Ireland in 2014 as a prevention measure due to the presence of DSPs in mussels measured during monitoring program.

South and North America

In 2001, several cases of DSP occurred in the Gulfs San José and Nuevo (Argentina) [32]. In 2002, approximately 40 poisonings were reported in the Chubut Province (Argentina) [32]. These outbreaks occurred following consumption of blue mussels and clams from the North-Patagonian gulfs and samples were shown to contain diarrhetic shellfish toxins up to 94 µg DTX-1/kg meat as detected by LC-MS/MS. This value is below the current regulatory limit and is assumed not to cause adverse health effects, but DTX-1 might have been depurated between the incident and the time of collection of the sample from the production site, and therefore the measured samples might not have been representative for the eaten samples (toxin distribution over individual mussels).

In 2002, consumption of mussels (Mytilus chilensis) led to 50 cases of food poisoning from which one person died in the geographic area of Chiloé Island (Chile) [33]. HPLC-FLD analyses confirmed the presence of both DSPs and PSPs in Mytilus chilensis above regulatory levels (reported PSP levels were 150000 µg STX-eq/kg). Two years later 26 patients were hospitalized in San Jose de la Mariquina (Chile) following consumption of Mytilus chilensis. Although patient's fecal analysis and the MBA were negative, HPLC-FLD and LC-MS analysis revealed levels of DTX-3 as high as 316.1 µg OA-eq/kg meat. DTX-3 is the generic term for all esterified forms of OA, DTX1 and DTX2. These esterified forms are known to be deconjugated into the original toxins after ingestion, in this case into DTX-1 [34]. In 2005, 35 patients with gastrointestinal complaints after having eaten Mytilus Chilensis were hospitalized in Puerto Montt Hospital (Chile). This outbreak was first associated with the enteropathogen Vibrio parahaemolyticus. However, 280 µg OA-eq/kg was detected by HPLC-FLD analysis and was considered to be the causative agent of the poisoning [35].

In 2011, three cases of intoxication following ingestion of highly contaminated mussels with DTX-1, with levels up to 1600 µg OA-eq/kg, were

reported in Washington State (USA) [36]. The same year, 62 cases of DSP due to consumption of Pacific coast mussels contaminated with diarrhetic shellfish toxins occurred on Salt Spring Island in British Columbia (Canada) [36]. Samples taken from this production area contained up to 860 µg OA-eq/kg. Both outbreaks led to implementation of routine monitoring of Dinophysis species on the outer Washington State coast and improved monitoring programs in Canada, respectively.

Asia

At the end of May 2011, more than 200 people in the Chinese cities of Ningbo and Ningde (near the coast of the East China sea) were intoxicated following ingestion of mussels (Mytilus galloprovincialis). ELISA results indicated the presence of OA or analogues at levels above the regulatory limit. Confirmation with LC-MS/MS revealed the presence of OA and DTX-1 at levels more than 40 times higher than the current European regulatory limits (6520 µg OA-eq/kg). In addition, these samples were shown to contain PTX-2 (max. 80 µg/kg), PTX-2 seco acids (max. 3750 µg/kg) and yessotoxins (max. 1600 µg YTX-eq/kg), but OA and DTX-1 were held responsible for the incident [37, 38].

Oceania

In 2000, one food poisoning due to consumption of clams (Plebidonax deltoides) harvested in Queensland (Australia) was reported. Initially the toxin held responsible for the adverse health effects in humans was PTX-2 seco acid [39]. This toxin is rapidly converted from PTX-2 in shellfish tissues [40], but as known from animal studies, this toxin shows little if any oral toxicity [41, 42]. Further investigation of the samples with LC-MS/MS led to the discovery of a total concentration of 253 µg OA-eq/kg which consisted out of 120 µg/kg esterified OA (DTX-3), 23 µg/kg OA and 110 µg/kg PTX2. In the Australia New Zealand Food Standards Code of 2003 the total concentration of OA and analogues including PTX2 should not exceed the 200 µg/kg [39, 43].

2.3. NSP: brevetoxins (PbTxs)

Neurologic shellfish poisoning (NSP) is characterized by symptoms varying from vomiting and nausea to partial paralysis and respiratory distress [4]. The toxin responsible for this poisoning are PbTx and its congeners, i.e. polyether compounds produced by species from the Karenia genus (Fig. 2.3). PbTxs bind to

voltage gated sodium channels (VGSCs) thereby inducing a Na^+ influx, resulting in persistent activation of excitable cells [44]. Most intoxications occurred through inhalation of aerosolized red-tide toxins but PbTxs can also accumulate in fish and shellfish.

No accumulation of PbTxs, either in fish or shellfish, has been reported in Europe [45, 46], and there is no literature on reported diseases in Asia and Oceania. In 2005, four cases of NSP were recorded in Florida (USA) following ingestion of clams harvested from an area closed due to a red tide bloom [47]. A year later, 13 people got ill from five separate incidents of NSP following consumption of clams collected in Florida [48]. Leftover clam samples were analyzed with ELISA and contained levels up to 42900 µg PbTx2-eq/kg which is far above the regulated level of 800 µg PbTx-2-eq/kg [4].

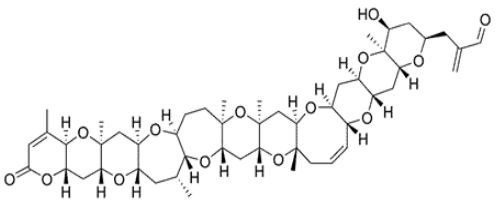

Figure 2.3. Chemical structure of brevetoxin-2 (PbTx-2)

2.4. PSP: saxitoxins (STXs)

Paralytic shellfish poisoning (PSP) is characterized by symptoms varying from nausea, vomiting, tingling of the mouth, slurred speech to paralysis. PSP can be fatal in extreme cases. The toxin responsible for this type of poisoning is the non-terpene alkaloid STX (Fig. 2.4) and STX analogues. STXs are mostly produced by dinoflagellates from the genera Alexandrium and Gymnodinium, but can also be produced by diatoms and cyanobacteria. STXs bind to VGSCs, inhibiting the influx of Na^+ ions and therefore inhibiting generation and propagation of action potentials in excitable cells [49].

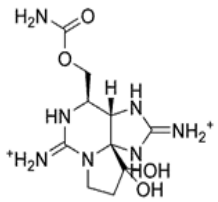

Figure 2.4. Chemical structure of saxitoxin (STX)

Europe

No human food posoning was reported in Europe since 2000 but toxin producing algae and contaminated seafood have been detected in monitoring programs [50]. In the Netherlands in 2012 there was a HAB of Alexandrium Ostenfeldii in a lake [51]. Due to the presence of high levels of PSP toxins a dog died.

South and North America

While puffer fish consumption is most of the time associated with tetrodotoxin poisoning (reviewed later in section 2.5.5), in 2002 21 cases of PSP occurred along the coast of Florida (USA) after consumption of puffer fish (Sphoeroides nephelus) containing up to 221040 µg STX-eq/kg tissue [52, 53]. This was analysed by the National Research Council (NRC) in Canada by using HPLC-FLD. The following two years in the USA, two cases of PSP in New Jersey, two in Virginia and two in New York were reported following consumption of puffer fish [52, 54-56]. Presence of STX and its analogs in these puffer fish samples was confirmed by using the MBA, a receptor binding assay, a mouse neuroblastoma cytotoxicity assay, ELISA, MIST Alert assay, HPLC and LC-MS analyses [52].

In November 2005, 45 cases of PSP were reported in the area close to Corinto (Pacific coast of Nicaragua). Collected samples tested positive in the MBA (all 15 mice died after peritoneal injection of a suspected shellfish sample) and the presence of STX in patients urine and collected shellfish was confirmed via the receptor binding assay and HPLC. The highest concentration of PSP toxins in clams was 4138 µg STX-eq/kg shellfish meat [57].

In June 2010, two confirmed and three suspected cases of PSP occurred following consumption of shellfish from Haines, Juneau and Kodiak (Alaska, USA) [58]. Death followed for two of these persons due to the food poisoning. Levels as high as 20440 µg STX-eq/kg meat for cockles and 8620 µg STX-eq/kg meat for butter clams were measured. In 2011, eight probable and five confirmed PSP cases were identified in Metlakatla (Alaska, USA) following consumption of diverse shellfish. Additional cases were reported in the same period, bringing the total number of affected consumers to 21. From these 21, eight had laboratory-confirmed PSP, and levels in eaten products were as high as 50370 µg STX-eq/kg meat [59]. In 2012, three suspected cases of PSP following consumption of shellfish (razor and butter clams Saxidomus gigantea) harvested in the Juneau area were reported to the Alaska Section of Epidemiology. Symptoms included tingling and numbness of the lips, tongue and face. More recently, in 2013 and

2014 in Alaska, two and one probable cases of PSP occurred after consumption of razor and butter clams, respectively [60, 61].

In October 2011, the Canadian Food Inspection Agency released a warning to avoid consumption of raw mussels, raw shell oysters and shucked oysters as these might contain PSP toxins [62].

Asia

According to the Japanese government, 338 people got intoxicated following consumption of puffer fish in Japan from 2000 to 2009. Among these cases, 23 deaths were reported. Toxin levels were not published. It is thus unclear whether the consumers suffered from PSP or TTX poisoning [4].

According to the Republic of the Philippines Department of Health, 83 people got intoxicated with paralytic shellfish toxins in 2011 in Bataan (Philippines) after eating seafood harvested in the Manila Bay. Two of these patients died [63, 64]. Two years later, in November 2013, seven cases of PSP were reported in Bataan following consumption of Gari spp. (sunset shell) [65]. Levels of measured PSP toxins were up to 2650 µg STX-eq/kg shellfish meat (detection method not mentioned) while the local limit is 600 µg STX/kg shellfish meat [66].

Oceania

In 2011, a fish farm employee was diagnosed with PSP after consumption of 12 fresh cooked wild mussels (Mytilus galloprovincialis). The mussels were cultivated in commercial fish cages in south eastern Tasmania, Australia [67]. At time of consumption there was a Gymnodinium Catenatum bloom and shellfish tested with the MBA showed levels up to 16000 µg STX-eq/kg during that period. According to New-Zealand health officials, 29 people got ill following consumption of Tuatua (Paphies subtriangulata) harvested in the Bay of Plenty in December 2012 [68]. Ten people required hospitalisation, making this outbreak one of the most severe PSP intoxication actually identified in the area. HPLC-FLD measurements revealed levels up to 31000 µg STX-eq/kg in the shellfish associated with the incident [69].

2.5. Other types of poisoning

2.5.1 AZP: azaspiracids (AZAs)

Symptoms associated with AZA poisoning are similar to those associated with DSP. However, neurological symptoms are also observed and therefore brought

the scientific community to classify AZAs in a separate group [70]. AZAs are polycyclic ether marine biotoxins (Fig. 2.5) produced by the genus Azadinium. Little is known about the mode of action of AZAs [71]. Detection of AZAs in shellfish is of great concern as the traditional lipophilic mouse bioassay has been shown not to be able to specifically pick up these toxins [72]. Therefore, an improved protocol is nowadays used for the detection of the lipophilic toxins including the AZAs with the mouse bioassay [73]. The difference in the protocol is the addition of a liquid-liquid partitioning step with dichloromethane or diethyl ether [74].

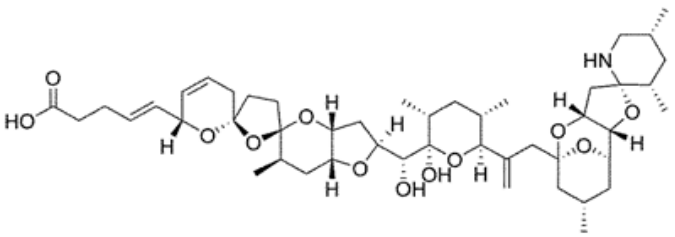

Figure 2.5. Chemical structure of azaspiracid-1 (AZA-1)

Europe

The first outbreak reported following consumption of seafood contaminated with AZAs is relatively recent. In 1995, at least eight people in the Netherlands got intoxicated following consumption of blue mussels (Mytilus edulis) harvested in Ireland. While symptoms were similar to those of DSPs, AZAs were found to be the causative agents [70]. In 2008 in France, 200 cases of AZP were reported following consumption of frozen mussels (Mytilus edulis) imported from Ireland [75]. Levels of AZAs measured by LC-MS/MS in mussels were above the EU regulatory limit of 160 µg AZA-1-eq/kg meat. In 2012 over 100 people turned ill in an elderly home in Belgium. Shellfish originating from Ireland (Castlemaine Harbour) were imported to Belgium via the Netherlands [76]. LC-MS/MS analysis of remaining mussels of this production area in the Netherlands revealed levels up to 1200 µg AZA-1-eq/kg (manuscript in preparation, data obtained from RIKILT Institute of Food Safety in the Netherlands).

South and North America

In 2008 in Washington (USA), two persons got ill after consuming frozen mussels (Mytilus edulis) produced in the Bantry Bay (Ireland) [77]. AZA levels measured by LC-MS/MS ranged from 86 to 244 µg AZA-1-eq/kg meat. Within the United States the same regulatory limit is set as in Europe, respectively at 160 µg AZA-

1-eq/kg meat.

2.5.2 CFP: ciguatoxins (CTXs), maitotoxins (MTXs), gambierol

Gastrointestinal (nausea, vomiting and diarrhea) and neurological (headache, paraesthesia, numbness and hallucinations) symptoms occur following CFP. In some individuals, symptoms lasted for years [78]. Toxins responsible for CFP are CTXs, MTXs and gambierol (Fig. 2.6), produced by the microalgae Gambierdiscus toxicus [79, 80]. CTXs bind to site 5 of VGSCs situated on the membrane of neuronal, muscular and to a lesser extent cardiac cells. CTXs have also been shown to interact with K^+ channels [81]. CFP is endemic in tropical and subtropical areas. Thousands of people are affected by CFP every year, making it the most common marine toxin poisoning [82]. For example, in Rarotonga (one of the Cook Islands) in 2010, an annual incidence of 1,058 per 10,000 individuals per year was reported [83]. Global warming is expected to raise CFP in Europe. In addition, fishery imports from Oceania contribute to ciguatera reaching Europe and the USA [84, 85]. Ciguatoxins are difficult to detect by chemical analytical methods due to the very low doses that already cause adverse effects and the scarce of affordable purified or semi purified standards. They can be detected by the MBA but also in vitro assays show very high sensitivity for these toxins.

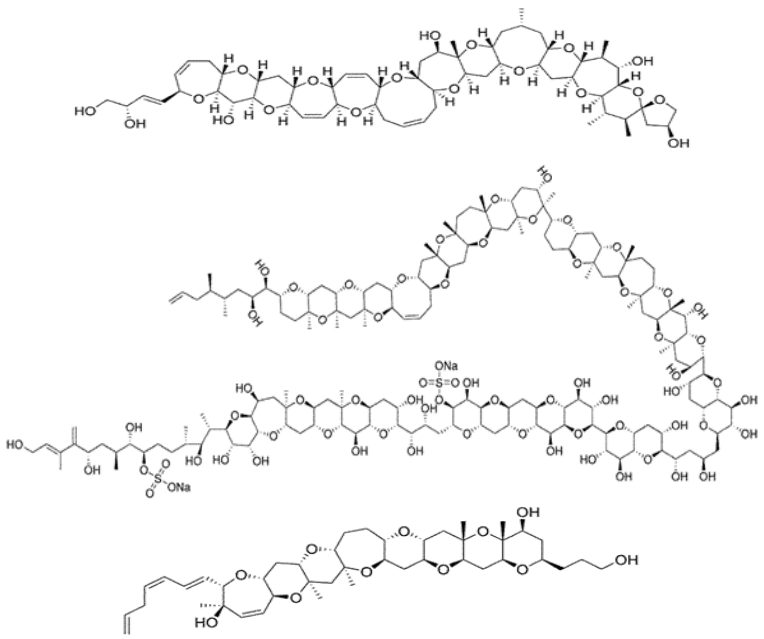

Figure 2.6. Chemical structure of ciguatoxin-1B, maitotoxin and gambierol

Europe

The EU legislation does not mention specific limits, the only reference made in EU legislation is that fish should not contain ciguatoxins. This means in practice a zero tolerance and that the detection limit of the applied technique is the decision level.

Two clusters of CFP occurred in Paris (France) in 2001. Both were related to consumption of Sphyraena barracuda and Lutjanus griseus caught in Guadeloupe (French West Indies). The first one involved 8 persons and the second two individuals. According to the authors, the presence of CTXs in both incidents was confirmed by the use of an MBA protocol specifically designed for the detection of toxicity due to ciguatoxins [85]. Based on visual observations of the mice (death time, loss in body weight) it could be concluded that the levels of ciguatoxin were the highest in the barracuda.

In 2009, in the port of Hamburg (Germany), 15 sailors presented gastrointestinal or neurological symptoms after having eaten fish (Caranx sexfasciatus and Cephalopholis miniata) caught in the Caribbean. The presence of CTXs in the fish was confirmed through LC-MS/MS analysis performed by the European reference laboratory in Vigo, Spain [86]. From 2000 to 2013 a total of 61 cases of CFP have been reported in Germany [87, 88]. More recently, 20 CFP cases were reported to the GIZ-Nord Poisons Center in Germany following consumption of fresh fish imported from South Indian fishing grounds. As analytical methods are scarce, the incidents are related to CFP mainly by the symptoms observed in the patients.

In 2004, multiple cases of CFP were observed on the Canary Islands (Spanish archipelago). Five persons who consumed amberjack (Seriola riviolana) exhibited symptoms including gastrointestinal and neurological disturbances. One fish sample was sent to the US Food and Drug Organization's Gulf Coast Seafood Laboratory where high levels (1 µg/kg) of Caribbean CTX-1 (C-CTX-1) were measured by LC-MS/MS [89]. In addition to C-CTX-1, two other potential toxins were detected in the fish sample by LC-MS/MS, but these toxins could not be identified. In 2008-2009, additional cases of CFP occurred in the Canary Islands, where C-CTX-1 was described as the causative agent upon LC-MS/MS analysis [90]. More recently, in April 2012, 16 cases of CFP were reported in Lanzarote (Canary Islands) following consumption of Seriola rivoliana [91]. From 2008 to 2012, in total 68 cases of CFP have been reported on the Canary Islands [92]. All of them occurred after ingestion of amberjack (Seriola spp.). According to the

general administration for public health of the Canary government (Dirección general de salud pública de Gobierno de Canarias) 16 cases of CFP were reported in 2013 in Spain (Canary Islands) following consumption of fish caught in the region of San Bartolomé [93, 94].

South and North America

During 2005 and 2006 on the Island of Culebra (Puerto Rico) 340 and 335 households were surveyed, respectively. The annual incidence of possible CFP was estimated to be 4/1000 persons per year [95].

During the summer of 2007, nine cases of CFP were reported in North California (USA) by the Centers for Disease Control and prevention (CDC) following consumption of amberjack purchased at a local market [96]. Extracts of fish tissue were tested in the mouse neuroblastoma neuro-2a assay and a level of 0.6 µg C-CTX-1(eq)/kg was estimated. A similar level of C-CTX-1 (0.6 µg/kg) was determined by LC-MS. Furthermore, from August 2010 till July 2011 the New York city department of Health and Mental hygiene reported 6 outbreaks of CFP with a total of 28 food poisonings. These poisonings occurred after consumption of grouper and barracuda. The highest level reported by a semi-quantitative mouse neuroblastoma neuro-2a assay was 1.9 µg C-CTX-1-eq/kg where the limit set by the FDA is 0.1µg C-CTX-1-eq/kg [97].

Asia

In 2000 in Israel 7 people got intoxicated following consumption of rabbitfish (Siganus spp.). Symptoms were similar to those of CFP although laboratory identification of CTX was not possible due to lack of fish tissue [98].

In Hong-Kong (China), 247 victims of CFP presented symptoms including gastrointestinal and neurological effects in 2004 and several cases were also reported in 2007 [99, 100]. An MBA designed for specific detection of CTXs was positive to CTXs for all samples collected during the time of the incidents.

In the city of Okinawa (Japan), 33 outbreaks due to CFP have been officially reported between 1997 and 2004 [101]. These outbreaks involved a total of more than 100 patients who consumed a large variety of seafood products including Variola louti, Lutjanus bohar, Lutjanus monostigma, Epinephelus fuscoguttatus, unidentified Lutjanus sp., Plectropomus areolatus, Oplegnathus punctatus, Epinephelus polyphekadion, Caranx ignobilis and moray eel. From 12 incidents there was left over meal which was tested in the MBA. The toxicity estimated by

the MBA was up to 0.8 mouse units (MU)/g which is far above the level to cause adverse effects (0.1 MU/g). LC-MS/MS analysis revealed the presence of CTX1B although due to extreme low concentrations present in fish (sub ng/g) it was not possible to obtain accurate reliable concentrations. In the south of Taiwan in 2005, six members of a family developed various symptoms such as nausea, vomiting, diarrhea and myalgia, followed by cardiotoxicity and neurotoxicity after having eaten barracuda eggs [102]. Cultures of stool samples from the victims were negative for Salmonella, Shigella, Campylobacter, Yersinia, and Vibrio. CTXs were confirmed to be present in the samples using a bioassay based on competitive binding with PbTx3 for voltage-dependent sodium channels using rat brain membranes. In 2006 in southern Taiwan two patients showed typical CFP symptoms following consumption of red snapper (Lutjanus bohar) [103]. The presence of CTXs was screened by using the MBA.

Oceania

From 2000 to 2008, a mean incidence rate of around 23 ± 6.5 cases of CFP per 10,000 people per year in French Polynesia was determined, with the highest average incidence of 140 cases/10,000 people for the period 2007-2008 in Raivavae (Australe Island in French Polynesia) [104]. These outbreaks were mostly due to consumption of carnivorous (68%), herbivorous (21.3%) and omnivorous (10.3%) fish [105, 106]. A survey of fish in the Raivavae lagoon by the receptor binding assay revealed concentrations up to 5.58 ng P-CTX3C-eq/g.

In total 52 cases, including three hospitalisations, have been reported between 2004 and 2008 in Guadeloupe (French Antilles) following consumption of giant trevally (Caranx ignobilis) and barracuda (Sphyranea). In Martinique (French Antilles) 93 cases of CFP, including 28 hospitalisations, have been reported between 1997 and 2007. Most cases were reported by general practitioners (information obtained from the Institut de veille sanitaire).

The CFP prevalence rate was established at 37.6% in Nouméa (New Caledonia) in 2005 mostly due to consumption of carnivorous fish [107]. In addition to fish consumption, four cases of CFP following shellfish consumption were reported in the same area. Nothing is mentioned on detection methodologies applied to confirm the presence of ciguatoxins in fish.

From 2007 to 2011, 2678 cases of CFP were reported in French Polynesia (Institut de la statistique de Polynésie, Institut Louis Malardé).

In June 2014, nine cases of CFP have been reported in Anaho bay in the island

of Nuku-Hiva, Marquesas archipelago (French Polynesia) following consumption of sea snail (Tectus niloticus) for which harvesting and consumption is forbidden (www.ciguatera-online.com, consulted on 13/02/2015).

According to Skinner et al., [108], 39677 cases from Pacific Island Countries and Territories were reported between 1998 and 2008. This represents not less than 194 cases of CFP/100,000 people per year, an increase of 60% compared to the reported annual incidence between 1973 and 1983 [108].

In 2014, a total of 33 cases of CFP were recorded in Townsville (Australia) following consumption of contaminated Spanish mackerel [109].

2.5.3 Cyclic imines: spirolides, gymnodimines, pinnatoxins, pteriatoxins

Cyclic imines induce neurological symptoms when administered intraperitoneally in mice. Spirolides (Fig. 2.10) are produced by the algae Alexandrium ostenfeldii. Gymnodimines are metabolites of the planktonic dinoflagellate Karenia selliformis [110]. Pinnatoxins are produced by Vulcanodinium rugosum and pteriatoxins are thought to be biotransformed from pinnatoxins in shellfish [111]. Although no information has been reported linking cyclic imines to human poisoning [112], these toxins can be detected using LC-MS/MS and are known to cause mouse death in the MBA. Based on their effects in the in vivo assays these compounds are of interest. Currently there is no clear effect of relatively high levels of cyclic imines in shellfish and human intoxication.

2.5.4 Palytoxin (PlTx) poisoning

PlTx poisoning is characterized by symptoms varying from chest pain to convulsion and in rare cases death due to respiratory paralysis following consumption of contaminated seafood [113]. PlTxs (Fig. 2.7) are produced by different genera of algae as well as corals [114]. PlTx is known to convert the Na^+/K^+-ATPase pump into a non-selective cation channel [115]. A detailed review on human risks associated with PlTx exposure can be found in [116].

Europe

In 2005 in Liguria (Italy) hundreds of cases of PlTx poisoning following ingestion of contaminated seafood or inhalation of vapours from plankton were reported [117-119]. LC-MS analysis revealed high levels of PlTx and one of its analogues ovatoxin-a [120]. Most outbreaks recently occurred following exposure to PlTxs

through dermal absorption (e.g. skin contact while swimming), in Germany [121] or the USA [116], or through inhalation [122].

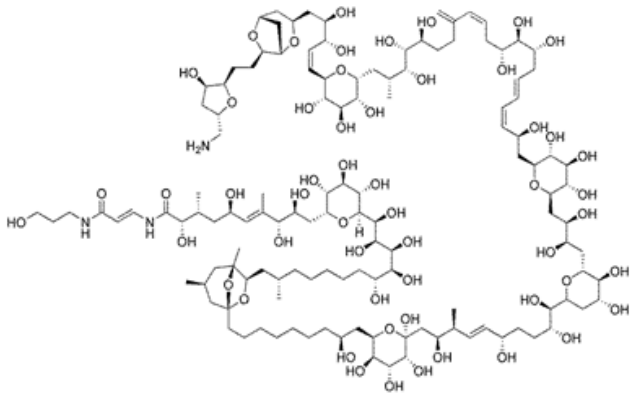

Figure 2.7. Chemical structure of palytoxin (PlTx)

Asia

At the end of 2000, eleven persons exhibited symptoms including severe muscle pain, low back pain and discharge of black urine following consumption of a PlTx (identified using an anti-PlTx antibody) contaminated serranid fish (Epinephelus sp.) in Kochi prefecture (Japan) [123].

2.5.5 Tetrodotoxin (TTX) poisoning

TTX poisoning induces paralysis of muscles and can be fatal especially through respiratory failure due to paralysis of respiratory muscles. TTX (Fig. 2.8) is mostly found in puffer fish and is produced by symbiotic bacteria such as Pseudoalteromonas tetraodonis, Pseufomonas spp. and Vibrio spp. Like STX, TTX exerts its toxicity through the binding to VGSCs, blocking Na^+ influx thereby preventing action potential generation and propagation in excitatory cells [124]. From 2001 to 2007, a total of 313 cases of TTX food poisoning (methods of detection not mentioned) were reported in Japan, with an average mortality rate of about 6% [125]. Puffer fish is forbidden on the European market. Like in Japan, puffer fish entering the American territory should be processed and prepared by specially trained and certified fish cutters. The presence of TTX in fish and shellfish is not only limited to Asia and the US as shown for example with the recent presence of TTXs in shellfish along the south coast of England [126].

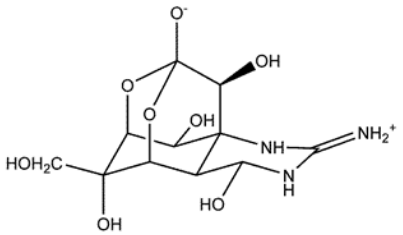

Figure 2.8. Chemical structure of tetrodotoxin (TTX)

Europe

The first European case of TTX poisoning was described by Fernández-Ortega et al. in Spain in 2010. The victim ingested part of a trumpet shellfish (Charonia sauliae) harvested from the Atlantic Ocean in the southern coast of Portugal during September 2007 and brought to the Spanish market [127]. LC-MS/MS confirmed the presence of TTX in the trumpet shellfish.

South and North America

Two cases of TTX poisoning were reported in Chicago (USA) in 2007 following consumption of soup with "monkfish" imported from China [128]. Chemical analyses by LC-MS/MS performed at the Food and Drug Administration revealed life-threatening levels of TTXs up to 9610 µg/kg.

Recently, in June 2014, two persons got intoxicated in Minneapolis (Minnesota, USA) following consumption of dried pufferfish. TTXs levels found by LC-MS/MS were as high as 72000 µg/kg in analyzed samples [129].

Asia

From 1996 to 2006, 280 cases of TTX poisoning following ingestion of horseshoe crab eggs (Carcinoscorpius rotundicauda) were reported in Chon Buri (Thailand) [130]. Among these cases, five patients died and one suffered from brain damage.

As mentioned above, according to the Japanese government, 338 people got intoxicated following consumption of puffer fish in Japan from 2000 to 2009. Toxin levels were not published therefore the incident could not be attributed to PSP or TTX poisoning specifically.

In 2001, four persons exhibited symptoms including general paresthesia, paralysis, coma, vomiting and aphasia following ingestion of snails (Zeuxis

sufflatus and Niotha Clathrata) in northern Taiwan [131]. Chemical analysis revealed high levels of TTXs up to 294 µg/snail. The same year, six cases of TTX poisoning following ingestion of puffer fish in the Taiwan Strait were reported [132]. From 2002 to 2004 six cases of TTX poisoning were reported in Tungkang (southern Taiwan) after consumption of gastropods (Oliva miniacea, Oliva mustelina, Oliva nirasei and Nassarius glans) [133]. The MBA was used to determine the toxicity and the presence of TTX was confirmed by LC-MS and GC-MS.

In 2001 in southern Zheijang (China), 30 cases of TTX poisoning following ingestion of snails (Zeuxis samiplicutus) were reported [131, 134]. In the same year, six individuals were intoxicated on Tungsa Island (Taiwan) following ingestion of snails (Nassarius glans) contaminated with TTX and its analogues 4-epiTTX and anhydro-TTX, two patients died. Toxicity was determined with the mouse bioassay and the toxins were confirmed in urine and blood in the patients using LC-MS [135, 136]. Levels found with the mouse bioassay were up to 5188 MU / snail and it is assumed by the authors that a dose of 10000 MU is lethal to human.

In Khulna (Bangladesh), 37 cases of likely to be TTX poisoning following puffer fish consumption were reported [137]. Of these 37 cases, eight patients died within a few hours following ingestion. TTX was not confirmed by means of any method.

Between 2005 and 2008, 13 cases of TTX poisoning were reported in Israel after consuming puffer fish (Lagocephalus sceleratus) which migrated from the indo-pacific region through the Suez canal [138]. TTX food poisoning was based on clinical symptoms and the confirmed consumption of puffer fish, analytical techniques and/or a bioassay was not applied.

In Japan from 2000 to 2007, more than 350 persons have been hospitalized following TTX poisoning amongst whom 18 died [125]. In 2007 in Nagasaki (Japan), one poisoning after the consumption of a small gastropod (Alectrion glans) was reported. High levels of TTX (600 MU/g meat) were measured [125].

In 2008, three people ate puffer fish (Lagocephalus inermis) purchased at a market in Nagasaki (Japan) and presented neurologic symptoms [139]. Levels of TTX present in the residual liver sample were as high as 1230 MU/g meat, and were therefore considered as “strongly toxic”. In the same year, three outbreaks occurred in Bangladesh due to consumption of cheap puffer fish sold on markets, resulting in the hospitalization of 141 patients [140]. Out of these 141 patients,

17 died [141]. The amount of TTX was estimated in patient blood and urine using ELISA. Furthermore HPLC-FLD revealed the presence of TTX and its analogues 4-epiTTX and 4,9-anhydroTTX in the consumed puffer fish.

3. Lessons learned

The above mentioned food poisonings underline to some extent a lack of awareness and knowledge about toxicity associated with seafood consumption and control of seafood products placed on the market. It is also clear that it may be difficult to prevent all food poisonings, especially for certain toxins (i.e. ciguatoxins). Most marine biotoxins cannot be destroyed by freezing or cooking and do not present any particular taste. Outbreaks tend to occur several times at the same place during well-defined periods, i.e. when the environmental conditions are favorable for HABs. Table 2.3 presents an overview of the main types of poisoning and the number of cases reported worldwide during the 21st century. Based on this overview it can be concluded that CFP and DSP are, according to the number of reported outbreaks, the types of poisoning that occur most often. Therefore these toxins may require specific attention. According to the literature presented in this review, while CFP and DSP are the most prevalent, TTX poisoning appears to be the most lethal one, followed by PSP.

While the occurrence of outbreaks in the last ten years already requires attention from a public health perspective, one should be aware that the literature currently available provides an underestimation of the real incidence of fish/shellfish poisoning due to marine biotoxins, especially because symptoms of poisoning are similar to allergic reactions and viral or bacterial infections [143, 144]. DSP is often associated with poisoning induced by Vibrio parahaemolitic and Bacillus cereus, which are routinely found in seafood [145]. Moreover, it is often difficult to associate observed symptoms with marine biotoxin poisoning as the contaminated food is often not available for confirmatory analysis, i.e. all is eaten. In addition to symptoms that are misclassified and linked to other types of disease, bias associated with a lack of clinical diagnostic tests and a lack of knowledge of clinicians also adds to the underestimation of (shell)fish poisoning [146]. To make sure that an adequate treatment is applied, the presence of similar symptoms with persons who consumed seafood should raise suspicion of fish/shellfish poisoning to clinicians [147].

Table 2.3: Main types of poisoning and number of cases reported worldwide during 21st century (2001-present).

Poisoning	Number of cases reported	Reference(s)
Amnesic shellfish poisoning	None after the 1987 outbreak in Canada	[18]
Diarrhetic shellfish poisoning	> 1200	[22-28, 33-37]
Neurotoxic shellfish poisoning	America : 17	[47, 48]
Paralytic shellfish poisoning	> 400	[52, 54-56, 58, 59, 67, 68, 142], Japanese government
Azaspiracid poisoning	> 200	[75, 77]
Ciguatera fish poisoning	> 3400	[86, 89, 90, 99-102], Institut de veille sanitaire, Institut Louis Malardé, ciguatera-online.com
Palytoxin poisoning	Few hundreds	[117-119]
Tetrodotoxin poisoning	> 500	[123, 127, 130-132, 134-141]

*: the numbers presented in this table are based on reported cases of human poisoning as opposed to estimated cases. They might therefore underestimate the actual occurrence of poisonings.,

Due to the lack of time-dependent data it is not possible to determine whether there is an increase in the incidence of shellfish poisonings, but the reported outbreaks clearly emphasize the risk associated with consumption of seafood, thus indicating a need for adequate prevention and surveillance. Besides being aware of the occurrence of outbreaks following consumption of contaminated seafood, one should keep in mind that other types of exposure are reported, e.g. inhalation and dermal contact, and that also freshwater biotoxins present a threat for human health, emphasizing the need for high throughput and sensitive techniques to detect these toxins in food but also water. Furthermore, it is clear from the data available that less severe cases are described in the literature from countries where monitoring programs and good healthcare systems are available. When monitoring is not in place and healthcare options are limited

only severe cases are reported (most often where death occurs). The problem is a global issue but cases are better reported in Europe, North America, Japan, Australia and New Zealand. This means that in certain parts of the world such as Africa, parts of Asia and the middle east, more effort should be taken to transfer knowledge, establish surveillance programs and communicate about risk associated with marine biotoxins.

4. Prevention and surveillance

Worldwide, surveillance and prevention of outbreaks rely on monitoring programs that are crucial for seafood producers. However, from a consumer and food safety perspective it is still necessary that further improvements are made, as food poisonings are still occurring substantially and more toxins (diversity) are found in worldwide coastal waters and seafood products. Although several tools have been developed to screen and identify possibly contaminated seafood, there is still an urgent need for sensitive high throughput assays for routine monitoring.

Human activity greatly contributes to the occurrence of algal blooms along our coasts, e.g. through loads of nutrients coming from areas with agricultural production and activities in petroleum production areas, the latter resulting in growth of dinoflagellates in for example the Gulf of Mexico [2, 148]. It might be possible to avoid such activities to limit the potential contamination of seafood. However, it is obviously impossible to modify parameters such as salinity, pH and temperature of the coastal waters to prevent that they are favorable to marine biotoxin producers. Therefore surveillance of the coasts is crucial in prevention programs. Besides monitoring the algae that produce marine biotoxins, moving batches of contaminated seafood to toxin-free areas helps detoxifying seafood. Also seasonal quarantine may prevent the harvesting and consumption of contaminated seafood. In addition, informing potential consumers about the risk of seafood consumption, based on observations of toxic blooms, i.e. consumer awareness, is required to further ensure food safety.

While red tides due to high concentrations of *Karenia brevis* can easily be detected, most toxic plankton blooms do not present such visual characteristics. Satellite monitoring of temperature allows for identification of areas where toxic blooms are likely to occur and while it does not predict contamination of seafood, it offers important information to understand HABs pattern and potentially prevent human poisoning. In such areas, monitoring programs should be intensified

in order to check if toxin levels in seafood are above regulatory limits. Despite regular controls of seafood, surveillance should also be more frequent during periods where marine biotoxins are more likely to be present in the food chain. Ideally, two different monitoring levels are implemented at the same time for the prevention of outbreaks: 1) monitoring of plankton species and of favorable conditions for growth (especially useful for seafood producers) and 2) screening of marine biotoxins in seafood harvested at specific locations, which is eventually of main importance for both producers and regulators (enforcement). The first level is of importance to know which potential toxins can be expected in the fish or shellfish but it should be mentioned that for some potential toxic algae there is not a clear correlation between the algae quantity (cells/liter) and the amount of toxin found in shellfish. Furthermore some algae (such as Azadinium), due to their relatively small size, are difficult to identify using conventional microscopy. But plankton monitoring appears to gain more attention and importance due to global warming, potentially resulting in increased occurrence of HABs or at so far unexpected locations. Besides the use of solid phase adsorption toxin tracking resins [41], advanced tools are currently developed and tested for real time monitoring of toxic algae, such as the Imaging Flow Cytobot [149] or the environmental sample processor developed by researchers from the Monterey Bay Aquarium Research Institute [150]. In the same line, algorithms to forecast toxic algae blooms are investigated [151, 152], besides HAB trackers like the US Integrated Ocean Observing System that allows for prediction of HAB spreading. The second level of monitoring still requires in vivo assays such as the MBA [153] or chemical methods such as HPLC-UV, LC-FLD and LC-MS/MS [7, 8]. The MBA involves use of experimental animals and gives high rates of both false positive and false negative results due to its lack of specificity [154]. This lack in specificity can have severe consequences, as shown for example with the event that happened in France in 2009, where the MBA was negative while the levels of OA were approximately eight times higher than the current EU limits [29]. Chemical analyses of marine biotoxins do not allow for the detection of unknown compounds and stand in need of standards that are barely available. In Europe, the MBA is forbidden for the detection of lipophilic toxins from 2015 onwards, except for the control of production areas (European Commission, 2011). It is also still allowed for detection of PSPs. Alternative tools for screening of marine biotoxins are thus urgently required. In vitro alternatives should allow for the screening of marine biotoxins at and below regulatory levels with high

throughput, low costs and high reproducibility. Efforts have already been made on the development of in vitro alternatives. Cell-based assays represent promising tools for ensuring food safety with respect to marine biotoxins, and the neuro-2a assay in particular, as it is able to detect a broad range of marine biotoxins including some that are not regulated at the moment [155-157]. A recent example is the use of multielectrode arrays with neural cells [158], allowing for the detection of a wide range of marine neurotoxins too. In the same line, surface plasmon resonance (SPR) technology resulted in the development of a multiplex SPR biosensor that is successful in detecting groups of marine biotoxins instead of individual toxins [159]. Furthermore, there are some commercial ELISA kits available on the market that show great potential for relatively cheap and fast screening by i.e. producers. Although for both the SPR and ELISA technique which are using antibodies there is a potential risk of missing certain analogues due to the limited cross-reactivity towards these analogues. In addition, one can expect the miniaturisation of some of these screening devices to bring the laboratory analysis on-site [160, 161], allowing producers to have a more rapid and efficient screening of sensible areas and allow the harvesting of safe seafood. These techniques are currently designed for first screening of seafood only and therefore chemical analyses are required for confirmation of the presence of specific marine biotoxins and identification/quantification of these toxins.

