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REVIEW

Biological methods for marine toxin detection

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Abstract The presence of marine toxins in seafood poses a health risk to human consumers which has prompted the regulation of the maximum content of marine toxins in seafood in the legislations of many countries. Most marine toxin groups are detected by animal bioassays worldwide. Although this method has well known ethical and technical drawbacks, it is the official detection method for all regulated phycotoxins except domoic acid. Much effort by the scientific and regulatory communities has been focused on the development of alternative techniques that enable the substitution or reduction of bioassays; some of these have recently been included in the official detection method list. During the last two decades several biological methods including use of biosensors have been adapted for detection of marine toxins. The main advances in marine toxin detection using this kind of technique are reviewed. Biological methods offer interesting possibilities for reduction of the number of biosassays and a very promising future of new developments.

Keywords Phycotoxin · Seafood · Shellfish poisoning · Biosensor · SPR

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Introduction

Marine toxins produced by phytoplankton accumulate in filter-feeding shellfish and finfish and can cause human intoxication, sometimes with lethal consequences. The protection of human health has prompted the establishment of legal limits for the toxin content of seafood destined for human consumption. The implementation of those limits requires the availability of detection methods for every group of toxins (regulatory limits and official detection methods are summarized in Table 1). Currently, the most commonly used detection method is the mouse bioassay, which is also the reference method for most marine toxin groups. The animal bioassays consist in administration of seafood extracts to laboratory animals and monitoring of the symptoms and time to death. Obviously, this method has raised ethical concerns related to the use of laboratory animals, but also has technical limitations, for example low sensitivity and high rates of false positives and negatives [1–3]. In recent decades, therefore, much effort has been focused on the development of alternative methods for detection of seaborne toxins. Although complete substitution of the mouse bioassay will be difficult to achieve because of the strict requirements of regulatory authorities in order to ensure human consumer protection, the alternative methods will at least help to reduce the number of bioassays.

The methods that can be currently used for detection of marine toxins could be classified into analytical methods, that enable unequivocal identification and quantification of the toxins as long as there are standards available, and the non-analytical methods, that do not enable identification of the different analogues of a toxin group present in the sample, but yield an overall estimate of toxin content, similarly to the mouse bioassay. Among the analytical methods, high-performance liquid chromatography with

Table 1 Production, distribution and regulatory limits of marine toxins

Toxin group	Producing organism	Distribution	Regulatory limit (per kg shellfish meat[a])	Official detection method	Refs.
Saxitoxin and derivatives	*Alexandrium, Gymnodinium, Pyrodinium.*	Worldwide	0.8 mg of STX equivalents	Mouse bioassay HPLC–FLD	[9, 12, 14, 15, 92]
Domoic acid	*Pseudonitzschia, Nitzschia, Chondria armata*	Worldwide	20 mg	HPLC–UVD	[14, 92]
Brevetoxin	*Karenia brevis*	Mexico, USA, and New Zealand	200 MU	Mouse bioassay	[9, 93]
Palytoxin	Corals, sponges. *Ostreopsis siamensis* and *Ostreopsis ovata*	Tropical and subtropical waters, Mediterranean Sea	Not regulated		
Ciguatoxin	*Gamberdiscus* Present in tropical and subtropical fish	Tropical and subtropical reefs	None. Fish containing ciguatoxin must not be placed on the market.	Mouse bioassay (not official)	[92, 94]
Tetrodotoxin	Present in tropical fish	Japan and other Asiatic countries	None. Commercialization of some fish species restricted in Asiatic countries		
Cyclic imines	Various		None	None	
Okadaic acid and dinophysistoxins	*Dinophysis* and *Prorocentrum*	Worldwide. Mainly Europe and Japan	0.16 mg okadaic acid equivalents (plus pectenotoxin)	Mouse bioassay	[9, 92]
Pectenotoxin	*Dinophysis*	Worldwide	0.16 mg okadaic acid equivalents (plus DSP toxins)	Mouse bioassay	[9, 92]
Yessotoxin	*Protoceratium reticulatum*, *Lingulodinium polyedrum*, *Gonyaulax spinifera*	Worldwide	1 mg yessotoxin equivalents	Mouse bioassay	[9, 92]
Azaspiracid	*Protoperidinium crassipes*	Ireland, United Kingdom, Spain, France, Norway, and Morocco	160 μg azaspiracid equivalents	Mouse bioassay	[9, 14, 92]

