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REVIEW

Comparison of analytical tools and biological assays for detection of paralytic shellfish poisoning toxins

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Abstract The paralytic shellfish poisoning toxins (PSTs) were, as their name suggests, discovered as a result of human poisoning after consumption of contaminated shellfish. More recently, however, the same toxins have been found to be produced by freshwater cyanobacteria. These organisms have worldwide distribution and are common in our sources of drinking water, thus presenting another route of potential human exposure. However, the regulatory limits for PSTs in drinking water are considerably lower than in shellfish. This has increased the need to find alternatives to the mouse bioassay, which, apart from being ethically questionable, does not have a limit of detection capable of detecting the PSTs in water at the regulated concentrations. Additionally, the number of naturally occurring PSTs has grown substantially since saxitoxin was first characterised, markedly increasing the analytical challenge of this group of compounds. This paper summarises the development of chromatographic, toxicity, and molecular sensor binding methodologies for detection of the PSTs in shellfish, cyanobacteria, and water contaminated by these toxins. It then summarises the advantages and disadvantages of their use for particular applications. Finally it recommends some future requirements that will contribute to their improvement for these applications.

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Introduction

Paralytic shellfish poisoning toxins (PSTs) are commonly produced by pelagic marine dinoflagellates, for example species of *Alexandrium*, *Gymnodinium catenatum*, and *Pyrodinium bahamense* var. *compressum*. Bioaccumulation of the toxins by the tissues of fish, molluscs, and crustaceans makes human consumers vulnerable to exposure to the toxins. There are many historical accounts of deaths or illness caused by consumption of shellfish contaminated with PSTs [1, 2].

More recently it was found that PSTs are also produced by freshwater cyanobacteria. They were first detected in these organisms in the early 1980s in a bloom of *Aphanizomenon* sp. in North America [3], although subsequent reports of PST production by this genus in the US have been rare. In Australia, *Anabaena circinalis* was known in the 1970s to be responsible for livestock deaths, with symptoms suggesting neurotoxicity [4], but it was not until the early 1990s that this organism was shown to produce PSTs [5]. A range of PSTs were identified including saxitoxin (STX), gonyautoxins (GTXs), and C-toxins [5–8] but, in contrast with the PST variants previously found in dinoflagellates, the C-toxins were almost always the predominant variant present. Meanwhile, in Brazil, *Cylindrospermopsis raciborskii* was also found to produce these toxins [9]. Lagos et al. [9] detected several PST analogues in three different Brazilian strains of *Cylindrospermopsis raciborskii,* isolated from a pond in São Paulo State. They identified neosaxitoxin (neoSTX)

and STX in one strain, and STX and the GTX 2/3 isomers in the other two. Subsequently, Molica et al. [10] have provided evidence for further unidentified PST-like compounds in these strains. These unidentified compounds behave chemically and chromatographically like PSTs, and cell extracts produce effects in the mouse typical of PSTs but with a potency much greater than can be explained by the content of known PSTs [9, 10]. Extracts of these strains also were much more toxic in the neuroblastoma assay than could be explained by LC–MS–MS quantification of known PSTs [11].

In contrast with the situation in the US, *A. circinalis* and *C. raciborskii* are widespread in Australia and Brazil, respectively, and are regularly found in large numbers in sources of drinking water. In fact, the reason they were discovered in Australia was a massive *Anabaena* bloom that occurred in 1991 along 1000 km of the Darling River that caused extensive stock deaths and necessitated the declaration of a state of emergency, with the army called in to provide purified water [12]. In Brazil, Funil Reservoir which supplies drinking water for Rio de Janeiro, had more than 100,000 cells of *Cylindrospermopsis raciborskii* per mL, and during the same month, more than 250,000 cells of total cyanobacteria per mL. HPLC analysis showed concentrations of 3.48 μg STXeq L^{-1} [13]. PSTs can persist in fresh water for three months and some congeners can undergo chemical transformations during this time, becoming more toxic [14]. Their bioaccumulation in edible freshwater shellfish has been demonstrated [15].