Finally, surveillance of contaminations in countries that cannot afford the costs associated with the implementation of surveillance programs should be undertaken by qualified and trained staffs that would quickly react by taking measures in order to avoid further contamination. It is not rare that general practitioners call poison centers because of a lack of knowledge to be able to treat their patients. Therefore, effort should be put on informing and educating health personnel that might encounter cases of fish/shellfish poisoning. Identification of the cause is one aspect, gaining insight about transmission mechanisms and subjects at risk is another item to consider. By knowing what food affected subjects consumed, it is possible to go back to the source of the problem and eventually prevent other human poisonings. Historical information regarding previous outbreaks should also not be forgotten, as in a large majority of the cases outbreaks tend to happen several times at the same place during a relatively short period of time. In addition, educating people about not harvesting seafood during red tides still represents one of the most efficient tools to prevent fish or shellfish poisoning.

Based on the data on outbreaks collected in this review, the need to replace the MBA and the threat that new emerging marine biotoxins represent, more efforts should be put on the development of in vitro effect-based assays that would allow for the sensitive and specific detection of a wide range of marine biotoxins in seafood, including those that are still unknown.

5. Summary and perspectives

The present paper reviewed data on human poisonings following seafood consumption in the last decade (2001-2015) and suggests actions to be taken in order to better address the food safety concerns posed by marine biotoxins associated with HABs.

The summarized data underline the large number of poisonings occurring worldwide following consumption of seafood, leading to unnecessary deaths. As described, CFP and DSP are of major concern as thousands of persons got intoxicated since 2000 (Tab. 2.3). The numbers only cover the reported cases and therefore call for cautiousness while deriving trends to be used for designing appropriate prevention and monitoring programs. Prevention of seafood poisoning is best achieved through monitoring of HABs in combination with testing seafood to be introduced on the market, thus avoiding consumption of fish or shellfish. Further protection is achieved by advices from authorities regarding fish and shellfish harvesting and consumption during algal blooms. Authorities play an active role in developed countries but much less in developing countries, where no advisory system is in place. Implementing such a program would contribute to the reduction of the outbreak incidence. There are not enough data to draw conclusions about the differences between developed and developing countries, but when comparing the CFP incidence in the Pacific Islands between the 1973-1983 and 1998-2008 periods [108], the 60% increase is certainly not only due to an increase in cases, but also to better monitoring programs and therefore a higher number of reported outbreaks.

Online community tools are currently being developed to ensure the completeness of reported cases and give a better picture of the real incidence of seafood poisoning. CFP is for example better reported in French Polynesia thanks to an initiative from the Malardé Institute (see www.ciguatera-online.com for more information). There are still cases of poisoning following consumption of seafood forbid to harvest and efforts should be made in order to better

communicate about species forbidden to eat because of food safety concerns related to marine biotoxins. Surveillance is crucial and better means of reporting food poisonings are primordial to have a precise description of where these poisonings took place and which seafood and biotoxins were involved, allowing for development of more specific prevention plans.

6. Conclusion

Marine biotoxins represent a threat for human health. As shown in this review, thousands of poisonings following consumption of seafood contaminated with marine biotoxins were reported in the 21st century, emphasizing the need for carrying on/developing surveillance programs to identify the presence of HABs. In addition to chemical analyses allowing for confirmation of the presence and quantification of marine biotoxins for which standards are available, sensitive and high throughput effect-based in vitro assays should be developed and validated for the screening of these biotoxins and those that are yet unknown or for which standards are not available to protect consumers. Such tests should also become available to less developed countries regarding the occurrence pattern of outbreaks. The interplay between such tests and chemical analytical techniques will allow the identification of thus far unknown toxins and add to our understanding of their occurrence and associated adverse effects.

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CHAPTER 3

Marine neurotoxins: state-of-the-art, bottlenecks and perspectives for mode of action based methods of detection in seafood

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Abstract

Marine biotoxins can accumulate in fish and shellfish, representing a possible threat for consumers. Many marine biotoxins affect neuronal function essentially through their interaction with ion channels or receptors, leading to different symptoms including paralysis and even death. The detection of marine biotoxins in seafood products is therefore a priority. Official methods for control are often still using in vivo assays, such as the mouse bioassay. This test is considered unethical and the development of alternative assays is urgently required. Chemical analyses as well as in vitro assays have been developed to detect marine biotoxins in seafood. However, most of the current in vitro alternatives to animal testing present disadvantages: low throughput and lack of sensitivity resulting in a high number of false negative results. Thus, there is an urgent need for the development of new in vitro tests that would allow the detection of marine biotoxins in seafood products at a low cost, with high throughput combined with high sensitivity, reproducibility and predictivity. Mode of action based in vitro bioassays may provide tools that fulfil these requirements. This review covers the current state-of-the-art of such mode of action based alternative assays to detect neurotoxic marine biotoxins in seafood.

1. Introduction

Marine biotoxins are naturally occurring chemicals produced by microscopic algae. Particular phytoplankton can either induce ecological problems by producing marine biotoxins that cause widespread killing of sea life or affect humans through different routes of exposure (oral, respiratory, skin). Regarding the global warming and its potential effect on the incidence of harmful algal blooms, the presence of marine biotoxins in the environment and the human food chain represents a growing concern for public health.

Marine biotoxins can accumulate either in fish and/or in shellfish (Table 3.1). The consumption of contaminated shellfish can lead to several major types of poisoning: amnesic shellfish poisoning (ASP), diarrhetic shellfish poisoning (DSP), neurologic shellfish poisoning (NSP) and paralytic shellfish poisoning (PSP). A fifth syndrome, azaspiracid poisoning (AZP) has been characterized during the last twenty years [1, 2]. Azaspiracids essentially elicit the same symptoms as observed in DSP but in the mouse bioassay they also induce neurotoxicity [3]. Another syndrome, ciguatera fish poisoning (CFP), differs from the ones cited above as the toxins accumulate in fish instead of shellfish (Table 3.1). Palytoxins (PlTxs) are currently associated with CFP but recent evidence suggests that these toxins should be excluded from this group [4]. NSP is caused by brevetoxins, but most of the other types of poisoning i.e. ASP, PSP, AZP and CFP affect neuronal functioning too. Because marine biotoxins represent a potential threat for consumers EU limits have been established by the European Commission [5-7] (see Table 3.1).

Table 3.1. Main marine neurotoxins in seafood, types of seafood affected and EU regulatory limits in the EU.

Toxin	Main toxins in seafood	Type of seafood affected	Current EU limits
Azaspiracids	AZA-1,2,3	Bivalve shellfish	160 µg AZA eq/ kg SM
Gambierol	Gambierol	Fish	NR
Cyclic imines	13-desmethyl spirolide C 13,19-didesmethyl spirolide Gymnodimine, gymnodimine-C Pinnatoxin-E, F and G	Bivalve shellfish	NR
Palytoxin	PlTx, ovatoxins	Bivalve shellfish, fish	NR
Ciguatoxins	C-CTX-1, C-CTX-2, CTX1B, CTX2, CTX3	Fish	a
Brevetoxins	PbTx-1,2,3,6,7,9,10,COOH, cysteine and glycine metabolites	Bivalve shellfish	NR
Saxitoxin	STX and a wide range of analogues	Bivalve shellfish	800 µg PSP/ kg SM
Tetrodotoxin	TTX	Bivalve shellfish, fish	b
Domoic Acid	DA	Bivalve shellfish, fish	20 mg DA/kg SM
Maitotoxin	MTX	Fish	NR

SM: shellfish meat. NR: Not regulated. a: Fishery products containing biotoxins such as ciguatoxin or muscle-paralysing toxins must not be placed on the market. However, fishery products derived from bivalve molluscs, echinoderms, tunicates and marine gastropods may be placed on the market if they have been produced in accordance with Section VII and comply with the standards laid down in Chapter V, point 2, of that Section [5]. b: Fishery products derived from poisonous fish of the following families must not be placed on the market: Tetraodontidae, Molidae, Diodontidae and Canthigasteridae [5].

The official methods for detecting marine biotoxins in seafood products are currently in vivo assays such as the mouse bioassay (MBA) [6] or chemical methods such as HPLC-UV, LC-FLD and LC-MS/MS [8, 9]. For PSP toxins the official methods are the MBA and the Lawrence method based on LC-FLD detection [9]. As stated in the regulation No 2074/2005, HPLC-UV is the official method of detection for ASP toxins. The MBA assay results in a high number of false positive and negative results, and is considered unethical making development of alternative assays urgently required [10]. In Europe, the use of the MBA for lipophilic toxins is forbidden from 2015 onwards, except for the control of production areas [8].

In Europe, LC-MS/MS techniques such as developed by Gerssen et al. [11] are now inter-laboratory validated as an alternative routine method to the in vivo assays for the detection of lipophilic marine biotoxins in seafood [8, 9]. However, these techniques are expensive and do not allow detection of presently unknown marine biotoxins.

Thus, there is an urgent need for the development of new in vitro tests that would allow the detection of marine biotoxins in seafood products at a low cost, with high throughput combined with high sensitivity, reproducibility and predictivity. Mode of action based in vitro bioassays may provide tools that fulfil these requirements.

The objective of this review is to present an overview of the state-of-the-art on mode of action based assays developed for the detection of marine neurotoxins in seafood products and, based on this, to identify data gaps regarding certain toxins and bottlenecks to be solved in the future when developing more high throughput, specific and sensitive assays. The review starts with a concise overview of the modes of action of the marine neurotoxins of most concern for the modern food chain.

2. Modes of action of marine neurotoxins that may be present in the modern food chain

This section focuses on the modes of action of marine neurotoxins including effects on ion channels and receptors (Figure 3.1). This knowledge can be the basis for the development of mode of action based bioassays.

2.1. Brevetoxins (PbTxs): Mode of action

NSP is caused by PbTxs, produced by Chattonella cf. verruculosa and different species from the Karenia genus. Algal blooms responsible for the production of PbTxs are clearly identifiable because of their specific color, causing the so-called red tide [12, 13]. PbTxs (Figure 3.2) are lipid soluble marine neurotoxins which essentially accumulate in shellfish but also in fish. Ingestion of contaminated seafood leads to gastrointestinal disturbances and to partial paralysis [1].

PbTxs bind to the voltage gated sodium channels (VGSCs) on the site 5. This binding does not lead to blocking but instead to a persistent activation of the channels [14, 15] leading to a prolonged Na^+ entry into the cells. This Na^+ entry will trigger a calcium (Ca^{2+}) influx from the Na^+/Ca^{2+} exchanger inducing excitatory

cellular responses. Eventually, the cells may not be excitable anymore, leading to paralysis [16]. The Ca^{2+} entry induced by PbTx-1 occurs through at least three routes, i.e. the N-Methyl-D-Aspartate (NMDA) receptor ion channel, the L-type voltage gated calcium channels (VGCCs) and the Na^{+}/Ca^{2+} exchanger [17].

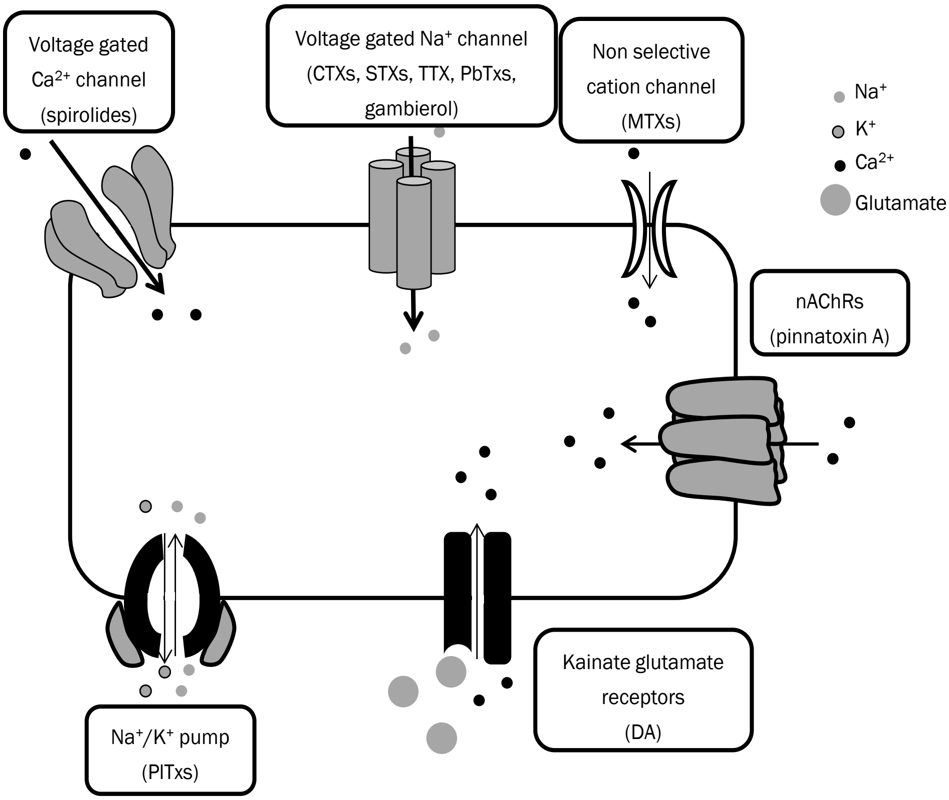

Figure 3.1. Schematic representation of the principal targets of marine neurotoxins. The different targets of marine neurotoxins including ion channels and pump as well as receptors are included.

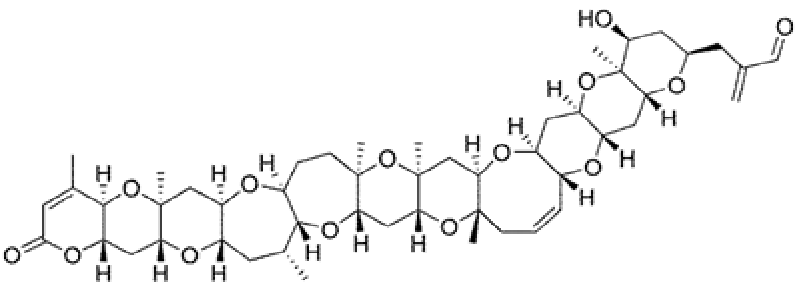

Figure 3.2. Chemical structure of PbTx2.

2.2. Saxitoxins (STXs)/Tetrodotoxins (TTXs): Mode of action

STXs (Figure 3.3) are produced by several algae species from the genera Alexandrium and Gymnodinium and accumulate in shellfish. STXs induce PSP. TTXs (Figure 3.4) are essentially produced by bacteria in the puffer fish but also in other fish species and shellfish [18]. STXs and TTXs cause paralysis that could be fatal in extreme cases.

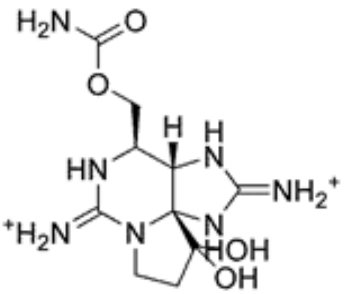

Figure 3.3. Chemical structure of STX.

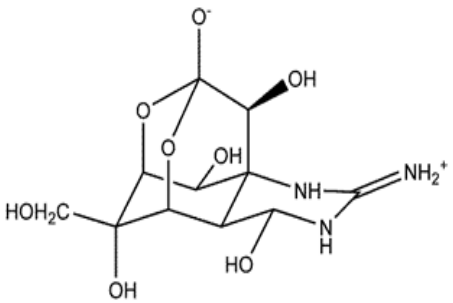

Figure 3.4. Chemical structure of TTX.

Both TTXs and STXs are targeting the VGSC by binding to one of its subunits (site 1) [19]. Under normal conditions VGSCs undergo a conformational change when detecting a change in ion concentration inside the cell, resulting in the opening of the channel and a sodium (Na^+) influx. This induces membrane depolarization and regulates the excitability of the cells. Binding of TTXs and STXs to the VGSCs blocks their ion conductance which prevents membrane depolarization and transmission of the action potential. The blockade of the Na^+ current by these toxins is reversible. Different subtypes of VGSCs exist of which some are sensitive

to TTXs/STXs while others are resistant [20]. TTX was also found to be able to prevent the opening of NMDA receptors and VGCC in neurons thus reducing the Ca^{2+} entry and its neurotoxic consequences [21].

2.3. Palytoxins (PlTxs): mode of action

PlTxs (Figure 3.5) are complex compounds produced by different genera of algae as well as corals [22]. Following ingestion of contaminated fish or shellfish, symptoms vary from chest pain to convulsions and death which may occur in rare cases within minutes after ingestion.

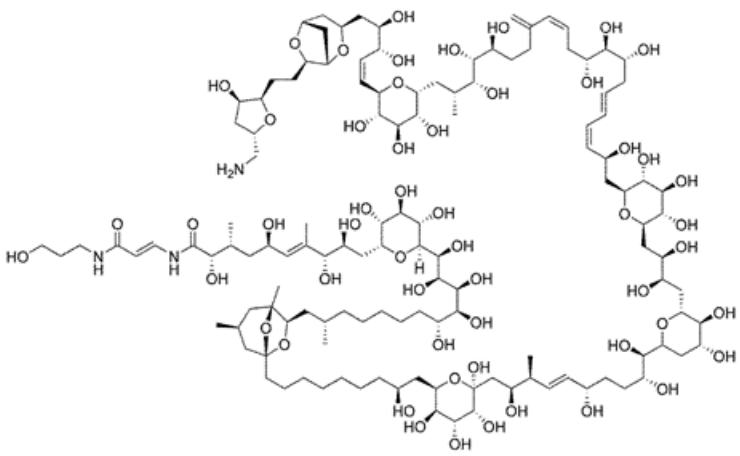

Figure 3.5. Chemical structure of PlTx.

PlTxs bind to the Na^+/K^+-ATPase pump [23, 24]. At its normal state, this pump carries ions against their concentration gradients through the use of ATP exerting a simultaneous efflux of 3 Na^+ and influx of 2 K^+ ions. PlTxs convert the ion pump in a non-selective cation channel allowing ions flowing following their concentration gradients [24]. As a consequence, PlTxs induce a Na^+ influx and a K^+ efflux causing membrane depolarization [25]. The Na^+ influx induces a secondary increase of intracellular Ca^{2+} concentrations, due to the activity of Na^+/Ca^{2+} exchangers, and leads to a further perturbation of ion homeostasis and alteration of the membrane potential, disturbing cell communication in excitable tissues [26, 27]. More in-depth studies on the mode of action of PlTxs have been performed as well. Among them, Vale et al. [28] investigated the role of different mitogen-activated protein kinases (MAPKs) in the cellular

effects induced by PlTxs in cultured neurons including cytosolic [Ca^{2+}] increase, intracellular pH decrease and cytotoxicity. The authors concluded that MAPK pathways are strongly associated with the cytosolic [Ca^{2+}] increase as well as the cytotoxicity induced by PlTxs.

2.4. Ciguatoxins (CTXs): mode of action

CTXs (Figure 3.6) are produced in fish as a result of biotransformation and acid-catalysed spiroisomerisation of gambiertoxins made by the microalga Gambierdiscus toxicus. CTXs cause CFP, a seafood poisoning occurring in tropical areas. This poisoning is mostly characterized by cardiovascular and neurological disturbances such as dizziness, numbness and tingling of the mouth and digits. Recently discovered around the European coasts, these toxins have awakened both public health concerns and scientific interest.

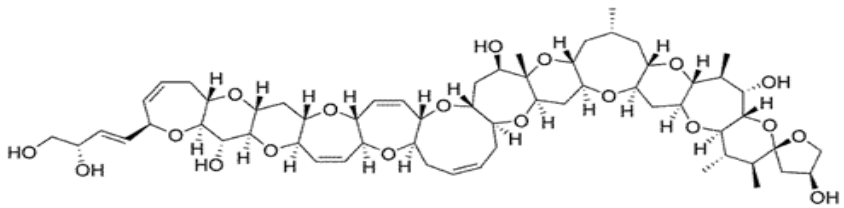

Figure 3.6. Chemical structure of P-CTX1.

The mode of action of CTXs is related to their effect on excitable tissues. Like PbTxs, CTXs bind to the VGSCs on site 5, leading to changes in the gating properties of these channels resulting in enhanced Na^+ entry. The affinity of CTXs for the VGSCs depends on the CTX analogue. For example, CTX1B has an affinity for VGSC that is 30 times higher than that of PbTxs while CTX4A and CTX4B have about the same affinity as PbTxs [29].

2.5. Maitotoxins (MTXs): mode of action

MTXs (Figure 3.7) are produced by Gambierdiscus toxicus [30]. MTXs accumulate in fish inducing CFP and the same symptoms as induced by CTXs. The major event in the mode of action of MTXs is hypercalcaemia. MTXs increase Na^+ and Ca^{2+} entry into both excitable and non-excitable cells [31]. However, it is not yet clear which channels are targeted by MTXs. One idea is that MTXs target non-selective cation channels (NSCCs) [32]. Estacion et al. [33] and Wisnoskey et al. [34] proposed the following mechanism underlying MTX toxicity: the activation

of NSCCs permeable to cations causing a $[Ca^{2+}]i$ increase which is followed by the opening of a cytolytic/oncolytic pore allowing molecules of less than 800 Da to enter the cell and final formation of a glycine-sensitive cytolytic pore leading to cell lysis. Wang et al. [35] investigated how NSCCs are activated by MTXs and identified the activation of Na^+ hydrogen exchangers as playing an important role in MTX-induced toxicity in rat cortical neurons. Na^+ hydrogen exchangers are membrane proteins involved in regulating different cell processes and their over activation in the central nervous system leads to ischemic injuries, stroke and other excitotoxic events [35].

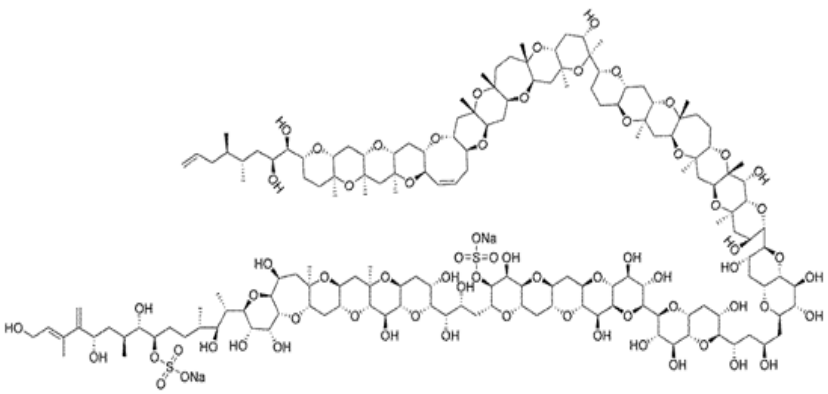

Figure 3.7. Chemical structure of MTX.

2.6. Gambierol: mode of action

Gambierol is produced by Gambierdiscus toxicus [36]. Like MTXs, gambierol (Figure 3.8) is structurally similar to CTXs. Gambierol is thought to affect two types of voltage-gated ion channels: Na^+ [37, 38] and K^+ channels [39, 40]. Symptoms following ingestion of fish contaminated by gambierol are similar to those induced by CTXs implying that gambierol may be also responsible for CFP.

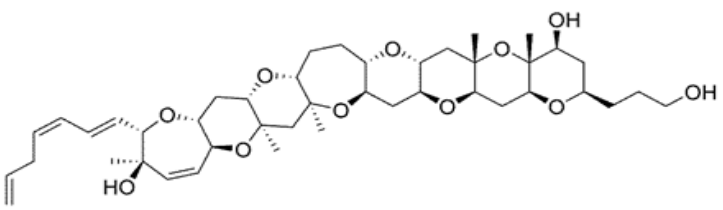

Figure 3.8. Chemical structure of gambierol.

Inoue et al. [38] observed that gambierol inhibited the binding of [3H]PbTx-3 to site 5 of the VGSC indicating that gambierol targets the site 5 of the VGSC. A more recent study from LePage et al. [41] showed that the elevation of $[Ca^{2+}]$ i following exposure to PbTx-2 could be at least partially inhibited by gambierol indicating that gambierol acts as an antagonist of neurotoxin site 5 on neuronal VGSCs. In another study, Louzao et al. [37] showed that gambierol induced a Na^+ depolarizing effect characterized by an excessive influx of Na^+ in human neuroblastoma cells. Gambierol also induced an increase of cytosolic Ca^{2+} in the same neuroblastoma cells when extra Na^+ was added to the medium.

2.7. Azaspiracids (AZAs): mode of action

This group of toxins has been identified by Satake et al. [42] following consumption of AZA contaminated shellfish in the Netherlands in 1995. The marine algae responsible for the production of AZAs is belonging to the Azadinium species. AZA-1 (Figure 3.9), AZA-2 and AZA-3 are the most prevalent AZAs found in shellfish (Table 3.1). Neurological symptoms including convulsions have been observed following exposure to AZAs. The molecular target of AZAs is still unknown.

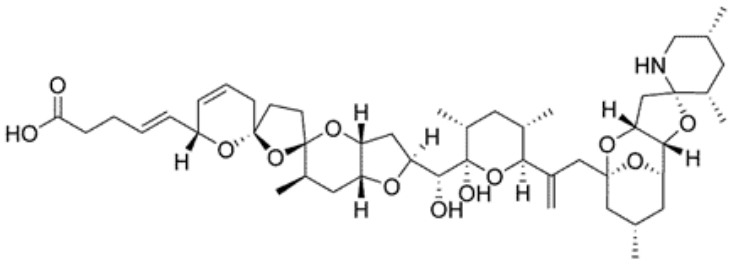

Figure 3.9. Chemical structure of AZA1.

Exposure of human lymphocytes to AZA-2 and AZA-3 led to an increase in cytosolic cyclic adenosine monophosphate levels [43]. Alfonso et al. [44] reported that natural AZA-1 to AZA-5 are able to modulate cytosolic Ca^{2+} levels in human lymphocytes. More recently, Cao et al. [45] found that AZA-1 increased lactate dehydrogenase (LDH) efflux, induced nuclear condensation and stimulated caspase-3 activity in murine neocortical neurons.

Kulagina et al. [46] showed, using a whole-cell patch clamp technique, that the effect of AZA-1 on the synaptic transmission does not involve voltage-gated channels as this biotoxin did not act on the voltage-gated Na^+, K^+ and Ca^{2+}

currents in cultured spinal cord neurons from primary cultures.

2.8. Cyclic imines: spirolides, gymnodimines, pinnatoxins, pteriatoxins: mode of action

Cyclic imines have been detected in the early 1990s when performing routine bioassays with oysters, scallops and mussels. When administered intraperitoneally as in the MBA, cyclic imines induce a cascade of neurological symptoms within a few minutes. Marine biotoxins belonging to the cyclic imine group are macrocyclic compounds with imine and spiro-linked ether moieties. No information has been reported linking cyclic imines to human poisoning [47]. Spirolides (Figure 3.10) are produced by the algae Alexandrium ostenfeldii. Gymnodimines are metabolites of the planktonic dinoflagellate Karenia selliformis [48]. One of the most characterized neurotoxins from the cyclic imine group is pinnatoxin, produced by marine dinoflagellates. The organism producing pinnatoxins is Vulcanodinium rugosum and pteriatoxins are thought to be biotransformed from pinnatoxins in shellfish [49].

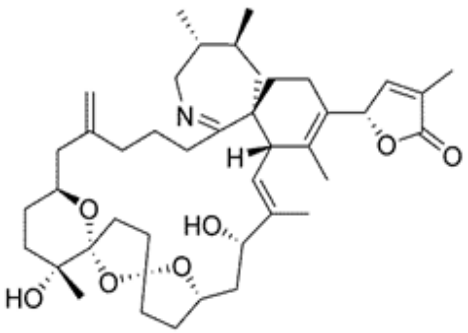

Figure 3.10. Chemical structure of SPX1.

Information on the neurological mode of action of spirolides is quite limited essentially because of the poor availability of pure standards or toxic materials. What is known is that the toxins from the cyclic imines group are inhibiting the nicotinic and muscarinic receptors at the central and peripheral nervous system level as well as at the neuromuscular junctions [50]. Araoz et al. [50] described that pinnatoxin A inhibits various subtypes of nicotinic acetylcholine receptors and selectively interacts with the human neuronal α7 subtype of nicotinic receptors. According to Hu et al. [51] and Uemura et al. [52], spirolides are capable of

weakly activating L-type Ca^{2+} channels.

2.9. Domoic acid (DA): mode of action

DA (Figure 3.11) is produced by the red algae Chondria armata and some marine diatom species of the genus Pseudo-nitzschia. This marine toxin induces ASP following ingestion of contaminated fish or shellfish. Symptoms vary from nausea, vomiting to seizures, coma and eventually death. One of the most important intoxication occurred in Canada in 1987 [53].

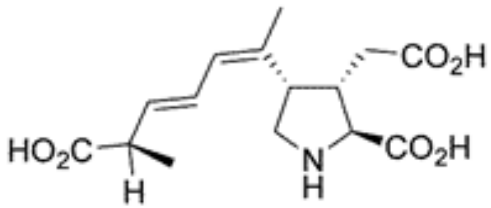

Figure 3.11. Chemical structure of DA.

DA is structurally related to kainic acid which is an analogue of the neurotransmitter and excitatory amino acid L-glutamate. DA is known to be an agonist of the kainate receptor, a non-NMDA ionotropic glutamate receptor [54, 55]. It exerts its toxicity through the activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainic acid subtype of glutamate receptors [56]. Administration of DA in the hippocampus of rats induced a neuronal firing increase [57, 58], indicating that DA induces an excitatory response in neuronal cells.

Qiu et al. [59] studied the involvement and the time-dependent contribution of NMDA, non-NMDA and metabotropic-type glutamate receptors in DA-induced neuronal cell death using rat mixed cortical cell cultures. The results suggested that DA neurotoxicity is governed by the simultaneous involvement of all these glutamate receptor types.

Because glutamate release is mediated through elevation of Ca^{2+}, Berman et al. [60] monitored the intracellular Ca^{2+} accumulation in rat cerebellar granule neurons in vitro following DA exposure and observed a rapid and concentration-dependent elevation of $[Ca^{2+}]i$, responsible for neuronal degeneration. This elevation of $[Ca^{2+}]i$ is known to cause glutamate release that subsequently overstimulates NMDA receptors, leading to cell death [61].

In summary, most marine neurotoxins target either ion channels or specific

receptors. The development of mode of action based assays in which the final endpoint is related to the binding of the marine neurotoxins to ion channels represents a way to follow. Most marine neurotoxins ultimately modulate intracellular Ca^{2+} levels or bind to receptors such as glutamate receptors or acetylcholine receptors. Therefore, monitoring of Ca^{2+} fluxes and/or the binding to glutamate receptors or acetylcholine receptors may prove to present useful endpoints for mode of action based assays for the detection of marine neurotoxins in seafood.

3. Methods for detection of marine neurotoxins in the food chain

At present, the actual method for the detection of most marine biotoxins in seafood relies on the MBA although more and more laboratories are moving towards chemical testing as for marine biotoxins regulated at the European level there are now alternative chemical methods available. Since the 1st of July 2011, the LC-MS/MS method is the reference method for the detection of lipophilic marine biotoxins in Europe. The MBA has a low reproducibility, is not highly-sensitive, gives a high number of false positive and negative results and is highly unethical [6]. Thousands of mice are killed each year for the detection of marine biotoxins in seafood and the MBA will be forbidden from 2015 onwards in Europe for the detection of lipophilic toxins [8]. The LC-MS/MS technique is expensive and does not allow the detection of currently unknown marine biotoxins.

Therefore, effort has been put in developing alternative methods based either on chemical properties of the different marine biotoxins or on their specific modes of action. When developing such assays one should take into account that most marine biotoxins exert their neurotoxicity through the binding to ion channels or receptors as described in the previous section. Because contaminated seafood generally contains more than one marine biotoxin, mode of action based assays will provide an excellent tool for the screening for the presence of mixtures of marine biotoxins and/or of unknown analogues within groups with chemical diversity.

This part of the review will summarize the major types of assays currently available for detection of neurotoxic marine biotoxins including in vivo assays, chemical analyses, biochemical assays and cell-based assays, some of the latter two being already based on modes of action. The different marine neurotoxins

and their methods of detection are summarized in Table 3.2.

Table 3.2. marine neurotoxins, their molecular target and methods of detection in seafood.

Toxin	Molecular target	In vivo or chemical assays	Mode of action based assays
Azaspiracids	?	LC-MS, LC-FLD	NA
Gambierol	Voltage-dependent Na^+ and K^+ channels	LC-MS	NA
Cyclic imines	Muscarinic and nicotinic AChRs, VGCC	LC-MS	Receptor-binding assay
Palytoxin	Na^+/K^+ ATPase pump	MBA, LC-FLD	Neuro-2a
Ciguatoxins	Voltage-dependent Na^+ channels	MBA, LC-MS, ELISA	Neuro-2a
Brevetoxins	Voltage-dependent Na^+ channels	MBA, LC-MS, ELISA	Neuro-2a
Saxitoxin/Tetrodotoxin	Voltage-dependent Na^+ channels	MBA, LC-MS, LC-FLD RIA, ELISA	Receptor-binding assay, neuro-2a
Domoic Acid	Non-NMDA glutamate receptors	LC-MS, LC-UV, ELISA	NA
Maitotoxin	Ca^{2+} channels	LC-MS	Neuro-2a

NA: not available.

3.1. In vivo assays

Two in vivo assays are available for the detection of marine biotoxins in seafood: the mouse or rat bioassay (MBA and RBA). The MBA was developed in Japan and has been adjusted, resulting in different protocols [62, 63]. This in vivo assay consists of intraperitoneally injecting an extract from suspect seafood to mice. The endpoint measured is the mortality of the animals monitored from 24 to 48 hours. Every regulated toxin can be detected through the MBA although different extraction procedures should be used (Table 3.2). The RBA was developed in the early 1980s [64, 65]. This assay consists of feeding rats with suspect seafood and observing them during 16 hours in order to detect any potential diarrheic disorders. This validated assay is used only for the detection of DSPs and AZAs and therefore does not allow for the detection of neurotoxic marine biotoxins [66]. Besides being highly unethical, both in vivo assays give a high rate of false positive and negative results [47]. In addition, different results are obtained

depending on whether the extract is from the whole body of the seafood or from the hepatopancreas [67, 68].

Because of the disadvantages of these in vivo assays, the European Commission has decided to emphasize the need for developing alternative methods. Some countries do not have LC-MS/MS equipment installed at the moment and are therefore still using the MBA for lipophilic marine biotoxins. In Europe, the in vivo tests currently used for the detection of lipophilic marine biotoxins in seafood, i.e. the MBA and RBA, will be forbidden from 2015 onwards, except for the control of production areas [8].

3.2. Chemical analyses

The detection of marine biotoxins in seafood through chemical analyses is mostly based on chromatography techniques (Table 3.2). Chemical techniques are promising but still need to be improved. Pure analytical standards and reference materials are barely available hampering the further development of analytical methods. Weighing the pros and cons of the current in vivo assays, the European Commission [8] stated that the LC-MS/MS method validated at the European level under the coordination of the European Union Reference Laboratory on marine biotoxins should be used as the reference technique for the detection of lipophilic marine biotoxins. However, only well-defined toxins can be detected whereas unknown marine biotoxins and marine biotoxins that are not well defined remain generally undetected by chemical analyses. The method can detect lipophilic marine biotoxins at levels below their allowed limits but there is no routine protocol for marine biotoxins that do not yet have regulatory limits. In Europe only ASP and PSP toxins as well as lipophilic marine biotoxins are regulated and specific regulation apply for TTXs and CTXs [5]. Therefore within Europe there is no inter-laboratory validated LC-MS/MS method routinely applied for the detection of for example PbTxs, PlTxs, CTXs and cyclic imines.

LC-FLD and the HPLC-UV techniques are the official methods for the detection of PSP and ASP toxins, respectively [69]. According to regulation No 1664/2006, STX and any of its analogues for which standards are available should be tested. Despite the fact that the LC-FLD is very sensitive, if results are challenged then the MBA is the method to be used according to the European Commission [69].

3.3. Immuno- and biochemical assays

A number of groups developed immunoassays for the detection of marine

biotoxins. These immunoassays can be divided in radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). Both assays are based on antibodies directed against the marine biotoxin of interest. These antibodies are either labeled with a radioactive isotope (RIA) or linked to an enzyme. Garthwaite et al. [70] developed an ELISA screening system allowing for the detection of toxins causing ASP, NSP and PSP in addition to the classic DSP toxins. This system was shown to be sensitive enough for the detection of ASP, NSP, PSP and DSP toxins at the regulatory levels. Oguri et al. [71] developed a sandwich ELISA assay using mouse monoclonal antibodies (MAbs) that was capable of detecting pacific CTX3C with a limit of detection of about 5 ng/mL and with no cross-reactivity against other marine biotoxins such as PbTx1 and 2, OA or MTX. A comparable method has been developed by Tsumuraya et al. [72, 73] where the authors produced MAbs against pacific CTX1B, CTX3C and 51-hydroxyCTX3C and obtained similar detection limit and specificity as Oguri et al. [71]. Boscolo et al. [74] proposed a sandwich ELISA assay as a promising tool for the detection of PlTx in mussel extracts, algal samples and seawater. The level of detection was low and the limit of quantification even lower than the LC-MS/MS. Neither cross-reactivity with other marine biotoxins nor matrix effects were reported. In addition, this assay had a lower limit of quantification than the LC-MS/MS technique currently implemented at the European level.

