

Review

Biosensors to detect marine toxins: Assessing seafood safety

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Abstract

This article describes the different types of marine toxins and their toxic effects, and reviews the bio/analytical techniques for their detection, putting special emphasis to biosensors. Important health concerns have recently appeared around shellfish (diarrhetic, paralytic, amnesic, neurologic and azaspiracid) and fish (ciguatera and puffer) poisonings produced by different types of phycotoxins, making evident the urgent necessity of counting on appropriate detection technologies. With this purpose, several analysis methods (bioassays, chromatographic techniques, immunoassays and enzyme inhibition-based assays) have been developed. However, easy-to-use, fast and low-cost devices, able to deal with complicated matrices, are still required. Biosensors offer themselves as promising biotools, alternative and/or complementary to conventional analysis techniques, for fast, simple, cheap and reliable toxicity screening. Nevertheless, despite the wide range of seafood toxins and the already rooted biosensing systems, the literature on biosensors for phycotoxins is scarce. This article discusses the existing biosensor-based strategies and their advantages and limitations. Finally, the article gives a general overlook about the regulation toxin levels and monitoring programmes currently established around the world concerning seafood safety.

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Keywords: Marine toxins; Seafood; Shellfish poisoning; Toxicity; Biosensors

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1. Introduction

Marine microalgae, especially phycotoxin producers such as several species of dinoflagellates and diatoms, are one of the main problems in the exploitation of marine resources around the world [1]. Phycotoxins are toxic compounds that enter into the food chain as components of the phytoplankton. Shellfish ingest these toxins and act as vectors, transmitting them to humans; and not only shellfish: several marine carnivores, such as some fish species and crabs, may also act as vectors. Table 1 reviews the marine phycotoxin producer algal species, as well as already detected contaminated organisms and the toxin occurrence in the world.

Phycotoxins accumulate in the digestive glands of shellfish without causing any toxic effect on it. However, when humans consume a sufficient amount of contaminated seafood (phycotoxins are odourless and tasteless), intoxication occurs. Until now, seven different types of poisoning have been identified, each one being responsible for different symptoms:

- diarrhetic shellfish poisoning (DSP);
- paralytic shellfish poisoning (PSP);
- amnesic shellfish poisoning (ASP);
- neurologic shellfish poisoning (NSP);
- azaspiracid shellfish poisoning (AZP);
- ciguatera fish poisoning (CFP);
- puffer fish poisoning.

Usually, only acute seafood intoxications are reported, long-term health effects due to chronic exposition being generally incorrectly attributed. Moreover, communities living in coastal areas have seen their marine living resources threatened by toxic episodes. Hence, marine toxins are a serious concern not only for public health, but also for environmental protection, fishing industry and tourist economy.

Year in, year out, the frequency and intensity of harmful algal blooms (HABs) (also called “red tides” due to the red-brown pigmentation of waters), the number of new toxins, of geographical areas affected and of intoxication episodes dramatically increase. Microalgal blooming is a complex phenomenon, not completely elucidated, resulting from stringent climatic and ecological conditions [3]. The toxin release mechanism is also poorly understood and it seems to be a defence mechanism against other organisms or harmful environmental conditions [4]. Despite the existence of both classical and emergent analysis techniques for phycotoxin detection, monitoring and control

of algal blooms and toxin production episodes are still at an early stage. Their consolidation will require the overcoming of several limitations, since none of the current technologies is ideal. Moreover, more data about the biology, ecology and dynamics of harmful algae are essential; and this will only be possible with the development and implementation of reliable detection methods.

This paper describes the structural characteristics and toxic effects of marine phycotoxins, and reviews the current analysis methods, with their advantages and disadvantages, putting special emphasis on biosensors, since these bioanalytical tools may be the key to solve the applicability problem and to assure the consumer protection.

2. Classification and toxic effects

Marine toxins are non-proteinaceous compounds of low molecular weight, which widely differ in the chemical structures, physical properties and mechanisms of action, producing different effects on contaminated shellfish consumers. Some classifications have been proposed, the most accepted being that based on the syndromes caused by the involved toxins. However, some phycotoxins do not fit in such classification, and other groups have been included according to the name of the first identified toxin of the group (azaspiracid) or the name of the contaminated fish (“*cigu*” and puffer fish). Other toxins, more difficult to classify, have been grouped into a miscellaneous section. Below, their main structural characteristics and toxic effects are described [2,5–7]. In Fig. 1, the chemical structure of the most characteristic marine toxins is drawn.

2.1. Diarrhetic shellfish poisoning (DSP) toxins

DSP toxins are a group of thermostable polyether and lipophilic compounds, which includes okadaic acid (OA) and its acidic derivatives named dynophysistoxins (DTXs). The main symptom of human intoxication is diarrhoea, although other effects are also relevant, such as nausea, vomiting and abdominal pain. The typical onset period of symptoms ranges from 30 min to a few hours after consumption of contaminated shellfish. Patients can show gastrointestinal disorders up to 3–4 days, afterwards being completely recovered. Hospitalisation is rarely required and no human deaths have been reported. The proposed OA mechanism of action, allegedly similar to that of DTXs, is based on the inhibition of protein phosphatases (PPs), enzymes

Table 1

Main marine toxins, their source organisms, and infected organisms and countries where their presence has been revealed*