[a] Whole body or any part edible

fluorimetric detection (HPLC–FLD), high-performance liquid chromatography with ultraviolet detection (HPLC–UVD), liquid chromatography–mass spectrometry (LC–MS), and liquid chromatography–tandem mass spectrometry (LC–MS–MS) techniques are available for the detection of most groups of toxins, including okadaic acid and derivatives, pectenotoxins, yessotoxins, azaspiracids, brevetoxins, cyclic imines, saxitoxin and derivatives, domoic acid, and ciguatoxins [4–13]. The detection of domoic acid by HPLC–UVD is actually the reference method for the detection of the amnesic toxin group in many countries given the low sensitivity of the mouse bioassay for this toxin [9, 14]. The detection of paralytic shellfish poisoning (PSP) toxins by HPLC–FLD using the so-called Lawrence method has been recognized recently as an official method in the legislation of many countries [8, 15]. The methods reviewed in this paper are non-analytical techniques. None of them can identify the analogues of a toxin group present in a sample, but they overcome one of the main limitations of the analytical methods. Unlike analytical methods, non-analytical methods do not require the availability of certified standards of the different compounds of a toxin group. Unfortunately the lack of certified standards has been an important limitation in the marine toxin field.

A constantly increasing number of toxin groups and analogues, the scarce amount of toxins available worldwide, and the dependence on natural, unpredictable toxic blooms to obtain the raw material for purification and standard production are probably the main reasons for the lack of certified standards. The current classification of marine toxins is based on their chemical structure. Among the most dangerous marine toxins are the neurotoxic compounds belonging to the saxitoxin and derivatives group, that induce paralytic shellfish poisoning (PSP), the domoic acid (DA) and derivatives group, that cause amnesic shellfish poisoning (ASP), ciguatoxins, that are responsible for ciguatera poisoning (CTP), tetrodotoxin, that causes puffer fish related poisoning, and the palytoxins. Although other groups of toxins are not life threatening, their presence in seafood destined for human consumption causes human sickness and important economic losses both in health care and aquaculture. Okadaic acid (OA) and derivatives, the yessotoxins, the pectenotoxins, the brevetoxins, and the azaspiracids can be included in this category. Some other groups have been described recently, and, although they are not regulated and no human intoxication has been reported, it is not yet clear to what extent they might pose a threat to human health. An example of toxins with these characteristics would be the

cyclic imines gymnodimine and the spirolides, although the pinnatoxins, another group of cyclic imines, seem to be actually related to human intoxication.

The methods reviewed in this paper use biological components for detection of marine toxins. The techniques have been classified considering the nature of the biological component. A distinction has been made between immuno-based techniques, which are based on the recognition of the chemical structure of the toxins, receptor-based techniques, which are based on the mechanism of action of the toxin by measuring the interaction with a natural target but often do not provide information about toxin-induced functional changes, and cell-based or tissue-based techniques that measure toxic effects on biological functions. Biosensors are a relatively recent addition to the array of marine toxin detection methods and a special focus has been made on these within the four sections because of the increasing application of this kind of technology in the marine toxin field. A biosensor is an analytical device incorporating a biorecognition element intimately associated with or integrated within a transducer that converts the response into an electrical signal. During the last two decades the development of biosensor technologies has offered excellent tools for detection of food contaminants [16–18]. Probably the most common classification of biosensors is based on the transducer platform used for transformation of a biological response into an electrical signal, which can include electrochemical (potentiometric, amperometric, impedance), piezoelectric, thermal, or optical (reflectrometric interference spectroscopy, interferometry, optical waveguide lightmode spectroscopy, total internal reflection fluorescence, surface plasmon resonance …) biosensors [18, 19]. However, biosensors can also be classified considering the biological component, which could be nucleic acids, enzymes, antibodies, receptors, cell organelles or whole cells and tissues [16–20] and is often a better indicator of the kind of information provided by the method. The mechanisms of action of the marine toxins mentioned in this review are summarized in Table 2 together with reported toxicity to humans and animals when available. Table 3 contains a summary of the detection limits of the most sensitive and practical detection methods reviewed in this paper.