Beside immunoassays, receptor-binding assays have been developed for the detection of marine neurotoxins in seafood. These receptor-binding assays can be considered as a first example of mode of action based assays for the detection of marine neurotoxins. Van Dolah et al. [75] developed a receptor assay for the detection of DA using the glutamate receptor 6 kainate receptor situated on the membrane of SF9 insect cells. Recently, Van Dolah et al. [76] conducted a collaborative study on a receptor binding assay for PSP toxins. This assay is based on the ability of contaminated extracts to compete with [3H] STX for the binding to VGSC from rat brain membrane preparations. The reduction in [3H] STX binding is proportional to the amount of toxin present in the seafood. This assay showed a good repeatability and reproducibility and was sensitive enough to detect PSP toxins at the current regulatory limits. Aráoz et al. [77] investigated the suitability of a receptor-binding assay for the detection of cyclic imines based on their competitive inhibition of biotinylated-α-bungarotoxin binding to nicotinic acetylcholine receptors. This assay was shown to adequately detect neurotoxins targeting nicotinic receptors with a high sensitivity and reproducibility. Following

the same principle, Rodriguez et al. [78] developed a nicotinic acetylcholine receptor/Luminex-based assay capable of detecting 13-desmethyl spirolide C in a sensitive way (10-6000 µg/kg of shellfish).

It can be concluded that immunoassays represent an interesting alternative especially because they present a high sensitivity and low limit of detection due to high affinities between antigens and antibodies [79]. The ELISA assay developed by Boscolo et al. [74] for the detection of PlTx in seafood is promising especially because of a high sensitivity, a high specificity and a limit of quantification lower than that of the current technique applied i.e. LC-MS/MS. Finally, the receptor-binding assay developed by Van Dolah et al. [76] has been validated according to the association of analytical communities (AOAC) guidelines and will most likely be put in practice as a routine in the near future.

3.4. Cell-based assays

Cell-based bioassays offer the advantage to study altered cellular or biochemical functions. In most assays developed up to now for the screening of marine biotoxins in seafood the endpoint assessed is general cytotoxicity. These assays could be performed either in cell lines, which present the advantage of not requiring animal use, or in primary cell cultures which still require the sacrifice of experimental animals. In addition to this, while chemical or immunological analysis allows for the detection of known toxins, cell bioassays offer the advantage to permit the detection of unknown toxins.

Louzao et al. [80] developed a fluorometric technique using human neuroblastoma BE(2)M17 cells. As PSP toxins induce changes in membrane potential due to the blockade of VGSCs, the authors used bisoxonol as a probe that distributes across the plasma membrane in a potential-dependent manner. The assay was able to detect PSP toxins in contaminated extracts with a limit of detection of 0.2 µg STX di-HCl-eq/100 g of seafood which is much lower than the regulatory limit threshold of 400 ng/mL (equivalent to 80 µg STX di-HCl-eq/100 g of seafood). This assay is therefore specific and sensitive enough to detect PSP toxins at the current regulatory level.

Kogure et al. [81] developed an assay capable of detecting marine biotoxins activating VGSCs. In this assay the mouse neuroblastoma cell line neuro-2a is exposed to pure toxins or extracts containing VGSC-activating marine biotoxins and the final read out is cell viability, assessed via the MTT assay (Figure 3.12). This assay presents several advantages enabling its use as a first screening

assay of marine biotoxins in seafood as it is relatively inexpensive compared to other assays developed, able to detect marine biotoxins at the current regulatory levels, and it uses an easy readout i.e. a colorimetric endpoint (MTT). However, cell viability can be altered by multiple factors including culture conditions and other toxins present in extracts leading to false positives. Therefore cell viability may not be the most appropriate endpoint for such an assay.

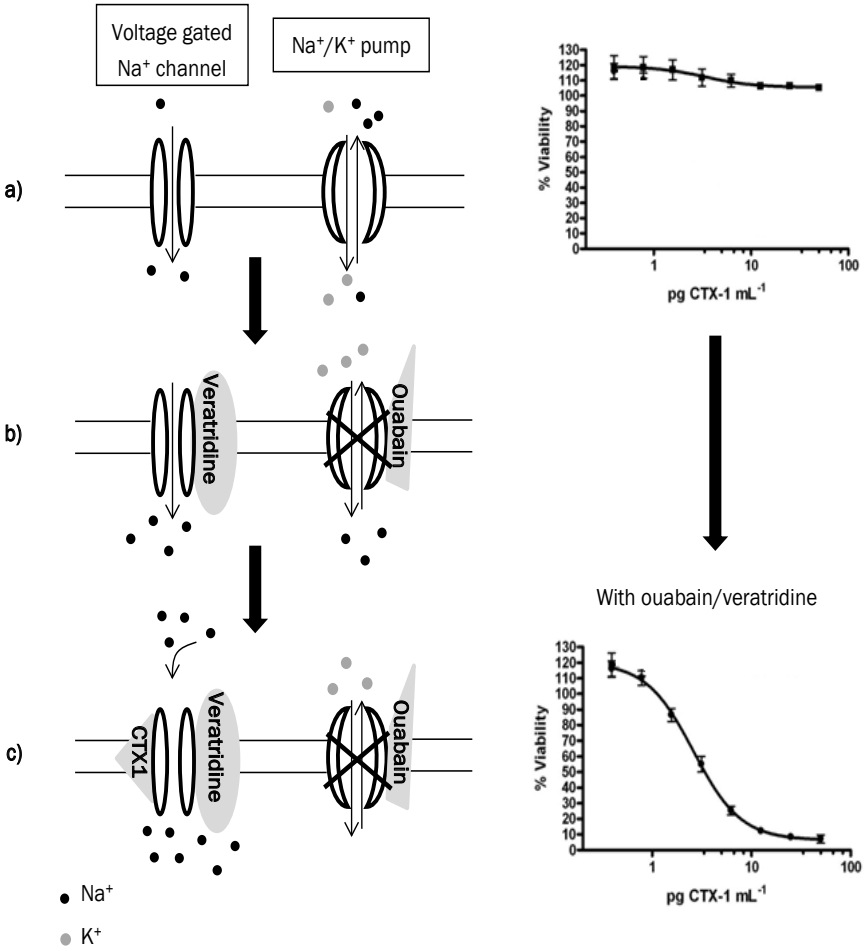

Figure 3.12. Schematic representation of the neuro-2a assay. a) At the normal state the VGSC allows the entry of Na^+ and the Na^+/K^+ ATPase pump permits the outflow of 3 Na^+ and the entry of 2 K^+ to maintain cell membrane potential. CTX1B (CTX-1) alone enhances Na^+ entry in the cells but this does not decrease cell viability since the Na^+/K^+ ATPase pump counteracts the increase of Na^+. b) Veratridine induces permanent activation the VGSC and ouabain blocks the Na^+/K^+ ATPase pump, leading to an increase of intracellular Na^+ levels inducing limited cytotoxicity (less than 20% decrease in MTT result). c) In the combination with veratridine (0.01 mM) and ouabain (0.1 mM), CTX1B dramatically increases intracellular Na^+ levels leading to cytotoxicity (EC_{50} = 3 pM). Cell viability curves are modified from Caillaud et al. [89].

Manger et al. [82] developed a method based on flow cytometry on neuro-2a cells incubated with fluorescent voltage-sensitive dyes. This assay aims at detecting STXs and improving the sensitivity of the neuroblastoma assay described above. Adding flow cytometry to the classic neuroblastoma assay shortened analysis times. Instead of measuring the cell viability, this additional technique allowed to study a functional endpoint, i.e. changes in membrane potential. Improvements such as the inclusion of changes in membrane potential represents an important advance in developing an accurate and sensitive in vitro alternative. Although Manger et al. [82] focused on the detection of STXs, other marine neurotoxins having similar modes of action will also be detected with this assay.

Bovee et al. [83] developed a tailored microarray platform for the detection of marine biotoxins in seafood. The authors selected 17 genes differentially regulated in human intestinal Caco-2 cells upon exposure to AZA-1 and DTX-1. Five out of the 17 genes showed clear signals enabling fingerprinting AZA-1 and DTX-1 and therefore allowing their detection in seafood.

The combination of different cell lines for the detection of multiple marine biotoxins has been investigated by Sérandour et al. [84], and Ledreux et al. [85]. The authors aimed at pre-validating cell-based assays for the screening of shellfish extracts contaminated by lipophilic marine biotoxins. This integrated testing strategy included three cell lines, i.e. a human liver cell line (HepG2), a human intestinal cell line (Caco2) and a mouse neuroblastoma cell line (neuro-2a). These three cell lines have been chosen to cover the main target organs for marine biotoxins. Cytotoxicity was chosen as endpoint. This assay adequately detected certified reference calibration solutions of OA, AZA-1 and PTX-2.

4. Bottlenecks and data gaps

At the current state-of-the-art several bottlenecks and data gaps still exist in the development of alternative testing strategies for the detection of marine neurotoxins, and these can be summarised as follows. An overview of these bottlenecks and data gaps of in vitro assays is presented in Table 3.3.

Receptor-binding assays are capable of detecting toxins at the regulatory levels but only allow for the detection of the toxin that will interfere with the probe selected for monitoring receptor activity. Thus these assays are inappropriate for the detection of toxins that do not bind to these receptors or unknown toxins present in seafood extracts containing mixtures of marine biotoxins. In addition,

some of the receptor-binding assays require the use of membrane preparations of rat brain cells and are therefore not suitable as in vitro alternatives to in vivo assays.

Table 3.3. Overview of the bottlenecks and data gaps of in vitro assays for the detection of marine neurotoxins in seafood.

Technique	Bottlenecks/data gaps
Chemical analyses	Not capable of detecting unknown toxins present in the seafood. No routine protocol for toxins that do not yet have regulatory limits. Pure analytical standards and reference materials barely available or expensive.
Receptor-binding assay	Many probes required to detect a wide range of marine neurotoxins. No detection of unknown marine neurotoxins. Some assays require animal use. Reference materials barely available or expensive.
Cell-based assays	Most of them measure cytotoxicity while cytotoxicity can be due to external factors. Limited number of modes of action covered. Lack of specificity but suitable as screening assays. Reference materials barely available or expensive.

Most cell-based bioassays for the detection of marine biotoxins rely on cytotoxicity as final readout [81, 84, 85]. Despite the fact that in these assays cytotoxicity occurs in response to exposure to marine biotoxins, it yields a risk that the cytotoxicity is not caused by marine biotoxins but due to external factors (culture conditions, mixture of chemicals, other compounds present in the extracts). Moreover, no international standard operation procedure has been validated at the moment. Other cell-based assays detect functional endpoints such as changes in membrane potential, limiting the risk of false positive results. Cell bioassays developed so far aim at detecting essentially PSP toxins targeting VGSCs and have been testing for cytotoxicity and only a limited number of modes of action. This could be partly due to the lack of reference material commercially available and when available, these toxins are expensive. Because a large number of marine neurotoxins target ion channels or neurotransmitter receptors, one should take this into account when designing new mode of action

based assays for the screening of marine neurotoxins. To be high throughput, such assays should ultimately express a large variety of ion channels and neurotransmitter receptors. Endpoints such as receptor binding and ion flux measurement or changes in membrane potential are most likely to be relevant for such assays. Finally, because seafood often contains mixtures of marine biotoxins with different modes of action integrated testing strategies (i.e. a range of assays) are promising approaches to cover a wide range of modes of action of marine biotoxins.

Mode of action based bioassays lack specificity. However, specificity is not the first requirement for such bioassays that are expected to be used in future screening programmes as a first Tier to detect possible contaminated samples that will then require further analytical testing such as LC-MS/MS in second Tier testing. For this first Tier testing, one should develop an assay with a broad sensitivity and specificity to allow for the detection of a wide range of marine biotoxins. Identification and quantification will follow in second Tier testing for only the samples that test positive in the bioassays. Cell-based assays are in most cases less expensive and more practical than LC-MS/MS for a first screening tool.

Despite the fact that a couple of bioassays are available for the detection of marine biotoxins in seafood products, there is still a long way to go to either validate these assays or develop new high throughput techniques that are highly sensitive and capable of detecting known marine biotoxins at the levels established by regulatory authorities as well as unknown marine biotoxins at a level that would not cause any harmful effect to humans.

Future studies should focus on the identification of molecular biomarkers using for example omics approaches, as proposed by Rossini [86] and Hogberg et al. [87] and put into practice by Bovee et al. [83]. This will not only allow for high throughput screening of marine biotoxins but will also provide more insight in the modes of action of these toxins which are for some of them still not completely known.

Furthermore, it is of interest to note that no correlation is observed when comparing the LD_{50} obtained in the in vivo MBA following exposure to different marine biotoxins and their EC_{50} in the in vitro neuro-2a assay (Figure 3.13). One reason for this discrepancy could be that LD_{50} reported in different studies vary and thus be inaccurate [47]. This is not due to gender or selected mouse strain used but most likely to handling of mice and source and handling of the

toxins [88]. Another reason for the discrepancy between in vivo LD_{50} and in vitro EC_{50} data could be that the in vitro models do not take absorption, distribution, metabolism and excretion (ADME) data into account. To overcome this apparent bottleneck of in vitro studies the combination of the in vitro studies with computer based modelling of the ADME processes by so-called physiologically based kinetic modelling of marine biotoxins may prove a way forward to address this issue.

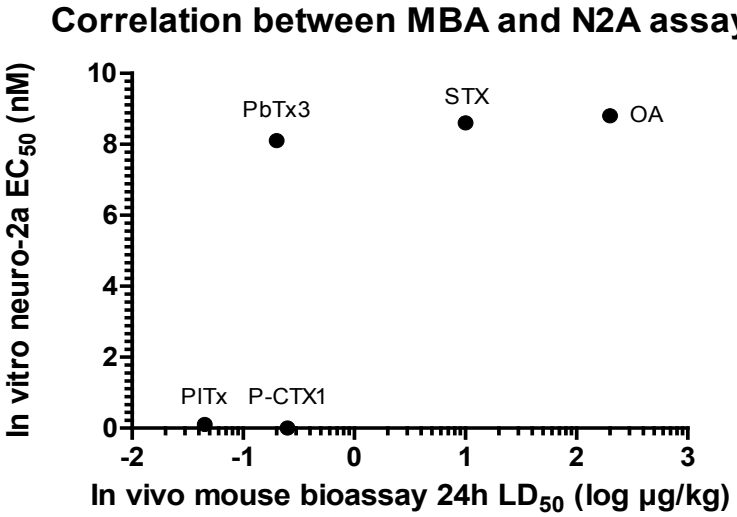

Figure 3.13. Comparison of the LD50 for selected marine biotoxins in the mouse bioassay and their EC_{50} in the neuro-2a assay. Neuro-2a EC_{50} values were obtained from Cañete and Diogène [90] and Caillaud et al. [89]. Intraperitoneal mouse bioassay LD_{50} values were obtained from various sources [91-95]. No correlation is observed.

Another issue to include in future development of alternative in vitro assays is the fact that each mode of action based assay is currently focusing on a unique mode of action. One should work on the development of a model covering a broad range of modes of action. Based on the modes of action described in this review, the ideal system would be capable of detecting modulators of different ion channels and of different specific receptors such as nicotinic acetylcholine receptors. To this end, neuronal cells expressing ion channels and receptors targeted by marine neurotoxins as well as cardiomyocytes also expressing a wide variety of different ion channels may represent promising model systems for the screening of marine neurotoxins in seafood. However, one should be careful when designing such assays as there is a multitude of ion channel subtypes and marine biotoxins can target a specific one whereas the model system of interest

may contain another subtype, thus providing a basis for the occurrence of false negatives.

Finally, mode of action based assays can especially be used as screening assays to detect whether an extract may contain marine biotoxins. Further chemical analysis is still needed to confirm and quantify marine biotoxins present in seafood.

5. Summary and perspectives

Even when not classified as neurotoxins because of a primary mode of action on organs different from the neurological system, a wide variety of marine biotoxins induces neurological symptoms. It is possible to group some of the toxins together, based on their mode of action: acting on ion channels or binding to specific receptors involved in neuronal processes. Based on these specific properties, mode of action based new assays seem to be a promising way forward to detect marine biotoxins in seafood. Given that at present most mode of action based cellular assays use cytotoxicity as the readout there is ample room for development of more specific mode of action based cellular bioassays.

From 2015 onwards, the use of animals for the detection of marine biotoxins in seafood products will be banned in the EU, except for the control of production areas [8]. Thus, there is an urgent need for the development of new in vitro tests that would allow the detection of marine biotoxins in seafood products at a low cost, with high throughput combined with high sensitivity, reproducibility and predictivity. Mode of action based in vitro bioassays may provide tools that fulfil these requirements.

Most mode of action based assays presently available allow for the detection of PSP toxins targeting the VGSCs and are not suitable for the screening of extracts containing mixtures of also other marine biotoxins. Other assays still need to be developed and validated to determine whether they are capable of detecting these other marine biotoxins at or below their regulatory limits.

Cell based bioassays present multiple advantages over currently available in vivo and chemical assays including the fact that they detect functional endpoints, do not require animal sacrifices and provide the potential to also detect as yet unidentified marine biotoxins based on their mode of action. However, these assays are primarily based on cytotoxicity instead of mode of action and thus sensitive to interference and false positives. A thorough knowledge of the

intracellular targets of marine biotoxins is important in the development of in vitro alternatives. For some marine neurotoxins the present knowledge on modes of action is still too limited and more research has to be done. Future studies should also focus on the identification of molecular biomarkers for example by using omics technologies.

In the present overview the data gaps and future perspectives and challenges to be tackled to further facilitate and validate the use of alternative testing strategies to replace the MBA for the detection of marine neurotoxins were defined.

Acknowledgements

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CHAPTER 4

In vitro detection of cardiotoxins or neurotoxins affecting ion channels or pumps using beating cardiomyocytes as alternative for animal testing

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Abstract

The present study investigated if and to what extent murine stem cell-derived beating cardiomyocytes within embryoid bodies can be used as a broad screening in vitro assay for neurotoxicity testing, replacing for example in vivo tests for marine neurotoxins. Effect of nine model compounds, acting on either the Na^+, K^+, or Ca^{2+} channels or the Na^+/K^+-ATPase pump, on the beating was assessed. Diphenhydramine, veratridine, isradipine, verapamil and ouabain induced specific beating arrests that were reversible and none of the concentrations tested induced cytotoxicity. Three K^+ channel blockers, amiodarone, clofilium and sematilide, and the Na^+/K^+-ATPase pump inhibitor digoxin had no specific effect on the beating. In addition, two marine neurotoxins i.e. saxitoxin and tetrodotoxin elicited specific beating arrests in cardiomyocytes. Comparison of the results obtained with cardiomyocytes to those obtained with the neuroblastoma neuro-2a assay revealed that the cardiomyocytes were generally somewhat more sensitive for the model compounds affecting Na^+ and Ca^{2+} channels, but less sensitive for the compounds affecting K^+ channels. The stem cell-derived cardiomyocytes were not as sensitive as the neuroblastoma neuro-2a assay for saxitoxin and tetrodotoxin. It is concluded that the murine stem cell-derived beating cardiomyocytes provide a sensitive model for detection of specific neurotoxins and that the neuroblastoma neuro-2a assay may be a more promising cell-based assay for the screening of marine biotoxins.

Introduction

During the last two decades, the in vitro embryonic stem cell test (EST), in which murine D3 cells are induced to differentiate into beating cardiomyocytes formed in attached embryoid bodies (EBs), has been successfully implemented and validated by the European Centre for the Validation of Alternative Methods (ECVAM) for the assessment of embryo toxicity [1]. Where embryo toxicity is assessed by the effect of a compound on the differentiation process, the present study uses the beating cardiomyocytes within EBs as a model to study effects of neurotoxic compounds. The mechanism behind the contractions of the cardiomyocytes involves Na^+, Ca^{2+}, K^+ channels and the Na^+/K^+-ATPase pump. These channels and pump are also involved in the generation of the action potential in neuronal cells and are important for a wide range of physiological processes, including intracellular messaging, regulation of cell volume, regulation of gene expression, synaptic transmission, and cardiac excitation-contraction coupling [2]. The blockade or opening of these ion channels results in a disturbance of the ion homeostasis that in turn affects the physiology and action potentials of the cells. Figure 4.1 describes which channels are involved in the generation of action potentials both in cardiac and neuronal cells [3]. Although action potentials in the heart (both in pacemaker cells and in cardiomyocytes) and neurons are driven by similar ionic fluxes (Na^+, K^+ and Ca^{2+}), different ion channel subtypes exist, resulting in different specificity and sensitivity towards different neurotoxins [4]. However, despite the differences, murine embryonic stem cell-derived beating cardiomyocytes might be a promising model for the detection of neurotoxic compounds, including marine neurotoxins, because Na^+, Ca^{2+} and K^+ channels and the Na^+/K^+-ATPase pump are the target of a wide range of neurotoxic compounds [5-7]. Marine biotoxins are naturally occurring chemicals produced by microscopic algae. They accumulate in fish and shellfish and therefore represent a threat for consumers. The current standard for the detection of marine biotoxins in seafood is the in vivo mouse bioassay (MBA), in which mice are injected with sample extracts and death is the final readout. More recently, a chemical analytical LC/MS-MS method was EU approved/accepted but many countries still use the MBA, as the chemical analysis method is not able to detect all known toxins and misses unknown toxins. Extensive information on marine biotoxins can be found in recent reviews [8, 9]. In line with the 3R concept of Russell and Burch, alternative in vitro assays to replace in vivo

testing are urgently needed as in vivo tests are considered as highly unethical [10]. In the case of marine biotoxins, the mouse test will be forbidden from 2015 onwards, except for the control of production areas where seafood produced is intended for future consumption [11].

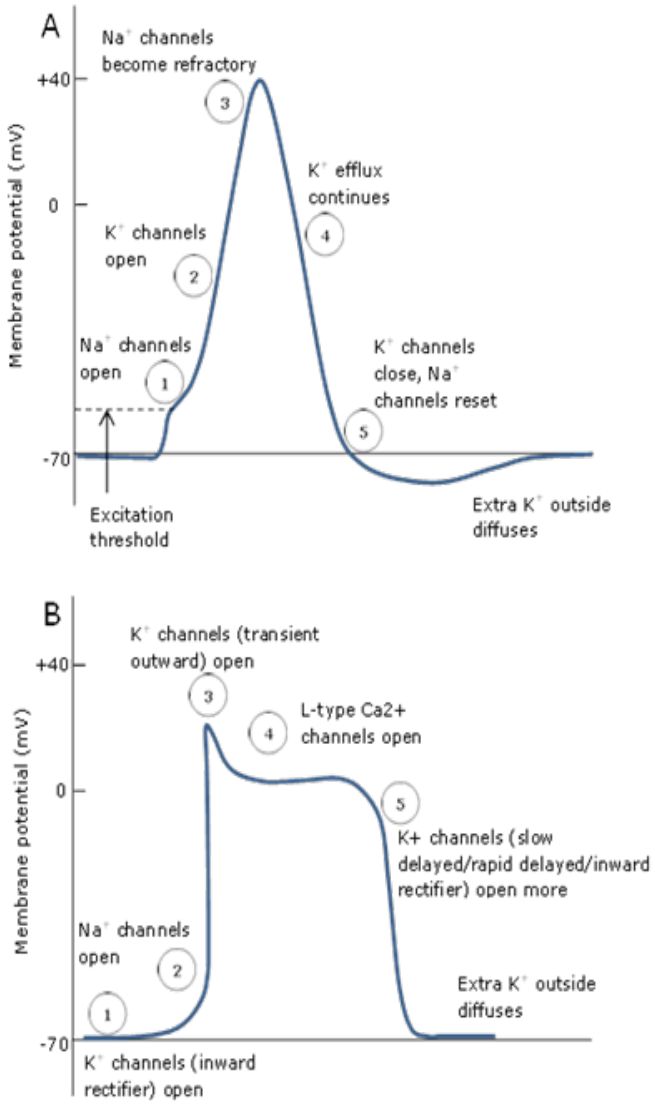

Figure 4.1: Overview of ion channels and fluxes involved in the generation of action potentials in A) neuronal cells and B) cardiomyocytes (based on [2, 3]). A) A stimulus increases the membrane potential above the excitation threshold until +40 mV thanks to Na^+ influx. At +40 mV an efflux of K^+ ions brings the membrane potential back to -70 mV. B) K^+ and Na^+ influxes raise the membrane potential from -70 to +20 mV. Na^+ channels close and L-type Ca^{2+} channels open, causing a plateau. K^+ channels open decreasing the membrane potential back to its initial value of -70 mV.

Maltsev et al. (1994) showed that all basic cardiac-specific channels are present in murine embryonic stem cell-derived cardiomyocytes [12]. Since neurons and cardiomyocytes share many ion channels [13] and a wide range of neurotoxic compounds are known to act on ion channels, we hypothesized that beating cardiomyocytes might be suitable as an in vitro tool to detect the potential neurotoxic effects of compounds, including marine neurotoxins. Based on these considerations the aim of the present study was to assess whether and to what extent embryonic stem cell-derived cardiomyocytes could be used as an in vitro assay for the screening of neurotoxic compounds. To this end cardiomyocytes formed in EBs were exposed to different neurotoxins, using the contractility (i.e. the inotropy) of the cardiomyocytes within the EBs as a read-out. Nine model neurotoxins were selected that are known to affect either one of the three types of channels or the Na^+/K^+-ATPase pump (Tab. 4.1). In addition to these nine model neurotoxins, two commercially available pure marine neurotoxins were tested: saxitoxin (STX) and tetrodotoxin (TTX) (Na^+ channel blockers) in order to establish whether this assay may be of value as a replacement of the in vivo assays currently used for the screening of marine biotoxins in seafood. Moreover, the nine model compounds and STX and TTX were also tested in the neuroblastoma neuro-2a assay in order to compare the sensitivity of the embryonic stem cell-derived cardiomyocytes to the sensitivity of the neuro-2a cells, as the latter is currently regarded as a promising cell-based assay for the screening of marine biotoxins in seafood [8, 14].

Materials and Methods

Chemicals

Amiodarone, clofilium, digoxin, diphenhydramine, isradipine, ouabain, sematilide, verapamil, and veratridine were purchased from Sigma-Aldrich (St. Louis, USA) and dimethylsulfoxide (DMSO) was obtained from Acros Organic (New Jersey, USA). STX was purchased from the National Research Council (Montreal, Canada) and TTX from Latoxan (Valence, France). Compound stock solutions were prepared in DMSO.

Table 4.1. Inhibition of beatings in contractile embryonic stem cell-derived cardiomyocytes and effect on the viability in the neuro-2a assay.

Compound	Mode of action	References[a]	% cell viability at the noted drug concentration (differentiated cardiomyocytes)[b]	EC_{50} differentiated cardiomyocytes[c]	EC_{50} neuro-2a[c]
DPH	Na^+ channel blocker	[15, 16]	150 μM: 104 ± 14	45 μM	> 100 μM
Veratridine	Na^+ channel opener	[17, 18] (EC_{50}: 85 μM)	100 μM: 114 ± 6	35 μM	90 μM
Isradipine	Ca^{2+} channel blocker	[19]	100 μM: 113 ± 23	15 μM	> 100 μM
Verapamil	Ca^{2+} channel blocker	[20, 21] (EC_{50}: 19 μM)	1 μM: 106 ± 10	100 nM	190 nM
Sematilide	K^+ channel blocker	[22]	500 μM: 95 ± 10	> 400 μM	> 300 μM
Clofilium	K^+ channel blocker	[23]	500 μM: 51 ± 0.25	207 μM due to cytotoxicity	150 μM
Amiodarone	K^+ channel blocker	[24]	60 μM: 54 ± 1	> 60 μM	80 μM
Ouabain	Na^+/K^+ AT-Pase blocker	[25] (EC_{50}: 370 μM)	600 μM: 86 ± 4	257 μM	220 μM
Digoxin	Na^+/K^+ ATPase inhibitor	[26]	160 μM: 64 ± 4	> 150 μM	> 100 μM
Saxitoxin	Na^+ channel blocker	[27]	No cytotoxicity up to 1 μM	4 μM	With o/v: 11 nM
Tetrodotoxin	Na^+ channel blocker	[28]	No cytotoxicity up to 10 μM	10 μM (with veratridine)	With o/v: 10 nM

[a]The references stand for the modes of action of the selected model compounds and the EC_{50} values are mentioned when available. [b]The percentage of cell viability was determined at concentrations above those which elicited effects on beatings of cardiomyocytes following a 24 hour exposure to the compounds. [c]The EC_{50} values were calculated using a non-linear regression model. o/v: ouabain/veratridine.

Cell lines and cell culture

The murine-derived embryonic stem cell line D3 was kindly donated by Johnson & Johnson (Beerse, Belgium). The cells were cultured in flasks of 25 cm^2 (Corning Inc., Cambridge, USA) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Breda, The Netherlands) supplemented with 20% (v/v) heat inactivated fetal calf serum (FCS) (BioWhittaker, Maryland, USA), 1% (v/v) non-essential amino acids (Invitrogen), 50 U/mL penicillin/50 µg/mL streptomycin (P/S) (Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.1 mM ß-mercaptoethanol (Sigma-Aldrich). To prevent cell differentiation, 1000 U/mL murine leukemia inhibitory factor (Sigma-Aldrich) was added to the medium when cells were seeded. The cells were cultured in a humidified atmosphere (37 °C, 5% CO_2) and subcultured three times a week. The cells were detached when reaching 80% confluence using non-enzymatic dissociation buffer (Sigma-Aldrich).

Neuro-2a cells (LGC standards, Middlesex, UK) were cultured in flasks of 75 cm^2 using 10% FBS/Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 1% 5000 U/mL penicillin/5 mg/mL streptomycin (P/S) (Invitrogen), 2 mM L-glutamine (Invitrogen) and 1 mM sodium pyruvate (Sigma-Aldrich). Cells were cultured at 37 °C, 5% CO_2 and subcultured 3 times per week. The cells were detached when reaching 80% confluence using trypsin (Sigma-Aldrich).

Embryonic stem cell test

The embryonic stem cell test was performed as described previously [29] with some minor modifications. For the differentiation process, hanging drops of 20 µl cell suspension (3.75.104 cells/mL) were prepared at day 0 on the cover of 96-well plates. Phosphate buffered saline (250 µL) was put in each well in order to maintain humidity and prevent evaporation of the hanging drops. Cells in hanging drops were allowed to differentiate for 3 days in the humidified atmosphere (37 °C, 5% CO_2), and at day 3, the cell aggregates formed (called EBs) were transferred to bacterial petri dishes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) and incubated for 3 days. At day 5, the EBs were plated in 96-well plates (Corning, 1 EB/well) and incubated for 5 days. The cardiomyocytes started beating on day 10.

At day 13, when most EBs have beating areas, beating EBs were incubated with the neurotoxic compounds for 1 h (37 °C, 5% CO_2). Ten beating EBs per concentration were exposed to the different compounds selected (final DMSO

solvent concentration of 0.25%) and the number of remaining beating EBs after one hour incubation was scored by visual inspection (beating arrest). Subsequently, cells were washed and the medium was replaced by medium without the test compound, and the EBs were incubated for an additional hour after which the contractility of the EBs was assessed again to evaluate recovery. At least three independent experiments with six replicates were performed per concentration for each compound. In the case of STX, five instead of ten EBs were exposed and the number of beating EBs were scored after 20 hours of incubation instead of one hour. The control for STX was a solution of HCl (0.003 M) as commercial STX is dissolved in HCl. Because TTX alone did not affect the beatings and TTX, being a sodium channel blocker, has an opposite mode of action as veratridine which acts as a sodium channel activator, two experimental designs were tested to evaluate whether TTX could prevent beating arrest following veratridine exposure: ten EBs were exposed to 10 µM of TTX for 5 minutes and then exposed to 100 µM of veratridine for one hour and in another experiment, ten EBs were exposed to 100 µM of veratridine for 1 h followed by an exposure to 10 µM of TTX for an additional hour.

Cell viability

Cell viability was assessed with the WST-1 assay by measuring mitochondrial activity (Roche, Woerden, The Netherlands) for embryonic stem cell-derived cardiomyocytes. The viability of the cardiomyocytes within EBs was assessed 24 h after exposure to the different compounds and vehicle. To this end, 20 µL of WST-1 solution was added to each well containing 200 µL of medium. After incubation for 3 h (37 °C, 5% CO_2), the absorbance was determined spectrophotometrically at 450 nm. The mitochondrial activity was expressed as percentage of the average of the vehicle control (DMSO solvent).

Neuro-2a assay

Cells were grown for 24 hours and subsequently exposed for 24 hours to the marine neurotoxins with and without a combination of ouabain/veratridine. Concentrations of ouabain/veratridine inducing about 80% cytotoxicity were selected, in order to evaluate the toxin's ability to oppose or prevent the cytotoxicity induced by this Na^+ channel opener (veratridine) and Na^+/K^+-ATPase pump blocker (ouabain). Cell viability of neuro-2a cells was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

(Sigma-Aldrich) measuring the mitochondrial activity. Absorbance was read on a spectrophotometer at 570 nm and the mitochondrial activity was expressed as percentage of the average of the vehicle control (DMSO).

Data analysis

In this study, the EC_{50} of a compound is the concentration at which 50% of the maximum inhibiting effect on the contractility of the cardiomyocytes was induced. The EC_{50} values and their 95% confidence intervals (CI) were calculated using GraphPad Prism (San Diego, CA). When no inhibition of the contractility occurred, no EC_{50} value could be calculated (Tab. 4.1).

For the establishment of dose-response curves and the determination of EC_{50} values for the neuro-2a assay, cell viability for each concentration of each model compound or marine neurotoxin after a 24 hour exposure was measured at least in duplicate. In this assay, the EC_{50} is the concentration at which 50% of the cytotoxicity occurs following exposure to the model compounds and the concentration at which 50% of the cytotoxic effect caused by ouabain/veratridine is opposed following exposure to the marine neurotoxins.

Results

With the embryonic stem cell test (EST), the proportion of beating EBs is the read-out and cell viability was assessed by the WST-1 assay as the MTT yielded unreliable results (due to loss of cells during the removal of the medium), while in the neuro-2a assay, the cell viability is the read-out and was assessed by the MTT assay as the neuro-2a cells were well attached to the bottom of each well. Moreover, in the neuro-2a MTT assay, STX and TTX and the nine neurotoxic model compounds were also tested in combination with a dose of ouabain/veratridine that caused a 80% decrease in cell viability in order to determine whether the toxin was able to oppose the effect, i.e. to counteract the cytotoxicity induced by ouabain/veratridine [14]. However, none of the nine neurotoxic model compounds was able to oppose the cytotoxicity induced by ouabain/veratridine, and these results are not further discussed or shown.

Na^+ channels

The Na^+ channel blocker diphenhydramine inhibited beating of cardiomyocytes at and above a concentration of 25 µM (EC_{50} = 45 µM, Tab. 4.1). A concentration

of 100 µM elicited beating arrests of all EBs (Fig. 4.2A). This beating arrest was completely reversible, as upon refreshing the medium, all EBs started to beat again. Veratridine induced a concentration-dependent decrease of the proportion of beating EBs at concentrations at and higher than 25 µM (EC_{50} = 35 µM, Tab. 4.1). When the cells were exposed to 60 and 100 µM, all EBs stopped beating. After a recovery period of 1 h, every EB that stopped beating after exposure to 60 µM of veratridine started beating again, while for 100 µM 80% of the EBs recovered (Fig. 4.2B). The cell viability was not affected by both compounds according to the results obtained with the WST-1 assay (Tab. 4.1).

Figure 4.3A and 4.3B show that in the neuro-2a assay, diphenhydramine slightly decreased cell viability while veratridine induced a concentration-dependent decrease in cell viability (EC_{50} = 90 µM, Tab. 4.1)
The embryonic stem cell-derived cardiomyocytes are thus more sensitive to diphenhydramine and veratridine than the neuro-2a cells.

Ca^{2+} channels

Figure 4.2C shows that the Ca^{2+} channel blocker isradipine at concentrations of 20 µM and higher caused beating arrests in every exposed EB (EC_{50} = 15 µM, Tab. 4.1). After exposure to up to 30 µM isradipine, all EBs recovered after refreshment of the medium. After exposure to 60 or 100 µM israpidine, 50% of the EBs recovered after medium refreshment. The other Ca^{2+} channel blocker, verapamil, induced beating arrests at concentrations from 100 nM onwards. Where 100 nM of verapamil induced beating arrest in half of the EBs, all EBs stopped beating at 300 and 1000 nM (EC_{50} = 100 nM, Tab. 4.1). All EBs recovered at 100 nM, while 50% recovered at 300 nM and 600 nM. At concentrations of 1000 nM and higher, the cells did not recover anymore (Fig. 4.2D). The WST-1 tests showed that cell viability was not affected by the tested concentrations of isradipine or verapamil (Tab. 4.1).

Figure 4.3C and 4.3D show that in the neuro-2a assay, isradipine up to 100 µM had no effect on the cell viability, while verapamil at concentrations of 30 nM and higher induced a decrease in cell viability.

The determined EC_{50} values show that the embryonic stem cell-derived cardiomyocytes are more sensitive for isradipine than neuro-2a cells, and also slightly more sensitive for verapamil (Tab. 4.1).