Toxin	Source organisms	Infected organisms	Countries
DSP toxins	<i>Dinophysis acuminata</i> , <i>D. acuta</i> , <i>D. caudata</i> , <i>D. fortii</i> , <i>D. hastata</i> , <i>D. mitra</i> , <i>D. norvegica</i> , <i>D. rotundata</i> , <i>D. sacculus</i> , <i>D. tripos</i> , <i>Gonyaulax polyhedra</i> , <i>Phalacroma rotundatum</i> , <i>Protoceratium reticulatum</i> , <i>Protoperidinium oceanicum</i> , <i>P. pellucidum</i> , <i>Prorocentrum arenarium</i> , <i>Prorocentrum belizeanum</i> , <i>Prorocentrum concavum</i> (or <i>Prorocentrum maculosum</i>), <i>Prorocentrum lima</i> , <i>Prorocentrum redfieldi</i>	Clams, mussels, oysters, scallops	Europe: Belgium, Croatia, Denmark, France, Germany, Greece, Ireland, Italy, The Netherlands, Norway, Portugal, Spain, Sweden, United Kingdom; Africa: South Africa North America: Canada, USA Central and South America: Argentina, Brazil, Chile, Mexico, Uruguay; Asia: China, India, Japan, The Philippines, The Russian Federation; Oceania: Australia, New Zealand
PSP toxins	<i>Alexandrium andersonii</i> , <i>A. catenella</i> , <i>A. cohorticula</i> , <i>A. fraterculus</i> , <i>A. fundyense</i> , <i>A. minutum</i> (or <i>A. excavata</i>), <i>A. tamarensis</i> , <i>Aphanazomenon flos-aquae</i> , <i>Gymnodinium catenatum</i> , <i>Pyrodinium bahamense</i> , <i>Spondylus butler</i> , <i>Zosimos acnus</i>	Clams, crabs, cockles, cods, copepods, gastropods, herrings, lobsters, mackerels, mussels, ormers, oysters, puffer fishes, salmon, scallops, starfishes, whales, whelks	Europe: Denmark, France, Germany, Ireland, Italy, The Netherlands, Norway, Portugal, Spain, Sweden, United Kingdom; Africa: Morocco, South Africa, Tunisia; North America: Canada, USA; Central and South America: Argentina, Brazil, Chile, Guatemala, Mexico, Trinidad y Tobago, Uruguay, Venezuela; Asia: China (also Hong Kong and Taiwan), India, Japan, Malaysia, The Philippines, Thailand, Timor-Leste; Oceania: Australia, New Zealand
ASP toxins	<i>Alsidium corallinum</i> , <i>Amphora coffaeiformis</i> , <i>Chondria armata</i> , <i>C. baileyana</i> , <i>Nitzschia navis-varingica</i> , <i>Pseudo-nitzschia australis</i> , <i>Pseudo-nitzschia fraudulenta</i> , <i>Pseudo-nitzschia multiseriata</i> , <i>Pseudo-nitzschia multistriata</i> , <i>Pseudo-nitzschia pseudodelicatissima</i> , <i>Pseudo-nitzschia pungens</i> , <i>Pseudo-nitzschia seriata</i> , <i>Pseudo-nitzschia turgidula</i>	Anchovies, clams, crabs, gastropods, lobsters, mackerels, mussels, oysters, scallops	Europe: Belgium, Denmark, France, Ireland, Italy, The Netherlands, Norway, Portugal, Spain, United Kingdom; North America: Canada, USA; Central and South America: Argentina, Chile, Mexico; Asia: Japan; Oceania: Australia, New Zealand
NSP toxins	<i>Chattonella antiqua</i> , <i>Chattonella marina</i> , <i>Fibrocapsa japonica</i> , <i>Gymnodinium breve</i> (or <i>Ptychodiscus breve</i>), <i>Heterosigma akashiwo</i> , <i>Raphidophyceae</i>	Clams, mullets, mussels, oysters, tunas, whelks	Europe: France, Germany, Greece, The Netherlands, Portugal, The Russian Federation, Spain, United Kingdom; Africa: South Africa; North America: Canada, USA; Central and South America: Brazil, Mexico; Asia: China (also Hong Kong), Japan, Korea, Malaysia; Oceania: Australia, New Zealand
AZP toxins	<i>Protoceratium crassipes</i> , <i>Protoperidinium</i>	Mussels, oysters	Europe: Ireland, Norway, Portugal, United Kingdom
CFP toxins	<i>Gambierdiscus toxicus</i> , <i>Gymnodinium sangienseum</i> , <i>G. polyedra</i> , <i>Ostreopsis lenticularis</i> , <i>Prorocentrum concavum</i> , <i>Prorocentrum mexicanum</i> , <i>Prorocentrum rhathytum</i>	Amberjacks, barracudas, bonefishes, carrangs, Chinamanfishes, crabs, emperors, groupers, hogfishes, jacks, javelin fishes, mackerels, marine snails, moray eels, mullets, parrotfishes, porgys, shrimps, snappers, swordfishes, surgeon fishes, triggerfishes, tunas, wrasses	Europe: France, Germany, Italy, The Netherlands; Africa: Madagascar; North America: Canada, USA; Central and South America: Anguilla (United Kingdom), The Bahamas, Chile, Cuba, Dominican Republic, Guadeloupe (France), Haiti, Jamaica, Martinique (France), Mexico, Puerto Rico (USA), Saint Barthelemy (France), Saint Martin (France – The Netherlands), Saint Vincent (United Kingdom), Venezuela, Virgin Islands (USA); Asia: China, Fiji, French Polynesia (France), New Caledonia (France), Israel, La Reunion (France), several South Pacific Islands; Oceania: Australia, New Zealand, Tonga

*See Ref. [2] for specific references.

that play an important role in the protein dephosphorylation in cells. These toxins bind to the receptorial site of PP1 and PP2A, blocking their activity. As a consequence, hyperphosphorylation of the proteins that control sodium secretion by intestinal

cells and of cytoskeletal or junctional moieties that regulate solute permeability is favoured, causing a sodium release and a subsequent passive loss of fluids, responsible for a diarrhoeic episode [8]. Studies carried out with animals also show OA and