Seven freshwater cyanobacterial species have so far been clearly identified as PST producers by isolation into monospecific culture: *Anabaena circinalis* (Nostocales) in Australia [12, 16], *Lyngbya wollei* (Oscillatoriales) in USA [17], *Cylindrospermopsis raciborskii* (Nostocales) in Brazil [9, 10], *Planktothrix sp.* (Oscillatoriales) in Italy [18], and *Aphanizomenon gracile, Aph. issatschenkoi,* and an unidentified *Aph. sp.* (Nostocales) in the US, Portugal, and China [3, 19–21]. Paralytic shellfish toxins have also been identified in Danish freshwater cyanobacterial blooms dominated by *Anabaena lemmermannii* [22], and more recently, Rapala et al. [23] have shown that this species is also associated with PST occurrence in Finnish freshwater sites. The likelihood that toxin genes move between organisms by horizontal gene transfer suggests that it is impossible to predict which species, if any, will produce the toxins in a given location [24]. In Brazil, blooms of toxic *C. raciborskii* have been recorded in different aquatic ecosystems [25, 26] and most of the isolated strains are PST producers. The discovery of PST analogues in freshwater cyanobacteria was very important because they occur in our sources of drinking water, and are more acutely toxic than microcystins, which are the most frequently detected cyanobacterial toxins in blooms worldwide [27].

The PSTs are a range of alkaloids based on the 3,4,6-trialkyltetrahydropurine skeleton. Modification of this basic structure by addition of carbamoyl, *N*-sulfocarbamoyl, hydroxyl, or sulfate groups produces a great diversity of toxins with much variation in charge and other chemical properties. More recently, *Lyngbya wollei* toxins and *Gymnodinium catenatum* toxins have been identified in which the carbamoyl group is replaced by a methyl or hydroxybutyrate moiety, respectively [28]. A total of about 30 structural variants are known, although usually only perhaps 5–10 are produced by any one organism. As already mentioned, evidence for other, as yet uncharacterised, PSTs has also been found. The different variants have widely differing toxicities [29]. *N*-Sulfocarbamoyl toxins have only low toxicity, but can be chemically or biologically converted to more toxic forms. In fact, both chemically and biologically catalysed inter-conversion of PST variants is a common occurrence [30]. Because of this diversity of structures and toxicities, the accurate detection and quantification of the PSTs is a substantial challenge.

The original regulatory limit for neurotoxicity in shellfish was established after the Sommer and Meyer study in 1937 [31], and because the toxic agent was not known, it was expressed in terms of the time to death of a treated mouse rather than the quantity of the toxic agent. It was only in 1957 that Schantz and co-authors isolated and purified a toxic product from clam and mussel tissues using acid extraction and ion-exchange chromatography [32]. The structure of STX was finally established using NMR in 1971 [33]. Although the structure was still to be elucidated, in 1959 STX was used in the mouse bioassay (MBA) to provide a regulatory limit in terms of STXequivalents per 100 g shellfish [34, 35]. Thus, in contrast with most regulated toxic substances, regulations for PSTs in shellfish are based on the characteristics of the bioassay rather than a formal risk assessment of the purified toxin [31].

Current seafood regulations (e.g. EU directive 91/492/EEC, [36, 37]) set the maximum acceptable level of PSTs in shellfish at 80 μg STXeq g^{-1} in most countries, although Mexico uses 30 μg STXeq/100 g and the Philippines use 40 μg STXeq/100 g [38]. Although these limits provide a minimum margin for safety, they cannot be made lower because of the LOD of the MBA (approximately 40 μg STXeq/100 g shellfish), which remains the official method of PST toxicity determination in most countries. Despite these difficulties, Wekell et al. [31] state that the 80 μg STXeq/100 g shellfish limit has proven to be protective of human health. Nevertheless, regulation of these toxins in drinking water has taken a different route. Here, a risk assessment based on documented human toxicity was used to determine a *lowest observed adverse effect level* and a standard 10-fold uncertainty or safety factor was applied to derive a *no observed adverse effect level*. From this was