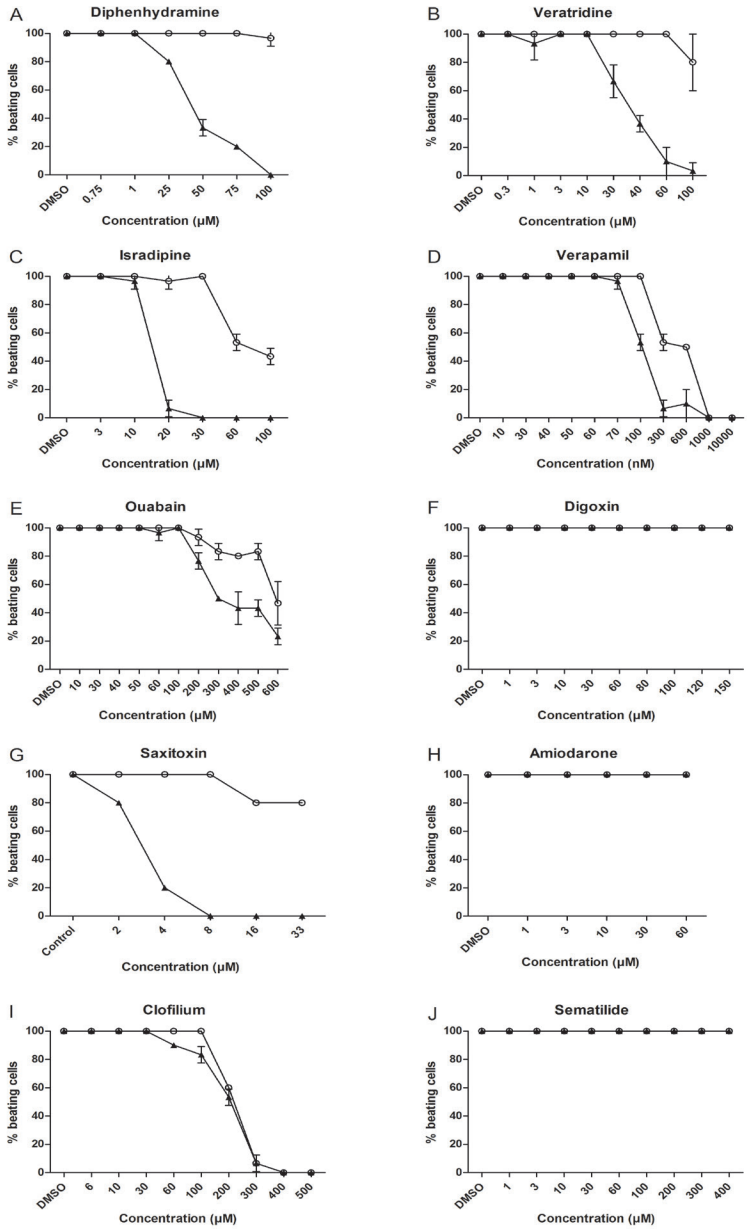

Figure 4.2: Effect of A) the Na^+ channel blocker diphenhydramine, B) the Na^+ channel opener veratridine, C-D) the Ca^{2+} channel blockers isradipine and verapamil, E-F) the Na^+/K^+-ATPase blockers ouabain and digoxin, G) the Na^+ channel blocker STX, H-I-J) the K^+ channel blockers amiodarone, clofilium and sematilide on the beating of embryonic stem cell-derived cardiomyocytes. Upon exposure (curve with triangles) the EBs were incubated with fresh medium for an additional hour to assess the recovery (curve with open circles). The data are presented as the mean of 3 experiments ± SD. 0.3% DMSO or HCl, used as solvent controls, had no effect on cell viability. At least three independent experiments with ten replicates were performed per concentration for each compound.

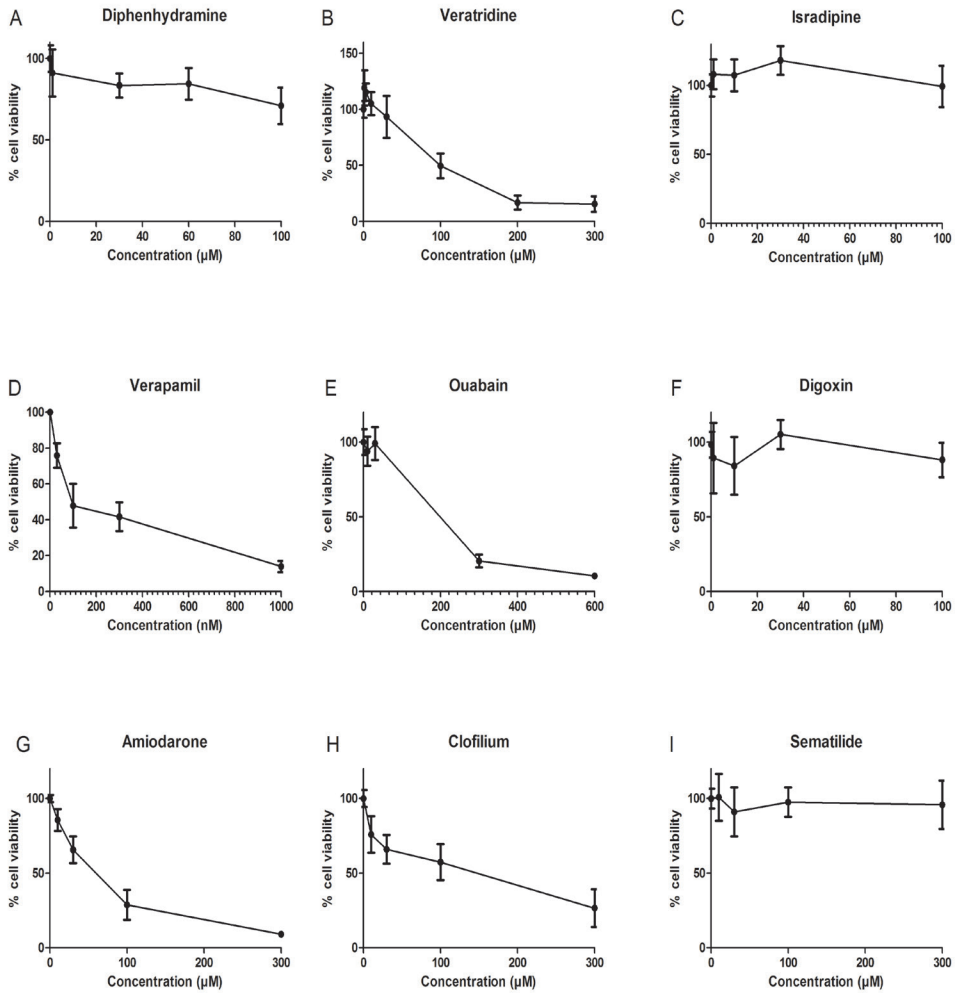

Figure 4.3: Effect of A) the Na^+ channel blocker diphenhydramine, B) the Na^+ channel opener veratridine, C-D) the Ca^{2+} channel blockers isradipine and verapamil, E-F) the Na^+/K^+-ATPase blockers ouabain and digoxin, G-H-I) the K^+ channel blockers amiodarone, clofilium and sematilide on the cell viability of neuro-2a cells. The cells were exposed for 24 hours and cell viability was assessed using the MTT assay. At least three independent experiments with six replicates were performed per concentration for each compound.

K^+ channels

None of the K^+ channel blockers induced beating arrests of the EBs at non-cytotoxic concentrations: amiodarone up to 60 µM, clofilium up to 100 µM and sematilide up to 400 µM had no effect on the beating (Fig. 4.2H-I-J). The cell viability was affected by amiodarone but not by sematilide according to the results obtained with the WST-1 assay (Tab. 4.1). However, higher concentrations of clofilium from 100 µM onwards induced cytotoxicity (WST-1 assay, Tab. 4.1), and the resulting beating arrests were thus not due to the K^+ channel blockade. In accordance with this observation, none of the EBs exposed to high levels of clofilium recovered (Fig. 4.2I).

In the neuro-2a assay, amiodarone and clofilium at and above a concentration of 10 µM induced a decrease in cell viability, while sematilide had no effect up to 300 µM (Fig. 4.3G-H-I).

The neuro-2a cells are thus more sensitive to amiodarone and clofilium than the embryonic stem cell-derived cardiomyocytes, while both cell assays were insensitive for sematilide (Tab. 4.1).

Na^+/K^+-ATPase pump

Figure 4.2E shows the effects of the Na^+/K^+-ATPase pump blocker ouabain on the beating of the cardiomyocytes. Concentrations up to 100 µM of ouabain did not induce a beating arrest in the EBs. At concentrations of 200 µM and higher, ouabain induced a concentration-dependent decrease in the percentage of beating cells (EC_{50} = 257 µM, Tab. 4.1). While most EBs recovered from the 300 µM exposure, almost none of the EBs that stopped beating at 600 µM recovered after the medium refreshment. It should be mentioned that the beating rates of some EBs that did not stop beating after exposure to ouabain (up to 100 µM) were largely increased (increased beating frequency not quantified) and this increased beating frequency may also reflect neurotoxicity. The Na^+/K^+-ATPase pump inhibitor digoxin did not affect the beating of the cells up to 150 µM (Fig. 4.2F). According to the outcomes of the WST-1 assay, the viability of the cardiomyocytes was not affected by ouabain but slightly affected by digoxin at 160 µM (Tab. 4.1).

Ouabain at concentrations of 300 µM and higher induced a decrease in cell viability of neuro-2a cells and digoxin up to 100 µM did not affect cell viability of neuro-2a cells (Fig. 4.3E and 4.3F). The two assays thus display a similar sensitivity towards these Na^+/K^+-ATPase pump inhibitors.

Marine biotoxins: Proof of principle

Figure 4.2G shows the effects of the commercially available Na^+ channel blocker STX on the beating of cardiomyocytes after 20 hours of exposure. As no beating arrest was observed after a 1 hour exposure to STX, the EBs were checked every hour for 6 hours and after 20 hours. STX only induced a concentration dependent decrease in the beating of the cardiomyocytes after a period of 20 hours (EC_{50} = 3.9 µM, Tab. 4.1). Every EB exposed to concentrations of STX up to 8 µM recovered, while 80% of them recovered at 16 and 33 µM. TTX up to 60 µM did not alter the beating of cardiomyocytes after an exposure of 24 hours. Neither STX nor TTX had an effect on the viability of embryonic stem cell-derived cardiomyocytes (WST-1 assay, Tab. 4.1).

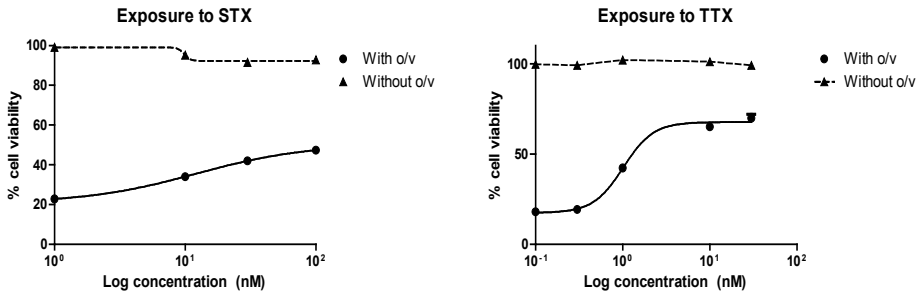

Figure 4.4: Effect of the Na^+ channel blockers saxitoxin and tetrodotoxin on the viability of neuro-2a cells, with (plain line) or without (dashed line) ouabain/veratridine. The cells were exposed to ouabain/veratridine and STX or TTX at the same time. The cell viability of the cells exposed to ouabain/veratridine alone was about 20%. No cytotoxicity was induced by STX and TTX without ouabain/veratridine (dashed line). At least three independent experiments with six replicates were performed per concentration for each compound.

Because TTX and veratridine have opposite modes of action, TTX being a Na^+ channel blocker and veratridine a Na^+ channel activator, it was hypothesized that TTX could prevent the beating arrest induced by veratridine. Indeed, exposing the cardiomyocytes to 100 µM of veratridine for 1 hour, resulting in a beating arrest of all EBs, and then adding TTX to a final concentration of 10 µM in each well during an additional hour, resulted in a 100% recovery of the beating. In addition, when the cells were pre-treated with 10 µM of TTX during 5 minutes and then exposed to 100 µM of veratridine for one hour, none of the EBs stopped beating.

Without ouabain/veratridine STX and TTX did not induce cytotoxicity in the neuro-2a cells. However, when co-incubated with ouabain/veratridine, STX and TTX increased cell viability in the neuro-2a cells at and above 10 nM and 3 nM

respectively (Fig. 4.4). These data show that the neuro-2a cells are more sensitive for these marine biotoxins than cardiomyocytes (Tab. 4.1).

Discussion

Diphenhydramine has been reported to block Na^+ channels in neurons at a concentration of 100 µM [16]. Without a Na^+ influx, cardiac cells cannot generate action potentials and cardiomyocytes are therefore not able to beat. However, diphenhydramine did not decrease cell viability of neuro-2a cells, indicating that either neuro-2a cells do not express the Na^+ channel subtypes targeted by diphenhydramine or blockade of these channel subtypes does not affect cell viability.

The Na^+ channel opener veratridine causes an abnormal entry of Na^+ ions followed by a secondary increase of the Ca^{2+} concentration, leading to beating arrests because the Na^+ channel is unable to close [30]. The sensitivity of the murine embryonic stem cell-derived beating cardiomyocytes to veratridine in the present study, i.e. EC_{50} = 35 µM, is comparable to what has been found by Yanagita et al. in 2003, reporting an increase of Na^+ influx by veratridine with an EC_{50} of 85 µM in adrenal chromaffin cells [18]. The neuro-2a assays showed a similar sensitivity to veratridine, as cell viability was affected with an EC_{50} of 90 µM.

Verapamil blocks the L- and T-type Ca^{2+} channels, while isradipine blocks only the L-type Ca^{2+} channels [31, 32]. In 1994, Keith et al. obtained an EC_{50} of 19 µM for the synaptosomal inhibition of the Ca^{2+} influx by verapamil in rat cortical neurons [21]. The embryonic stem cell-derived beating cardiomyocytes represent a more sensitive assay for the detection of verapamil, as specific beating arrests were observed for verapamil with an EC_{50} of 100 nM, which is also lower than the one obtained with the neuro-2a assay (EC_{50} = 190 nM). This indicates that embryonic stem cell-derived cardiomyocytes cells are relatively sensitive to verapamil. The EC_{50} of isradipine (15 µM) for inducing beating arrests was much higher than that for verapamil, most likely because this compound only blocks the L-type Ca^{2+} channels. Isradipine had no effect on the cell viability of neuro-2a cells. Together these data suggest that the neuro-2a cells only express the T-type Ca^{2+} channels, making them sensitive to verapamil in the µM range, but do not express the L-type Ca^{2+} channels, making them insensitive to isradipine. Murine cardiomyocytes express both the L- and T-type Ca^{2+} channels and are therefore

sensitive to both verapamil and isradipine.

The K^+ channel blockers amiodarone, clofilium and sematilide had no specific inhibiting effect on the beating cardiomyocytes. The K^+ channel isoforms on the surface of the murine embryonic stem cell-derived cardiomyocytes might be resistant to amiodarone, clofilium and sematilide or, in the case of clofilium, the concentrations that elicited beating arrests in the murine cardiomyocytes are too close to the concentrations that elicited general cytotoxicity. The neuro-2a assay was also insensitive to sematilide, but amiodarone and clofilium were found to affect cell viability.

The Na^+/K^+-ATPase pump blocker ouabain elicited specific beating arrests and decreased cell viability of neuro-2a with a similar sensitivity. The EC_{50} of 257 μM obtained in the present study for ouabain is in line with the findings of Kagiava et al. [25] who showed that ouabain elicited neurotoxicity with an EC_{50} of 370 ± 18 μM in the mouse myelinated sciatic nerve fibres. Digoxin had no effect either on the beating (inotropy) of the cardiomyocytes in the EBs or on the cell viability of neuro-2a cells. This was expected as digoxin only decreases the function of the Na^+/K^+-ATPase pump and does not block it, However, digoxin appeared to affect beating frequency (chronotropy) in the cardiomyocytes which was not included as a read out in our test.

STX and TTX block Na^+ channels and therefore the cells cannot generate action potentials, reflecting a mode of action similar to that of diphenhydramine [33]. The incubation time with STX required to elicit beating arrests was 20 hours, while for all model neurotoxins tested one hour was sufficient. This difference may be due to the fact that STX may only partially block the Na^+ type channels expressed by the cardiomyocytes, resulting in residual amounts of Na^+ ions still entering the cells causing shortage of Na^+ and effects on the beating process only upon prolonged exposure. TTX did not have any effect on the beating up to 60 μM. However, none of the cardiomyocytes stopped beating with the combination of 10 μM TTX with 100 μM of the Na^+ channel opener veratridine, a concentration that stopped the beating of all EBs, indicating that co-exposure of the EBs to TTX with veratridine is suitable for detecting TTX. This finding is in line with the observation that TTX has been shown to block veratridine-induced effects [34]. STX or TTX had no effect on cell viability of neuro-2a cells without addition of ouabain/veratridine. However, neuro-2a cells swell and eventually lyse upon exposure to a combination of ouabain/veratridine that enhances sodium influx [35]. The Na^+ channel blocker STX has been shown to protect neuro-2a cells

from the action of ouabain/veratridine [14]. Both STX and TTX prevented to a certain extent cytotoxicity induced by ouabain/veratridine in neuro-2a cells. This indicates that the main voltage gated sodium channels present in neuro-2a cells are TTX-sensitive while in the cardiomyocytes these channels are less sensitive to TTX and most likely belong to the Nav1.5 channel subtypes [36].

Certain compounds affect channels that are not expressed on the surface of the murine cardiomyocytes such as the N- and R-type Ca^{2+} channels and will thus not be detected. Since K^+ channel blockers tested negative their integrity was confirmed by showing their activity towards other endpoints. Amiodarone inhibited neuronal activity in rat cortical neurons [37]. Measuring effects on the beating rate or on the generation of action potentials as additional parameters, using for example multielectrode arrays [38], will most likely result in a more sensitive assay and might even result in an assay able to detect a wider range of neurotoxins. Ultimately, a model capable of detecting an extensive range of marine neurotoxins shall present a large variety of ion channels/pumps as well as neuronal receptors, the principal targets of such toxins.

In line with the findings from Maltsev et al. (1994) the data provided in the present study show that the murine cardiomyocytes have functional Na^+, Ca^{2+} channels and Na^+/K^+-ATPase pump and that beating murine cardiomyocytes can be used as a model to detect specific neurotoxic effects of compounds on Na^+, Ca^{2+} channels and the Na^+/K^+-ATPase pump, but not on K^+ channels [12]. The inhibition of beatings in the murine cardiomyocytes was shown to be reversible and to occur at concentrations below those affecting cytotoxicity, which implies that these effects are not due to overall cytotoxicity providing a more specific read out for neurotoxicity than the cytotoxicity endpoint as determined in the neuro-2a assay. This is an advantage of the cardiomyocytes assay over the neuro-2a cells, providing a way to avoid detection of false positives for neurotoxicity. Moreover, for the nine neurotoxic model compounds tested, the cardiomyocytes were generally somewhat more sensitive for those compounds that affect Na^+ and Ca^{2+} channels, but less sensitive towards the compounds that affect K^+ channels. The sensitivity of cardiac cells towards neurotoxins suggests that when neurotoxins affect ion channels or pumps they will also most likely exhibit cardiotoxicity.

However, while beating is a more specific endpoint than cytotoxicity, the process behind the differentiation of embryonic stem cells into cardiomyocytes is time consuming and labour intensive, hampering the implementation of such assay for screening purposes. Nevertheless, EC_{50} values obtained with neuro-2a

cells were in the same order of magnitude as that of the cardiomyocyte data, which are based on a more specific endpoint than cytotoxicity, and therefore seem to validate the cytotoxicity endpoint used in the neuro-2a mouse neuroblastoma assay. In addition, a striking difference in sensitivity was observed for STX and TTX, for which the stem cell-derived cardiomyocytes were not as sensitive as the neuroblastoma neuro-2a assay, as the EC_{50} values for STX and TTX were almost three orders of magnitude lower in the neuro-2a assay. Sensitivity in the low nanomolar range, as obtained in the neuro-2a assay, is required to detect these marine biotoxins in contaminated samples. Beating cardiomyocytes might also not be sensitive to neurotoxins with modes of action other than affecting ion channels or pumps as for example binding to specific neuronal receptors.

In summary, this is the first study proposing a cardiac model for neurotoxicity testing. Despite its limitations, the assessment of the beating arrest in murine embryonic stem cell-derived cardiomyocytes represents an interesting tool for the screening of compounds for their neurotoxic properties. Murine stem cell-derived cardiomyocytes provide a sensitive model for the detection of specific neurotoxins and the neuroblastoma neuro-2a assay appears to be a more promising cell-based assay for the screening of marine biotoxins.

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CHAPTER 5

Exploration of new functional endpoints in neuro-2a cells for the detection of marine neurotoxins

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Abstract

Marine neurotoxins accumulate in seafood and therewith represent a threat for consumers. At the European level, the use of in vivo bioassays is banned from 2015 onwards, except for the control of production areas for the detection of unknown toxins. Development of in vitro alternatives is thus urgent. Cytotoxicity in the neuro-2a assay has been shown a promising in vitro alternative testing strategy. However, given that cytotoxicity does not represent a specific endpoint and may be sensitive to confounding factors the current study aims at investigating the suitability of functional endpoints as alternatives to cytotoxicity in the neuroblastoma neuro-2a assay for the detection of marine neurotoxins. Microarray analyses were performed following exposure of neuro-2a cells to three pure marine neurotoxins (palytoxin (PlTx), saxitoxin (STX) and tetrodotoxin (TTX)) in order to identify genes that are specifically up- or down-regulated by one or more of these neurotoxins and that can subsequently be used as biomarkers for screening purposes. In addition to microarrays, the voltage dependent fluorescent probe bisoxonol was used to assess changes in cellular membrane potential induced in neuro-2a cells by the above mentioned marine neurotoxins. Biomarkers based on mRNA expression were detected for PlTx but not for STX and TTX. On the other hand, STX and TTX decreased the fluorescence of bisoxonol while PlTx showed no effect in this test. When using cytotoxicity as the read out the neuro-2a assay detects PlTx, STX and TTX at similar concentrations. Therefore it is concluded that the newly investigated endpoints in the neuro-2a assay, although being mode of action driven, are not preferred over cytotoxicity as a final endpoint in a suitable broad and sensitive bioassay for the detection of marine neurotoxins in real practice.

Introduction

Marine neurotoxins are produced by particular phytoplankton species [1, 2]. Shellfish accumulate these neurotoxins through filter-feeding, representing a threat to human health after consumption. In vivo assays such as the mouse bioassay (MBA) and chemical analyses including high-performance liquid chromatography (HPLC), liquid chromatography coupled with fluorescence detection (LC-FLD) and tandem mass spectrometry (LC-MS/MS) are currently used to screen marine neurotoxins in seafood in order to ensure food safety [3-5].

The MBA is banned in the EU from 2015 onwards, except for the control of production areas for the detection of unknown toxins and when the outcomes of official analytical methods are equivocal [5]. Chemical analyses are sensitive but are expensive, not sensitive enough for detecting ciguatoxins below their regulatory limits and do not allow for the detection of unknown marine neurotoxins while the required pure analytical standards are not always available. Therefore, there is a need for the development of alternative testing strategies enabling high throughput and sensitive detection of marine neurotoxins in seafood.

The most promising in vitro method developed up-to-now in terms of sensitivity and applicability is the neuroblastoma neuro-2a assay [6, 7]. This neuro-2a assay is based on cytotoxicity evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that is based on mitochondrial activity. Cañete and Diogène [8] reported that the neuro-2a assay allows for the detection of not only Na^+ channel blockers/openers but also of a wide range of marine neurotoxins and therefore represents a promising model for the screening of these neurotoxins in seafood [8]. The current limitation of this assay lies in the fact that the readout, i.e. cytotoxicity, is not specific and could be affected by external factors such as handling conditions and variation in pH or temperature.

The present study investigated whether the neuro-2a assay can be improved by implementing additional functional endpoints such as the effect of marine neurotoxins on gene expression. Omics technologies offer the possibility to identify modes of action, discover biomarkers and screen for natural contaminants present in the food, thus potentially reducing if not replacing animal testing [9, 10]. Among omics technologies, transcriptomics allows for the assessment of effects induced by toxic compounds on the expression of whole genome mRNAs. Transcriptomics has therefore the potential to identify biomarkers following

exposure of sensitive cells to toxic compounds and natural contaminants. In the field of marine biotoxins, transcriptomics has been applied in a few studies. Lefebvre et al. [11] observed up-regulation of genes involved in apoptosis and down-regulation of genes involved in protein synthesis in zebrafish following exposure to domoic acid [11]. Ryan et al. [12] described that exposure of mice to a sub-lethal dose of ciguatoxin affected hundreds of genes [12]. In vitro, using a small-scale dedicated microarray, Bovee et al. [13] identified five genes that were differentially regulated in human intestinal Caco-2 cells upon exposure to azaspiracid AZA-1 and dinophysistoxin DTX-1 [13].

Besides transcriptomics, monitoring of changes in membrane potential constitutes a promising approach in addition to general cytotoxicity. In this regard, Louzao et al. [14] investigated the suitability of the fluorescent probe bis(1,3-dibutylbarbituric acid) trimethine oxonol (bisoxonol) for detecting changes in membrane potential induced by sodium channel activators in human neuroblastoma BE(2)-M17 cells [14]. This assay permitted detection and quantitation of ciguatoxin (CTX-3C) and brevetoxins (PbTx-3) in a sensitive way (nM range, fulfilling the detection limits as required by authorities) [14].

The aim of the present study is to define and validate alternative functional endpoints for the current neuro 2a assay detecting cytotoxicity for the screening of marine neurotoxins [15]. To this end, we investigated whether transcriptomics and/or fluorescence-based measurements would improve the sensitivity and specificity of the current neuro-2a assay, thus limiting the number of potential false positives and negatives with the current cytotoxicity measurement. The identification of genes over- or under-expressed following exposure to pure marine neurotoxins commercially available, i.e. the Na^+/K^+-ATPase inhibitor palytoxin (PlTx) and the Na^+ channel blockers saxitoxin (STX) and tetrodotoxin (TTX) was investigated using microarrays. Given the promising results obtained with the use of fluorescent probes for monitoring changes in membrane potential for the screening of marine neurotoxins in seafood [14, 16], effects of the above mentioned neurotoxins on the fluorescence of bisoxonol (membrane potential-dependent probe) were also assessed.

Materials and methods

Chemicals

Veratridine, gramicidin and the voltage sensitive bis(1,3-dibutylbarbituric acid)

trimethine oxonol [DiBAC4(3)] (bisoxonol) dye were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

PlTx was purchased from Wako Chemicals (Neuss, Germany), STX from the National Research Council (Montreal, Canada) and TTX from Latoxan (Valence, France).

0.1% Acetic acid (HAc) was used as a solvent control for gene expression analysis and 0.25% DMSO was used as solvent control for fluorescence-based assay. Both solvents had no effect on cell viability.

Neuroblastoma neuro-2a culture

The mouse neuroblastoma neuro-2a cell line was purchased from ATCC (Beerse, Belgium). Neuro-2a cells were cultured in flasks of 75 cm^2 (Corning, Schiphol-Rijk, The Netherlands) in Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Invitrogen, Breda, The Netherlands) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (BioWhittaker, The Netherlands), 50 U/mL penicillin/50 µg/mL streptomycin (P/S) (Invitrogen). The cells were detached using trypsin (Invitrogen). The cells were cultured in a humidified atmosphere (37 °C, 5% CO_2) and sub-cultured three times per week.

Cell viability

Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). Cells were grown for 24 h in 96-well plates and subsequently exposed to different concentrations of the tested toxins for 24 h. Next, 20 µL of MTT solution were added to each well containing 200 µL of medium. After incubation for 40 min (37 °C, 5% CO_2), the medium was discarded and 200 µL of DMSO were added to each well. Absorbance was then read at 570 nm and mitochondrial activity was expressed as percentage vs. the average of the vehicle control (0.1% HAc).

Statistical significance between exposure and control groups was assessed by one-way ANOVA with a Dunnett's post hoc test using GraphPad Prism (San Diego, CA). Data are expressed as mean ± SD.

RNA isolation and quality control

The culture medium was removed following a 16-hour exposure to PlTx, STX and TTX. Cells were lysed in 300 µL cell lysis buffer (RLT) (Qiagen, Venlo, The Netherlands) supplemented with 10% β-mercaptoethanol and stored at -80 °C

until further processing. RNA was isolated with the Qiagen QIAshredder kit and purified using the miRNeasy Mini kit (Qiagen) according to the manufacturer's protocols. RNA yield was assessed spectrophotometrically (NanoDrop 2000, Isogen Life Science, De Meern, The Netherlands). RNA quality was determined before sending the samples to ServiceXS B.V. (Leiden, The Netherlands) by automated gel electrophoresis using Biorad's Experion system (Veenendaal, The Netherlands). Samples with RNA Quality Index (RQI) values > 8 were considered to be of sufficient quality.

Microarray

The Quality control, RNA labeling, hybridization and data extraction were performed at ServiceXS B.V.. The RNA concentration was measured using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A). The RNA quality and integrity was determined using Lab-on-Chip analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.) and/or on the Shimadzu MultiNA RNA analysis chips (Shimadzu Corporation, Kyoto, Japan). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's specifications with an input of 200 ng total RNA. Per sample, 20 ng of the obtained biotinylated cRNA samples were hybridized onto the mouse Illumina BeadChip (Illumina, Inc., San Diego, CA, U.S.A.). Each BeadChip contains eight arrays. Hybridization and washing were performed according to the Illumina Manual "Direct Hybridization Assay Guide". Scanning was performed on the Illumina iScan (Illumina, Inc., San Diego, CA, U.S.A.). Image analysis and extraction of raw expression data was performed with Illumina GenomeStudio v2011.1 Gene Expression software with default settings (no background subtraction and no normalization).

Fluorescence measurements

Fluorescence experiments were adapted from Louzao et al. [16]. Briefly, neuro-2a cells were seeded in 96-microwell plates (CellStar, the Netherlands) in a total volume of 200 µL per well (± 15.000-20.000 cells/well). After 24 hour incubation without treatment, 10 µL of 4 µM bisoxonol were added and plates were incubated for 10 minutes to allow distribution of the probe in the cell membrane. The fluorescence was measured using a Spectramax M2 microplate-reader (Molecular Devices, Berkshire, United Kingdom) at 540 nm (excitation)

and 560 nm (emission) for 10 minutes. Then, neuro-2a cells were exposed to 40 µM veratridine in order to depolarize the cells through inhibition of the inactivation of the voltage gated sodium channels (VGSCs), leading to Na^+ influx. After ten minutes of measurement, 10 µL of different concentrations of the marine neurotoxins were added to each well, and fluorescence was measured for ten minutes. At the end of each experiment, 10 µL of a 10 µg/mL gramicidin solution were added to induce complete cell depolarization.

Statistical significance between exposures to the different compounds/toxins was assessed by Student t-test using GraphPad Prism. Data are expressed as mean ± SD.

Data analysis

Illumina microarrays (MouseRef-8 v2.0) contain 25600 spots representing 19100 unique gene IDs. Noise due to spots that are not or very low expressed was reduced using floor values for the data. To this end, all spots with an intensity lower than 70 were corrected and assigned an intensity of 70. The intensity values were then 2log mean centered. 2log ratios vs. the average of all arrays were calculated for each spot. Thereafter, 2log ratios of treatments vs. the average of the control samples (0.1% HAc) were determined.

Unsupervised hierarchical clustering was performed with the publicly available programs Cluster (uncentered correlation; average linkage clustering) and Treeview [17].

Functional interpretation of differentially expressed genes for each marine neurotoxin tested was performed using Consensus Path DB (CPDB) analysis [18]. CPDB is available at http://cpdb.molgen.mpg.de and combines and compares the results of multiple pathway databases. Pathways with a p-value < 10-5 were considered significant.

Results

Cell viability

PlTx had no effect on cell viability up to 1 pM while 3 pM and 5 pM PlTx induced a 25% and 50% decrease in cell viability, respectively. The highest concentrations of STX and TTX tested, 33 nM and 10 nM respectively, had no effect on cell viability (Fig. 5.1). Concentrations of STX and TTX were the highest concentrations attainable based on the available stock and the setup of the experiment.

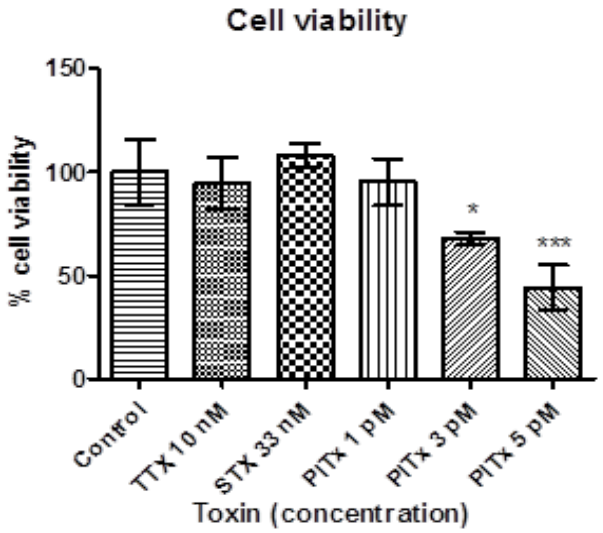

Fig. 5.1. Effect of tetrodotoxin (TTX), saxitoxin (STX) and palytoxin (PlTx) on cell viability of neuro-2a cells. Cell viability is expressed as percentage vs. the solvent control (0.1% acetic acid). Data are shown as mean ± SD (n = 3). *, P < 0.05; ***, P <0.001 compared to the solvent control.

Gene expression analysis

Sub-cytotoxic concentrations of PlTx (1 pM), STX (33 nM) and TTX (10 nM) were selected for gene expression analysis in order to identify specific mode of action-related genes as opposed to cytotoxicity-related genes (Fig. 5.1).

Hierarchical clustering was performed to compare the responses of the three marine toxins to each other. Since the effect on mRNA expression of each marine toxin was assessed in duplicate, genes were selected on being affected in at least two microarrays. Taking an up- or down-regulation of at least 1.62-fold (2log 0.7), 497 genes, represented by 588 spots fulfilled this criterion. A heatmap of these 497 genes is shown in Figure 5.2. PlTx affected the majority of these genes (clusters 2, 3 and 4), while much less genes were affected by STX and TTX (clusters 1 and 4). Based on this repression or induction value of 1.62, 1 pM PlTx induced up- or down-regulation of 433 genes, 33 nM STX induced up- or down-regulation of 21 genes while 10 nM TTX induced up- or down-regulation of 43 genes (Fig 5.2). In total, 4 clusters were identified, of which the genes were analyzed for overrepresentation in pathways using CPDB. No pathways were significantly affected in clusters 1 and 4, which contained the majority of STX and

TTX affected genes. Also no pathway was significantly affected in cluster 2, where most genes were down-regulated by PlTx. PlTx significantly induced five pathways in cluster 3: osteoclast differentiation ($p < 3 \times 10\text{-}7$), TNF signaling pathway ($p < 7 \times 10\text{-}7$), toxoplasmosis ($p < 10\text{-}6$), HTLV-I infection ($p < 3 \times 10\text{-}5$), and signaling by FGFR1 fusion mutants (myeloproliferative syndrome) ($p < 3 \times 10\text{-}5$).

For selection of genes suitable as biomarker, a threshold of at least 3-fold up- or down-regulation in both of the two replicates was used since we consider this as an appropriate value that can be reliably assessed by methods like qPCR or Luminex, techniques suitable for high throughput analyses. For STX and TTX, none of the genes fulfilled this criterion: STX also did not up- or down-regulate any gene more than two-fold in both replicates and TTX only induced the expression of four genes more than 2-fold in both replicates: Erich5, Rhbdl2, Masp2 and Tcf25, but did not down-regulate any gene more than 2-fold in both replicates. PlTx up-regulated seven genes more than 3-fold in both replicates: Lgi1, Angpt2, Prkg2, Gpr12, Egr2, Fam78b and Megf10 (Tab. 5.1). PlTx did not down-regulate any gene more than 3-fold in both replicates. Thirty-five genes were between 2- and 3-fold down-regulated by PlTx. Overall, although no biomarker was found for STX or TTX, seven genes are potential biomarkers of PlTx exposure.

The seven genes mentioned in Table 5.1 are not overrepresented in any pathway according to CPDB analysis. The function of one of these genes (Fam78b) is still unknown while five other genes (Lgi1, Angpt2, Prkg2, Egr2 and Megf10) are involved in cell survival, apoptosis and neuronal development [19-21]. The last up-regulated gene, Grp12, is known to promote neurite outgrowth [22].

Table 5.1. Up- or down-regulated genes following exposure to the marine neurotoxins PlTx, STX and TTX.

Marine neurotoxin	**Specifically affected genes (more than 3-fold change in expression)**	**Function**	**Reference**
PlTx	Angpt2 (up-regulated)	Angiopoietin-2: antitumor activity, may induce cell apoptosis	[19]
	Egr2 (up-regulated)	Early Growth Response 2: encodes for transcription factor involved in formation and maintenance of myelin	[24, 25]
	Prkg2 (up-regulated)	Protein Kinase, CGMP-Dependent, Type II: regulation of neuronal development and proliferation of cells	[39, 40]
	Fam78b (up-regulated)	Unknown	-
	Megf10 (up-regulated)	Multiple EGF-Like_Domains: plays a role in cell adhesion, motility, proliferation and apoptosis	[21, 26]
	Lgi1 (up-regulated)	Leucine-Rich, Glioma Inactivated 1: may play a role in neuroblastoma cell survival	[20]
	Gpr12 (up-regulated)	G Protein-Coupled Receptor 12: promotes neurite outgrowth and blocks myelin inhibition	[22]
STX	-	-	-
TTX	-	-	-

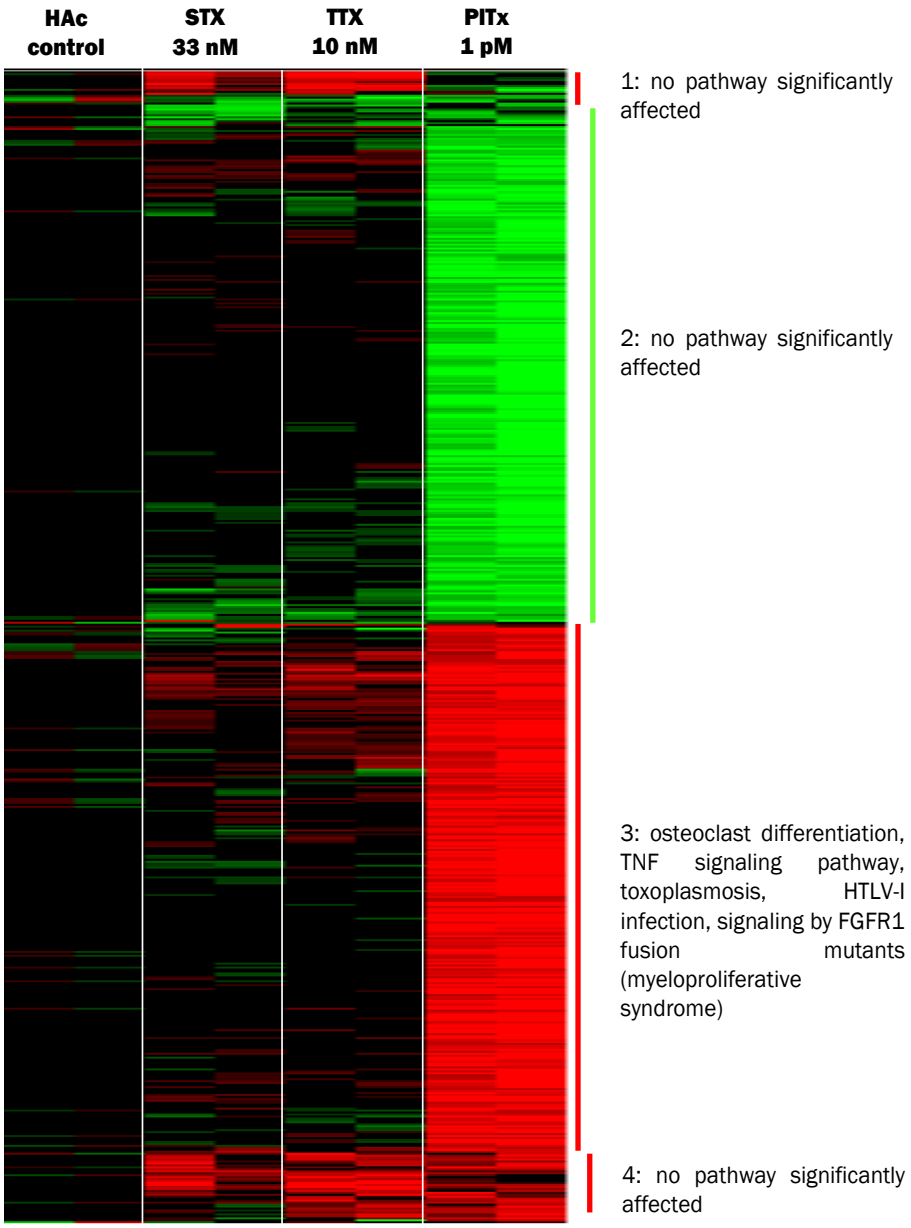

Fig. 5.2. Differential gene expression in neuro-2a cells following exposure to 1 pM palytoxin (PITx), 33 nM saxitoxin (STX) and 10 nM tetrodotoxin (TTX). Since the effect on mRNA expression of each marine toxin was assessed in duplicate, genes were selected on being affected in at least two microarrays. Taking an up- or down-regulation of at least 1.62-fold (2log ratio ≥ |0.7|), 497 genes, represented by 588 spots fulfilled this criterion and are indicated in red when down-regulated or green when up-regulated, respectively. This selection led to a total of 588 spots representing 497 genes. Pathways significantly affected within sub-clusters are indicated at the right. Additional pathways are mentioned in the text. A maximal red or green color indicates ≥1.62-fold up- or down-regulation versus the average of the control (0.1% acetic acid (HAc)). Red: up-regulated, green: down-regulated, black: unchanged expression.