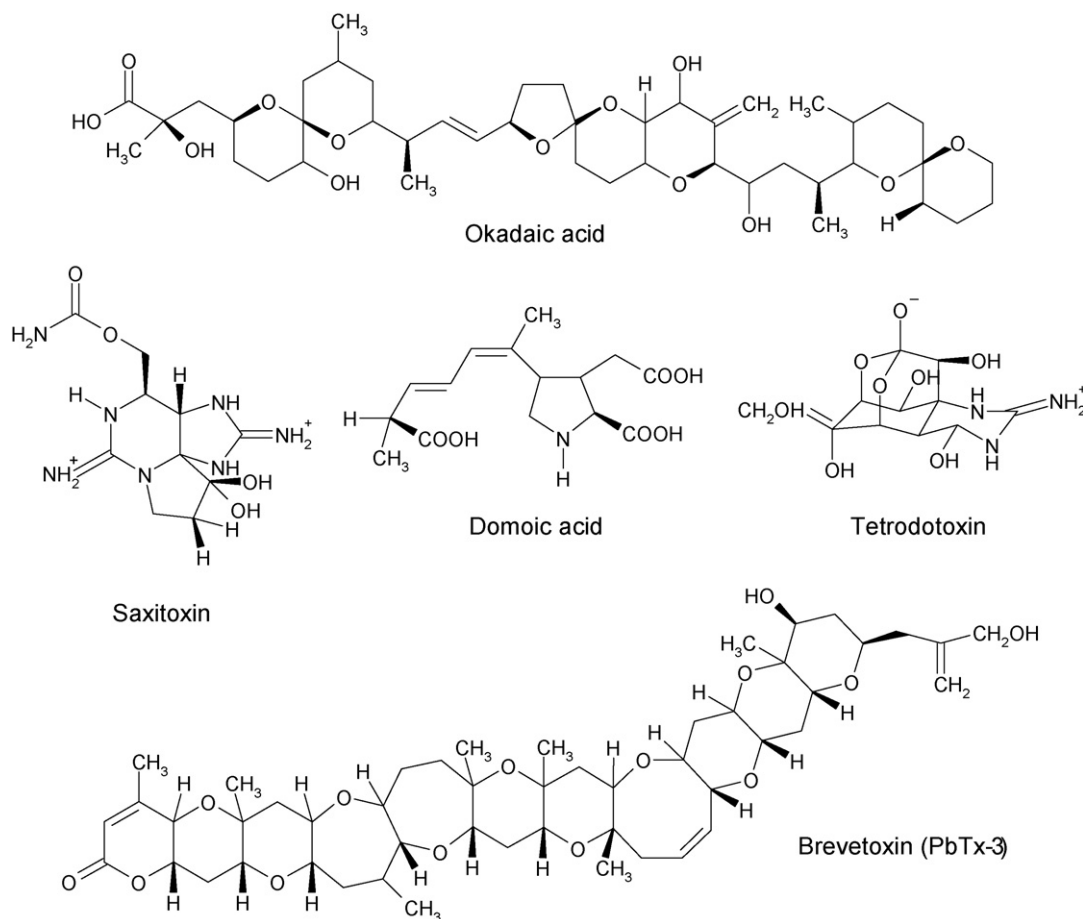


Fig. 1. Chemical structure of the most characteristic marine toxins.

DTXs as potent tumour promoters and possible mutagenic and immunotoxic agents.

Pectenotoxins (PTXs) and yessotoxins (YTXs) have been usually classified into this group; however, neither their effects nor their structures correspond to those of DSP toxins. PTXs are neutral polyether lactone compounds with hepatotoxic character, while YTXs are sulfated polyether compounds causing cardiac muscle damage or liver damage when desulfated. Little information exists about these toxins, making unclear if they pose a real health threat to humans.

2.2. Paralytic shellfish poisoning (PSP) toxins

Due to their world-wide incidence, PSP toxins pose the most serious threat to public health and cause an immeasurable economic damage. They are water-soluble and thermostable tetrahydropurine compounds, which can be further divided into four groups: carbamate, *N*-sulfo-carbamoyl, decarbamoyl and deoxydecarbamoyl. Among them, the carbamate saxitoxin (STX) is the most toxic. Dominant symptoms are neurological, the gastrointestinal ones (nausea, vomiting and diarrhoea) being less usual. These symptoms appear between 15 min–10 h after contaminated shellfish ingestion, starting with slight tingling, numbness in mouth and extremities, prickly sensation in

fingertips and toes, lips and skin burning, dizziness, floating sensation, headache, ataxia and fever. However, it is often reported a noticeable calm and serenity in intoxicated patients. Severe poisoning is characterised by general muscular incoordination, dysmetria and respiratory distress that can lead to death within 2–25 h. These toxins block the sodium channels, affecting the propagation of the action potential. Only symptomatic treatment exists, based on adequate ventilation and gastric lavage when no vomiting has occurred spontaneously.

2.3. Amnesic shellfish poisoning (ASP) toxins

ASP toxins include domoic acid (DA), a potent neurotoxin water-soluble acidic amino acid, and its derivatives. DA is a kainoid excitatory neurotransmitter that binds to specific receptor proteins causing depolarisation of the neuronal cells and their subsequent rupture. Fifteen minutes after consumption of contaminated shellfish, first symptoms appear, both gastrointestinal (nausea, vomiting, diarrhoea, abdominal cramps) and neurological (memory loss). These symptoms may be combined with optical problems, such as disconjugate gaze, diplopia and ophthalmoplegia, which are resolved within 10 days. In case of severe intoxications, important neurological deficits are usually described, involving confusion, mutism, seizures,

autonomic dysfunction, lack of response to painful stimuli and uncontrolled crying or aggressiveness, sometimes leading to coma and death. Compounds blocking the domoate-sensitive receptors, such as magnesium ions, organic antagonists (such as D-2-amino-5-phosphonovalerate) and dissociative anesthetics (phencyclidine), have been considered as antidotes for domoate poisoning.

2.4. Neurologic shellfish poisoning (NSP) toxins

The main group of NSP toxins is that of brevetoxins (PbTx). They are thermostable and stable at acid pH, lipid-soluble, cyclic polyether compounds. Some analogue metabolites formed by the shellfish itself (BTXs), as well as some phosphorus-containing ichthyotoxic compounds similar to anti-cholinesterases, are also considered as NSP toxins. Intoxication by PbTx shows effects 30 min–3 h after their ingestion, including gastrointestinal symptoms, hot-to-cold temperature reversal phenomenon, numbness, tingling, cramps, hypotension, arrhythmias, seizures, paralysis and coma. Furthermore, the wave action causes the lysis of the fragile *Gymnodinium breve* organism, creating toxic aerosols that contribute to conjunctival irritation and asthma-like symptoms, effects that rapidly disappear. Respiratory problems are believed to be due to the binding of PbTx to the receptor site of the sodium channel, the subsequent enhancement of the inward flow of sodium ions into the cell, and the release of synaptic neurotransmitters, such as acetylcholine. The aerosolised PbTx may also be associated to acute and chronic immunologic effects, due to their inhibitory effect on the lysosomal proteinases cathepsins found in phagocytic cells.