Immuno-based methods

Antibody-based techniques, for example the enzyme-linked immunosorbent assay (ELISA), have been widely used to develop marine toxin detection methods, despite the difficulty of toxin-specific antibody production because of the small amount of pure toxins available worldwide for most of the groups. ELISAs are available for detection of most toxin groups, including okadaic acid, saxitoxin, azaspiracids, brevetoxins, domoic acid, yessotoxin, ciguatoxins, palytoxins, and tetrodotoxin [21–31]. Apart from this extended technique, several immunosensors have also been adapted for detection of marine toxins. An immunosensor is a biosensor that detects the presence of the analyte of interest by measuring its binding to a specific antibody.

Different biosensor technologies have been used for immunodetection of marine toxins. Most of the marine toxin immunobiosensor assays are designed as competition assays in which the toxin in the sample competes for binding to the antibody with immobilized toxin. Electrochemical detection is commonly used in the development of immunosensors. The electrical signal is usually generated by the electroactive product of an enzymatic reaction and detected by screen-printed carbon electrodes. One of the components of the enzymatic reaction is bound to the antibody; it is, for example, common to use alkaline phosphatase-labelled antibodies. Amperometric and differential pulse voltametry immunosensors are available for the detection of okadaic acid, brevetoxins, domoic acid, and tetrodotoxin [21, 32–35]. The sensitivity of these methods enables the detection of these toxins in the range of current regulatory limits. The sensitivity for okadaic acid reaches the pg mL^{-1} range; for the brevetoxin PbTx-3 the sensitivity is in the ng mL^{-1} range; and for domoic acid detection limits were 2 and 5 ng mL^{-1} in two different studies. Recent advances in the design of this kind of immunosensors provide a substantial improvement in sensitivity using an enzymatic recycling system for signal amplification in an amperometric immunosensor [21]. This technology was developed for the immuno-detection of okadaic acid, but could also be used to improve detection limits for other groups of marine toxins. The electrochemical immunosensors developed for domoic acid show good performance and recovery rates in mussel matrix, enabling on-site detection. However, no information is provided about matrix interference for the other methods.

In addition to electrochemical immunosensors, optical immunosensors can also be used for detection of some groups of marine toxins. The most extended optical biosensor in the marine toxin field is the surface plasmon resonance (SPR)-based biosensor. SPR-based immunosensors are available for detection of diarrheic shellfish poisoning (DSP) toxins, PSP toxins, and domoic acid [36–41]. The SPR-based immunosensor for PSP toxins is capable of detecting most PSP analogues at concentrations a factor of five lower than the current regulatory limit, with a detection limit for saxitoxin of 0.3–0.7 ng mL^{-1}, depending on the choice of antibody. However, the cross-reactivity of the antibodies raised against PSP toxins is usually low for the *N*-1-hydroxylated members of the group [23, 38, 39, 42]. Actually, some performance tests with incurred shellfish samples suggest that this low cross-reactivity