Fluorescence measurements

In the absence of veratridine, neither STX nor TTX had an effect on the fluorescence of bisoxonol (data not shown). Veratridine, through the inhibition of the inactivation of the VGSCs, induced a significant increase in fluorescence of bisoxonol prior to exposure to the different marine neurotoxins (Fig. 5.3). A significant decrease in bisoxonol fluorescence was observed after addition of the Na^+ channel blockers STX and TTX. PlTx affects a different target, i.e. the Na^+/K^+-ATPase pump, and therefore the use of veratridine is not required. Palytoxin at 30 pM did not significantly affect the fluorescence of bisoxonol. Gramicidin was included as positive control since this compound is known to induce pores in the cell membrane leading to a complete depolarization. Gramicidin added at the end of each testing cycle indu0ced a high increase of bisoxonol fluorescence indicating that the neuro-2a cells were still responsive. Besides PlTx, STX and TTX, the marine neurotoxin domoic acid was tested and it did not have any effect on the fluorescence of bisoxonol (data not shown).

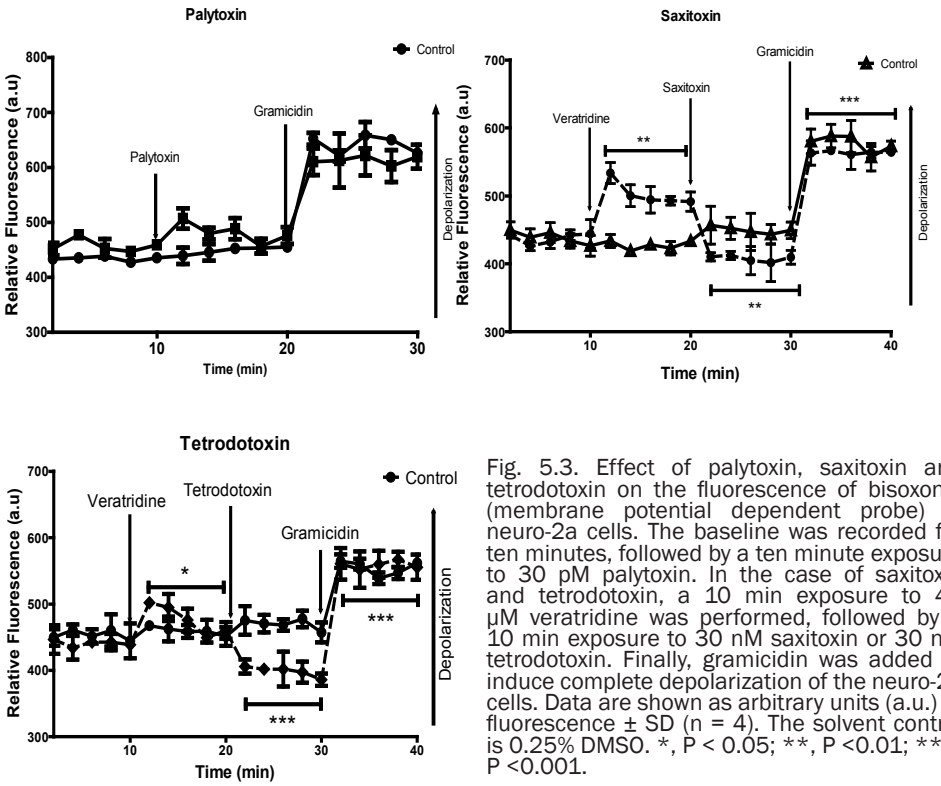

Fig. 5.3. Effect of palytoxin, saxitoxin and tetrodotoxin on the fluorescence of bisoxonol (membrane potential dependent probe) in neuro-2a cells. The baseline was recorded for ten minutes, followed by a ten minute exposure to 30 pM palytoxin. In the case of saxitoxin and tetrodotoxin, a 10 min exposure to 40 µM veratridine was performed, followed by a 10 min exposure to 30 nM saxitoxin or 30 nM tetrodotoxin. Finally, gramicidin was added to induce complete depolarization of the neuro-2a cells. Data are shown as arbitrary units (a.u.) of fluorescence ± SD (n = 4). The solvent control is 0.25% DMSO. *, P < 0.05; **, P <0.01; ***, P <0.001.

Discussion

The aim of the present study was to investigate the suitability of gene expression and fluorescence measurements as alternative to the current endpoint based on cytotoxicity in the neuroblastoma neuro-2a assay for the detection of marine neurotoxins. Seven biomarkers based on mRNA expression were detected that can serve as potential biomarkers of exposure to PlTx. In contrast, no biomarker gene could be identified for STX and TTX. In addition, STX and TTX significantly decreased the fluorescence of bisoxonol while PlTx did not affect bisoxonol fluorescence.

PlTx binds to the Na^+/K^+-ATPase pump, inhibiting its activity and converting it into a non-selective ion channel thereby affecting ion homeostasis [23]. The seven genes that were more than 3-fold up-regulated by PlTx are involved in cell survival, apoptosis and neuronal development. Increased expression of Lgi1 and Angpt-2 has been reported to reduce proliferation and trigger apoptosis of neuroblastoma cells [19, 20]. Egrf2, also known as Krox20, is involved in the process of myelination in embryonic dorsal root ganglia glial cells and the peripheral nervous system in mice [24, 25]. Furthermore, MEGF10 binds to apoptotic neurons and participates in their clearance through phagocytosis [21, 26]. G Protein-Coupled Receptor 12 (Gpr12), affects neuronal development and promotes neurite outgrowth [22]. Interestingly, the Na^+/K^+-ATPase pump is involved in controlling neurite outgrowth as well [27]. The induction of Gpr12 expression might therefore be a compensation response for the inhibition of the Na^+/K^+-ATPase pump. An increase in neurite outgrowth has also been observed in sensory ganglia following exposure to digoxin that is known to have a similar mode of action as PlTx [28]. Of the five pathways affected by PlTx, the Tumor Necrosis Factor (TNF) signaling pathway is the most relevant one as the other pathways are not specific to neuronal cells. TNF is a major proinflammatory mediator with the capacity to induce apoptosis. In stress conditions, such as following exposure to xenobiotics, TNF is recognized as a reactive cytokine that plays a crucial role in tissue regeneration and expansion [29]. Induction of increased mRNA levels of TNF and other inflammation-related proteins by PlTx has also been reported in human macrophages [30].

STX and TTX are known Na^+ channel blockers. These toxins can be detected using the neuro-2a assay using the capability of STX and TTX to counteract the effect of the Na^+ channel opener veratridine and the Na^+/K^+-ATPase affecting

agent ouabain [8]. More specifically, STX and TTX are known to affect all VGSC subtypes (although Nav 1.5, 1.8 and 1.9 are relatively STX/TTX-resistant), thus one could expect genes from the Na^+ channel (SCN) family to be affected [31, 32]. However, none of these genes was differentially regulated. The very limited response of the neuro-2a cells is most probably related to the fact that in the present study the cells were exposed without addition of veratridine, i.e. the Na^+ channels on the surface of the neuro-2a cells are already closed and the effect of the Na^+ channel blockers STX and TTX will thus be limited. However, we could not perform the transcriptomics analyses in the presence of veratridine at concentrations used in the neuro-2a assay when measuring cytotoxicity as the endpoint, since at this concentration veratridine might induce cytotoxicity which is not desirable for the identification of specific gene biomarkers.

In line with the results from Louzao et al. [14], in the absence of veratidine neither STX nor TTX affected the fluorescence of bisoxonol. In the presence of veratridine, STX and TTX significantly decreased the fluorescence of bisoxonol. While only slight changes in fluorescence of bisoxonol were observed following exposure to PlTx, STX and TTX significantly affected the fluorescence of bisoxonol at nanomolar concentrations. The neuro-2a assay with cytotoxicity as final endpoint allows the detection of PlTx at the low pM range (EC_{50} value of 0.1 nM) and the detection of STX and TTX at the low µM range (EC_{50} value of 8.6 nM and 10 nM, respectively) [8, 33]. Although TTX is forbidden on the European market, the concentration of STX tested, equivalent to 180 µg STX/kg shellfish meat when taking into account the extraction method for preparation of seafood extracts for testing in the neuro-2a assay, is below the current European regulatory limit for STX, i.e. 800 µg STX/kg shellfish meat [34]. Therefore, the fluorescence-based approach is sensitive enough for the detection of STX for regulatory purposes. As the cytotoxicity-based neuro-2a assay does not allow for the detection of domoic acid, we also investigated whether the fluorescence-based endpoint would allow for its detection. However, domoic acid had no effect on the fluorescence of bisoxonol which can be explained by the absence of its primary target, i.e. the N-methyl-D-aspartate (NMDA) receptor in neuro-2a cells [35]. In the present study, a plate reader was used to measure the fluorescence during time. This method has its limitations, as responses in multiple wells cannot be measured at the same time, hampering the real-time kinetic measurements of highly transient changes in membrane potential caused by marine neurotoxins [36]. In addition to responding slowly to changes in membrane potential (in minutes due to its

movement across the membrane while some of the marine neurotoxins elicit changes within seconds) and providing low temporal resolution, bisoxonol also interacts with cytosolic proteins giving rise to increased fluorescence signal [37, 38]. This slow response to changes in membrane potential is not desirable when testing marine neurotoxins that elicit fast transient changes. Multi-well real time kinetic measurements using for example a fluorescence imaging plate reader (FLIPR, capable of reading an entire plate within one second) can capture the transient changes in membrane potential or perturbation in ion fluxes induced by marine neurotoxins much more precisely.

In conclusion, measuring changes in membrane potential with bisoxonol is suitable for the detection of STX and TTX but not of PlTx. In addition, biomarkers based on mRNA expression were identified that could be used in a bioassay to detect PlTx but not STX and TTX. Thus, none of these techniques alone is suitable for the detection of each of these three toxins. Given the fact that the neuro-2a assay with cytotoxicity as a readout is able to detect PlTx, STX and TTX at similar concentrations as the ones detected by the new functional enpoints, it is concluded that the newly investigated endpoints in the neuro-2a assay are not preferred over cytotoxicity as a final endpoint in a suitable broad and sensitive bioassay for the detection of marine neurotoxins.

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CHAPTER 6

Detection of marine neurotoxins in food safety testing using a multielectrode array

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Abstract

At the European level, detection of marine neurotoxins in seafood is still based on ethically debated and expensive in vivo rodent bioassays. The development of alternative methodologies for the detection of marine neurotoxins is therefore of utmost importance. We therefore investigated whether and to what extent a multielectrode array (MEA) approach can be used as an in vitro alternative for screening of marine neurotoxins potentially present in seafood. This MEA approach utilizes rat cortical neurons comprising a wide range of ion channels/pumps and neurotransmitter receptors targeted by marine neurotoxins. We tested the effects of neurotoxic model compounds, pure marine neurotoxins and extracts from contaminated seafood on neuronal activity of rat cortical neurons cultured on commercial 48-well plates to increase throughput. We demonstrate that the MEA approach has a sensitivity of 88% (7/9 model compounds, 6/6 pure marine neurotoxins and 2/2 marine neurotoxins present in seafood extracts were correctly identified) and a good reproducibility compared to existing in vitro alternatives. We therefore conclude that this MEA-based approach could be a valuable tool for future food safety testing.

Introduction

Harmful algal blooms, characterized by rapid proliferation of particular phytoplankton species, negatively impact living organisms by producing marine biotoxins [1, 2]. These biotoxins can accumulate in fish and shellfish, thereby representing a threat for human consumers. Due to the global warming, the occurrence of algal blooms and associated biotoxins will most likely increase. Monitoring programs are therefore required and regulatory levels have been set by the European Commission for several marine biotoxins to ensure food safety and public health.

The detection of marine biotoxins is currently performed through in vivo assays, such as the mouse bioassay (MBA), and chemical methods, including high-performance liquid chromatography (HPLC), liquid chromatography coupled with fluorescence detection (LC-FLD) and tandem mass spectrometry (LC-MS/MS) [3, 4]. Besides requiring a large number of experimental animals, the MBA results in a high number of false positive and false negative results [5] due to a lack of specificity and the interference of free fatty acids with the outcome. The use of the MBA will be forbidden in Europe from 2015 onwards for the screening of lipophilic toxins, except for "the periodic monitoring of production areas for detecting new or unknown marine toxins" [6]. The LC-MS/MS method validated at the European level presents low limits of detection varying from 0.041 to 5.1 ng/mL depending on the toxin, the type of columns and conditions (acid, basic or neutral) used [7, 8]. LC-MS/MS based analysis should be used as the reference technique for the detection of lipophilic marine biotoxins in Europe. However, only well-defined toxins can be detected, whereas unknown marine biotoxins and marine biotoxins that are not well defined remain generally undetected by chemical analyses. The LC-MS/MS method can detect lipophilic marine biotoxins at levels below their allowed limits, but no routine protocol has been developed for marine biotoxins that do not yet have regulatory limits. LC-FLD and the HPLC-UV techniques are the official methods for the detection of PSP and ASP toxins, respectively [9]. According to regulation No 1664/2006, saxitoxin (STX) and its analogues for which standards are available should be tested by the LC-FLD technique. LC-FLD is very sensitive but if results are challenged then the MBA is the method to be used according to the European Commission [9]. The available chemical methods are highly sensitive, but require expensive instruments and do not allow for the detection of unknown marine biotoxins or marine biotoxins for

which no reference material is available. The development of alternative assays for the detection of marine biotoxins at a low cost, with high throughput, high sensitivity and reproducibility, and biological relevance is therefore of utmost importance.

A wide range of marine biotoxins is able to target the neuronal system. Consumption of seafood contaminated with such marine neurotoxins may result in mild symptoms such as dizziness, numbness and tingling of the mouth and digits, but also paralysis and in severe cases death [10]. These symptoms are the result of biotoxin-induced perturbation of cellular homeostasis, alterations in ion channel and neurotransmitter receptor function, and subsequent changes in neuronal activity [11]. A suitable model for the detection of such marine neurotoxins should therefore cover a large number of different ion channels and neuronal receptors in a functional network that allows for real-time monitoring of neuronal activity as an integrated measure of inter- and intracellular signaling.

The multielectrode array (MEA) has been developed in the field of electrophysiology to study in vitro neuronal activities by measuring local field potentials of electrically active neuronal cells [12, 13]. MEAs typically consist of an electrode array containing 16 to 64 electrodes. In the most advanced systems, multi-well MEA may contain up to 48 times 16 electrodes, i.e. a total of 768 electrodes. The multi-well MEA allows for a higher throughput than traditional electrophysiological (patch-clamp) recordings and for measurements from active neuronal networks rather than single cells [14]. MEA recordings have been recently introduced for neurotoxicological research to study the effect of chemicals on neuronal activity [15]. Hogberg et al. (2011) [16] successfully detected neurodevelopmental toxicity in primary cultures of rat cortical neurons induced by the marine toxin domoic acid.

Given the urgent need for alternative testing strategies, the lack of standard material for chemical analytical methods, the fact that chemical analyses do not detect all marine biotoxins and the wide range of marine neurotoxins that can be present in seafood it is essential to develop an alternative bioassay able to detect a wide range of neurotoxins with high sensitivity based on their biological activity [17]. Therefore, the aim of the present study was to investigate whether a MEA-based assay can be utilized as a highly sensitive and high throughput bioassay for the detection of marine neurotoxins in seafood to ultimately replace the in vivo MBA. We exposed cultured rat neonatal cortical cells to nine reference compounds known to have similar modes of action as marine neurotoxins, i.e. the

blockade/opening of specific ion channels and pumps (amiodarone, clofilium, digoxin, diphenhydramine (DPH), isradipine, ouabain, sematilide, verapamil, and veratridine). As a proof of principle, 6 pure marine neurotoxins (brevetoxin-3 (PbTx-3), domoic acid (DA), pacific ciguatoxin-1 (PCTX-1), palytoxin (PlTx), saxitoxin (STX) and tetrodotoxin (TTX)), one extract from mussels contaminated with STX and one extract from fish contaminated with TTX were tested. The sensitivity of the MEA to detect marine neurotoxins was compared to the sensitivity of the neuro-2a assay, which is considered to be a promising cell-based assay for the screening of marine neurotoxins [17, 18].

Materials and methods

Chemicals

Amiodarone (≥ 98%), clofilium (> 97%), digoxin (≥ 95%), DPH (≥ 98%), isradipine (≥ 98%), ouabain (≥ 95%), sematilide (≥ 99%), verapamil (≥ 99%), and veratridine (≥ 90%) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Stock solutions of model compounds were prepared in dimethylsulfoxide (DMSO, ≥ 99.9%) which was obtained from Merck (Schiphol-Rijk, The Netherlands).

STX and DA (> 95%) were purchased from the National Research Council (Montreal, Canada). Pacific CTX-1 (> 90%) was purchased from the University of Queensland (Queensland, Australia), PlTx (> 90%) from Wako Chemicals (Neuss, Germany) and PbTx-3 (> 95%) and TTX (> 96%) from Latoxan (Valence, France). STX was dissolved in water while the other marine neurotoxins were dissolved in methanol. An extract from mussels contaminated with STX was donated by Prof. Dr. Ana Gago Martínez from the European Union Reference Laboratory for Marine Biotoxins (EURLMB, Vigo, Spain). An extract from fish (Lagocephalus lunaris) contaminated with TTX was donated by Dr. Othman Muhamad from the Fisheries Biosecurity Centre Kuantan (Pahang, Malaysia).

A final concentration of 0.3% DMSO was used as a solvent control for all model compounds, while a final concentration of 0.3% methanol was used as control for DA, PCTX-1, PbTx-3, PlTx, and 1% water was used as control for STX and TTX.

Rat neonatal cortical culture

Experiments were approved by the Ethical Committee for Animal Experiments of Utrecht University and were in accordance with Dutch law. Primary cultures

of rat cortical neurons were prepared from postnatal day (PND) 0-1 Wistar rat pups. Pups were decapitated and cortices were rapidly dissected on ice. Cortices were minced into small pieces with scissors and scalpel. Small pieces of cortex were further dissociated mechanically by gentle trituration and filtered through a cell strainer (BD Falcon, 100 µm nylon). Cells were resuspended in dissection medium, i.e. Neurobasal-A supplemented with sucrose (14 g/500 mL), 200 mM L-glutamine (Gibco, Bleiswijk, Netherlands), 2.5 mM glutamic acid (Sigma-Aldrich, Zwijndrecht, Netherlands), 10% fetal bovine serum (FBS, Gibco), and 1% of a solution containing 10000 units/mL of Penicillin and 10000 µg/mL of Streptomycin (Gibco).

The cell-containing medium was centrifuged for 5 min at 800 rpm and supernatant was removed. Cells were diluted in dissection medium and seeded on poly-L-lysine-coated 48-well MEA plates (Axion Biosystems Inc., Atlanta, USA) at a density of approximately 1x105 cells/well. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2.

At day in vitro (DIV) 1, the dissection medium was replaced by glutamate medium, i.e. Neurobasal-A supplemented with sucrose (14 g/500 mL), 200 mM L-glutamine, 2.5 mM glutamic acid, 2% B-27 (Gibco) and 1% Penicillin/Streptomycin. On DIV4, glutamate medium was replaced by FBS culture medium, i.e. Neurobasal-A supplemented with sucrose (14 g/500 mL), 200 mM L-glutamine, 10% fetal bovine serum, and 1% Penicillin/Streptomycin (Gibco).

Spontaneous activity in rat neonatal cortical neurons was measured at DIV8 to test the viability and proper development of the neuronal cultures. All experiments were carried out at DIV11, and were performed on at least three different cultures i.e. three different days.

Multi-electrode array recordings

Each well in the 48-well plate (MEA plate) contains 16 nano-textured gold microelectrodes (~40-50 µm diameter; 350 µm center-to-center spacing) with four integrated ground electrodes, yielding a total of 768 channels (Axion Biosystems Inc., Atlanta, USA). Electrodes are much larger than the cell soma and therefore one electrode records activity from multiple neurons i.e. from a network of inhibitory and excitatory cells that is spontaneously active. Signals were recorded using a Maestro 768-channel amplifier with integrated heating system, temperature controller and data acquisition interface (Axion Biosystems Inc., Atlanta, USA). Axion's Integrated Studio (AxIS 1.7.8) was used to manage

data acquisition.

Spontaneous electrical activity in rat cortical cultures was recorded at DIV11 at a constant temperature of 37ºC. MEA plates were allowed to equilibrate in the Maestro for 5-10 minutes prior to recordings of electrical activity. Each recording consisted of a 30 min baseline recording of spontaneous activity, followed by addition of the test compounds and a subsequent 30 min recording to determine the effect of the toxins compared to baseline spontaneous activity (paired comparison).

Channels were sampled simultaneously with a gain of 1200x and a sampling frequency of 12.5 kHz/channel using a band-pass filter (200 Hz - 5000 Hz), resulting in raw data files. Afterwards, raw data files were re-recorded to obtain Alpha Map files for further data analysis in NeuroExplorer (see section 2.4). During the re-recording, spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a variable threshold spike detector set at 7 times standard deviation of the internal noise level (rms) on each electrode.

At the end of the experiments, MEA plates were cleaned for re-use by rinsing with MilliQ® and overnight incubation with 0.05% Trypsin-EDTA. Subsequently, plates were washed with Milli-Q®, filled and incubated with ethanol overnight, washed with ethanol and placed upside down (lid on) at 55ºC overnight.

Data analysis

Spike count files (Alpha Map files) generated from MEA recordings were loaded into NeuroExplorer® software (Nex Technologies, Madison, USA) for further analysis of the percentage of active wells (defined as ≥ 1 active electrode), the percentage of active electrodes (defined as ≥ 2 spikes/min) per well, and the average mean spike rate (MSR; spikes/s/electrode) per active electrode. To determine effects of toxin exposure, only electrodes with stable baseline activity were used. To select stable electrodes, the 30 min baseline recording was divided in windows of 10 min and the MSR of the 30 min period was compared with the MSR during the 10 min windows. If the MSR in a 10 min window was larger/smaller than the average MSR ± 2xSD of active electrodes in each well, electrodes were considered unstable and were excluded for further analysis.

Effects of the toxins were calculated per well as follows: MSR per electrode was averaged per well and effects of toxins were calculated as percentage change compared to baseline. Next, the effect of toxin was expressed compared

to control wells. Electrodes were considered outliers if their MSR >average MSR ± 2xSD in each well and were removed after data analysis (~2%). For each condition 9-25 wells from three independent isolations were used. Statistical significance between exposure and control groups was assessed by one-way ANOVA with a Dunnett's post hoc test using GraphPad Prism (San Diego, CA). Data are expressed as mean ± SEM from N wells or n electrodes.

Results

Basal characteristics of cortical cultures

During the 30 min baseline recording, spontaneous neuronal activity could be recorded in ~85% of the wells (N = 1141) at DIV11. From these active wells (N = 992), 4922 out of 15872 electrodes presented stable neuronal activity higher than 2 spikes/min, yielding an average % of active electrodes/well of 30.45 ± 1.61%. From these active and stable electrodes, the MSR ranged from 0.03 to 13.09 spikes/s (average 0.9 ± 0.01; N = 992 and n = 4922).

The 30 minutes exposure period was divided into three segments of 10 minutes to determine whether compounds have different early, late or transient effects. Since effects of toxins gradually developed over a time course of several minutes, the last 10 minutes of the 30 min recording were taken as a basis for evaluating the toxin-induced effect on MSR.

In this study, none of the solvent controls had an effect on the neuronal activity when compared to the baseline.

Model compounds

Model compounds have been selected to represent multiple modes of action, comparable to those of marine neurotoxins, such as specific inhibition or opening/activation of ion channels or pumps. These ion channels and pumps are involved in the maintenance of cellular homeostasis as well as in the generation of action potentials and are thus likely to interfere with neuronal activity.

DPH, a sodium (Na^+) channel blocker, decreased the neuronal activity at 10 µM by 72% ± 4% ($P < 0.001$) with an EC_{50} of 5 µM (Fig. 6.1A). Neuronal activity was completely suppressed at 100 µM. The Na^+ channel opener veratridine also decreased neuronal activity; 36% ± 9% inhibition at 0.01 µM ($P < 0.001$) with complete cessation of neuronal activity at 0.1 µM (EC_{50} = 0.01 µM, Fig. 6.1B).

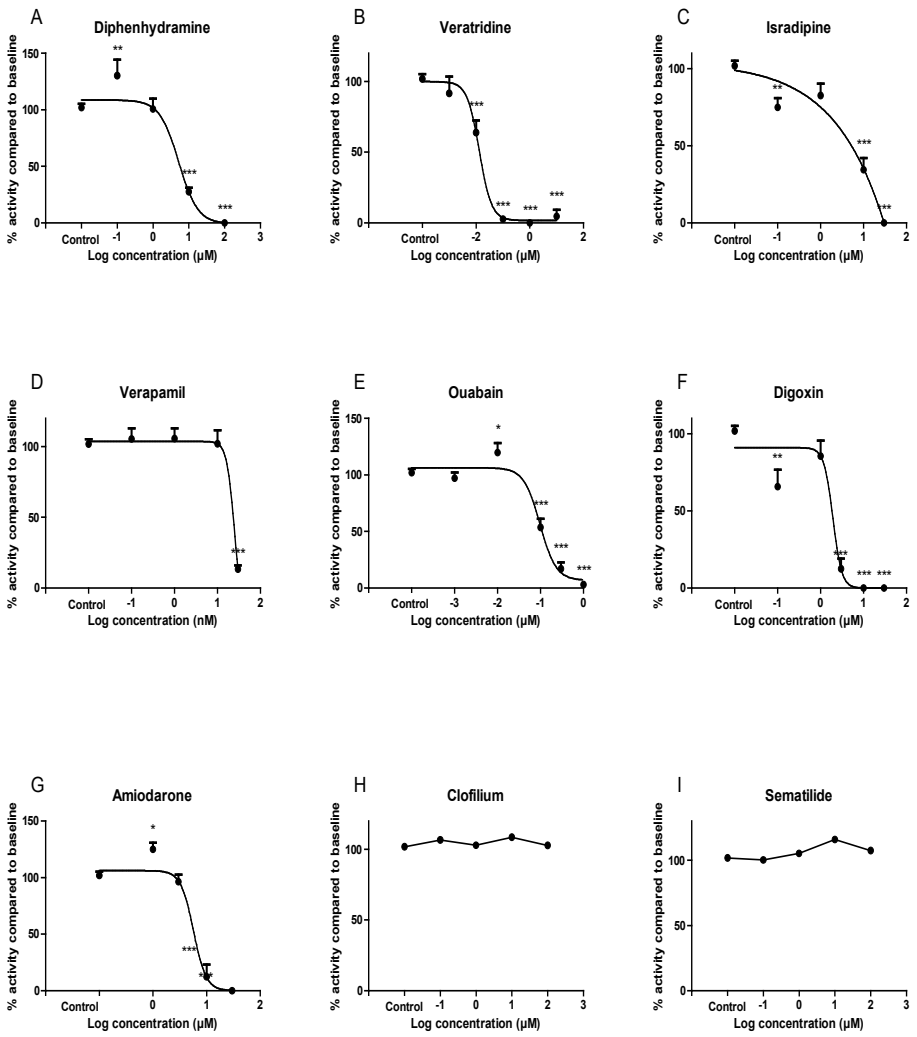

Figure 6.1. Effect of model compounds including the Na^+ channel blocker diphenhydramine (A), the Na^+ channel opener veratridine (B), the Ca^{2+} channel blockers isradipine (C) and verapamil (D), the Na^+/K^+-ATPase blockers ouabain (E) and digoxin (F), and the K^+ channel blockers amiodarone, clofilium and sematilide (G) on neuronal activity of rat cortical neurons. Results are expressed as average mean spike rate ± SEM during exposure as a fraction of the baseline MSR (N = 9-25; n = 13-123). *, P < 0.05; **, P <0.01; ***, P <0.001 compared to the respective solvent controls.

The calcium (Ca^{2+}) channel blockers isradipine and verapamil reduced neuronal activity from 10 μM (by 65% ± 8%; P < 0.001) and 30 nM (by 87% ± 3%; P < 0.001) onwards, respectively, with EC_{50} values of 9 μM and 25 nM (Fig. 6.1C-D).

Similarly, ouabain and digoxin, which respectively block or decrease the

activity of the Na^+/K^+-ATPase, decreased neuronal activity at 0.1 µM (by 45% ± 8%; P < 0.001) and 2 µM (by 87% ± 7%; P < 0.001), respectively, with EC_{50}s of 0.1 µM and 1.4 µM. Neuronal activity was completely suppressed by 0.3 µM ouabain and 30 µM digoxin (Fig. 6.1E-F).

Amiodarone, a potassium (K^+) channel blocker, induced a concentration-dependent decrease of neuronal activity with an EC_{50} of 6 µM. At 30 µM, amiodarone completely suppressed neuronal activity (Fig. 6.1G). In contrast, the K^+ channel blockers clofilium and sematilide had no effect on the neuronal activity (tested up to 100 µM) (Fig. 6.1H-I).

Comparison between the data obtained with the MEA and the data obtained with the neuroblastoma neuro-2a cell line from Nicolas et al. (submitted) is depicted in Table 6.1.

Table 6.1. Comparison of the sensitivity of the multielectrode array with the neuro-2a assay for testing model compounds.

Model compound	**EC_{50} neuro-2a assay *,§**	**LOD neuro-2a assay**	**EC_{50} multielectrode array §**	**LOD multielectrode array**
Diphenhydramine	NE up to 150 µM	> 150 µM	5 µM	Between 1 and 10 µM
Veratridine	90 µM	30 µM	0.01 µM	0.01 µM
Isradipine	NE up to 100 µM	> 100 µM	9 µM	0.1 µM
Verapamil	190 nM	30 nM	30 nM	Between 10 and 30 nM
Ouabain	220 µM	100 µM	0.1 µM	0.1 µM
Digoxin	NE up to 100 µM	> 100 µM	1.4 µM	0.1 µM
Amiodarone	80 µM	10 µM	6 µM	Between 3 and 10 µM
Clofilium	150 µM	30 µM	NE up to 100 µM	> 100 µM
Sematilide	NE up to 100 µM	> 100 µM	NE up to 100 µM	> 100 µM

* The EC_{50} values and LOD of the neuro-2a assay have been obtained from Nicolas et al., submitted.
§ The EC_{50} values were calculated using a non-linear regression model.
LOD: Limit of detection. NE: No effect

Pure marine neurotoxins

The marine neurotoxins tested in the present study have been selected depending on their modes of action, occurrence in seafood and commercial availability. STX and TTX, both Na^+ channel blockers, elicited a concentration-dependent decrease of neuronal activity of rat cortical neurons in the nanomolar range. 1 nM STX decreased neuronal activity by 65% ± 8% ($P < 0.05$; EC_{50} = 0.5 nM) and 5 nM TTX decreased neuronal activity by 63% ± 7% ($P < 0.05$; EC_{50} = 4 nM). Both neurotoxins induced a cessation of the neuronal activity at 10 nM (Fig. 6.2A-B).

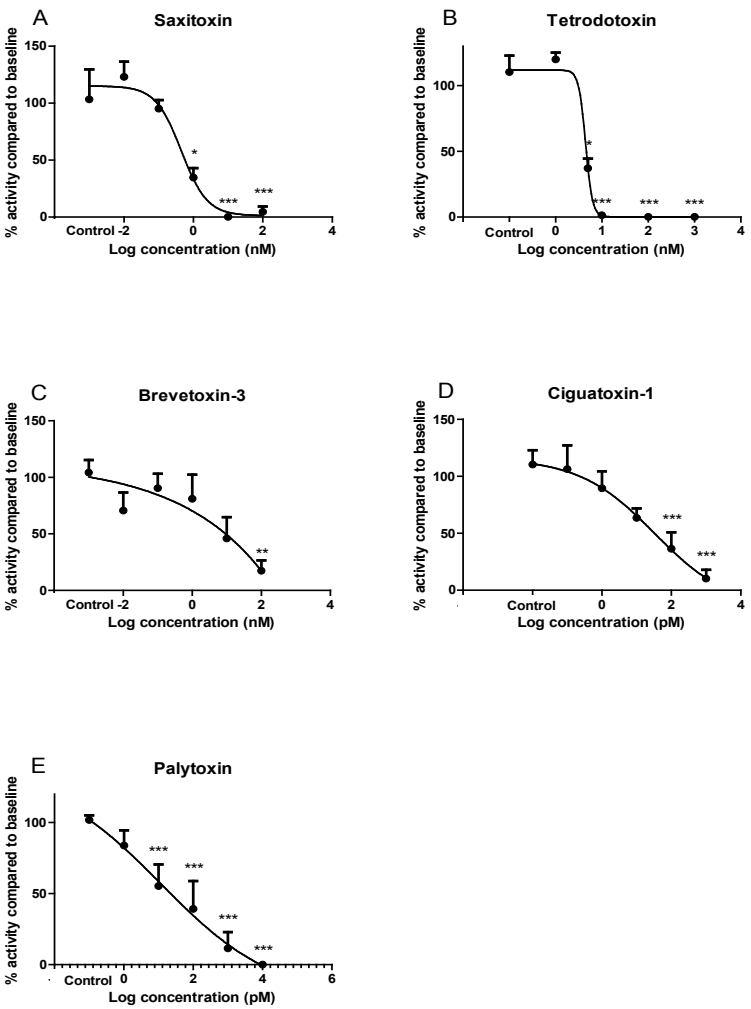

Figure 6.2. Effect of the marine neurotoxic Na^+ channel blockers saxitoxin (A) and tetrodotoxin (B), the Na^+ channel opener brevetoxin-3 (C), the Na^+ channel binder ciguatoxin-1 (D), and the Na^+/K^+-ATPase blockers palytoxin (E) on neuronal activity of rat cortical neurons. Results are expressed as average mean spike rate ± SEM during exposure as a fraction of the baseline MSR (N = 9-15; n = 17-59). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to the respective solvent controls.

The Na^+ channel opener PbTx-3 decreased neuronal activity of rat cortical neurons with an EC_{50} of 8 nM. At 100 nM, neuronal activity was almost completely suppressed (82% ± 9% decrease; $P < 0.05$) (Fig. 6.2C). PCTX-1, which is also known to interact with voltage-gated Na^+ channels, decreased neuronal activity of rat cortical neurons at 100 pM by 63% ± 14% ($P < 0.05$) with an EC_{50} of 33 pM (Fig. 6.2D). At 1 nM PCTX-1 induced complete cessation of neuronal activity.

The Na^+/K^+-ATPase blocker PlTx reduced neuronal activity of rat cortical neurons in a concentration dependent manner, i.e. by 54% ± 15% at 10 pM ($P < 0.05$) and by 88% ± 11% at 1 nM ($P < 0.001$) with an EC_{50} of 12 pM (Fig. 6.2E). At 10 nM, PlTx completely suppressed neuronal activity.

Finally, the glutamate receptor agonist DA reduced neuronal activity by 92% ± 2% at 1 µM with an EC_{50} of 0.4 µM. At 10 µM DA induced complete cessation of neuronal activity ($P < 0.001$; data not shown).

Comparison between the data obtained with the MEA and the data obtained with the neuroblastoma neuro-2a cell line from Nicolas et al. (submitted) is depicted in Table 6.2.

Table 6.2. Comparison of the sensitivity of the multielectrode array with the neuro-2a assay for testing marine neurotoxins.

Marine biotoxin	**EC_{50} neuro-2a assay***	**LOD neuro-2a assay**	**EC_{50} multielectrode array****	**LOD multielectrode array**
Pacific ciguatoxin-1	3 pM	1 pM	33 pM	1 pM
Saxitoxin	9 nM	1 nM	Pure: 0.5 nM Extract: 4 nM	< 0.5 nM
Tetrodotoxin	10 nM	1 nM	Pure: 4 nM Extract: around 10 nM	1 nM
Brevetoxin-3	8 nM	6 nM	8 nM	1 nM
Palytoxin	40 pM	30 pM	12 pM	1 pM
Domoic acid	NE	NE	0.4 µM	0.1 µM

* The EC_{50} values and LOD of the neuro-2a assay have been obtained from Cañete and Diogène (2008) [18] and Nicolas et al. (submitted).
** The EC_{50} for the multielectrode-based assay were calculated using a nonlinear regression model.
LOD: Limit of detection. NE: No effect

Contaminated extracts

The contaminated extracts were chosen based on availability and possible comparison with the pure marine neurotoxins tested in this study. Blank mussel extracts, used as controls, did not have any effect on neuronal activity. Extracts from mussels/fish contaminated with STX and TTX elicited a concentration-dependent decrease in neuronal activity (Fig. 6.3A-B). The extract from contaminated mussels (1.6 mg STX/kg mussel based on chemical analysis, i.e. a concentration of 5.55 µM) diluted to a final concentration of 5 nM STX completely suppressed neuronal activity ($P < 0.001$). The extract from contaminated fish flesh (300 µg TTX/kg fish flesh based on chemical analysis, i.e. a concentration of 0.9 µM) diluted to a final concentration of 9 nM TTX decreased neuronal activity by 42%. The curves obtained with extracts correlate well with the curves obtained with the pure marine neurotoxins, allowing for an estimation of the amount of toxins present in tested extracts. For example, 10 nM of pure STX fully suppressed neuronal activity while when present in extract, a concentration of 5 nM STX induced complete cessation of the neuronal activity.