2.5. Azaspiracid shellfish poisoning (AZP) toxins

AZP toxins are relatively thermostable nitrogen-containing compounds, azaspiracid, formerly called Killary toxin-3 (KT3), being the most important one. Intoxication by AZP toxins causes symptoms similar to those of DSP toxins (nausea, vomiting, severe diarrhoea and stomach cramps); however, neurotoxic symptoms are quite different. It is also important to mention their potential hepatotoxic effect.

2.6. Ciguatera fish poisoning (CFP) toxins

A global health concern exists about CFP toxins, since the CFP is the most commonly found fish-borne poisoning syndrome. Different lipid-soluble polyether compounds are involved in CFP, being ciguatoxins (CTXs) the main responsible. These toxins follow a mechanism of action similar to that of PbTx, showing different affinities for the receptor site of the sodium channel depending on the specific toxin. As a result of the sodium ion influx, cellular mechanisms act trying to exchange sodium for calcium ions. It is thought that the cardiovascular effects are due to the cardiac muscle contraction caused by the increase of the local intracellular calcium concentration. Similarly, diarrhoea is attributed to this transport of calcium in intestinal epithelial cells. From the first few minutes to

30 h, symptoms appear: diarrhoea, vomiting, nausea, abdominal pain, numbness and tingling of the mouth, hands and feet, hot-to-cold temperature reversal phenomenon, cramps, temporary blindness, joint pain, cyanosis, depression, anxiety, hypotension, bradycardia, ataxia and paralysis. Whereas neurological symptoms can be resolved within weeks, other symptoms may persist for months or even years. Apart from gastric lavages, mannitol has been proved to reduce the severity and duration of neurological symptoms, if administered in the acute phase. It is important to notice the possibility to transfer the toxins to the foetus during pregnancy or to infants via breast milk. The second group of CFP toxins is the one of maitotoxins, which affect the calcium channels. These toxins, however, do not accumulate in shellfish at levels dangerous for humans. Scaritoxin, apparently corresponding to a mixture of two different CTXs, would be the third CFP toxin. Its ingestion initially causes symptoms resembling to those of CTXs, and 5–10 days later, equilibrium failure and marked locomotor ataxia can appear. Finally, some authors consider palytoxin as another CFP toxin, suggesting different mechanisms of action not fully clarified yet.

2.7. Puffer fish poisoning toxins

Tetrodotoxin and derivatives are thermostable water-soluble molecules, considered to be bacterial in origin. They are found in puffer, porcupine and toby fish. Like STX, these toxins block sodium channels of excitable membranes, causing tingling, numbness, floating sensation, nausea, vomiting, diarrhoea, muscle and respiratory paralysis, and leading to death within the 6 h after its ingestion. Although no antidote is available, administration of fluids and anti-cholinesterases can help the patients.

2.8. Other marine toxins

This section includes a miscellaneous group of toxins that must be considered separately:

- Other polyether toxins and bioactive polyoxygenated compounds: amphidinolides (macrolides with extremely potent cytotoxicity), amophidinol (a potent antifungal and haemolytic polyhydroxypolyene compound), prorocentrolide (a nitrogenous polyether lactone) and goniodomin A (an anti-fungal polyether lactone).
- The named *fast acting toxins*, which contain nitrogen: gymnodimine, spirolides and pinnatoxins. Up to now, gymnodimine is the only toxin that has proved to affect humans, but all of them may warrant concerns for human health.
- Neosurugatoxin and prosurugatoxin, responsible for vision dimness, pupil abnormal dilatation, thirst, lip numbness, speech disorders, constipations and dysuria.
- The macroalgal toxins polycavernoside A, a glycosidic macrolide, and debromoaplysiatoxin, compound responsible for severe dermatitis of swimmers and having tumour-promoting activity.

3. Analysis methods

3.1. Bioassays

3.1.1. *In vivo* assays

Among the different bioassays, the *in vivo* mouse bioassay is the most commonly used. This bioassay is based on the administration of suspicious samples to mice, the evaluation of the lethal dose and the toxicity calculation according to reference dose response curves, established with reference material [9,10]. It provides an indication about the overall toxicity of the sample, as it is not able to differentiate among individual toxins. Apart from being laborious and time-consuming, results are poorly accurate, non-specific non- or semi-quantitative, and change from a laboratory to another and even from an animal to another. Moreover, the mouse bioassay suffers from ethical implications. Despite the drawbacks, this bioassay is useful as preliminary toxicity screen and is still the method of reference for almost all types of marine toxins (ASP toxins not included), being official only for PSP toxins [11].

3.1.2. *In vitro* assays

Cytotoxicity assays are based on morphological changes in cells, such as neuroblastoma cells, rat hepatocytes, human epidermoid carcinoma cells, intestinal epithelial cell lines, Buffalo-green-monkey-kidney cells, larynx carcinoma cell lines and mammalian fibroblasts, when exposed to DSP toxins. These changes can be detected by microscopy [12,13], by spectrophotometry with (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) [13,14] or by fluorescence measurements of the F-actin levels [15–17]. In fact, the actin-disrupting effect of OA in cultured cells may be implied in the loosening of tight junctions *in vivo*; consequently, there is a direct link between PP inhibition and cytoskeletal changes. Cytotoxicity assays are easy to perform and economical. However, they are subjective, time-consuming, and confusing results may appear in the presence of toxin mixtures.

Other cytotoxicity tests are the neuroreceptor assays based on the binding of PSP, NSP and CFP toxins to voltage-sensitive sodium channels in nerve cells. Usually, neuroblastoma cells are used due to the high number of sodium channels within their cell wall. Ouabain, which inhibits the sodium release from the cell, and veratridine, which slightly opens the sodium channels, are usually added to the culture prior to the experiment, in order to enhance the specificity. The toxins bind to the receptors with an affinity proportional to their toxic potency. Whereas tetrodotoxins and PSP toxins block the sodium channels, promoting cell survival, PbTx_s and CFP toxins open them, promoting cell death. The effect of the toxins on the sodium channels has been monitored by microscopy [18], spectrophotometry [19] and fluorescence [20]. Although sodium channel assays are more sensitive than the mouse bioassay, they are not cost-effective for routine screening.

Receptor binding assays have also been developed for the detection of the ASP toxins. DA is able to bind the kainite/quisqualate glutamate receptor [21]. This method is rapid and sensitive enough. Since endogenous glutamate present

in shellfish may interfere in the measurements, a glutamate decarboxylase has been incorporated to a pre-treatment step, improving the limit of detection and the selectivity of the assay [22].