Table 2 Toxicity and mechanism of action of marine toxins

Toxin group	Biological target	Mechanism of action	Toxicity to humans	Toxicity to animals[a] ($LD_{50,}$ or minimum LD)
Saxitoxin and derivatives	Voltage-dependent sodium channel, site 1	Blockage of sodium influx and excitable cell depolarization	Neuronal and gastrointestinal symptoms. Paralytic shellfish poisoning (PSP). Lethal	Neurotoxic. (LD≈6 μg kg^{-1})
Domoic acid	Kainate receptors	Activation of kainate receptors	Neurotoxic. Amnesic shellfish poisoning (ASP). Lethal	Neurotoxic (3.6 mg kg^{-1})
Brevetoxin	Voltage-dependent sodium channel α-subunit, site 5	Sustained sodium influx and depolarisation of neural membranes	Neurotoxic shellfish poisoning (NSP). Gastrointestinal and neurological symptoms	Neurotoxic (PbTx-2 LD_{50}: 200 μg kg^{-1} i.p., 6600 μg kg^{-1} p.o.)
Palytoxin	Sodium potassium ATPase	Pumps turn into non-selective ion channels which alters membrane potential	Gastrointestinal symptoms, paresthesia of the extremities, myalgia, respiratory distress. Lethal Exposure to aerosol induces respiratory symptoms.	Neurotoxic (LD_{50} 0.025–0.45 μg kg^{-1}, i.p.)
Ciguatoxin	Voltage-dependent sodium channel α-subunit, site 5		Neurological, gastrointestinal and cardiac symptoms. Ciguatera fish poisoning (CFP). Lethal	Neurotoxic (LD 0.45 μg kg^{-1} i.p.)
Tetrodotoxin	Voltage dependent sodium channel, site 1		Neurotoxic. Similar to PSP. Lethal	Neurotoxic (LD_{50} 9 μg kg^{-1} i.p., 334 μg kg^{-1} p.o.)
Cyclic imines -Spirolides -Gymnodimine -Pinnatoxins…	Unknown for most of them. Nicotinic acetylcholine receptor (GYM and SPX)	Unknown for most of them. Nicotinic acetylcholine receptor antagosim (GYM and SPX)	Unknown. Gastrointestinal (pinnatoxins)	Neurotoxic by i.p. Lower by p.o. (Gym: 80–96 μg kg^{-1} i.p.) (13-des C SPX: 5–8 μg kg^{-1} i.p., 150 hg kg^{-1} p.o.)
Okadaic acid and dinophysis toxins	Protein phosphatases PP2A and PP1	Inhibition of phosphatases	Diarrheic shellfish poisoning (DSP). Gastrointestinal symptoms	Diarrheic (OA: 192–225 μg kg^{-1} i.p., 1–2 mg kg^{-1} p.o.)
Pectenotoxin	Unknown, actin?	Disruption of the actin cytoskeleton	Unknown	Hepatotoxic by i.p. administration (219 μg kg^{-1}) None by oral administration.
Yessotoxin	Phosphodiesterase	Unknown	Unknown	Cardiotoxic by i.p. (100 μg kg^{-1}). None by oral administration.
Azaspiracid	Unknown	Unknown	Diarrheic. Azaspiracid poisoning (AZP)	Diarrheic and neurotoxic. (0.2 mg kg^{-1} i.p., 0.25 mg kg^{-1} minimum lethal dose p.o.)

[a] LD values of representative compound of the group

Abbreviations: i.p., intraperitoneal administration; p.o., oral administration

with some analogues can be overcome by the complex mixture of toxins from this group present in naturally contaminated samples. A comparison with mouse bioassay, ELISA, and HPLC–FLD methods suggests that this biosensor assay could be used as a screening method for PSP toxin detection in shellfish samples [38, 43, 44]. The SPR-based assays for domoic acid have a detection limit of the order of 0.1 ng mL^{-1} [40, 41]. One of these has optimum performance with shellfish matrixes using a simple extraction with methanol, enabling detection in the range of μg kg^{-1} of meat [41]. The problem of immuno-based techniques is the lack of correlation between the cross-reactivity of the antibody with the different analogues of a toxin group and the relative toxic potency of those compounds. This problem has been recently solved for okadaic acid, dinophysistoxin-1, and dinophysistoxin-2 with the development of a monoclonal antibody for which cross-reactivity with these three compounds in buffer and shellfish extract matches the toxic potency of the molecules [37]. This antibody was used for optimization of DSP toxin detection in an SPR-based biosensor. A different optical biosensor based on the measurement of chemiluminescence integrated into a flow-injection analysis system was also adapted for the immunodetection of okadaic acid [45].