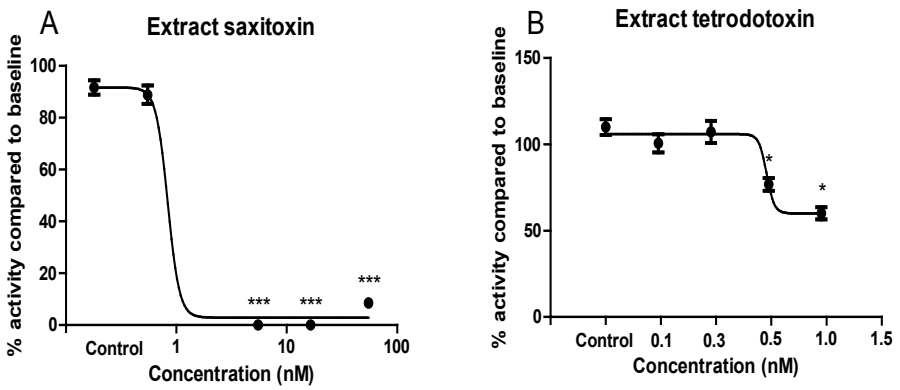

Figure 6.3. Effect of extracts from mussels contaminated with saxitoxin (1.6 mg saxitoxin/kg mussel, i.e. a concentration of 5.55 µM) (A) and extracts from fish contaminated with tetrodotoxin (300 µg tetrodotoxin/kg fish flesh, i.e. a concentration of 0.9 µM) (B) on neuronal activity of rat cortical neurons. Controls are extracts from blank mussels. Results are expressed as average mean spike rate ± SEM during exposure as a fraction of the baseline MSR (N = 10-18; n = 55-127). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to the respective solvent controls.

Discussion

Our MEA approach allowed for the successful detection of the majority of the model compounds and marine neurotoxins tested. The MEA performed well with a sensitivity of 88% (7/9 models compounds, 6/6 pure marine neurotoxins and 2/2 marine neurotoxins present in seafood extracts were detected). Only the two K^+ channel blockers clofilium and sematilide (up to 100 µM) could not be detected with this approach.

The Ca^{2+} channel blockers isradipine and verapamil decreased neuronal activity (Fig. 6.1), which is expected since the blockade of Ca^{2+} channels results in the inhibition of neurotransmitter release [19].

DPH, STX and TTX block voltage-gated Na^+ channels (VGSCs). Since Na^+ influx is required for the generation of action potentials, these compounds were expected to decrease neuronal activity as shown in Figs 6.1-6.2. In the present study, STX was not only detected as pure standard, but also when present in an extract derived from a complex matrix such as mussel (Fig. 6.3). It is thus possible to determine which concentration of STX was approximately present in the original extract by comparing its effect on neuronal activity with the effect obtained with pure standard. Similarly, TTX could be detected as pure standard as well as when present in a contaminated fish sample. However, full inhibition of neuronal activity could not be achieved due to the relatively low TTX concentration in the used extract (300 µg/kg fish). TTX is forbidden on the European market, requiring levels as low as possible to be detected. The MEA approach has a similar sensitivity as the neuro-2a assay and none of the assays developed up to now allows for screening of very low levels of TTX. Based on comparison to data from chemical analysis and dose-response curves obtained for pure standards of STX and TTX in the MEA assay, it can be concluded that the MEA approach allows for quantification of these marine neurotoxins in extracts from contaminated seafood. Additional types of matrices, i.e. from different seafood products, should be studied further (and included as blank extract controls) in order to extend the applicability of the MEA approach to a wide range of food products present on the market.

Ouabain and digoxin respectively block or decrease the activity of the Na^+/K^+-ATPase pump, thereby interfering with the normal efflux of Na^+ ions and influx of K^+ ions [20, 21]. PlTx also induces an accumulation of Na^+ via interference with the Na^+/K^+-ATPase pump [22]. Though this is expected to depolarize the

neuronal membrane and increase neuronal activity, ouabain, digoxin, and PlTx reduced neuronal activity in the MEA approach (Figs 6.1-6.2). Similarly, veratridine and PbTx-3 can be expected to increase membrane depolarization by preventing the inactivation of Na^+ channels [23], whereas PCTX-1 may induce depolarization by lowering the threshold for opening VGSCs by binding to the receptor-site 5 of VGSCs [24]. These compounds also reduced neuronal activity in the MEA approach (Figs 6.1-6.2). In line with our findings, similar effects for some of the compounds tested in this study, i.e. verapamil, DPH, ouabain and veratridine, have been observed previously [25-27]. It thus appears that a large number of compounds reduce neuronal activity in cortical neurons cultured on a MEA, including compounds that induce accumulation of Na^+ ions. The reduction in neuronal activity by the latter compounds may be due to desensitization of the neurons. Alternatively, it is possible that there is an overabundance of inhibitory GABAergic neurons. Since all neurons will be equally activated by these compounds, the MSR will decrease if there are more activated inhibitory neurons than activated excitatory neurons. Irrespective of the underlying mechanism, the MEA approach allowed for efficient detection of these compounds.

On the other hand, amiodarone was the only K^+ channel blocker that could be detected with this MEA approach. Clofilium targets the human Ether-à-go-go-Related Gene (hERG), Slick (sequence like a calcium-activated K^+ channel) and Slack (sequence like an intermediate conductance K^+ channel) K^+ channels [28, 29] and sematilide targets the delayed rectifier K^+ channels [30]. All these K^+ channels are supposed to be present in rat cortical neurons, but these compounds possibly only block the K^+ channels only partially similar as what has been reported for the blockade of delayed rectifier K^+ channels by 1 mM thiamine which just slightly inhibited the delayed rectifier K^+ current in rat cerebral cortical cultures [31]. Similarly, the K^+ channel blocker 4-aminopyridine, in combination with bicuculline, did not have any effect on neuronal activity of rat cortical neurons [32]. The reason that amiodarone can be detected using the MEA approach may be due to the fact that in addition to blockade of K^+ channels, amiodarone also has an effect on Na^+ and Ca^{2+} channels [33]. The ability to detect K^+ channel modulators is not of utmost importance as no marine neurotoxins that target primarily voltage-dependent K^+ channels have been reported so far.

The sensitivity of our MEA approach is about 88%, close to the 87% found by McConnell et al. (2012) [15] who revealed that the MEA is suitable for screening of neurotoxic compounds having a wide variety of modes of action using a chemical

training set. In addition to the other five marine neurotoxins tested in the current study and in line with previous findings [16], domoic acid decreased MSR with ~ 92% at 1 μM (data not shown) and was thus detected with high sensitivity. It is expected that other marine biotoxins such as azaspiracids (known to induce neurological symptoms) will also interfere with neuronal activity and therefore will be identified with this MEA approach. Seafood contaminated with mixtures of marine neurotoxins with similar modes of action will likely give additive effects (concentration-addition), though this notion requires further testing. This is of importance from a food safety perspective as the presence of several toxins present at levels just below the (chemical) detection limit may still exert adverse effects when combined.

As previously shown by Novellino (2011) [34], MSR is a simple and effective parameter to identify neurotoxicity. Each MEA experiment takes approximately one hour and allows for simultaneous recording of 48 different samples, making the MEA a suitable tool for fast screening that will allow laboratories to screen for a wide range of marine neurotoxins in a limited amount of time prior to allowing seafood on the consumer market. Moreover, the MEA performed better than the in vitro neuroblastoma neuro-2a, which is currently considered a promising in vitro assay for the detection of marine biotoxins in seafood (Table 6.1). Both in vitro assays were equally sensitive for detecting the tested marine neurotoxins, but the MEA was more sensitive than the neuro-2a assay for the tested model compounds and even allowed for the detection of some model compounds (digoxin, DPH and isradipine) that were negative in the neuro-2a assay. Furthermore, the MEA platform is based on the measurement of functional endpoints, relevant for mode of action based assays [35], thereby limiting the risk of false positive results. As a result the MEA might be better suited for the screening of real samples than the neuro-2a assay. This is also demonstrated with DA, which was detected in the MEA, but not in the neuro-2a.

The variation in MEA recordings is relatively large, possibly due to the (varying) presence of different cell types with different preparation. It should be noted though that the presence of different cell types is a clear advantage as subpopulations of neurons can respond in a different manner to stimuli [36] due to e.g. differences in expression profiles of receptors and ion channels. The heterogeneity in cell types therefore allows for the detection of compounds with very diverse modes of action.

The mouse bioassay will be partially banned from 2015 onwards, but will still

be used for the control of production areas for the detection of unknown marine biotoxins [4]. For an in vitro assay to ultimately replace the in vivo MBA for the detection of marine neurotoxins in seafood, it has to be fast, reliable, easy and sensitive. The costs for MEA recordings are relatively high, possibly hampering implementation. However, these costs can be considerably reduced by re-using the plates as already done in the present study (see material and method section). Because rat cortical neurons do not comprise all different types of ion channels that could be targeted by marine neurotoxins, it would be of added value to combine this approach with another cell type such as cardiomyocytes cultured on MEA plates to ultimately replace the MBA. The current MEA approach still requires the use of animals, though to a lesser extent than the current mouse bioassay and it induces less suffering of the animals. With the current procedure, approximately 10 seafood extracts could be tested per animal, while the MBA requires at least three animals for the screening of only one seafood extract, suggesting an about 30-fold reduction in animal use with the MEA approach. The possibility to use neuronal cell lines with sufficient spontaneous neuronal activity, such as neurons derived from human induced pluripotent stem cells (iPS) that could be generated from exfoliated renal epithelial cells, is currently under investigation (see e.g. [37]) and, if successful, will further reduce animal use for food safety testing for the presence of marine biotoxins.

In conclusion, we demonstrated that the MEA approach successfully detected the majority of model compounds and all marine neurotoxins tested, with some of them being shown to test positive both as pure standards and in extracts. The MEA approach is able to detect DA and has a slightly higher sensitivity than the neuroblastoma neuro-2a assay, which is currently considered as the most promising assay for in vitro detection of marine biotoxins in seafood. The MEA approach is thus a promising tool for the screening of marine neurotoxins present in seafood products. Successful implementation of this approach can contribute to the reduction of the number of animals required for the screening of marine biotoxins in seafood and is a valuable important step towards ensuring seafood consumers safety with a lower number of experimental animals.

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CHAPTER 7

Broad and animal free in vitro detection of marine biotoxins in seafood products

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Abstract

Due to the drawbacks of both the in vivo mouse bioassay and the chemical analytical methods for the detection of marine biotoxins, the European Food Safety Authority has pointed out the need for developing alternative animal friendly methods. One option is based on in vitro tests able to detect marine biotoxins in seafood products at relevant levels, including both regulated known toxins and those still unknown. The neuro-2a assay, measuring cytotoxic effects as determined by the MTT assay, is considered one of the most promising cell-based in vitro bioassays for such broad screening. In the present study, the applicability of this assay was assessed by testing a broad range of marine biotoxins and 100 samples (17 negatives and 83 naturally contaminated) that were also analysed by LC-MS/MS. All regulated lipophilic marine biotoxins and marine biotoxins exerting neurotoxicity, including paralytic shellfish poisons (PSPs), amnesic shellfish poisons (ASPs) and neurologic shellfish poisons (NSPs), were tested. Analogues of these toxins were also tested to demonstrate whether this cell-based bioassay can potentially detect unknown marine biotoxins too. The cell line showed great sensitivity towards all toxins and their analogues, except for the ASP domoic acid. Extracts of both blank and contaminated seafood samples, including two samples positive in the in vivo mouse bioassay, showed a good agreement with official LC-MS/MS methods. At the same time some false-positive results were obtained that suggest the presence of unknown toxins. The results strongly support the applicability of the neuro-2a assay to real samples in a daily routine setting which would allow the replacement of the mouse bioassay. Finally, a screening strategy that allows the detection and identification of specific classes of marine biotoxins is presented.

Introduction

Marine biotoxins are naturally occurring compounds mostly produced by certain algae. Marine biotoxins can affect human health mainly through foodborne intoxications, e.g. consumption of contaminated seafood, and occasionally through direct exposure to seawater aerosols [1]. Consumption of seafood contaminated with marine biotoxins may result in relatively mild symptoms such as diarrhoea, dizziness, numbness and tingling of the mouth and digits, but also paralysis and in severe cases even death [2]. Several major types of poisoning are described: diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurologic shellfish poisoning (NSP) and paralytic shellfish poisoning (PSP). A fifth syndrome, azaspiracid poisoning (AZP) has been characterised during the last twenty years [2, 3]. Due to the consequences of global and regional climate changes it is expected that the occurrence, patterns and chemistries of marine biotoxins will change. As a result marine biotoxins present a factor of growing concern [4, 5]. To avoid intoxications, monitoring is obligatory (Regulation No 854/2004) and limits have been set by the European Commission (Regulation No 853/2004) for ASP and PSPs, as well as several lipophilic marine biotoxins (mainly DSPs and azaspiracids (AZAs)). Furthermore, the European Food Safety Authority evaluated the toxicity of the various classes of marine biotoxins (EFSA) [6].

The detection of marine biotoxins in seafood products is currently performed by both in vivo assays and chemical analyses [6-8]. The main in vivo assay is the mouse bioassay (MBA), where mice are intraperitoneally injected with a seafood extract with lethality as the critical endpoint [9-11]. In Europe, ASPs and PSPs as well as several lipophilic marine biotoxins (mainly DSPs and azaspiracids (AZAs)) are regulated. HPLC-UV is the official method for the detection of ASPs (domoic acid) [7]. The official methodfor the detection of PSPs (saxitoxin (STX) and its analogues) is the mouse bioassay (MBA) but the internationaly recognized pre- or post-column oxidation HPLC-FLD methods can be used as alternatives [7, 12]. According to regulation No 1664/2006, STX and any of its analogues for which standards are available should be tested. Despite the fact that the HPLC-FLD methods are very sensitive to test for the presence of STX, if results are challenged, the MBA should be used according to the European Commission [12]. For the detection of lipophilic marine biotoxins the reference method at the moment is the EURL-MB LC-MS/MS method [7, 8], [13].

Besides being highly unethical, the MBA gives high rates of false positive and false negative results [6, 14]. For instance, it lacks specificity as very low levels of spirolides (SPXs) can already cause death of mice within minutes [15] while these levels do not impair human health (probably due to the route of administration). Moreover, in Europe, the use of the MBA is forbidden from 2015 onwards, except for STX analyses and for the control of production areas and relaying areas for the detection of new or unknown marine biotoxins [8]. In addition, there is no LC-MS/MS or other chemical analytical method that is routinely applicable for the broad detection of marine biotoxins. Also. ciguatoxins (CTXs) and neurotoxic brevetoxins (PbTxs) are for example missed. The most sensitive LC-MS/MS method developed so far for the detection of pacific CTX-1 (P-CTX-1) can detect this toxin with a limit of detection of 0.2 µg P-CTX-1-eq/kg seafood, while the FDA regulatory limit is 0.01 µg P-CTX-1-eq/kg seafood. According to Yogi et al. [16], the LC-MS/MS is capable of detecting P-CTX-1 below 0.01 µg P-CTX-1-eq/kg seafood but this could not be confirmed due to lack of certified standards. Moreover, for many toxins certified standards and reference materials are barely available or not available at all, making the use of chemical analytical methods for detecting all marine toxins very difficult if not impossible and also hampers the further development of these analytical methods. There are e.g. at least 24 PSP type saxitoxins [17], 13 DSP type okadaic acid-ester derivatives [18], 90 DSP type yessotoxins [19], 15 NSP type brevetoxins [20] and around 30 AZP type azaspiracids [21]. Furthermore, analytical methods are per definition unable to predict toxicity of complex mixtures (despite the fact that several marine biotoxins have toxic equivalencyfactors). As a result, many countries are hesitating to rely on these methods only.

Because of the drawbacks of the MBA and the chemical analytical methods, the European Food Safety Authority emphasised the need for developing alternative animal friendly methods. Thus, there is an urgent need for the development of in vitro tests that allow the detection of marine biotoxins that are currently known and those which might emerge in the future. Biochemical assays and especially cell-based bioassays have the potential to fulfil these requirements [22]. Among the biochemical assays developed for the detection of known marine biotoxins, the immunoassays, both radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA), seem the most promising. The major drawback is that these immune-based methods only allow detection of one specific toxin or at the best several toxins that are structurally related,

although often the cross reactivity towards analogues is unknown and cannot be predicted for unknowns. Cell-based bioassays, however, offer the advantage to potentially detect both analogues as well as unknown toxins. Most cell-based bioassays for the screening of marine biotoxins in seafood, assess cytotoxicity as endpoint. The neuro-2a assay is considered as one of the most promising cell-based bioassays for the broad screening of marine neurotoxins [22, 23] and is already used in routine for the detection of PbTxs and CTXs [24, 25]. To our knowledge it is not used for routine testing of shellfish for DSPs and PSPs, and as such as an alternative for the MBA.

In the present study, initially a comparison was made between the use of murine neuroblastoma cells (neuro-2a) and murine neuroblastoma x rat glioma hybrid cells (NG108-15) for a broad range of marine biotoxins as determined by the MTT assay. In addition, a new clean-up procedure was tested in order to reduce positives due to matrix effects of lipophilic extracts. All regulated lipophilic marine biotoxins, i.e. okadaic acid (OA), dinophysistoxin-1 (DTX-1), azaspiracid 1 (AZA-1), pectenotoxin-2 (PTX-2) and yessotoxin (YTX) were tested. In addition, analogues of OA, AZA-1 and YTX were tested, i.e. DTX-2, AZA-2, AZA-3, and 1a-homo yessotoxin (hYTX), in order to demonstrate whether these cell-based bioassays can potentially detect unknown marine biotoxins too. Further testing of several marine neurotoxins, i.e. the NSP brevetoxin-3 (PbTx-3), the ASP domoic acid (DA), the PSPs saxitoxin (STX) and tetrodotoxin (TTX), palytoxin (PlTx) and pacific ciguatoxin (P-CTX-1), was carried out with the neuro-2a assay only, as this assay turned out to be slightly more sensitive than the NG108-15 assay. Also all commercially available analogues of STX were tested. Next, extracts of both blank and contaminated seafood samples, including two fish samples positive in the MBA, were prepared and tested in the neuro-2a assay in order to examine whether this assay is applicable to real samples in a daily routine setting. At the end, a screening strategy combining the neuro-2a assay with analytical methods and additional confirmatory assays was set-up that would allow replacement of the MBA.

Materials and methods

Reagents and standards

Certified reference materials (CRMs) of AZA-1 (1.24 ± 0.07 µg/mL), AZA-2 (1.28 ± 0.05 µg/mL), AZA-3 (1.04 ± 0.04 µg/mL), DTX-1 (15.1 ± 1.1 µg/mL), DTX-

2 (7.8 ± 0.4 µg/mL), OA (13.7 ± 0.6 µg/mL), PTX-2 (4.40 ± 0.13 µg/mL), YTX (5.6 ± 0.2 µg/mL), hYTX (5.8 ± 0.3 µg/mL), STX (19.8 ± 0.4 µg/mL), dcSTX (16.7 ± 0.5 µg/mL), NeoSTX (20.7 ± 1.1 µg/mL), dcNeoSTX (8.0 ± 0.3 µg/mL), GTX1&4 (32.9 ± 0.9 µg/mL), GTX2&3 (62.4 ± µg/mL), GTX5 (21.1 ± µg/mL) and dcGTX2&3 (50.1 ± 1.8 µg/mL) were purchased from the National Research Council, Institute for Marine Biosciences (NRC CNRC) (Halifax, Canada). DA was purchased from Tocris Bioscience (Bristol, UK). P-CTX-1 was purchased from the University of Queensland (Queensland, Australia), PlTx from Wako Chemicals (Neuss, Germany) and PbTx-3, PbTx-9 and TTX from Latoxan (Valence, France). Ouabain and veratridine were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). DMSO, formic acid (98-100%), ammonium hydroxide (25%), acetic acid and acetone were obtained from Merck (Darmstadt, Germany) and methanol from Biosolve (Valkenswaard, The Netherlands). Acetonitrile (Ultra LC-MS), methanol (Ultra LC-MS) and water (Ultra LC-MS) were purchased from Actu-All (Oss, The Netherlands). Except for STX, dcSTX, NeoSTX, dcNeoSTX, GTX1&4, GTX2&3, GTX5, dcGTX2&3 and TTX that were prepared in water, stock solutions of the biotoxin standards were prepared in DMSO after evaporation of the original solvent.

Samples

Fifty samples, potentially naturally contaminated with DSPs and 17 samples potentially naturally contaminated with PSPs were kindly donated by Dr. Carlos García from the Faculty of Medicine (Universidad de Chile, Santiago, Chile). Two samples from fish contaminated with CTX were kindly donated by Dr. Ronel Biré (French Agency for Food, Environmental and Occupational Health & Safety (ANSES), France). In addition, in-house samples (both blank and validation samples) used for previous validation studies at RIKILT Institute of Food Safety were tested.

Preparation of extracts

Before extracting the toxins from the shellfish or fish material the materials were homogenized. Intact shellfish material was homogenized with a T25 Ultra Turrax mixer at 24000 rpm (IKA® Works Inc., Wilmington, NC, USA), intact fish tissue with a common grinding machine.

Lipophilic marine biotoxins (i.e. DSPs and AZPs).

One gram of shellfish homogenate was vortex mixed with 3 mL methanol for one min. Subsequently, the extract was centrifuged for 5 min at 2000 × g. The supernatant was transferred to a volumetric flask and the residue was extracted twice with 3 mL methanol. After the third extraction the volume of the collected supernatant was adjusted to 10 mL with methanol. For the neuro-2a assay an SPE clean-up was applied which was not necessary for the LC-MS/MS analysis.

PSP toxins.

One gram of (shell)fish homogenate was vortex mixed with 0.6 mL water containing 1% (v/v) acetic acid and was placed in an oil bath at 110 ºC for 5 min. Subsequently, the extract was centrifuged for 5 min at 3600 × g. The supernatant was transferred to a graduated conical tube. The pellet was extracted for a second time with the same amount of extraction solvent by vortex mixing and the extract was centrifuged for 5 min at 3600 × g. The supernatant was transferred to the same conical graduated tube and was adjusted to 2 mL with water.

Ciguatoxins.

Five grams of fish homogenate were mixed for 1 h with 15 mL acetone using a tumbling machine. The extract was centrifuged for 5 min at 3600 × g and the supernatant was decanted into a tube.

Clean-up by solid phase extraction (SPE)

Lipophilic marine biotoxins.

A 4.8 mL aliquot of the crude methanolic shellfish extract was diluted with 1.2 mL Milli-Q water and extracted twice with 6 mL n-hexane in order to remove matrix substances that could lead to false positive test outcomes. The hexane washes were discarded and the combined aqueous methanolic extract was further diluted with Milli-Q water to a final volume of 10 mL. Solid phase extraction (SPE) using a StrataTM-X cartridge (200 mg/6 mL; Phenomenex, Torrance, CA) previously conditioned with 4 mL methanol/water (30:70 v/v) was applied to the aqueous methanolic extract. Subsequently, the cartridge was washed with 8 mL methanol/water (20:80 v/v) and the toxins were eluted from the cartridge with 4.8 mL methanol. The eluate obtained was evaporated to dryness under a stream of nitrogen gas and reconstituted in 20 µL DMSO.

PSP toxins.

Solid phase extraction (SPE) using a StrataTM-X cartridge (200 mg/6 mL) was applied to the crude extract. The cartridge was conditioned with 6 mL methanol followed by 6 mL water. Then 1 mL of the crude extract was applied to the cartridge and the effluent was collected in a conical graduated tube. Subsequently, the cartridge was additionally eluted with 2 mL water, the effluent was collected in the same conical graduated tube. Vacuum was applied to dry the cartridge in order to retrieve all eluent, thereafter the eluent volume was adjusted to 4 mL with water. This extract was partly kept for the LC-MS/MS analysis and partly for the neuro-2a assay (diluted 400 times).

Cell culture and exposure

Neuroblastoma neuro-2a cells were purchased from the American Type Culture Collection (ATCC; CCL-131) and cultured in 75 cm^2 culture flasks containing 15 mL RPMI-1640 medium (R0883, Sigma-Aldrich) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) of a 100 mM sodium pyruvate solution and 1% (v/v) of a 200 mM L-glutamine solution. NG108-15 cells were obtained from ATCC (HB-12317) and cultured in 75 cm^2 culture flasks containing 30 mL Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS and 2% (v/v) of 50× HAT supplement (5 mM hypoxanthine, 20 µM aminopterin and 0.8 mM thymidine). Both cell lines were routinely maintained in a humidified incubator at 37 °C under 5% CO_2 and sub-cultured three times per week (dilution 1/14) up to approximately 90% confluence.

Neuro-2a and NG108-15 cells were seeded separately into 96-well plates with an initial density of 25,000 and 14,000 cells/well, respectively. After growing the cells for 24 h, exposure to increasing concentrations of pure marine biotoxins or sample extracts was performed in quadruplicate in 200 µL medium for 24 h. For screening cytotoxic effects caused by DSPs, the culture medium was renewed with fresh medium containing test compound or sample extract dissolved in DMSO. For screening neurotoxic effects, ouabain and veratridine at concentrations that produce a 80% or 20% decrease in cell viability were added to each well in combination with the test compound or sample extract. The final DMSO concentration in the medium was kept at 0.25% (v/v) for all standards and samples. Ouabain and veratridine at 0.3 mM and 0.03 mM respectively, decreasing cell viability by 80% were used for STXs (saxitoxin, decarbamoylsaxitoxin, decarbamoylgonyautoxin-2 & -3, gonyautoxin-1 & -4,

gonyautoxin-2 & -3 and neosaxitoxin) and TTX. Ouabain and veratridine at 0.13 mM and 0.013 mM respectively, decreasing cell viability by 20% were used for PlTx, P-CTX-1, PbTx-3 and PbTx-9. At the end of the exposure, cell viability was measured using the MTT assay.

Cell viability assay (MTT)

Briefly, 60 µL of MTT (final concentration of 0.8 mg/mL), dissolved in serum free medium was added to the wells. After 30 min incubation at 37 °C, the medium was removed and the formed formazan crystals were dissolved in 100 µL DMSO. The absorbance was measured at 540 nm and corrected for background absorption at 650 nm. EC_{50} values were determined using a non-linear regression model (GraphPad Prismsoftware version 5.04, San Diego, CA).

Chemical analysis

Lipophilic marine biotoxins (i.e. DSPs, AZPs).

Chromatographic separation was achieved using a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA). The system consisted of a binary solvent manager, sample manager and a column manager. The column temperature was kept at 60 °C and the temperature of the sample manager was kept at 10 °C. A 5 µL injection volume was used. Mobile phase A was water and mobile phase B was acetonitrile/water (9:1 v/v), both containing 6.7 mM ammonium hydroxide. A flow rate of 0.6 mL/min was used. A gradient started at 30% B and after 0.5 min was linearly increased to 90% B in 3 min. This composition was kept for 0.5 min and returned to 30% B in 0.1 min. An equilibration time of 0.9 min was allowed prior to the next injection. The effluent was directly interfaced in the electrospray ionisation (ESI) source of the AB Sciex QTrap 6500 mass spectrometer (Ontario, Canada). The mass spectrometer operated in both ESI negative and positive ionisation by rapid polarity switching. For each toxin two transitions were measured.

PSP toxins.

Chromatographic separation was achieved using the same type of chromatographic system as described above. The only difference is the column temperature that was kept at 40 °C. For the analysis of the STXs a 10 µL injection volume was used. Mobile phase A was water and B was acetonitrile, both containing 50 mM formic acid. The analytical column used for STXs was a Tosoh Bioscience

TSKgel Amide-80 column (250x2 mm. 5 µm particles). A flow rate of 0.2 mL/min was used. A gradient started at 30% A and after one minute was then linearly increased to 95% A in 7.5 minutes. This composition was kept for 5 minutes and returned to 30% A in 0.5 minute. An equilibration time of 6 minutes was allowed prior to the next injection. The effluent was directly interfaced in the ESI source of the Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, US). The mass spectrometer operated in ESI positive ionisation mode and for each toxin two transitions were measured.

Results

Effects of individual marine biotoxins

Lipophilic marine biotoxins

Figure 7.1 shows the effect of several lipophilic marine biotoxins on the cell viability of neuro-2a cells. All lipophilic marine biotoxins and their analogues induced a concentration dependent decrease in viability of neuro-2a and NG108-15 cells (Tab. 7.1). Although AZAs caused a decrease in cell viability at low concentrations and can thus be detected at low concentrations, these toxins reduced cell viability to relative MTT levels of about 40%, while DTXs, YTX, OA and PTX-2 were able to further reduce the MTT activity.

As the neuro-2a cells were somewhat more sensitive to the lipophilic marine biotoxins than the NG108-15 cells, neuro-2a cells were further used for testing the other groups of marine biotoxins and the sample extracts.

Table 7.1. Summary of the effect of lipophilic marine biotoxins on the viability of murine neuroblastoma neuro-2a cells and murine neuroblastoma x rat glioma hybrid NG108-15 cells.

Toxin	CAS no.	EC_{50} (nM)	
		Neuro-2a	NG108-15
Azaspiracid-1	214899-21-5	1.0	2.6
Azaspiracid-2	265996-92-7	2.9	4.5
Azaspiracid-3	265996-93-8	1.5	4.3
Dinophysistoxin-1	81720-10-7	5.2	8.4
Dinophysistoxin-2	139933-46-3	28.8	28.9
Okadaic acid	78111-17-8	24.5	22.9
Pectenotoxin-2	97564-91-5	72.8	nd
Yessotoxin	112514-54-2	1.6	3.4
1-Homoyessotoxin	196309-94-1	1.1	2.7

nd: not determined

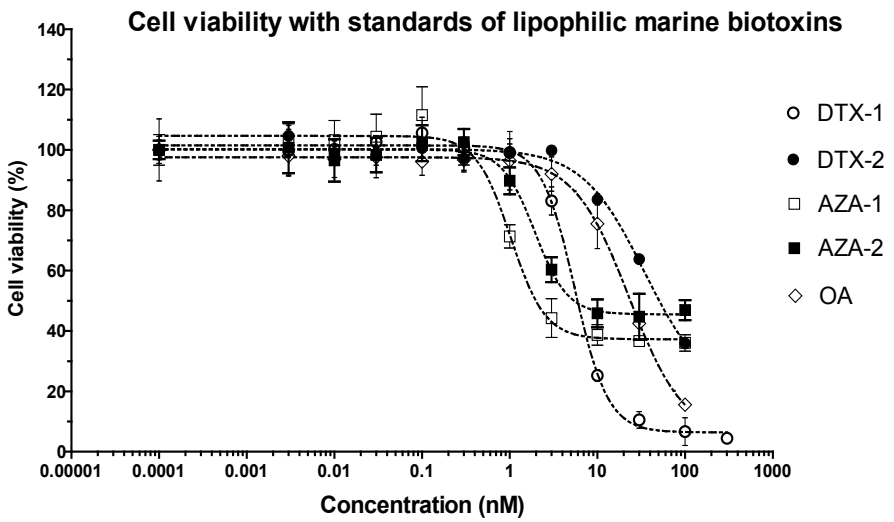

Figure 7.1. Effects of several lipophilic marine biotoxins on the viability of neuro-2a cells as measured with the MTT assay compared to the average of the solvent control (0.25% DMSO). AZA: azaspiracid; DTX: dinophysistoxin; OA: okadaic acid. Data are expressed as mean ± SD (n = 4).

Marine neurotoxins

Two designs were used for the screening of marine neurotoxins. The first one involved the use of high concentrations of ouabain and veratridine (o/v) eliciting approximately 80% cytotoxicity (20% cell viability) according to the MTT assay, aiming at detecting voltage gated sodium channels (VGSCs) blockers which counteract the cytotoxic effect of o/v. This procedure will thus detect STX and its analogues (decarbamoylsaxitoxin, decarbamoylgonyautoxin-2 & -3, gonyautoxin-1 & -4, gonyautoxin-2 &-3 and neosaxitoxin) and TTX [26]. The Na^+ channels on the surface of neuro-2a cells are closed and therefore the blockade of these channels by marine biotoxins would not be detected without the use of o/v. The second experimental set-up was based on the use of low concentrations of o/v inducing 20% cytotoxicity (80% cell viability) and was meant to detect PlTx, P-CTX-1, PbTx-3 and PbTx-9. In this case o/v is needed to inhibit the Na^+/K^+-ATPase pump so that the influx of Na^+ induced by some marine biotoxins leads to further cytotoxicity.

Figure 7.2a is an example of the first design, i.e. testing a compound on neuro-2a cells in which o/v treatment reduces the viability to 20%. Without o/v, STX does not decrease the viability, showing that STX alone is not toxic to the neuro-2a cells at the concentrations tested, but STX is able to counteract the cytotoxic effect of o/v. Figure 7.2b is an example of the second design, i.e. testing a compound or sample extract with (80% cell viability) or without (100% cell viability) o/v, showing that P-CTX-1 alone is not toxic to the neuro-2a cells, but is able to cause cytotoxicity at very low concentrations (pM range) when combined with a low dose of o/v that causes 80% cell viability according to the MTT assay. Figure 7.2c shows the effect of PlTx, showing that this marine biotoxin is already toxic to neuro-2a cells in the low pM range without the addition of o/v.

Table 7.2 gives a summary of all marine neurotoxins tested. Except PlTx, none of the marine neurotoxins tested had an effect on cell viability without o/v. When co-incubated with a concentration of o/v inducing approximately 80% cytotoxicity, STXs and TTX increased cell viability in the neuro-2a cells in a concentration dependent manner by counteracting the effect of o/v. When co-incubated with a concentration of o/v inducing approximately 20% cytotoxicity, P-CTX-1, PbTx-3 and PbTx-9 further decreased the viability in the neuro-2a cells in a concentration dependent manner.

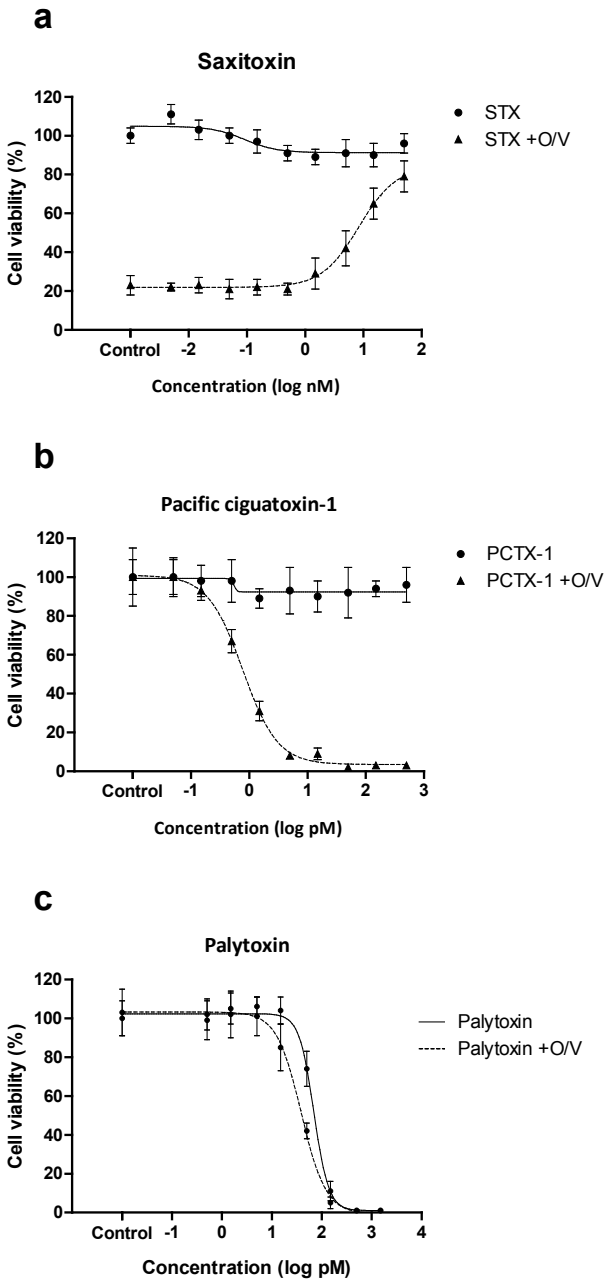

Figure 7.2. Effects of certain marine neurotoxins on the viability of neuro-2a cells as measured with the MTT assay: a) saxitoxin (STX) with or without a high concentration of ouabain/veratridine (o/v) (20% cell viability); b) pacific ciguatoxin-1 (P-CTX-1) with or without a low concentration of o/v (80-100% cell viability); and c) palytoxin (PlTx) with or without a low concentration of o/v (80-100% cell viability). 0.25% DMSO was used as solvent control. Data are expressed as mean ± SD (n = 4).

Table 7.2. Summary of the effect of marine neurotoxins on the viability of neuroblastoma neuro-2a cells in the presence of o/v as indicated.

Toxin	CAS no.	EC_{50} (nM)	o/v
Brevetoxin-3	85079-48-7	8	80% cell viability
Brevetoxin-9	155751-73-8	8.4	80% cell viability
Decarbamoylgonyautoxin-2 & -3	86996-87-4 87038-53-7	94.8	20% cell viability
Gonyautoxin-1 & -4	60748-39-2 64296-26-0	22.6	20% cell viability
Gonyautoxin-2 & -3	60508-89-6 60537-65-7	29.3	20% cell viability
Neosaxitoxin	64296-20-4	6.8	20% cell viability
Pacific Ciguatoxin-1	11050-21-8	0.9 pM	80% cell viability
Palytoxin	77734-91-9	39.2 pM	80% cell viability
Decarbamoylsaxitoxin	58911-04-09	21.6	20% cell viability
Saxitoxin	35554-08-6	8.2	20% cell viability
Tetrodotoxin	4368-28-9	18	20% cell viability
Domoic acid	14277-97-5	NE	-

NE: no effect. o/v: ouabain/veratridine

Marine biotoxins present in seafood extracts

The current EU limits for the regulated marine biotoxins and the above determined sensitivities of the neuro-2a assay for these toxins were used to calculate the sample amount and the dilution of the prepared sample extract in the cell culture medium. For example, regarding the lipophilic marine biotoxins, as all toxins should be detected at levels below their established limits, the worst case is the allowed level of 160 µg OA-eq/kg, as this is the lowest allowed level and the cognate toxins (OA, DTX and PTX) display the highest EC_{50} values. As a consequence, the test will be relatively sensitive for samples contaminated with YTXs as these have a relatively high EU limit of 3750 µg YTX-eq/kg and display low EC_{50} values in the neuro-2a assay (Tab. 7.1).