3.2. Analytical techniques

Chromatographic techniques have been widely developed for the detection of seafood toxins. These techniques allow separation, highly selective identification and sensitive quantification of the different toxins present into a sample. Moreover, limits of detection are generally one order of magnitude lower than those obtained with the mouse bioassay. However, it is well known that they require expensive equipment and trained personnel, and that they are laborious and time-consuming. The lack of standards is also a serious difficulty, since non-certified or unknown toxins cannot be evaluated. Other related problems that may appear during analysis are peak spreading, poor resolution and need of a continuous calibration. Quilliam [23] gives a very complete overview of the different chromatographic methods for marine toxin detection.

LC is the AOAC official method for DA and PSP toxins [24,25]. High performance liquid chromatography (HPLC) is usually coupled to UV or fluorimetric detection. Since most of the toxins do not possess a chromophore, toxin derivatisation methods are usually required. In order to make it official also for other seafood toxins, it will be necessary to standardise the current methodologies. Liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) are gaining increasing success due to the efficient toxin separation, high selectivity, high sensitivity (limits of detection five times lower than those obtained with the mouse bioassay), wide working range, accurate and precise quantification, ease-of-use and rapidity [26]. Another emergent and promising analytical technique is capillary electrophoresis, consisting of a fused silica capillary usually coupled to a UV or fluorescence detector [27–29]. Although only a few nanolitres of sample are required for the analysis, the current detection limits are still too high and research advances will be necessary to make it useful for screening purposes.

3.3. Immunoassays

Immunoassays are based on the affinity recognition between antibodies and antigens. The most commonly found format is the enzyme-linked immunosorbent assay (ELISA), where enzymes are used as labels to detect the interaction between polyclonal or monoclonal antibodies and toxins. There is a compromise between highly specific antibodies, able to detect a specific toxin, and less specific antibodies, able to detect all the members of a toxic family. Hence, cross-reactivity may be an advantage or a limitation, depending on the purpose. Colorimetric immunoassays have been developed for DSP [30], PSP [31], ASP [32], NSP [33] and CFP toxins [34]. Very interesting is the work performed by Garthwaite et al. [35], which integrates ELISAs for DSP, PSP, ASP and NSP toxins into a same screening battery. Less commonly found, due to obvious hazardous problems, are

radioimmunoassays (RIAs), developed for OA [36], PbTx-2 and PbTx-3 [37], and CTX [38].

Immunoassays seem to be a promising tool for routine detection and quantification of shellfish toxins, due to the high sample throughput and relative low cost. Moreover, they require neither sophisticated and expensive equipment nor skilled personnel. Several kits and dipsticks are already commercially available: DSP-Check® (<http://www.sceti.co.jp/english/index.asp>), OA-Check (<http://www.mitsubishichemical.com/index.html>) and Rougier Bio-Tech® (<http://www.erfabiotech.com/index.htm>) for DSP toxins, Ridascreeen (<http://www.r-biopharmrhone.com/index.html>) for PSP toxins, and Biosense® (<http://biosense.no/index.asp>) for ASP toxins. Immunoassays have already been envisaged by the European Community as possible alternative to the mouse bioassay [39], due to the higher sensitivities.

3.4. Enzyme inhibition-based assays

Enzymatic inhibition-based assays are very useful as rapid screening methods. The main enzymatic method is based on the PP inhibition and it has been developed for OA and dinophysistoxin 1, the only PP inhibiting marine toxins. The enzyme inhibition can be detected by several methods: radioisotopic assays allow low limits of detection but they suffer from sophisticated labelling procedures and hazardous problems; colorimetric assays are simple, cost-effective and sensitive enough [40,41]; fluorimetric assays lead to limits of detection lower than those obtained by colorimetry but require more sophisticated and expensive equipment [42,43]. Mountfort et al. [44] have improved this assay, overcoming the lack of sensitivity towards the ester derivatives of OA and other analogues, and reducing the incidence of false negatives. Due to the high sensitivity and the agreement with the mouse bioassay and chromatographic techniques, PP inhibition assay with fluorimetric detection has been considered by the European Community as possible alternative to the mouse bioassay for the detection of DSP toxins [39].

3.5. Biosensors

Increasing and serious concerns about seafood safety and human health protection have made evident the necessity of more rapid, robust, specific and sensitive analytical methods for the detection of phycotoxins. The biotool of the future should be able to deal with complicated matrices and, sometimes, toxin mixtures. Biosensors may not solve all the limitations of the current methods but will contribute with added advantages, since they combine the high affinity of the biochemical interactions, resulting in high sensitivities and low limits of detection, with the possible miniaturisation, portability and automation of the devices, which make them interesting for in situ monitoring. Moreover, biosensors are characterised by the simplicity of use, even for non-skilled personnel, and the low cost. At present, biosensors should be seen as bioanalytical tools for preliminary screening the toxicity of a sample. If the sample is considered suspicious, complementary analytical techniques should be used

in parallel in order to provide an accurate toxin determination and quantification.

3.5.1. Sodium channel-based biosensors

The effect of PSP toxins (STX, gonyautoxin and tetrodotoxin) as sodium channel blockers has been exploited for the development of a tissue biosensor [45,46]. The authors covered a Na⁺ electrode with a frog bladder membrane, rich in sodium channels, and integrated into a flow cell. Investigating the transport of Na⁺ ions, they could detect the toxin presence. The toxicity levels of the toxins correlated with those determined with the mouse bioassay and, in the tetrodotoxin case, the biosensor was able to detect concentrations more than one order of magnitude below the limit of the detection of the bioassay.

A particular case is the neuronal network biosensor developed by Kulagina et al. [47], which exploits the effect of STX and PbTx-3 on the extracellular action potentials. The biosensor was constructed by growing cultured mammalian neurons from spinal cord tissue of embryonic mice over a 64-site microelectrode array (Fig. 2). Despite the distinct actions of these two toxins on the nervous tissue (STX inhibits propagation of action potentials and PbTx-3 enhances activation of the sodium channels), both inhibited mean spike rate of spinal cord neuronal networks. The detection limits for STX and PbTx-3 were, respectively, 12 and 296 pg mL⁻¹ in buffer and 28 and 430 pg mL⁻¹ in 25-fold-diluted seawater. These extremely low values (approximately 30 000 times below the mouse bioassay detection limit for STX and 300 times below the regulatory limit for PbTx-3) are due to the extremely high sensitivity of the spinal cord networks. Additionally, the array responded to the presence of toxin-producing algae but not to the presence of non-toxin isolates of the same algal genera. Although this generic approach cannot fully identify or quantify individual toxins, its application as screening tool is clearly justified.