Immunosensors have several advantages, for example low cost, ease-of-use, speed, no need for highly-trained laboratory personnel, automation, reproducibility, robustness, sensitivity, and portability, that make them excellent tools for field tests. However, the lack of antibodies for some toxin groups and the lack of correlation between cross-reactivity profiles and toxic potency for groups of toxins that have often more than 20 analogues are important problems that still remain to be solved. Immunosensors are based on antigen–antibody interactions and the ability of an

Table 3 Biological detection methods for marine toxins

Biological method	Bioreactive element	Toxin	Detection limit	Matrixes
Electrochemical biosensor	Antibody	Okadaic acid	pg mL^{-1}	−
		brevetoxin	low ng mL^{-1}	−
		tetrodotoxin	low ng mL^{-1}	−
		domoic acid	ng mL^{-1}	+
	Protein phosphatase 2A	Okadaic acid	<ng mL^{-1}	−
Optical biosensor	Antibody	Okadaic acid	Low ng mL^{-1}	+
		saxitoxin	<ng mL^{-1}	+
		domoic acid	<ng mL^{-1}	+
	Phosphodiesterase	Yessotoxin	μg mL^{-1}	+
		brevetoxin	μg mL^{-1}	−
Cell biosensor	Neuronal networks	Tetrodotoxin	pg mL^{-1}	−
Tissue biosensor	Frog bladder	PSP toxins tetrodotoxin	<pg mL^{-1} Low pg mL^{-1}	+
Receptor-based assays	Protein phosphatase 2A	Okadaic acid	Low ng mL^{-1}	+
	Nicotinic AChR	Spirolides and gymnodimines	ng mL^{-1}	+
	Phosphodiesterase	Yessotoxin	Low μg mL^{-1}	+
Cytotoxicity-based assay	Multiple cell models and functional readouts	Palytoxin	<ng mL^{-1}	+
		okadaic acid	<ng mL^{-1}	+
		saxitoxin	<μg mL^{-1}	+
		tetrodotoxin	<μg mL^{-1}	+
		ciguatoxin	μg mL^{-1}	+
Electrophysiology-based assay	Voltage-dependent Na channel expressing cells	Saxitoxin tetrodotoxin	pg mL^{-1}	+
Membrane potential hanges by fluorimetry	Voltage-dependent Na channel expressing cells	Saxitoxin	Low ng mL^{-1}	+

antibody to interact with a toxic molecule is not related to the mechanism of action of the toxin, which is the consequence of toxin binding to a target or receptor in the organism. Consequently, quantification of the toxin content of a sample using immunosensors or any antibody-based methods is not usually an accurate measurement of the toxicity of the sample. Therefore, immunoassay results should not be reported as representative toxin equivalents. In the marine toxin field the terminology "toxin equivalent" is used to report the results of animal bioassays and the regulatory limits are established in representative toxin equivalents, and in both cases the term refers to toxicity equivalence. The use of this terminology to report the results of immuno-based detection would be misleading, because currently the different compounds of each toxin group cannot be identified by immuno-detection and there is no way to obtain a correlation with toxicity.

Receptor-based methods

Receptor or target-based techniques use the natural targets of the toxins to detect their presence. In this section we have included those methods that use a target macromolecule or a cell fraction containing the biological target of the toxin as the bioelement for toxin detection. At this point we should make a distinction between those methods that measure the effect of the toxin on the activity of a biomolecule and those methods that measure the interaction of the toxin with a biomolecule. The information that the receptor interaction-based methods provide is limited, because there is no indication of the effect/intrinsic activity of the toxin on the receptor. However, receptor-based methods are clearly related to the mode of action of the toxin. Actually, the receptor–toxin interaction is the first step in the cascade of events that produce toxicity. Receptor-based techniques (the term "receptor" will be used in this review to refer to biological targets in general) are less common than immuno-based techniques in the field of marine toxins. For many marine toxin groups the biological targets are plasma membrane proteins, and therefore their handling, is more complicated and their stability is poor compared with the antibodies. In other cases, the target has not been described yet. Despite the difficulties of working with receptors, receptor-based methods have some features that make them very attractive

for toxin detection. On the one hand, their ability to detect the different toxic compounds is usually related to their toxic potency, because they use the biological target as the biorecognition element. As well as immunosensors, these techniques do not require certified standards of each member of the group for estimation of sample toxicity. Therefore they lack the two main drawbacks of immunosensors and analytical techniques, although in exchange the robustness of immunosensors is difficult to match and identification and quantification of every toxic compound is not possible. The sensitivity and specificity of receptor-based biosensors depend on the biological target chosen for the development of the method.