Blank samples and samples contaminated with lipophilic marine biotoxins

Compared to the general extraction and clean-up for lipophilic marine biotoxins from shellfish samples, an additional extraction step with hexane (see experimental section) was introduced to eliminate matrix effects, that would otherwise result in high percentages of false positive outcomes in the neuro-2a assay. Subsequently, seven blank mussel

samples and one mussel sample containing a high amount of YTX were extracted and tested in the neuro-2a assay (without o/v). Figure 7.3a shows that the 7 blank sample extracts did not have any effect on the cell viability of neuro-2a cells and that the sample contaminated with 1332 µg YTX-eq/kg clearly reduced cell viability. Diluting five times the blank sample extracts only resulted in slightly higher MTT values, whereas diluting the YTX contaminated sample resulted in a strong increase of the MTT activity, indicating that the diluted extract was much less toxic than the undiluted extract. Based on the data obtained with blank samples (Fig. 7.3a), an "arbitrary" decision limit (CCα) was set at an MTT value of 1.1; samples with an MTT value above this decision limit are classified as negative (safe) and samples resulting in MTT values below this decision limit are classified as suspect (potentially unsafe). Figure 7.3b shows the results of eight samples contaminated with lipophilic marine biotoxins that were previously analysed by LC-MS/MS. All samples, except sample 5, resulted in MTT values below the decision limit and are classified as suspect, while sample 5 is classified as negative (n). Table 7.3 shows the amounts of lipophilic marine biotoxins present in each sample as determined by LC-MS/MS and the classification according to the neuro-2a assay. Table 7.4 gives a summary of the actual EU-limits and the EFSA recommended levels. From these data it becomes clear that the seven samples that were screened as suspect in the neuro-2a assay contained toxin levels above or just below the EU-limits, while sample 5 that was screened negative in the bioassay only contained low amounts of AZAs (well below the regulatory limit of 160 µg AZA-eg/kg). The bioassay classification of these samples was thus in line with the levels measured with LC-MS/MS and as expected the test is relatively sensitive for samples contaminated with YTXs.

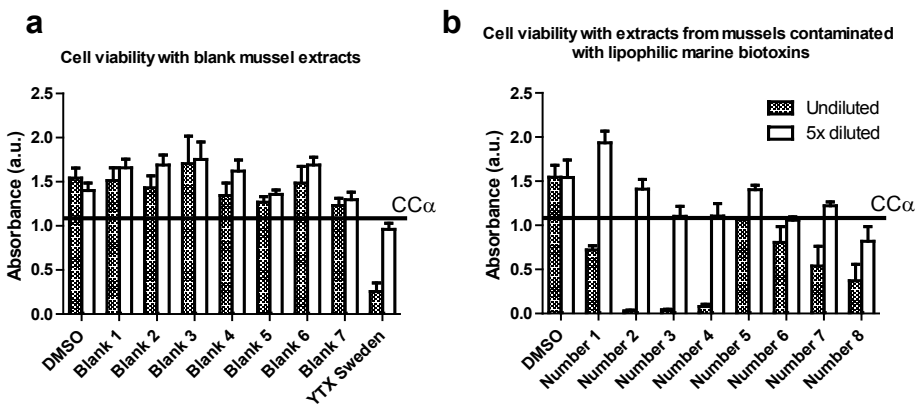

Figure 7.3. Effect on the viability of neuro-2a cells (as measured with the MTT assay) of: a) seven blank mussel extracts and one extract from mussels contaminated with yessotoxin (YTX); and b) eight extracts from mussels contaminated with lipophilic marine biotoxins. An "arbitrary" decision limit (CCα) (as defined in the text) of 1.1 was used and 0.25% DMSO was included as a control in each experiment. Data are expressed as mean ± SD (n = 3). a.u.: absolute unit.

Table 7.3. Levels of lipophilic marine biotoxins in seafood samples compared to the outcome of the neuro-2a assay.

Sample number	OA DTXs	AZAs	YTXs	PTXs	Levels of marine biotoxins*	Outcome of the neuro-2a assay
1	x				69.6 µg OA/kg 135.7 µg DTX-2/kg	Suspect
2	x		x		28 µg OA/kg 79.3 µg DTX-1/kg 25.8 µg DTX-2/kg 290.4 µg YTX/kg 191 µg 45OH-YTX/kg	Suspect
3	x	x			243 µg OA/kg 53.8 µg DTX-2/kg 1094.3 µg AZA-1/kg 259 µg AZA-2/kg 615.6 µg AZA-3/kg	Suspect
4	x	x		x	619 µg AZA-1/kg 145 µg AZA-2/kg 463 µg AZA-3/kg	Suspect
5		x			11 µg AZA-1/kg 8 µg AZA-2/kg 5 µg AZA-3/kg	n
6			x		0.55 mg YTX/kg 0.22 mg 45OH-YTX/kg	Suspect
7			x		0.21 mg YTX/kg 0.12 mg 45OH-YTX/kg	Suspect
8			x		0.47 mg YTX/kg 0.15 mg 45OH-YTX/kg	Suspect

* Levels of marine biotoxins were determined by LC-MS/MS.
n: negative. AZA: azaspiracid, DTX: dinophysistoxin, OA: okadaic acid, PTX: pectenotoxin, YTX: yessotoxin.
n: negative.

Table 7.4. Groups of lipophilic marine biotoxins and their current EU limits in seafood.

Groups of lipophilic marine biotoxins	Members	EU limit	EFSA recommended levels
Okadaic acid	OA esters DTX-1 esters DTX-2 esters PTX-2	160 µg OA-eq/kg	45 µg OA-eq/kg 120 µg PTX-eq/kg for PTX-2
Azaspiracids	AZA-1 AZA-2 AZA-3	160 µg AZA-eq/kg	45 µg AZA-eq/kg
Yessotoxins	YTX 45-OH-YTX	3750 µg YTX-eq/kg	3750 µg YTX-eq/kg

AZA: azaspiracid, DTX: dinophysistoxin, OA: okadaic acid, PTX: pectenotoxin, YTX: yessotoxin

Next, 50 samples obtained from Chile were tested in the neuro-2a assay without o/v and analysed by LC-MS/MS for lipophilic marine biotoxins contents (AZAs, DTXs, OA, PTXs, YTXs). Table 7.5 gives a summary of the obtained results. Out of the 50 seafood samples, numbers 4, 6, 17, 25, 28, 30, 34 and 45 were screened as suspect in the neuro-2a assay (Tab. 7.5). All these samples presented relatively high amounts of YTX-eq (> 500 µg YTX-eq/kg seafood) except sample 30. Samples 17 and 45 contained even levels above the limit of 3750 YTX-eq/kg. Four of these samples (17, 28, 34 and 45) contained also low levels of OA-eq (but well below the limit) and none of the 50 samples contained PTXs or AZAs. There is thus a very good correlation between the neuro-2a bioassay screening and the LC-MS/MS analysis. The only exceptions are samples 30 and 38. Sample 38 was screened negative, but contained a relatively high amount of 1787 µg YTX-eq/kg. This is not a false negative, as the EU-limit is 3750 µg YTX-eq/kg. Sample 30 is an interesting sample. This sample was clearly suspect in the bioassay but according to the LC-MS/MS contained only YTX and at a relatively low level. This sample might contain a yet unknown DSP analogue, missed by LC-MS/MS. Overall, the bioassay classification of these samples was thus correct, and one sample was flagged for additional efforts to possibly identify a yet unknown toxin.

In addition, extracts were prepared from eight samples previously used in a validation study of the LC-MS/MS method [27]. Figure 7.4 shows the results as obtained in the neuro-2a bioassay and a summary of the bioassay and LC-MS/MS

results is given in Table 7.5. Seven out of eight validation samples contaminated with levels of AZA, OA or YTX above regulatory limits elicited a decrease in cell viability of neuro-2a cells below that of the “arbitrarily” set decision limit and were thus correctly classified as suspect (Fig. 7.4). The sample that did not have any effect on neuro-2a cells contained an AZA-eq level above the EU-limit of 160 µg AZA-eq/kg. This is thus a false negative screening outcome.

Table 7.5. Summary of the effect of seafood products obtained from Chile and validation samples on the viability of neuroblastoma neuro-2a cells and the amounts of lipophilic marine biotoxins as measured by LC-MS/MS.

Sample	OA/DTXs/ PTX-2 (µg OA-eq/kg)	YTXs (µg YTX-eq/kg)	Outcome neuro-2a assay	Sample	OA/DTXs/ PTX-2 (µg OA-eq/kg)	Yessotoxin (µg YTX-eq/kg)	Outcome neuro-2a assay
1			n	26		2.1	n
2			n	27		3.8	n
3		9.9	n	28	5.7	3471.8	Suspect
4		477.4	Suspect	29		2.7	n
5			n	30		185.4	Suspect
6		660.2	Suspect	31			n
7			n	32			n
8			n	33			n
9			n	34	5.7	2098.1	Suspect
10		47	n	35			n
11		191.7	n	36			n
12			n	37			n
13			n	38		1787.3	n
14		49.9	n	39			n
15			n	40		35.2	n
16			n	41			n
17	27.5	4026.9	Suspect	42			n
18		27.4	n	43			n
19		117.3	n	44			n
20			n	45	2	4682.6	Suspect
21		5.4	n	46		23.4	n
22			n	47			n
23		701.4	n	48		121.7	n
24		34.5	n	49			n
25		654.7	Suspect	50			n

Validation samples

Sample	OA/DTXs/ PTX-2 (µg OA-eq/kg)	YTXs (µg YTX-eq/kg)	AZAs (µg AZA-eq/kg)	Outcome neuro-2a assay
S1	299	255	175	Suspect
S2	168	1702		Suspect
S3	243	1110	280	Suspect
S4	401		386	Suspect
S5	757	462		Suspect
S6	85	310	615	n
S7	1293	381	351	Suspect
S8	371			Suspect

n: negative; missing values means below limit of quantification (<LOQ)

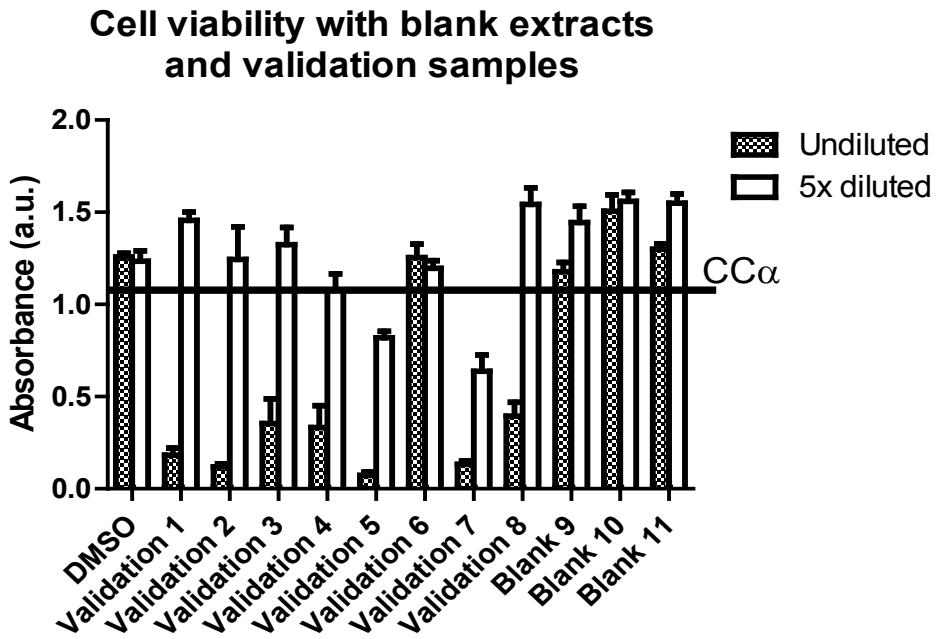

Figure 7.4. Effect of extracts of seafood products (validation samples 1-8) contaminated with okadaic acids/dinophysistoxins, yessotoxins and/or azaspiracids and blank seafood products (samples 9-11) on the viability of neuro-2a cells (as measured with the MTT assay). The decision limit was "arbitrarily" set (as defined in the text) and 0.25% DMSO was included as a control. Data are expressed as mean ± SD (n = 3). a.u.: absolute unit.

Blank samples and samples contaminated with marine neurotoxins

Seventeen seafood samples obtained from Chile and that could potentially be contaminated with marine neurotoxins were also tested in the neuro-2a assay, using the specific clean-up and the protocols with and without o/v at high concentrations, i.e. decreasing cell viability by 80% (20% cell viability). Samples were also analysed by LC-MS/MS for PSPs. Here the data are expressed as % of cell viability as determined in the MTT assay for consistency with the use of o/v that decrease the cell viability to either 20% (80% cytotoxicity) or 80% (20% cytotoxicity). Figure 7.5 shows that extracts had no effect on the viability of neuro-2a cells without o/v. On the other hand, extracts from samples 2, 3, 4, 8, 10, 11 and 14 induced an increase of the cell viability above an "arbitrarily" set decision limit (based on the data obtained in the current test series with blank samples that were also tested with the LC-MS/MS), i.e. clearly counteracted the

cytotoxic effect of o/v. Table 7.6 gives a summary of the bioassay and LC-MS/MS results. Samples 4 and 8 contained STX-eq amounts above the established EU/US-limit of 800 µg STX-eq/kg, containing 1823 and 1282 µg STX-eq/kg seafood, respectively. Substantial levels of STX, but lower than the current EU and US regulatory limits, were also measured in positively screened samples 1, 2, 3, 11 and 14. Sample number 10 is very interesting, as no toxins were detected by LC-MS/MS, while according to the neuro-2a bioassay this was the most toxic sample tested. Overall, the bioassay classification of these samples was thus correct and one very interesting sample was flagged for additional efforts to possibly identify a yet unknown toxin.

Additionally, 14 lionfish (*Pterois*) samples obtained from the Dutch Caribbean area, one positive Malaysian fish sample (red snapper, *Lutjanus spp.*) contaminated with ciguatoxin, and two fish samples obtained from France (snapper and jack fish from Martinique) testing positive in the MBA were extracted and analysed in the neuro-2a assay with the second set-up, i.e. decreasing cell viability by 20% (80% cell viability) using low concentrations of o/v. This procedure is very sensitive in detecting ciguatoxin (Fig. 7.2b). Figure 7.6 shows the results of a pacific ciguatoxin-1 standard (positive control) and of lionfish samples 13 and 14, showing a clear response of the positive control and no response of the lionfish samples. All lionfish samples actually tested negative (data for samples 1-12 not shown). Figure 7.6 also shows the results of the sample contaminated with ciguatoxin (41) and the two French samples (211 and 421). It shows that the sample contaminated with ciguatoxin and the two French samples clearly suspect in the neuro-2a assay. Both French samples also tested positive in the MBA. Overall, the bioassay classifying the samples as suspect was correct. The lion fish samples classified as negative in the neuro-2a assay probably do not contain CTX and further experiments might be required for full confirmation. However, the latter is rather difficult as to date there is no analytical method capable of detecting CTX at regulated levels.

Table 7.6. Summary of the effect of seafood extracts on the viability of neuro-2a cells and levels of marine neurotoxins measured by LC-MS/MS.

Sample	Saxitoxin (µg STX*2HCl eq/kg)	Outcome neuro-2a assay
1	159	n
2	266	Suspect
3	383	Suspect
4	1822.7	Suspect
5	47	n
6	28	n
7	15	n
8	1281.7	Suspect
9	24.1	n
10	0	Suspect
11	392	Suspect
12	253.3	n
13	41	n
14	290*	Suspect
15	146	n
16	299.5	n
17	83.7	n

n: negative
* After reintegrating shifted peaks (STX, dcSTX and NeoSTX) the total concentration of sample 14 is 290 µg STX-eq /kg.

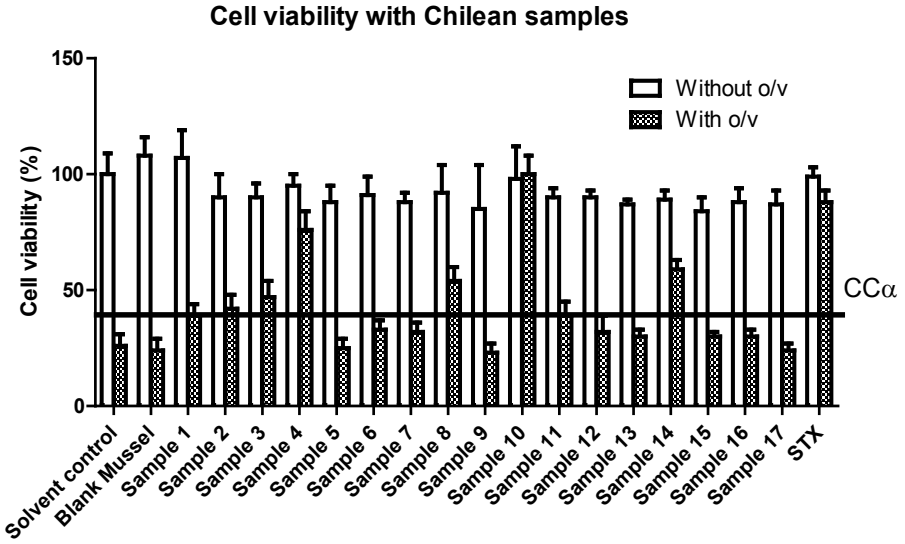

Figure 7.5. Effect of Chilean seafood extracts on the viability of neuro-2a cells as measured with the MTT assay, directed towards the detection of neurotoxic compounds. Extracts were tested with and without high concentrations of o/v (ouabain/veratridine), i.e. eliciting a 80% decrease in cell viability. The decision limit was "arbitrarily" set (as defined in the text) and 0.25% DMSO was included as a control. 12 nM saxitoxin (STX) was used as positive control. Data are expressed as mean ± SD (n = 3).

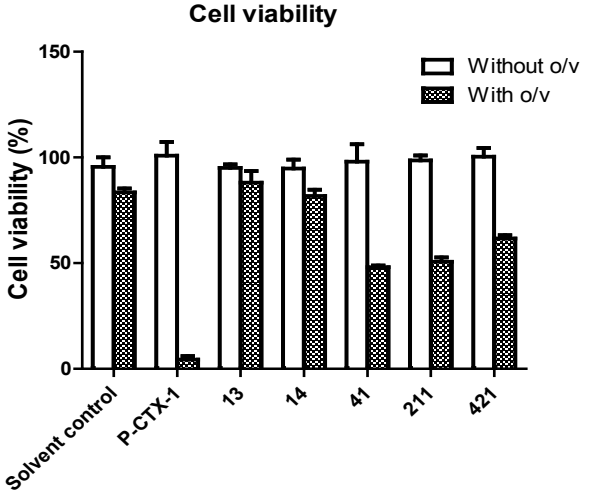

Figure 7.6. Effect of fish extracts on the viability of neuro-2a cells as measured with the MTT assay. Extracts were tested with and without low concentrations of o/v (ouabain/veratridine), i.e. eliciting a 20% decrease in cell viability. 0.25% DMSO was included as a negative solvent control. 3 pM P-CTX-1 were used as positive control. Data are expressed as mean ± SD (n = 3). 13, 14: lion fish samples, 41: fish sample contaminated with ciguatoxins, 211, 421: French fish samples of snapper and jack fish from Martinique responsible for intoxicating four and two people respectively in 2012, and positive in the mouse bioassay.

Discussion

The present study shows that the neuro-2a assay allows the detection of all regulated marine biotoxins at low concentrations and below the regulatory limits when present in seafood samples, except for the ASP domoic acid (DA). All analogues of both the lipophilic marine biotoxins and marine neurotoxins could be detected as well, indicating that this assay can potentially detect unknown marine biotoxins too. Lipophilic marine biotoxins induced a decrease in viability of neuro-2a and NG108-15 cells in a sensitive and concentration-dependent way. These findings are in agreement with the data obtained by Cañete and Diogène [23]. Marine neurotoxins and their analogues also affected the viability of neuro-2a cells, provided the cells were co-exposed with o/v. In the case of marine neurotoxins opening VGSCs or inhibiting the Na^+/K^+-ase pump, the cell viability is further decreased as the cytotoxic effect of low concentrations of o/v is enhanced. On the other hand, marine neurotoxins blocking Na^+ channels, such as STX and TTX, prevent cytotoxicity by opposing the effect of high concentrations of o/v, i.e. through opposite modes of action as those of o/v. PbTxs are lipophilic, thus the extracts prepared for lipophilic marine biotoxins should be tested in the neuro-2a assay with o/v as well when testing for these toxins.

Showing the performance on real samples both negative and positive for marine biotoxins is crucial for the neuro-2a assay to be implemented as a routine technique for replacement of the MBA. It is evident that positive samples should not be overlooked, but a too high incidence of false negative results also has serious consequences. Therefore, a large number of shellfish and fish samples were tested with clearly a bias on samples containing detectable levels of marine biotoxins, to some extent exceeding existing limits. A hexane extraction as a clean-up was introduced for testing samples for the presence of lipophilic marine biotoxins, i.e. DSPs and AZAs, in order to remove matrix effects leading to false positives. Such test results without this extra hexane clean-up were most probably caused by free fatty acids, also known to interfere with the outcome of the MBA [28]. This hexane extraction worked very well: blank samples did not affect cell viability anymore and an “arbitrary” decision limit (CCα) could be set that allowed the screening of real samples. Even the amplitude of the effect observed in the neuro-2a assay with positive samples (contaminated with lipophilic marine biotoxins) correlated well with the amount of toxins present in the seafood extracts as determined by LC-MS/MS. However, few samples elicited a different effect than expected when considering the amounts of measured marine biotoxins. Among the 66 samples screened for the presence of

lipophilic marine biotoxins, 22 were screened as suspect and 44 as negative, while LC-MS/MS identified 12 positives and 54 negatives. Only one sample was screened consistently as false negative, i.e. validation sample S6, containing AZAs above the regulatory limit of 160 µg AZA-eq/kg and therefore samples contaminated with AZAs might require more attention. Thus from the 22 samples screened as suspect in the neuro-2a bioassay, 11 turned out to be true positives. Most of the other 9 samples that were also screened as suspect in the neuro-2a bioassay turned out to contain clearly elevated levels of toxins, e.g. samples 1 and 2 from the first series (Tab. 7.3) and samples 28 and 34 from Chile (Tab. 7.5) which contained levels just below the limit. An exception was sample 30 that was obtained from Chile. This sample was clearly suspect in the bioassay, but only contained a low level of YTX-eq. This sample might contain yet unknown analogues of lipophilic marine biotoxins, missed by LC-MS/MS. It is also possible that this sample is contaminated with PlTx, as PlTx is extracted with the lipophilic DSP and AZA toxins and is also able to decrease MTT activity without the addition of o/v.

Regarding the marine neurotoxins, among the 34 samples screened for the presence of NSPs and PSPs, eleven were evaluated as suspect and 23 as negative. No false negative was recorded according to LC-MS/MS analysis and five out of the eleven suspects were confirmed by either the LC-MS/MS (two) or the MBA (two). Five of the other six samples screened as suspect in the neuro-2a bioassay turned out to contain clearly elevated levels of saxitoxins. One sample was flagged, as no toxins were detected by LC-MS/MS, while according to the neuro-2a bioassay this was the most toxic sample tested for the presence of neurotoxins capable of opposing the effect of o/v.

Although the “arbitrarily” set decision limits turned out to work well, a more accurate establishment of the CCα should be made, i.e. by testing at least 20 blank samples. This is possible for the lipophilic marine biotoxins, as more than 20 blank samples have now been tested in this set-up. By calculating the decision limit as the mean of 20 blank samples minus three times the standard deviation, an MTT value of 1.2 was calculated, close to the “arbitrary” limit of 1.1 applied in this paper. If a decision limit of 1.2 were used instead of 1.1, the screening outcome of the samples tested would be the same. It should be noted that the hexane extraction will also eliminate esterified forms of OA and DTXs. Thus for application to real samples in the future, an hydrolysis step prior to the SPE clean-up should be incorporated as done with LC-MS/MS analysis.

Regarding marine neurotoxins, more blank samples have to be screened in the neuro-

2a assay for both o/v set-ups, as less than 20 blank samples have been screened is these set-ups (14 blanks for each set-up now). In addition, the response of the assays upon the presence of two toxins with opposite modes of action needs to be checked, as they might counteract each other's effects, which could theoretically result in false negatives. However, this is highly unlikely, as for instance PbTxs can inhibit the response of STXs, but the extraction procedures are designed such that these toxins do not end up in the same sample extract, i.e. PbTxs end up in the lipophilic sample extract (together with the DSPs and AZAs), while STXs end up in the extract containing all other neurotoxins. PbTxs might thus interfere with the screening outcome of the other lipophilic marine biotoxins, but as PbTxs are tested in a different set-up (with o/v), it is clear whether or not the sample is contaminated with PbTxs and whether the interference with the other lipophilic biotoxins, i.e. DSPs and AZPs, was possible.

The neuro-2a bioassay is thus demonstrated to be a suitable alternative for the broad screening of seafood products. Besides its sensitivity towards a large variety of marine biotoxins, it allows a non-animal, quick and high throughput (24 h, 96-well plate set-up) detection of these toxins in seafood and thus fulfils the requirements for such a test to be used as a first screening approach in a routine set-up. It is also affordable compared to current alternatives, such as LC-MS/MS and the Lawrence method, the official methods for DSPs and PSPs respectively. Moreover, unlike the current analytical and immuno-based alternatives, the neuro-2a assay will most likely be able to detect emerging risks and yet unknown marine biotoxins. Two samples were flagged that might contain such yet unknown marine biotoxins. Both samples will be further analysed in order to show the additional value of this effect based screening approach.

While the neuro-2a assay offers the possibility to screen a wide range of marine biotoxins, it is unable to pick up the regulated ASP domoic acid or non-regulated cyclic imines such as spirolides. This can be explained by the fact that neuro-2a cells have a low expression of (especially in an undifferentiated state as used in the present study) or do not present the receptors at all that are targeted by these groups of toxins, i.e. N-Methyl-D-aspartate (NMDAR) targeted by DA or acetylcholine (AChR) receptors targeted by spirolides, therefore preventing their toxic effects on such cells [29, 30]. To overcome the problem with DA, the neuro-2a assay should be used as a base of an integrated testing strategy, together with an ELISA for DA for example [31]. No test is currently available for the detection of spirolides, certainly also because these toxins are currently not regulated, and there is thus no urgent

need to include them in regular monitoring approaches.

Here we propose a new screening strategy that enables a full replacement of the MBA (Fig. 7.8). Seafood samples are first screened with the neuro-2a assays for lipophilic marine biotoxins (without and with o/v at low concentrations) and for marine neurotoxins (with the two set-ups with o/v as explained and applied in this study) and an ELISA based dip stick test for detection of DA. If the outcomes of the in vitro neuro-2a assays and the DA dip stick are negative, the seafood sample is considered to be safe and can be placed on the market. When a sample is found as suspect in one of these tests, i.e. is potentially unsafe, it is further analysed with chemical analytical methods (LC-MS/MS). When chemical analyses confirm the suspected first screening outcome and reveal levels of marine biotoxins above current regulatory limits, the sample is unsafe and cannot be placed on the market. In case chemical analyses reveal levels below current regulatory levels, but can explain the suspected screening outcome, seafood can be placed on the market. If the outcome of the neuro-2a assays or DA dip stick is positive, but no known marine biotoxin is detected with chemical analyses, additional methods for the detection and identification of yet unknown marine biotoxins are required. For lipophilic marine biotoxins (except PbTxs and PlTxs) assays based on gene expression analysis of exposed human intestinal Caco-2 cells, i.e. PCR and Luminex methods are currently under pre-validation ([32] and Bodero et al., unpublished data). Such assays can give a better insight in the specific class of toxins present in the sample. For marine neurotoxins (including PbTxs and PlTxs) the multielectrode array-based approach described by Nicolas et al., in which neuronal activity of rat cortical neurons is recorded, is proposed [33]. This latter method still requires the use of animals but the use of cell lines recently proved to be suitable with this approach as well [34]. If the presence of an unknown toxin is confirmed by these additional bioassays, untargeted time of flight mass spectrometry (ToF-MS) or high resolution mass spectrometry (HRMS) analysis in combination with bioassay guided fractionation procedures are needed to identify the toxin. Two samples were flagged in the current study that should go through such an additional effort in order to identify potentially unknown toxins.

In conclusion, an integrated in vitro testing strategy comprising bioassays and biosensors combined with chemical analyses for identification and quantification, offers the opportunity to replace the MBA for routine detection of marine biotoxins in seafood. These assays are high throughput, easy to perform, relatively affordable and they potentially allow the detection of currently unknown marine neurotoxins too and might even result in less false negatives and false positives than the MBA.

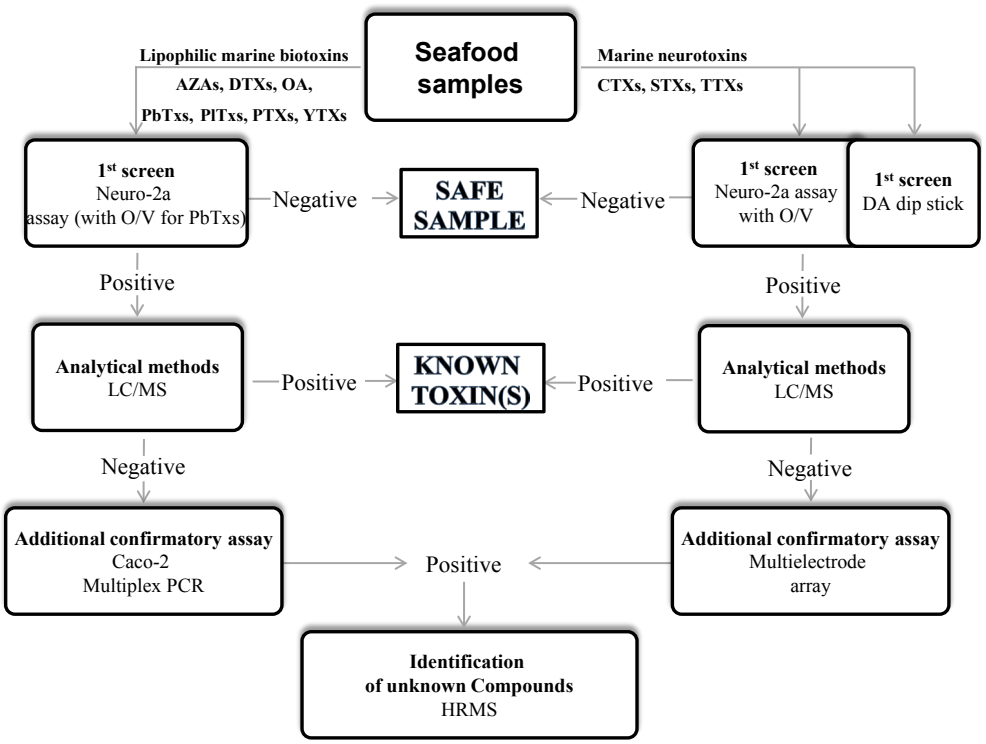

Figure 7.8. An integrated non-animal in vitro testing strategy for the detection of marine biotoxins in seafood products, enabling a full replacement of the mouse bioassay. The proposed strategy combines first screen assays (neuro-2a assays and a DA dip stick), analytical methods and additional confirmatory assays (multielectrode array, PCR or Luminex and ToF/MS or hrMS). AZA: azaspiracid, CTX: ciguatoxin, DA: domoic acid, DTX: dinophysistoxin, OA: okadaic acid, PbTxs: brevetoxins, PlTx: palytoxin, PTX: pectenotoxin, STX: saxitoxin, TTX: tetrodotoxin, YTX: yessotoxin and o/v: ouabain/veratridine.

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Acknowledgements

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CHAPTER 8

General discussion, summary, list of abbreviations

Main findings and outline

Main findings

The research described in this thesis focused on the development of in vitro assays for the detection of marine neurotoxins. For most marine neurotoxins regulatory limits have been set and the detection of these toxins still heavily relies on the mouse bioassay (MBA). The MBA is forbidden since the 1st of January 2015, except for the periodic control of production areas, especially with regard to the detection of unknown marine biotoxins. The MBA is also still widely used for the detection of paralytic shellfish poisons (PSPs), as there is no official method currently validated for their detection, and for ciguatoxins (CTXs), for which there is no analytical method sensitive enough to detect them at the regulatory limit (**chapter 1 and 2**).

While analytical methods are sensitive for many marine biotoxins and allow for identification and quantification of these toxins, the use of analytical methods still presents several major drawbacks (**chapter 3**). Among these drawbacks are the facts that analytical methods do not allow for the detection of unknown toxins, are not sensitive enough for CTXs and are quite expensive to be used as routine screening methods. Therefore, a screening assay that allows the broad detection of known and unknown marine biotoxins with great sensitivity at affordable costs is highly needed. Mode of action cell-based assays offer this possibility. The present thesis focused on the development of such mode of action based bioassays for marine neurotoxins, i.e. amnesic shellfish poison (domoic acid (DA)), CTXs, neurologic shellfish poisons (NSPs), and PSPs, as an important subcategory of marine biotoxins. Most marine neurotoxins target ion channels/pumps or receptors present on the plasma membrane of excitatory cells. Thus, the suitability of cardiomyocytes for detection of the above mentioned marine neurotoxins was investigated. Using beating cardiomyocytes as a tool for neurotoxicity testing might raise questions at first instance, but the rationale behind it is clear: cardiomyocytes present a large variety of ion channels at the surface of their membrane and the model is thus most likely to be affected by these particular marine neurotoxins. As described in **chapter 4**, beating cardiomyocytes responded well to reference neurotoxic model compounds and PSPs, but unfortunately showed a lack of sensitivity towards marine neurotoxins hampering their applicability for monitoring of real samples. Cardiomyocytes were approximately 400 times less sensitive towards the Na^+ channel blockers

saxitoxin (STX) and tetrodotoxin (TTX), when compared to the neuro-2a bioassay. Cardiomyocytes most likely express Na^+ channel subtypes resistant to the above mentioned marine neurotoxins.

The neuro-2a assay is currently based on the assessment of the effect of compounds on the cell viability. Cell viability is not a specific endpoint and might also be affected by external factors, e.g. handling conditions, changes in pH or temperature and matrix effects. The suitability of alternative functional endpoints in neuroblastoma neuro-2a cells was therefore investigated in **chapter 5**. Gene expression in neuro-2a cells after exposure to palytoxin (PlTx), STX and TTX was analysed. And besides transcriptomics, changes in membrane potential were monitored using the fluorescent dye bisoxonol. Biomarkers based on mRNA expression were detected for PlTx but not for STX and TTX. STX and TTX decreased the fluorescence of bisoxonol while PlTx had no effect on this outcome. Thus, transcriptomics and changes in membrane potential are not preferred over cytotoxicity as final endpoints in the neuro-2a assay for a suitable broad and sensitive bioassay for the detection of marine neurotoxins.

One important drawback of the neuro-2a assay appears to be that it does not allow the detection of the regulated marine neurotoxin DA (**chapter 7**). Therefore, in **chapter 6** the multielectrode array (MEA) was used to assess the effect of marine neurotoxins, including DA, on the activity of rat cortical neurons, which are known to express N-methyl-D-aspartate (NMDA) receptors that are targeted by DA [1, 2]. The MEA showed high sensitivity and specificity towards the model compounds and marine neurotoxins tested (see **chapter 6**). This technique is still relatively expensive and requires animal testing, impairing its applicability as a first screen assay. However, it constitutes an interesting tool for confirmation of the presence of marine neurotoxins and the detection of DA.

The results described in **chapter 7** revealed that the neuroblastoma neuro-2a assay, with cytotoxicity as a final readout, offered a high sensitivity towards marine neurotoxins and allowed for the detection of the majority of marine neurotoxins and their analogues not only when tested as pure standards, but also when present in food matrices (mussels, crabs, oysters, clams). Among the assays developed in this thesis and those described in the literature, the neuro-2a assay is the most promising technique for the broad, sensitive and high throughput detection of marine neurotoxins in seafood. In addition, it was shown that the neuro-2a assay is also capable of detecting all regulated lipophilic marine biotoxins and their analogues, both as pure standards and when present

in seafood.

The present thesis also shows that none of the assays alone allows for the detection of all regulated marine neurotoxins and therefore an integrated testing strategy is required. Such an integrated testing strategy may include different bioassays, a DA-biosensor (receptor-based dip stick test) and analytical methods, together allowing the screening, identification and quantification of marine biotoxins in seafood products (see **chapter 7** and 4th section of this **chapter 8** for the complete strategy for monitoring and replacing the MBA). It is concluded that with this strategy, the current bottlenecks of in vivo assays and analytical methods can be overcome, since this strategy comprises assays that do not only allow the detection of all regulated marine toxins and potentially unknown toxins, but also permits their identification and quantification at sufficiently sensitive detection levels. Moreover, this strategy will eventually allow replacement of the MBA and is thus in line with European regulation 15/2011, and also fully supports the 3R concept proposed by Russel and Burch [3].

Outline of the discussion

Chapter 2 clearly demonstrates the need for monitoring programs, together with appropriate detection tools. Detection of marine neurotoxins in seafood still requires the use of assays that are not in line with the current food safety strategies in this field, aiming at reduction and replacement of the MBA. Current limitations associated with monitoring programs are first discussed in the present chapter. In addition a brief comparison of the in vivo assays (mostly the MBA) with the in vitro mode of action cell-based bioassays developed during the present PhD project is then given, once more emphasizing the possibilities for alternatives to animal experiments.

Based on the results obtained in this thesis and a parallel project on diarrhetic shellfish poisons, an integrated testing strategy is proposed in this overall discussion, including first screen bioassays and biosensors, analytical methods for confirmation, identification and quantification of known toxins and both bioassays and analytical methods for confirmation and identification of unknown toxins respectively. This integrated testing strategy aims at providing the most suitable strategy to be implemented routinely for the screening of marine biotoxins in seafood to ensure food safety.

Finally, the discussion presents an analysis of the societal impact and, as there is still work to be performed in the field covered by this thesis, future

perspectives are discussed.

Monitoring programs

As described in chapter 2, monitoring programs for marine biotoxins are effective and successful in most developed countries. However, there are still some points for improvements and some adjustments should be made.

First, legislation still lacks clear explanations about which marine biotoxins require regulatory limits. Some of the marine biotoxins that are not regulated threaten the safety of seafood consumers. One major issue lies in the fact that while azaspiracid-1 (AZA-1), AZA-2 and AZA-3 should be monitored in routine, AZA-17 and AZA-19 do not require any specific attention [4]. This is rather surprising, if one realizes that during cooking processes AZA-17 and AZA-19 are converted into AZA-1, AZA-2 and AZA-3 and thus represent a potential risk for consumers too [5]. Some countries already take this issue into account, by cooking the mussels before analysis [6, 7].