3.5.2. Immunosensors

Most of the biosensors developed for shellfish toxins are immunosensors, i.e. based on immunoassays. Tang et al. [48]

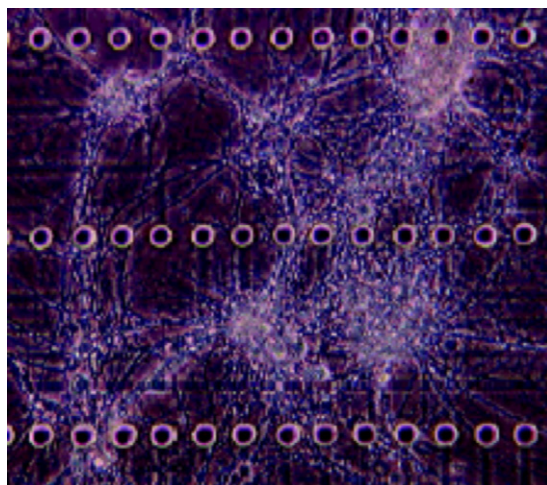


Fig. 2. Spinal cord neuronal network cultured over a microelectrode array substrate (reprinted from Ref. [47] with permission from ACS).

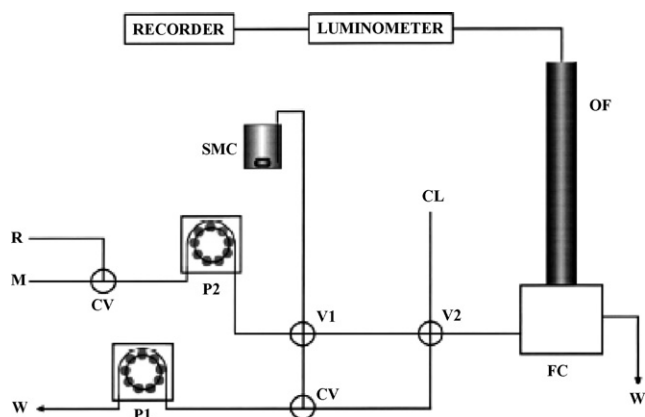


Fig. 3. Flow-system immunochemiluminescence set up. CL: chemiluminescent substrate solution, CV: connecting valve, FC: flow cell, OF: optical fibre, M: measurement buffer, P1 and P2: peristaltic pumps, R: regeneration solution, SMC: sample mixing cell, V1 and V2: injection valves, W: waste, B: optical fibre/flow cell interface (reprinted from Ref. [49] with permission from Elsevier).

developed a piezoelectric immunosensor for the determination of OA using a quartz crystal microbalance (QCM). Firstly, they used a competitive format, where free OA and anti-OA monoclonal antibody competed for immobilised OA-bovine serum albumin (BSA). The OA cross-linking immobilisation resulted in a good long-term storage lifetime of 38 days. However, the sensitivity and the limit of detection ($1.9 \mu\text{g mL}^{-1}$) were not satisfactory and they decided to use a non-competitive format with the antibody immobilised in a hydrogel. In this case, the sensitivity and the detection limit were improved by 80- and 524-fold, respectively.

A more sophisticated immunosensor for OA is that designed by Marquette et al. [49], integrated in a fibre optic-based chemiluminescence semi-automated flow injection system (Fig. 3). They immobilised OA-BSA on polyethersulfone membranes and performed a competition assay with free OA and horseradish peroxidase (HRP)-labelled anti-OA monoclonal antibodies. The use of such membranes was advantageous in terms of regeneration (a stable response was obtained under 34 measurements) and minimisation of non-specific antibody adsorption. This, combined to the high sensitivity of the chemiluminescence detection technique, led to very low limits of detection ($0.2 \mu\text{g OA}/100 \text{ g}$ of mussel homogenate, the fixed critical content being $40\text{--}60 \mu\text{g}/100 \text{ g}$). Moreover, the analyses were performed in only 20 min (conventional analysis techniques require 24–48 h) and the immunosensor was functional after 1-month storage.

Surface plasmon resonance (SPR) has also been used as detection technique in biosensing systems. In this direction, Yu et al. [50] developed an optical immunosensor for the detection of DA. The toxin was covalently linked to a mixed self-assembled monolayer (SAM)-modified SPR chip and the competition between free DA in solution and anti-DA monoclonal antibody was carried out. The detection limit was 0.1 ng mL^{-1} , much lower than that obtained with a conventional colorimetric ELISA (4 ng mL^{-1}), due to the high sensitivity of the SPR instrument and the elimination of the non-specific antibody adsorption on the SAM. The chip was successfully

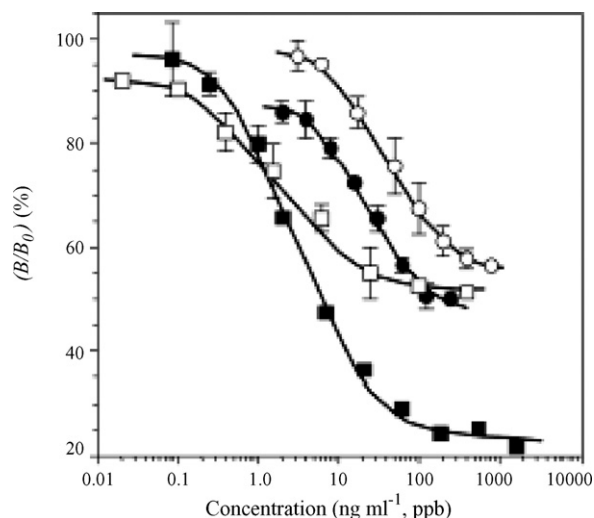


Fig. 4. Competitive immunoassays for the various seafood toxins using SPE with amperometric detection at +300 mV vs. Ag/AgCl. (○) Brevetoxin, (●) okadaic acid, (■) domoic acid and (□) tetrodotoxin (reprinted from Ref. [52] with permission from Elsevier).

regenerated with NaOH and had a long shelf-life time, being functional even after 2-month storage.