The protein phosphatase PP2A is the okadaic acid target used for development of receptor-based DSP detection techniques in designs suitable for a microplate assay. These assays are based on the well-known inhibition of PP2A enzymatic activity by okadaic acid and its analogues. There have been several reports of phosphatase inhibition assays used to evaluate the inhibition of the enzyme by the toxins present in a sample using protein phosphatase substrates that change their colorimetric or fluorescent properties after cleavage of a phosphate group by the phosphatase [46–49]. Moreover, the ability of PP2A-based assays to detect the different analogues of the DSP group correlates with their toxic potency [50] and also with other detection methods, for example the mouse bioassay or HPLC–FLD [51]. The phosphatase inhibition assays are fast, economic, sensitive (low ng mL^{-1} range) and perform well in shellfish extracts.

In the field of biosensors, three receptor-based assays have been developed for detection of DSP toxins using the protein phosphatase PP2A [52–55]. The three methods quantify toxin content by measuring inhibition of the enzymatic activity of PP2A using electrochemical detection. The simpler system is based on inhibition of PP2A immobilized on the surface of screen-printed electrodes and the electrochemical signal is generated by a PP2A substrate, catechyl monophosphate [54]. This method has been reported to have a detection limit of 6.4 ng mL^{-1} okadaic acid. In the other two methods the inhibition of PP2A occurs off-line in solution. The samples are then injected into a flow-injection analysis (FIA) system where a second enzyme (pyruvate oxidase) [52] or a bienzyme amplification system (alkaline phosphatase and glucose oxidase) [55] are immobilized. These two techniques provide higher sensitivity than the PP2A immobilization approach, being able to detect okadaic acid at concentrations of 0.1 ng mL^{-1} with the method that uses the pyruvate oxidase reporter [52] and of 30 pg mL^{-1} in the recently published method of the bienzyme amplification system [55]. The three techniques have enough sensitivity to detect the toxins in the range of the regulatory limit but their performance in shellfish matrixes has not been tested.

Receptor-based methods for yessotoxins use phosphodiesterases as the biological component. One of the available techniques consists in measurement of phosphodiesterase activation by yessotoxins using a fluorescent cAMP derivative [56]. This method was the starting point for the development of other techniques based on the detection of molecular interactions, such as biosensor assays and a direct fluorescence polarization assay [57]. Three biosensor-based techniques for the detection of yessotoxins have been published. Two of these were developed for detection of yessotoxins using a direct assay in which the interaction of yessotoxins with phosphodiesterases was measured in a resonant mirror biosensor or in an SPR-based biosensor [58–60]. These assays were capable of detecting yessotoxin in mussel matrix within the regulatory limit. Very recently, a SPR-based detection method for ladder-shaped polyether compounds, including yessotoxin and brevetoxin-2 (PbTx-2), was developed using an indirect assay in which the toxins in solution compete with immobilized desulfo-yessotoxin for binding to the phosphodiesterase [61]. This indirect assay has not been tested with shellfish matrixes and the data shown in this study point to a lack of specificity among different groups of toxins. In the case of PbTx-2, inhibition was achieved in the low μg mL^{-1} range, and, although theoretically this assay might also detect ciguatoxins (CTX) because of the structural similarity, the sensitivity shown for the other toxins suggests the method would not be able to detect CTX at levels low enough to guarantee consumer safety. The fluorescence polarization assay detects the presence of yessotoxin by the change of fluorescence polarization induced by binding of the molecule to a fluorescent phosphodiesterase. The sensitivity of the fluorescence polarization and the fluorescent cAMP assays (low μg mL^{-1} range) is enough to detect yessotoxin and its analogues in shellfish matrixes.