Besides AZAs, ciguatera fish poisoning poses a problem, as only one fish from a batch can be contaminated, while other fishes from the same batch are not. This means that prevention is often the best solution, i.e. ensuring that the products that might be contaminated with CTXs are not placed on the market. In other types of seafood, such as shellfish and crabs, it is possible to limit consumer exposure through suitable ways of sampling, i.e. through the testing of homogenates of multiple individuals. Before applying the different assays and methods from the newly proposed integrated testing strategy (see below), one should keep in mind that at the beginning a specific procedure for sampling is needed in order to protect seafood consumers as good as possible. However, to date there are no clear rules on how to sample seafood, while contamination of seafood depends on where the organism was situated in the water column. It is possible that mussels from a batch are declared as free from marine biotoxins, while some of the mussels from the batch are in fact contaminated [8]. The sampling methods for mycotoxins in Europe represents a good starting point to be taken into account and can be adapted to be included in the European Regulation for monitoring and detection of marine biotoxins in seafood [9].

Finally, an important issue in the multiple debates between experts in the field of HABs concerns the question whether one should assess the risk associated with HABs through measuring the amount of phytoplankton cells in the water or through measuring the amount of toxins present in seafood. The research

performed by Turki et al. already provides part of the answer [10]. The authors showed that there is no correlation between phytoplankton cell number and levels of toxin in shellfish. Therefore, cell count allows for applying prevention measures, but it most likely overestimates the production of toxins and therefore gives an incorrect assessment of the risk associated with consumption of potentially contaminated seafood. Screening seafood itself prior to market release is thus currently the best way for ensuring food safety. The following section briefly compares the in vivo assays routinely used for such monitoring programs with the in vitro mode of action cell-based bioassays developed during the present PhD project.

In vitro alternatives to replace the in vivo tests for the detection of marine biotoxins

The current in vivo assays, i.e. the MBA and to a lower extent the rat bioassay (RBA), are not in harmony with the actual scientific knowledge and present shortcomings that impair seafood consumer's safety. Because of the drawbacks and unethical aspects associated with the use of the MBA (Tab. 8.1), the European Commission decided to ban this assay from 2015 onwards, except for the periodic control of production areas for the detection of unknown toxins [3]. Furthermore, the MBA is still used for routine detection of PSPs as there are currently no alternative methods validated and available for the detection of these marine neurotoxins in seafood [11].

It is clear that the MBA is far from fulfilling the criteria for a routine assay to be applied for the detection of marine biotoxins worldwide. Besides requiring the use of a large number of animals, the detection limit depends on the strain of the animals, the levels of toxins are not correlated with death time, and matrix effects resulting in false positives are reported, i.e. nontoxic lipids are known to induce lethality of mice [12, 13]. In addition, and as mentioned in **chapter 2**, low levels of spirolides (far below levels that would be harmful to human) also lead to false positives in the MBA [14].

In vitro mode of action based bioassays present several advantages compared to the in vivo tests. They are high throughput, easy to perform, relatively affordable and they potentially allow the detection of currently unknown marine neurotoxins too and might even result is less false negatives and positives (Tab. 8.1). High throughput screening invites for innovation to push the limits of time constraint and labour intensity while screening a wide range of marine neurotoxins on a

routine basis. This has its limits for cell based assays, although they meet all criteria, exposure is performed overnight and results are thus not obtained at the day of arrival of the sample, but on the next day.

Table 8.1. Comparison between in vivo (mouse bioassay) and in vitro (cell-based) assays.

In vivo (mouse bioassay)	**In vitro (cell-based assays)**
Requires use of large amount of animals	No animals required
Detection limit depends on animal strain	Same cell line → standardized response
No linear relationship between toxin level and animal death time	Concentration-dependent effects
Labour intensive	High throughput
Expensive	Relatively affordable

Integrated testing strategy

Given the results obtained in the present thesis, it is clear that none of the assays alone will permit detection of all regulated marine neurotoxins. An integrated in vitro testing strategy is required to ensure seafood consumers' safety. In such an integrated testing strategy the methods developed in this thesis will contribute to replacing the MBA for a "first screen" and are complementary to the analytical techniques developed so far. An integrated in vitro strategy is also needed for the screening of marine biotoxins in seafood because there is a wide range of modes of action that cannot be covered by a single assay. **Chapter 7** described how a combination of the assays developed in the present thesis with assays developed in a parallel project on lipophilic marine biotoxins enables detection of all regulated marine biotoxins, resulting in a complete integrated testing strategy able to ensure seafood consumer safety. A scheme of this newly proposed strategy is shown in Figure 8.1 and consists of a tiered approach. The process begins with the preparation of different extracts from a single sample, which are subsequently used for a broad screening based on the neuro-2a assay for lipophilic toxins and CTXs/NSPs/PSPs/TTXs, and for a DA receptor based dip stick test (Tier 1). One should keep in mind that screening per definition results in false positives, as false negatives should be avoided. When a sample is classified as negative in these two in vitro assays it can be considered as safe, and the respective seafood can be placed on the market. If positive, the sample is further analysed with analytical methods (LC-MS/MS) (Tier 2). When analytical methods

show levels of marine biotoxins below their current regulatory limits, but can fully explain the positive bioassay result, seafood can be placed on the market. If the levels found are above regulatory limits, true positives, then the respective seafood is considered unsafe and cannot be placed on the market. Finally, if the outcome of the neuro-2a assay is positive, and the analytical methods do not detect any known marine biotoxins, additional methods for the detection and identification of the presence of yet unknown marine biotoxins in the sample are required (Tier 3). In the case of marine neurotoxins, such an additional method may consist of the MEA (**chapter 6**). In case of lipophilic marine biotoxins, PCR/ Luminex methods based on the expression of marker genes in Caco-2 cells appear to be promising methods and are currently under development/pre-validation in another PhD project (Marcia Bodero). If the presence of an unknown toxin is confirmed by these additional bioassays, untargeted time of flight mass spectrometry (ToF-MS) or high resolution mass spectrometry (HRMS) analyses in combination with bioassay guided fractionation procedures are needed to identify the toxin (chapter 7).

The following section describes the different Tiers of the integrated testing strategy in some more detail.

Tier 1: First screen assays

At Tier 1 first screen assays are used. A first screen assay should fulfil a series of criteria: it should be affordable (developing countries should be able to implement such an assay at the national level), high throughput (several batches of seafood to be screened every week in some areas), easy to perform (a wide range of people should be capable of performing such assays following a specific training), quick (seafood should be screened within a short time frame prior to being released on the market) and sensitive (marine biotoxins should be detected at the regulatory limits suggested by food safety authorities and implemented by regulatory bodies).

Among the assays developed and tested in this thesis, the neuroblastoma neuro-2a assay is the best candidate for a first screen assay (**chapter 7**). Besides being relatively easy to implement, it offers the possibility to screen a variety of seafood samples and allows for the detection of a wide range of marine biotoxins in a sensitive manner, i.e. at and below the current EU regulatory levels (Tab. 8.2).

Chapter 7 clearly demonstrates the applicability of the neuro-2a assay as

a routine method for the broad screening of marine neurotoxins and lipophilic marine biotoxins in a wide range of seafood matrices. Like the majority of cell-based assays available up to now for the detection of marine biotoxins in seafood, the neuro-2a assay does not allow for the detection of DA. Fortunately, simple kits are available for DA and can be used in parallel to the neuro-2a assay at low cost and in a high throughput manner (first screen assays, Fig. 8.1). No test is currently available for the detection of spirolides, certainly also because these toxins are currently not regulated.

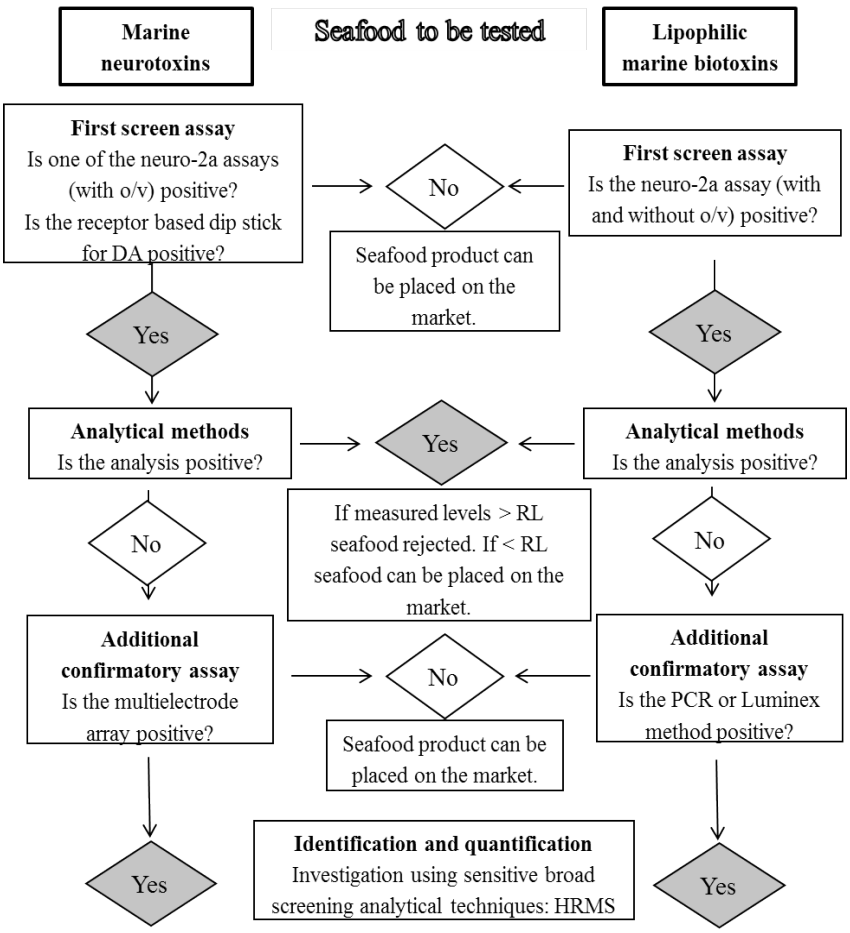

Figure 8.1. Framework for the testing of marine neurotoxins. This integrated testing strategy consists of a Tiered approach. Tier 1 comprises first screen assays, Tier 2 analytical methods and Tier 3 additional confirmatory assay and time of flight (ToF-MS) or high resolution (HRMS) mass spectrometry. Additional confirmatory assays for lipophilic marine biotoxins are currently under development by Bodero et al.. DA: domoic acid. RL: Regulatory limits.

Table 8.2. Current European and American regulatory limits compared to the EC_{50} values obtained in the neuro-2a assay.

Toxin	Current EU limits	Current US limits	EC_{50} values in neuro-2a assay	Estimated levels of toxins that can be detected in seafood based on EC_{50} values*
Palytoxin (PlTx)	NR	NR	39.2 pM	200 µg/kg SM
Ciguatoxins (CTXs)	a	0.01 µg P-CTX-1 eq/kg meat or 0.1 µg C-CTX-1eq/kg meat	P-CTX-1: 0.9 pM	1.6 µg P-CTX-1/kg meat
Brevetoxins (PbTxs)	NR	0.8 mg PbTx-2eq/kg SM	PbTx-3: 8 nM PbTx-9: 8.4 nM	PbTx-3: 13.8 mg/kg SM PbTx-9: 14.5 mg/kg SM
Saxitoxin (STX)	800 µg STX/kg SM	800 µg STX eq/kg SM	8.2 nM	3.9 mg/kg SM
Tetrodotoxin (TTX)	b	Importation of puffer fish products restricted	18 nM	9.2 mg/kg meat
Domoic Acid (DA)	20000 µg DA/kg SM	20000 µg DA/kg SM except in viscera of dungeness crab (30 mg DA/kg SM)	c	-

SM: shellfish meat. NR: Not regulated. *: based on the current extraction procedure and the setup of the neuro-2a assay. a: Fishery products containing biotoxins such as ciguatoxin or muscle-paralysing toxins must not be placed on the market. However, fishery products derived from bivalve molluscs, echinoderms, tunicates and marine gastropods may be placed on the market if they have been produced in accordance with Section VII and comply with the standards laid down in Chapter V, point 2, of that Section (for more information see [30]). b: Fishery products derived from poisonous fish of the following families must not be placed on the market: Tetraodontidae, Molidae, Diodontidae and Canthigasteridae [30]. c: the neuro-2a assay does not allow for detection of domoic acid but the multielectrode array does (EC_{50}: 0.4 µM).

8

Tier 2: Analytical methods

Samples tested positive in the bioassays of the Tier 1 should be further analysed by analytical methods for confirmation and identification of the marine biotoxjns actually present (analytical methods, Fig. 8.1). Only a few analytical methods are allowed as official methods for the screening of certain marine biotoxins. Among these methods, the EURL-MB LC-MS/MS method [15] and the pre or post-column

oxidation HPLC-FLD method are the official methods applied in Europe for the detection of lipophilic marine biotoxins (AZPs and DSPs) and PSPs in seafood, respectively [3, 16]. The EU official HPLC-UV method is used as a routine method for detection of ASP (DA). While analytical methods are very sensitive and allow for detection, identification and quantification of most regulated toxins, except for some marine biotoxins such as ciguatoxins (that are very potent and therefore require a detection at extremely low levels which cannot be achieved by analytical methods), they still present some major additional drawbacks. They require expensive pure standards that are barely or even not available, and do not allow for the detection of unknown toxins. Therefore, it is possible that a seafood extract is classified as positive in the neuro-2a assay and that the standard analytical methods are not able to detect a toxin that confirms the positive first screening result. This is why a Tier 3 with additional confirmatory assays, both bioassays and analytical methods, are crucial elements for the establishment of a suitable integrated testing strategy for the screening of marine biotoxins in seafood in order to replace the MBA.

Tier 3: Additional confirmatory assays

The neuroblastoma neuro-2a assay allows the detection of a wide range of marine biotoxins at sensitive levels. However, this assay was not capable of detecting DA. Luckily, a simple and cheap dip stick for the detection of DA is available for the first screen and the MEA for additional confirmation of the presence of marine neurotoxins in seafood. Besides allowing the detection of DA, the MEA permits the screening of marine biotoxins that affect neuronal activity and are not currently detected with analytical methods. The MEA is still expensive and this will most likely impair its implementation as a routine method for the screening of marine biotoxins at national levels. However, such confirmations are likely not often needed in real practice and samples containing unknown toxins might be sent to specialized laboratories equipped with the MEA and ToF-MS or HRMS techniques for further confirmation and identification of the marine biotoxins present in the suspected sample (additional confirmatory assay and identification and quantification, Fig. 8.1).

Groups of researchers are focussing on the development of analytical tools that allow the confirmation of the presence of marine biotoxins that are not detected with the official standard analytical methods mentioned in section 4.2. These methods are needed for a successful integrated testing strategy by allowing the identification and quantification of compounds that are presently

detected by the neuroblastoma neuro-2a assay, but cannot be confirmed by the current official methods.

Validation of the method

The assays described in this thesis are still in a developmental stage and may require adjustments and further optimisation before being implemented in routine testing. Other contaminants targeting ion channels and pumps might interfere with the outcome and thus might increase the amount of false positive results. The extraction procedure is therefore crucial. Before performing the assays, one should make sure that the extraction method is specific enough to exclude other compounds that could interfere with the outcome of the screening assays. Here we used an extra hexane extraction to get rid of undesired compounds in the procedure for lipophilic marine biotoxins. At the end, all the performed extractions seem to work well (**chapter 7**). However, additional samples need to be tested, especially samples that tested positive in the MBA as this is the assay we aim to replace, but also in order to set well established cut-off values. In addition, the response of the assays upon the presence of two toxins with opposite modes of action needs to be checked, as they might counteract each other's effects, which could theoretically result in false negatives. However, this is not very likely as for instance while STXs can inhibit the action of PbTxs, the extraction procedures are designed in a way that these toxins do not end up in the same extract due to the hydrophilic nature of STXs compared to the lipophilic PbTxs.

Results of the present thesis revealed that several samples from seafood contaminated with low levels of marine neurotoxins (below EU regulatory limits) tested positive in the neuro-2a assay (**chapter 7**). One might argue that such positives might represent a drawback for the applicability of the neuroblastoma neuro-2a assay as a first screen assay for the detection of marine biotoxins in seafood. However, one should keep in mind that an important prerequisite of a first screen assay is to keep the false negative rate as low as possible, even when that would be accompanied by a somewhat higher number of false positives. Moreover, the rate of false positives was rather low and these samples did contain substantial amounts of toxins (not far below the established regulated limits). In addition, the level of toxins can increase after processing the food, e.g. especially due to water loss (cooking), and therefore detection of the toxins at concentrations below the regulatory limits allows for early warning from a food safety perspective. The levels of toxins in seafood after cooking can be two times higher compared to the levels measured during routine monitoring [17]. Regulation is currently based on live shellfish, while processed products that might contain higher levels of toxins due to loss of water for example are also

placed on the market. These products thus require special attention. According to the European Food Safety Authority (EFSA), this variable should be taken into account when implementing official control systems [18]. EFSA also considers that the current EU regulatory levels protect less than 95% of the population (except for yessotoxins) [19].

While a validation assessment of the neuro-2a assay was performed and proved the assay to be successful (chapter 7), for implementation in routine screening one should focus next on the transferability of this assay first in a single-laboratory validation and subsequently in inter-laboratory studies. An arbitrary decision limit (CCα) for identification of positive samples that need further testing with analytical methods was chosen in chapter 7 and it is therefore necessary to test more extracts of blank samples in order to determine a more accurate CCα that could be used during routine testing. A single laboratory validation of the neuro-2a assay is acceptable for the implementation of such an assay in-house. At RIKILT, Institute of Food Safety in the Netherlands, the neuro-2a assay is now planned to run in parallel with the LC-MS/MS for a few months (starting at the end of 2015 or beginning of 2016) for in-house validation. The ultimate objective is to demonstrate that the bioassays can replace the LC-MS/MS for first screening of marine biotoxins. The LC-MS/MS would then be used if a sample is found positive in the neuro-2a assay or DA dip stick test (Fig. 8.1).

After being successfully validated in-house, the neuro-2a assay still requires an inter-laboratory validation to be implemented in other laboratories as an official routine tool for detection of marine neurotoxins. This implies a full validation exercise in at least three different laboratories, the evaluation by an external committee and the approval of a common protocol. Only after that and if the method is proven to show good results then the assay might be officially approved and legally implemented for routine screening. The choice of using the bioassays/DA dip stick combination or a broad LC-MS/MS as a first screen heavily depends on the situation in the specific countries, i.e. the problems occurring in their coastal waters. Countries with problems of all kind of known and unknown marine biotoxins might benefit from a biobased first screen, while countries facing problems with known lipophilic marine biotoxins only might benefit from an LC-MS/MS as a first screen.

Future outlook

This thesis focused on screening tools for seafood products, but part of the prevention of the occurrence of outbreaks following consumption of seafood contaminated with marine biotoxins lies in the maintenance and improvement of monitoring programs. In the Netherlands for example, monitoring tools such as resins (for instance solid phase adsorbent resins [20]) or real time monitoring equipment to record growth of populations of toxin-producing algae should be implemented. This extra "barrier" would allow for prioritization of sites where contamination of seafood is most likely to occur and would also permit seafood producers to allocate their resources more efficiently.

Besides monitoring programs, detection of marine biotoxins in seafood is of utmost importance. Based on the results presented in this thesis, transcriptomics in neuro-2a cells does not allow the identification of specific biomarkers that could be used for the broad detection of marine neurotoxins. Without external trigger the Na^+ channels remain close which could explain the absence of modifications in gene expression of neuro-2a cells following exposure to STX and TTX (**chapter 5**). Adding veratridine, i.e. inhibiting the inactivation of the Na^+ channels, might overcome this issue. However, it is also possible that the effects of veratridine mask those of STX and TTX and therefore do not allow the identification of specific biomarkers. The inability of transcriptomics to identify specific biomarkers holds not only for STX and TTX as described in **chapter 5**, but also for DA (data not shown). This result could have been expected as DA targets NMDA receptors and neuro-2a cells do not present these receptors [21]. However, because these receptors are present in mouse neuroblastoma x rat glioma hybrid NG108-15 cells, effects of DA on gene expression in these cells would probably result in the identification of biomarkers of exposure to DA.

While the assays developed in this thesis constitute promising tools as part of an integrated testing strategy proposed in this chapter and **chapter 7** for detection of marine neurotoxins in seafood (Fig. 8.1), further improvements can be applied and alternative applications can be explored. Some of these improvements include for example:

- While cardiomyocytes were not sensitive towards marine neurotoxins, they showed promising results with model neurotoxic compounds and therefore might be of interest from a chemical screening point of view, e.g. for REACH purposes.

- Although most of these techniques do not require the use of animals, the MEA-based approach still involved animal testing but to a lesser extent than the mouse bioassay. The use of embryonic stem cells with the MEA method is currently under investigation in order to fully replace animal use.
- Given the fact that neuro-2a cells are relatively sensitive to marine biotoxins, additional functional endpoints might be of interest using this cell line. Measuring neurite (neural projection from the cell body of a neuron, either an axon or a dendrite) outgrowth might be worthwhile to investigate as an extra functional endpoint that could be rapidly measured in a high throughput setup without the need of expensive equipment. This is supported by the results of chapter 5, i.e. the significant up-regulation by PITx of a gene promoting neurite outgrowth (Grp12), and the fact that voltage gated Na^+ channels (VGSCs) influence neuronal development. In line with these findings, brevetoxin-2, through the opening of VGSCs that in turn induces an influx of Ca^{2+} ions in the cells, enhanced neurite outgrowth [22, 23]. Similarly, the marine neurotoxin gambierol which inhibits voltage gated K^+ channels, stimulated neurite outgrowth in cerebrocortical neurons [24]. In addition, kainic acid, through the binding to NMDA receptors leading to subsequent increase in intracellular Ca^{2+} has been shown to decrease the length of the neurites of nociceptive-like dorsal root ganglion neurons [25]. Because DA has a similar mode of action as kainic acid, measuring neurite length in neuronal cells such as neuro-2a or rat pheochromocytoma PC-12 cells widely-used in such type of experiment could be a suitable alternative for the detection of this marine neurotoxin. Neurite outgrowth measurements should therefore be considered while exploring the suitability of innovative functional endpoints for the detection of a wide range of marine neurotoxins.
- In addition to implementing additional endpoints, the applicability of the bioassays developed in this thesis for confirmation of neuro-2a positive screened sample extracts, should be investigated, i.e. the MEA, use of marker genes and fluorescent probes. While we focused on the detection of specific marine neurotoxins, some of the models developed are most likely also able to detect marine biotoxins other than PSPs or NSPs such as those belonging to the cyclic imine group (pinnatoxins, 13-desmethyl spirolide C), but also okadaic acid, yessotoxins and azaspiracids, which do not directly affect the nervous system per se, but through a cascade of events that ultimately lead to neurotoxicity too and are therefore of interest from a food

safety perspective.

- Since the bioassays developed in this thesis are mostly neuronal-based models they will probably still miss some marine biotoxins for example those causing gastrointestinal disturbances. Therefore future work should focus on the development of in vitro assays for the screening of marine biotoxins inducing gastrointestinal disturbances, including the diarrhetic shellfish poison group, azaspiracids and yessotoxins as they represent an important source of intoxication and greatly contribute to the occurrence of outbreaks as described in chapter 2. To this end, and because the modes of action of some lipophilic marine biotoxins are still unknown, hampering the development of additional bioassays for confirmation purposes or as alternatives to the neuro-2a assay, additional insight into the modes of action of these marine biotoxins should be generated. The use of PCR- and Luminex-based approaches is currently under investigation and may provide novel bioassays to be included as additional confirmatory assa0ys in the integrated tested strategy presented in Fig.8.1 (Bodero et al., unpublished data).
- In addition, one should keep in mind that in addition to the marine biotoxins, also freshwater algal toxins represent a threat to humans. The World Health Organization released for example guidelines for drinking-water quality, requiring methods for the detection of these toxins in freshwaters [26]. Freshwater biotoxins and especially microcystins represent a threat for human health and have been detected in for example cyanobacterial supplements available on the market for human consumption. In Brazil patients died after having been treated with water contaminated with microcystins [27]. In addition to the water, microcystins can also accumulate in fish as demonstrated in Brazil where high levels of microcystins were measured in Tilapia flesh [28]. Besides freshwater toxins and especially microcystins, cyanobacterial supplements available on the market for human consumption also represent a threat for human health. It would be worthwhile to assess whether the assays described in this thesis can also be applied for the detection of freshwater biotoxins.

There is still some debate about whether to regulate some marine biotoxins or not and which regulatory limits could be considered safe for consumers. To partly solve this issue physiologically based pharmacokinetic (PBPK) modelling, which constitutes a valuable tool for risk assessors, allows to refine regulatory limits

and estimate whether seafood consumers are at risk when exposed to particular levels of marine biotoxins. For example, while PlTx is extremely potent in vitro and after intraperitoneal injection in the MBA, it does not induce adverse effects in rats after oral ingestion, which might be explained by his large molecular weight making it not readily absorbed from the gastrointestinal tract [29]. Additional insight can also be generated from the in vitro experiments described in this thesis using PBK modelling based reverse dosimetry. Extrapolating in vitro concentrations to in vivo doses would offer the possibility for regulators for example to prioritize the safety evaluation associated with specific groups of marine biotoxins and/or set relevant regulatory levels based on doses that would be harmful to humans.

Societal impact of this thesis

The detection of marine neurotoxins in seafood is not only important from a food safety perspective where the techniques presented in this thesis offer a more suitable alternative to the current assays applied but also currently involves the unnecessary use of animals and is therefore of importance from an ethical point of view.

Besides allowing the detection of regulated marine neurotoxins at and below regulatory limits, a mode of action based approach will potentially permit detection of yet unknown marine biotoxins with similar modes of action, therefore protecting the consumers from possible future threats. Combined with analytical tools, the assays described in this thesis are part of a future strategy that offers valuable protection to seafood consumers without the need for animal experiments.

Conclusions

This thesis describes a wide variety of innovative mode of action based assays that could be used for screening purposes and proposes an integrated testing strategy suitable for the current needs in terms of food safety associated with seafood consumption. In addition, the assays developed are in line with the 3R paradigm of Russel and Burch and therefore comply with the current European Regulation for the replacement of animal experiments in the field of marine neurotoxins detection in seafood.

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Summary

Marine biotoxins are naturally occurring compounds produced by particular phytoplankton species. These toxins often accumulate in seafood and thereby represent a threat to consumers. Regulatory limits have been set for lipophilic marine biotoxins (diarrhetic shellfish poisons (DSPs) and azaspiracids (AZPs)) and for most marine neurotoxins (amnesic (domoic acid (DA)), neurotoxic (NSPs), and paralytic shellfish poisons (PSPs)) and the detection of these biotoxins in seafood still heavily relies on the mouse bioassay (MBA). However, the MBA is forbidden since the 1st of January 2015, except for the periodic control of production areas, especially with regard to the detection of unknown marine biotoxins. Moreover, the MBA is also still widely used for the detection of paralytic shellfish poisons (PSPs), as there is no official method currently validated for their detection, and for ciguatoxins (CTXs), for which there is no analytical method sensitive enough to detect it at the regulatory limit (chapter 1 and 2).

Although analytical methods are sensitive to many marine biotoxins and allow for their identification and quantification, their use still presents several major drawbacks (chapter 3). They do not allow the detection of unknown toxins for example and are quite expensive to be used as routine screening methods. Therefore, a screening assay that allows the broad detection of known and unknown marine biotoxins with great sensitivity at affordable costs is highly needed. Mode of action cell-based assays offer these possibilities.

The present thesis focused on the development of such mode of action based bioassays for marine neurotoxins, i.e. CTXs, DA, NSPs, palytoxins and PSPs, as an important subcategory of marine biotoxins. Most marine neurotoxins target ion channels/pumps or receptors present on the plasma membrane of excitatory cells. The suitability of cardiomyocytes for detection of the above mentioned marine neurotoxins was first investigated. Using beating cardiomyocytes for neurotoxicity testing might raise questions at first instance, but the rationale behind it is clear: cardiomyocytes present a large variety of ion channels at the surface of their membrane and the model is thus most likely to be affected by these particular marine neurotoxins. As described in chapter 4, beating cardiomyocytes responded well to reference neurotoxic model compounds but unfortunately showed a lack of sensitivity towards PSPs, hampering their applicability for monitoring of real samples. Cardiomyocytes were approximately 400 times less sensitive towards the Na^+ channel blockers saxitoxin and

tetrodotoxin (STX and TTX), when compared to the neuro-2a bioassay.

The neuro-2a assay is currently based on the assessment of the effect of compounds on the cell viability. Cell viability is not a specific endpoint and might also be affected by external factors, e.g. handling conditions, changes in pH or temperature and matrix effects. The suitability of alternative functional endpoints in neuroblastoma neuro-2a cells was therefore investigated in **chapter 5**. Gene expression in neuro-2a cells after exposure to palytoxin (PlTx), saxitoxin (STX) and tetrodotoxin (TTX) was analysed. Besides transcriptomics, changes in membrane potential were monitored using the fluorescent dye bisoxonol. Biomarkers based on mRNA expression were detected for PlTx but not for STX and TTX. STX and TTX decreased the fluorescence of bisoxonol while PlTx showed no effect. When using cytotoxicity as the read out the neuro-2a assay detects these three neurotoxins at similar concentrations. Therefore it is concluded that the newly investigated endpoints in the neuro-2a assay are not preferred over cytotoxicity in a suitable broad and sensitive bioassay for the detection of marine neurotoxins in real practice.

The neuro-2a assay has one drawback: it is not sensitive to DA (**chapter 7**). Therefore, in **chapter 6**, the multielectrode array was used to assess the effect of marine neurotoxins, including DA, on the activity of rat cortical neurons, which are known to express N-methyl-D-aspartate (NMDA) receptors that are targeted by DA [1, 2]. The multielectrode array showed high sensitivity and specificity towards the model compounds and marine neurotoxins tested (**chapter 6**). This technique is still relatively expensive and requires animal testing, impairing its applicability as a first screen assay. However, it constitutes an interesting tool for confirmation of the presence of marine neurotoxins and the detection of DA.

It turned out that the neuroblastoma neuro-2a assay, with cytotoxicity as a final readout, offered a high sensitivity towards marine neurotoxins and allowed for the detection of the majority of marine neurotoxins and their analogues when tested as pure standards, but also when present in food matrices (mussels, crabs, oysters, clams). **Chapter 7** clearly demonstrated that among the assays developed in this thesis and those described in the literature, the neuro-2a assay is the most promising technique for the broad, sensitive and high throughput detection of marine neurotoxins in seafood. In addition, it was shown that the neuro-2a assay is also capable of detecting all regulated lipophilic marine biotoxins and their analogues, both as pure standards and when present in seafood.

Based on the results of this thesis, an alternative approach, i.e. integrated testing strategy, that can replace the current methods relying on animal testing for the screening of marine biotoxins in seafood products is presented. This integrated testing strategy, partly tested in **chapter 7** and further described in **chapter 8**, is based on the combination of bioassays and a DA-biosensor (receptor-based dip stick test) with analytical methods, allowing the screening, identification and quantification of marine biotoxins in seafood products. It is concluded that using this strategy, the current bottlenecks of in vivo assays and analytical methods can be overcome, since this strategy comprises assays that do not only allow the detection of all regulated marine toxins and potentially unknown toxins, but also permit their identification and quantification at sufficiently sensitive detection levels. Moreover, the strategy will eventually allow replacement of the MBA and is thus in line with European regulation 15/2011, and also fully supports the 3R concept proposed by Russel and Burch.

List of abbreviations

AChR: acetylcholine receptor
ADME: absorption, distribution, metabolism, excretion
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOAC: association of analytical communities
ARfD: acute reference dose
ASP: amnesic shellfish poisoning
ATP: adenosine-5-triphosphate
AZA: azaspiracid
AZP: azaspiracid poisoning
Ca^{2+}: calcium ions
CCα: decision limit
CFP: ciguatera fish poisoning
CGN: cerebellar granule neurons
CPDB: Consensus Path DB
CTX: ciguatoxin
DA: domoic acid
DiBAC: bis-(1,3-diethylthiobarbituric acid) trimethine oxonol
DIV: day in vitro
DMSO: dimethylsulfoxide
DPH: diphenhydramine
DSP: diarrhetic shellfish poisoning
DTX: dinophysistoxin
EB: embryoid body
EC_{50}: effect concentration 50
ECVAM: European Centre for the Validation of Alternative Methods
EFSA: European Food Safety Authority
ELISA: enzyme-linked immunosorbent assay
ESI: electrospray ionisation
EST: embryonic stem cell test
FAO: Food and Agriculture Organization
GC-MS: gas chromatography mass spectrometry
HAB: harmful algal bloom
HAc: acetic acid
HPLC-UV: high performance liquid chromatography with ultraviolet detection
HRMS: high resolution mass spectrometry
IC_{50}: inhibitory concentration 50
K^+: potassium ions
LC-FLD: liquid chromatography with fluorescence detection
LC-MS: liquid chromatography with mass spectrometric detection
LDH: lactate dehydrogenase
LOD: limit of detection
L-Type: long-lasting type
MAPK: mitogen-activated protein kinase
MBA: mouse bioassay
MEA: multielectrode array
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MTX: maitotoxin
MU: mouse unit
Na^+: sodium ions
NMDA: N-methyl-D-aspartate
NSCC: nonselective cation channels;
NSP: neurologic shellfish poisoning
N-Type: neural type
OA: okadaic acid
o/v: ouabain/veratridine
PBPK: physiologically based pharmacokinetic
PbTx: brevetoxin
PlTx: palytoxin
PND: postnatal day
PSP: paralytic shellfish poisoning
PP1, PP2A: protein phosphatase-1 and -2A
PTX: pectenotoxin
RBA: rat bioassay
RIA: radioimmunoassay
RL: regulatory limit
R-type: resistant type
SPE: solid phase extraction
SPX: spirolide
STX: saxitoxin
Tof/MS: time of flight mass spectrometry
TNF: tumor necrosis factor
TTX: tetrodotoxin
T-Type: transient type
VGCC: voltage-gated calcium channels
VGSC: voltage-gated sodium channels
YTX: yessotoxin

Appendix

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They begged me to write one or two short sentences about them, so here it is. Myrthe (yes Nacho, women first) I would like to thank you for making sure that at least once a day somebody would contradict me. You probably still do not believe it but I saw you changing (in a good or bad way, I leave it up to you to decide) during your PhD and I strongly believe that your office mates have something to do with that. If there would be one thing you should work on I would say: please, stop “baking” cakes from Dr. Oetker. Ignacio, or shall I say Nacho, I know that you are still disappointed that I did not offer you a position for doing your MSc thesis with me in 2012. Do not forget that it was for your own good: two crazy scientists working together is not a good combination. Thanks for teaching me how to (kind of) play guitar, but also drums (on pans, boxes or whatever we would find around), harmonica and a bit of keyboard. Besides this, we still have to finish the Olympic games so be prepared you are going to suffer. Lastly, I am looking forward to visiting you in Costa Rica.

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Curriculum vitae

Jonathan Nicolas was born on August 23rd, 1987 in Quimperlé, France. After receiving his bachelor degree in population biology, he pursued a master in biology and health with a specialisation in toxicology in Brest, France. During his MSc, Jonathan conducted an internship at the European Food Safety Authority in Italy where he provided scientific support to the team in charge of ensuring the safety of food additives and nutrient sources at the European level. Subsequently he started his PhD, a collaboration between the Division of Toxicology and RIKILT Institute of Food Safety, Wageningen UR. During his PhD he followed a series of postgraduate courses to be registered as a European toxicologist. He was involved in several activities as he was a member of the board committee and the education committee at the Division of Toxicology, Wageningen University, and was the president of the organizing committee of the international PhD excursion to the United Kingdom in 2013. Beside his involvement at the Division of Toxicology, Jonathan was a member of the VLAG PhD council that represents more than 370 PhD students.

List of publications

Nicolas, J., Wang, S., Gerssen, A., Klijnstra, M.D., Bodero, M., Portier, L., Reynaert, N., Hoogenboom, L.A.P., Biré, R., Hendriksen, P. J. M. and Bovee, T. F. H., Broad and animal free in vitro detection of marine biotoxins in seafood products. In preparation.

Nicolas, J., Bovee, T. F. H., Kamelia, L., Rietjens, I. M. C. M. and Hendriksen, P. J. M., Exploration of new functional endpoints in neuro-2a cells for the detection of marine neurotoxins. Submitted.

Nicolas, J., Hoogenboom, L.A.P., Hendriksen, P. J. M., Bodero, M., Bovee, T. F. H., Rietjens, I. M. C. M. and Gerssen, A., Marine biotoxins and associated outbreaks following seafood consumption: prevention and surveillance in the 21st century. Submitted.

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Sébert P, Mortelette H, Nicolas J., Amérand A., Belhomme M., Moisan C. (2011). In vitro aerobic and anaerobic muscle capacities in the European eel, Anguilla anguilla: Effects of a swimming session. Respir. Physiol. Neurobiol. 176(3), 118-22.

Overview of completed training activities

Discipline related courses

Cell toxicology, Postdoctoral Education in Toxicology (PET) (2012)
Toxicogenomics, PET (2012)
Pathobiology, PET (2012)
Epidemiology, PET (2012)
Laboratory animal science, PET (2012) & WUR (2015)
Mutagenesis/carcinogenesis, PET (2012)
Environmental toxicology, Wageningen University (WUR) (2013)

Meetings

International Conference on Harmful Algae (ICHA) (2014)
Annual meeting Society of Toxicology (SOT) (2015)
NVT conference (2013, 2014 & 2015)

General courses

Risk communication, PET (2012)
VLAG PhD week (2012)
Advanced management and marketing, WUR (2012)
Reviewing a scientific paper, WUR (2012)
How to give and receive feedback, Young AFSG (2012)
Entrepreneurship in and outside science, Startlife WUR (2014)

Optional

Preparing PhD research proposal
Attending scientific presentations (RIKILT and Division of Toxicology)
Organisation of and participation to the PhD excursion in 2013
VLAG PhD council meetings
Attending lunch lectures organized by VLAG PhD council
Online courses
Health in numbers: quantitative methods in clinical and public health research (edX - Harvard University)
Fundamentals of clinical trials (edX – Harvard University)
An introduction to operations management (Coursera – Wharton business school)
An introduction to financial accounting (Coursera – Wharton business school)
An introduction to marketing (Coursera – Wharton business school)
An introduction to corporate finance (Coursera – Wharton business school)
Developing Innovative Ideas for New Companies: The First Step in Entrepreneurship (Coursera - University of Maryland)
Law and the entrepreneur (Coursera – Northwestern University)
Gamification (Coursera – Wharton business school)
Organizational analysis (Coursera – Stanford)
Analyzing Global Trends for Business and Society (Coursera - Wharton business school)

Approved by Graduate School VLAG

Notes

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