Unlike QCM, SPR and chemiluminescence systems, electrochemical devices can be easily miniaturised, which make them interesting from a portability point of view. Carter et al. [51] developed an immunoelectrochemical biosensor for the detection of STX and PbTx, using glucose oxidase (GOD)-labelled antibodies and detecting the H_2O_2 produced. Much better is the use of alkaline phosphatase (ALP) as label and *p*-aminophenyl phosphate as enzyme substrate, since the reaction product, *p*-aminophenol, can be detected by amperometry at a lower working potential (+300 mV versus Ag/AgCl) avoiding non-desired current from oxidisable interferences. Following this strategy, OA, PbTx-3, DA and tetrodotoxin were measured with detection limits of 1.5, 1.0, 2.0 and 0.016 ng mL^{-1} , respectively [52–54]. In Fig. 4, the typical calibration curves for these toxins are presented. In addition to this, it is necessary to mention that they used low-cost and disposable screen-printed carbon electrodes and that the analysis time was 30 min. In a similar way but using differential pulse voltammetry (DPV), Micheli et al. [55] detected DA. However, the detection limit was higher (5 ng mL^{-1}) and the analysis time longer (150 min).

3.5.3. Enzyme inhibition-based biosensors

Hamada-Sato et al. [56] have recently developed a biosensor that combines the PP2A inhibition with the phosphate ion consumption by pyruvate oxidase (PyOx) into a flow injection analysis (FIA) system. However, the inhibition step is performed in a microtube and only the second enzyme is immobilised. Nevertheless, they measured OA with a detection limit of 0.1 ng mL^{-1} , the biosensor being 50 times more sensitive than ELISA.

Our group is currently working on the development of an electrochemical PP2A inhibition-based biosensor for the determination of OA. Colorimetric experiments with the enzyme

immobilised by entrapment with poly(vinyl alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP) on screen-printed carbon electrodes have demonstrated the viability of the approach and its applicability to the detection of the toxin in mussels. Our strategy is much simpler than the one mentioned above, since the enzyme inhibition is detected directly using appropriate PP2A substrates, electrochemically active only after the dephosphorylation by the enzyme.

3.5.4. Molecularly imprinted polymer (MIP)-based sensors

A particular case is that of MIP-based sensors. They are not properly biosensors, but sensors, since they do not use any biological molecule; however, they deserve to be mentioned in this review. Molecular imprinting technique is a generic technology that uses templates to provide polymers with specific recognition properties. Usually, the target toxin is mixed with a monomer and a cross-linker, and let it to polymerise under appropriate conditions. Then, the template is removed leaving the synthetic receptor, with a geometry and orientation of the functional groups complementary to the analyte. In this direction, Lotierzo et al. [57] synthesised a MIP for DA and performed a competitive assay with free DA and HRP-DA conjugate in a MIP-modified SPR chip. In terms of sensitivity, the detection limit of the MIP was three orders of magnitude higher compared to that of a monoclonal antibody (5 ng mL^{-1} in front of 1.8 ng mL^{-1}). However, the detection range was larger and the chip was easily regenerated, which made possible its continuous use over a period of 2 months. Despite the still low affinity constants, these synthetic receptors are promising as they have the advantages of stability, ease of preparation and compatibility with the micromachining technology.

3.5.5. Chemosensors

Like MIP-based sensors, chemosensors are not properly biosensors, since they are based on the photoinduced electron transfer (PET) sensing of the toxins by synthetic fluorophores, the toxin binding providing fluorescence enhancement. Anthracylmethyl [58], coumaryl [59] and acridinylmethyl [60] crown ethers have been synthesised and evaluated as fluorescence recognition molecules for STX. Anthracene-based crowns have the limitation of their poor solubility in water and the coumarin-based ones are not very appropriate due to trace impurities from the toxin. Nevertheless, acridinyl crowns have good toxin binding constants and are water-soluble. Additionally, they are selective to STX, not showing fluorescence enhancement in the presence of tetrodotoxin. At present, the detection limits are still slightly below those obtained by the mouse bioassay, but new fluorophores are being investigated. Although these recognition molecules have been used in solution, their immobilisation for the development of the optical fibre-based fluorescence sensor is in progress.

4. Regulations and monitoring

Maximum permitted phycotoxin levels widely vary depending on the country. Moreover, in some cases, they are adjusted to the limitations of the proposed detection method. The allowed

levels are usually given in μg of toxin equivalents/g, since not all the toxins belonging to a same group exhibit the same toxicity. Another frequently used concentration unit is the mouse units/g (MU/g), defined as the amount of intraperitoneally injected toxin causing the mouse death within 24 h.