Fluorescence polarization (FP) is a spectroscopic technique often used to detect molecular interactions. It is based on excitation of a fluorescent molecule with plane-polarized light and measurement of the degree of polarization of the emitted light, which is proportional to the rotational relaxation time. The rotational relaxation time is related to the molecular volume. When a small fluorescent molecule interacts with a big macromolecule there is an increase in the degree of polarization of the emitted light. Recently, another fluorescence polarization assay has been developed for the detection of gymnodimines and spirolides. In this case, an indirect assay detects the toxins by competition with fluorescent α-bungarotoxin for binding to nicotinic acetylcholine receptors (nAChR) [62]. This assay is specific and sensitive enough to detect gymnodimine-A and 13-desmethyl C spirolide in the ng mL^{-1} range, and it performs adequately in shellfish matrixes. Despite use of a membrane receptor, the stability of the reagents will allow method commercialization. Currently, we are working on

the development of a microplate chemiluminiscence method using competition of the spirolides for binding to the nAChR with biotin-label α-bungarotoxin. This new approach increases the sensitivity more than fourfold compared with the fluorescence polarization assay.

Other methods that use biological components for detection of brevetoxins and PSP toxins include receptor-binding assays [63–66]. However, the receptor-binding assays for these toxins developed to date use radioactive labelling, which is not acceptable for routine testing according to current laboratory practices. A recently available reagent that could be used in the future to detect tetrodotoxin, saxitoxin, ciguatoxin, and brevetoxin using biosensors or other biological methods is the recombinant human voltage-gated sodium channel included in lipid bilayers [67].

Cell-based assays

This section on cell-based assays includes those methods that measure the effect of a toxin or group of toxins on cell functionality. Therefore these methods require the use of living cells. The main drawback of cell-based assays is the need to maintain cell cultures, which is not a trivial problem for routine detection laboratories and can be even more complicated in particular cases such as neuronal networks. The alternative is to provide a viable eukaryotic cell by commercial distribution of the detection technique, which also entails an important technological challenge.

The methods that will be reviewed in this section can be divided in two groups, those that measure cell viability and those that measure a particular cell function. Compared with the methods that use receptor interaction for detection, cell-based methods have the advantage of detecting the effects of the toxic compounds on cell function and, therefore, they provide information about toxic potency based not only on the affinity of the toxins for their target but also on their intrinsic activity and efficacy. However, cell-based methods lose specificity as we move downstream in the cascade of events triggered by the toxins. In other words, if the functional readout is far from the toxin-receptor interaction in the signalling cascade, such as, for example, the decrease of the cell oxidative metabolism, which is a measurement of cell viability, different groups of toxins will probably have a similar effect. Cell-based functional methods can be very varied and multiple functional effects of toxins on eukaryotic cells have been described; most of the technologies, however, have not been adapted to perform routine detection of a high number of samples by non-highly-trained laboratory personnel. It is not in the scope of this review to provide a detailed description of all the functional effects that can be used to detect marine toxins. Methods have therefore been selected on the basis of either their practicality of use and/or their sensitivity.

In-vitro cytotoxicity-based methods have been developed for many groups of marine toxins, using different cell types and cytotoxicity markers. There are cytotoxicity-based assays for DSP toxins, pectonotoxins, palytoxins, PSP toxins, tetrodotoxin, ciguatoxins, brevetoxins, and multi-toxin detection systems [68–74]. Some of these methods have been optimized for detection in shellfish matrixes. These techniques based on cell viability are characterized by lack of specificity, but after solving some practical problems, this feature could be an advantage in the design of universal cell-based detectors for marine toxins. The specificity of palytoxin detection using cytotoxicity assays has been improved by using the inhibition of palytoxin-induced cell death by specific antibodies or ouabain (a Na^{+}/K^{+} pump inhibitor) for confirmation of palytoxin-related effects [72, 73]. These techniques usually measure the induction of cell death by marine toxins, but a different approach has been optimized for detection of tetrodotoxin and saxitoxin and analogues. These neurotoxins are detected by inhibition of veratridine/ouabain-induced cell death or haemolysis [74–76]. The functional readouts used as cytotoxicity markers are diverse, including vital dyes, metabolic activity markers, actin cytoskeleton labelling, morphology (microscopy) or nuclear stains combined with fluorescence microscopy. Some of these techniques are suitable for rapid screening, but in other cases they are not very practical for routine detection of a large number of samples, such as those techniques based on fluorescence microscopy.

Another functional method likely to be related to the induction of apoptosis is the detection of yessotoxin based on the measurement of an E-cadherin fragment named $ECRA_{100}$ by protein blot [77, 78]. This method, also, is not specific, because it detects azaspiracids with similar sensitivity (low ng mL^{-1} range) [79] and it is very lengthy for routine testing. Moreover, the assay is suitable for testing of shellfish extracts, although there is underestimation of yessotoxin related toxicity compared with HPLC.

Finally, there are some functional assays based on the measurement of changes in membrane potential and ion flux. All of these are designed to detect marine neurotoxins. Electrophysiological methods such as patch-clamp techniques can be used to detect PSP toxins with high sensitivity (23 pg mL^{-1} saxitoxin) [80, 81], but they are complicated to perform and not practical for routine testing. Alternatively, fluorescence assays based on membrane potential measurements in microplates have been optimized for detection of several groups of toxins including saxitoxin and derivatives, brevetoxins, and ciguatoxin [82–84]. Another functional method developed for the detection of PSP toxins and domoic acid is based on measurement of cytosolic calcium concentration using a fluorescent dye and a spectrofluorimeter [85].

Major technological advances during the last decades have made the development of cell-based biosensors

possible. In the field of marine toxins only two cell-based biosensors have been optimized for the detection of neurotoxins. Despite this currently limited availability of cell-based biosensors, this field offers a promising future as universal toxin detectors, because of the possibility of detecting different groups of toxins with a single assay based on cell function measurements [86]. As for the animal assays, identification of the different compounds present in a sample would not be possible using cell-based biosensors, but they could provide a general estimate of toxicity. Another possible option with cell-based technologies is that cells might be engineered to display a specific response. The sensitivity shown by these cell-based biosensors is usually very high, and device portability has already been achieved for some analytical approaches, although the sample-preparation procedures may also be a limitation for on-site analysis.

Neuronal network-based biosensors have been demonstrated to be useful for the detection of marine neurotoxins. Murine spinal cord neuronal networks cultured on microelectrode arrays constitute the biological component of a biosensor based on the measurement of extracellular potentials that was tested for the detection of tetrodotoxin [87]. Later, a similar experimental approach was demonstrated to have extremely high sensitivity for the brevetoxin PbTx-3 and saxitoxin in buffer and diluted seawater [88], with detection limits of 296 pg mL^{-1} for PbTx-3 and 12 pg mL^{-1} for saxitoxin. The performance of these neuronal network-based biosensors with shellfish matrixes is still to be tested.

Tissue-based methods

Only one tissue based-biosensor has been developed for detection of marine toxins, specifically for the detection of tetrodotoxin and saxitoxin. This biosensor combines frog bladder membranes, which have a high concentration of Na^+ channels, with an Na^+-specific electrode that measures the transport of Na^+ through the membrane and its dose-dependent inhibition by tetrodotoxin or saxitoxin [89–91]. This tissue-based biosensor is very sensitive, detecting tetrodotoxin and saxitoxin at concentrations of 2 pg mL^{-1} and 0.1 pg mL^{-1}, respectively, and it can detect different analogues of the PSP toxin group with a good correlation between detection efficiency and toxicity. This assay has been shown to be suitable for use with shellfish and finfish samples.

Concluding remarks

A varied array of biological methods is available for the detection of marine toxins. In recent years, great advances in the practicality of use, sensitivity, cross-reactivity, robustness, and seafood matrix tolerance, among other features, have provided methods that constitute possible alternatives to animal bioassays. Among the methods optimized for detection of regulated marine toxins in shellfish matrixes, the immunosensors for detection of PSP toxins, domoic acid, or DSP toxins, the protein phosphatase assays, and protein phosphatase-based biosensors for DSP toxins stand out for their performance and sensitivity.

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