- **PSP toxins:** The European Union directive 91/492/EEC permits a maximum level of PSP toxins of $80 \mu\text{g STX eq}/100 \text{ g}$ shellfish flesh [61]. This maximum permissible level (referred to PSP toxins in general or only to STX, in a variety of molluscs or specifically in bivalves) is also followed by countries such as Canada, USA, Chile, Guatemala, Venezuela, Morocco, Singapore, Australia and New Zealand [62–64]. Mexico and The Philippines lower this value to 30 and $40 \mu\text{g STX eq}/100 \text{ g}$ shellfish, respectively, due to the frequently reported PSP outbreaks [65]. In other countries, such as Argentina, Uruguay, Panama, China, Hong Kong, Japan and The Republic of Korea, a tolerance limit of $400 \text{ MU}/100 \text{ g}$ has been set [62,63]. The general accepted official method of analysis for PSP toxins is the mouse bioassay [11]. The European Union imposed a directive in January 1993 saying that a chemical detection method can be used in parallel but that if results are confusing, the reference method should be the biological method. The established maximum permitted levels leave a quite small margin of safety or even no margin at all. However, since the mouse bioassay presents a detection limit of approximately $40 \mu\text{g STX eq}/100 \text{ g}$ shellfish, it is not realistic and even nor practical to establish a lower tolerance level. In order to solve this problem, LC has been recently considered as official method for PSP toxins [25].
- **DSP toxins:** The European Commission agreed in 2002 to set a tolerable level for OA, DTXs and PTXs present at the same time in edible tissues of $160 \mu\text{g OA eq}/\text{kg}$ shellfish (equivalent to $40 \text{ MU}/\text{kg}$), and a maximum level of YTXs of $1 \text{ mg YTX eq}/\text{kg}$ shellfish [39]. Australia, New Zealand, Canada, Japan and The Republic of Korea recommend an amount of DSP toxins not exceeding $200 \mu\text{g OA eq}/\text{kg}$ shellfish, equivalent to $5 \text{ MU}/100 \text{ g}$ [64,66]. The mouse or rat bioassay is the reference method of analysis. Like for PSP toxins, the proposed limits led a quite small margin of safety or even no margin at all.
- **ASP toxins:** The European Union establishes an upper safe limit of $20 \text{ mg}/\text{kg}$ for the total ASP toxins content in the edible parts of molluscs [61]. This general guideline is applied also by Canada, USA and New Zealand [64]. The analytical technique used as reference method is HPLC [63,67].
- **NSP toxins:** A regulatory level of $80 \mu\text{g brevetoxin}/100 \text{ g}$ shellfish flesh, equivalent to $20 \text{ MU}/100 \text{ g}$ shellfish flesh, and the mouse bioassay as analytical method, have been adopted by USA and New Zealand as guidelines for the regulation of NSP toxins [68,69].
- **AZP toxins:** Although the European Commission decided in 2002 to set the maximum permitted level of AZP toxins in bivalve molluscs, echinoderms, tunicates and marine gastropods, to $160 \mu\text{g}/\text{kg}$; however, the lack of information about these AZP toxins seems to recommend a review of this limit [39]. In fact, this limit was set based on the fact that no lower

Table 2

Monitoring programmes used as primary preventive tools to protect public health and environment from marine toxins contamination*

Countries	Monitoring programmes
Argentina	Monitoring programme for mussel toxicity
Australia	Monitoring of mussels and algae
Brazil	Proposal of a pilot monitoring initiative, as a first step towards a national monitoring programme
Canada	Monitoring programme for <i>Alexandrium</i> spp., <i>Dynophysis</i> spp., <i>Prorocentrum</i> spp. and <i>Pseudo-nitzschia pungens</i>
Chile	Two different national toxicity monitoring programmes, established by The National Health Service (using bioassays) and by the Fisheries Research Institute in conjunction with universities
Denmark	Assessment of shellfish and monitoring of <i>Pseudo-nitzschia pungens</i> and <i>Gymnodinium</i> spp.
Ireland	Biotoxin monitoring programme based on weekly shellfish testing using DSP mouse bioassay, LC-MS for OA, DTX-2 and azaspiracids, and LC for DA, as well as phytoplankton analysis
Italy	Monitoring of NSP-producing algae
Japan	Monitoring programme for both plankton and shellfish and for the detection of <i>Dinophysis</i> spp. carried out by researchers from Prefectural Fisheries Experimental Stations
Malaysia	Two shellfish toxicity monitoring programmes, established by the Malaysian Department of Fisheries and by the Fisheries Research Institute of Penang
New Zealand	Biotoxin monitoring programme combining regular shellfish testing and phytoplankton monitoring
Korea	Bi-weekly assessment of plankton run by The National Fisheries Research and Development Institute, for the detection of ASP, PSP and DSP toxins, combined with monitoring of <i>Prorocentrum</i> spp.
United Kingdom	Programme co-coordinated by the Ministry of Agriculture, Fisheries and Food, based on testing bivalve mollusks (and some crustaceans) for the detection of PSP and DSP toxins by the mouse bioassay
USA	Sampling programme for DA conducted by The Department of Marine Resources and control programme for NSP-contaminated shellfish carried out by The Florida Department of Natural Resources
Uruguay	National monitoring programme for mussel toxicity and toxic phytoplankton

* See Ref. [2] for specific references.

limits can be detected by the mouse bioassay, proposed as the preferred method of analysis. However, a tolerable limit of 80 µg/kg seems to be more adequate, since it ensures no appreciable risk for human health.

- **CFP toxins:** Despite the high toxicity of CFP toxins, neither tolerance limits nor official methods of analysis have been set due to the extremely low detection limits required to ensure consumer safety. In most of the countries, included those of the European Union, USA and Australia, a directive has been imposed, forbidding the sale of some fishery products known to be potentially toxic with CFP toxins [62,70–72]. Nevertheless, a “safe” level of 0.01 µg/kg of fish flesh, a real challenge for the analyst, has been proposed [73,74].
- **Puffer fish poisoning toxins:** There is a European Commission directive that states that fish of the species *Tetraodontidae*, *Molidae*, *Diodontidae* or *Canthigasteridae* (puffer, porcupine and toby fish) may not be placed on the market [70].

The health risk caused by the possible accumulation of marine toxins in shellfish when sporadic algal blooms appear in areas where shellfish are traditionally gathered or commercially farmed, imposes the monitoring of possibly contaminated fishery products and of the marine environment. Many countries have implemented shellfish and seawater screening programmes, according to the established regulations, as primary preventive tools (Table 2).

5. Conclusions

This paper reviews the most important analysis techniques and the existing biosensor configurations that have been applied

to the detection of shellfish and fish toxins. Firstly, bioassays, analytical techniques, immunoassays and enzyme inhibition-based assays are discussed. Then, attention is focused on the existing biosensors. Although a few examples of sodium channel- and enzyme inhibition-based biosensors have been described, much more numerous are the immunosensors, probably due to the high sensitivities inherent to the affinity interactions. Among the different detection techniques, electrochemistry is in the first position, due to the attained low detection limits and the possibility to develop miniaturised and portable devices. Also interesting are the sensors based on non-biological recognition molecules, such as molecularly imprinted polymers or fluorescence crowns. These emerging strategies are still at their early age and work is in progress to improve their performance.

In summary, the existing biosensors seem to be highly promising as biotools for seafood toxicity screening, since they allow detection of phycotoxins with appropriate sensitivities. However, these devices still suffer from limitations that compromise their applicability and further work is required for their commercial exploitation. The combination of the existing knowledge with other interdisciplinary areas, such as nanoelectronics, bioelectronics, micromachining and microfluidics, will contribute to their consolidation.

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