calculated a health alert level in drinking water of 3 μg STXeq L^{-1} [39]. This concentration was later incorporated into the Australian Drinking Water Guidelines [40] and has become a mandatory legal requirement in Brazil [41]. New Zealand has also incorporated this concentration into its draft drinking water quality guidelines as a provisional maximum acceptable value [42]. Because it is assumed in the derivation of these guideline values that a person is exposed to 2 L of water per day, this equates to a tolerable exposure of 6 μg STXeq per adult per day. This is less than 1/10 the allowable amount of toxin persons can be exposed to if they consume 100 g shellfish. Although the drinking water regulations still lack any allowance for potential chronic effects, they nevertheless build in a level of conservatism that is lacking in the shellfish regulations. But this also poses an analytical challenge, because the MBA is not sensitive enough to detect the toxins at this concentration without preconcentration. The MBA LOD of 40 μg STX/100 g shellfish is equal to 0.2 μg STX mL^{-1} shellfish extract [31], or 200 μg STX L^{-1}, well above the 3 μg L^{-1} concentration allowable in drinking water. Thus, although MBAs continue to be widely used for monitoring seafood, in which bioaccumulation produces the high toxin concentrations needed for detection by this method, this is not practical for PST testing of drinking waters. Also, live animal assays are increasingly unpopular and have been banned in some jurisdictions. These considerations have led to assessment of many other chemical, biochemical, and toxicity assays developed for shellfish testing, for use with freshwater cyanobacteria or waters contaminated by them [11, 43]. The following review of methods therefore attempts to provide a sense of their historical development toward greater sensitivity while at the same time having to cope with ever more PST analogues and PST-producing organisms.

Analytical methods

As discussed above, the analytical challenges presented by PSTs include:

- up to 30 structural variants known with the prospect of further analogues yet to be discovered;
- analogues with different charge states because of their carbamoyl, *N*-sulfocarbamoyl, hydroxyl, and sulfate groups which can be affected by factors such as pH;
- analogues which can be chemically converted from one form to another with associated changes in toxicity; and
- analogues which are also subject to bio-transformation.

High performance liquid chromatography (HPLC) is widely used for separation of organic compounds and was one of the first analytical methods applied to PSTs. However, this technique also has some disadvantages, for example the need for pure and accurately quantified standards for all toxins of concern, the lack of a chromophore which requires the use of pre or post-column derivatization methods to produce fluorescent products, and the possibility of PST chemical conversions, especially during sample preparation. These conversions normally transform a less toxic variant into a more toxic form, and thus the real toxicity of the original sample becomes more difficult to determine. Thus the history of the development of analytical methods for PSTs is paralleled by a history of the discovery of increasing numbers of PST variants, which has driven the development of ever more sophisticated extraction and separation methods.

One of the earliest methods for STX was described by Bates and Rapoport [44]. Because of to the lack of a chromophore in STX, the method was based on the alkaline hydrogen peroxide oxidation of STX to a fluorescent compound. At that time the method was considered to be much more sensitive than the mouse bioassay, and they proposed its use for routine analysis of shellfish samples. However, the proposed method was very complicated, using several solvents and more than 10 steps for the chemical procedure. Some years later they improved their method, increasing its accuracy and reproducibility, but it remained very complicated [45].

The analytical problem increased when, in the 1970s, various groups found new compounds using methods such as X-ray diffraction, thin-layer chromatography (TLC) with fluorimetric detection, and high speed liquid chromatography (HSLC) [46–49]. Shimizu et al. [49] were the first group to describe the presence of GTX1, GTX2, and GTX3, isolated from a *Gonyaulax tamarensis* culture extract. Using a Sephadex G-15 or a Bio-Gel P-2 polyacrylamide gel column, and eluting with very dilute acetic acid, they separated STX from the GTX fraction. They then used HSLC to resolve the GTX peak into three different toxins. They also highlighted storage problems associated with the instability of some variants, observing the destruction or transformation of the GTXs when stored in a strongly acid medium and a decrease of the STX concentration upon storage. The structures of the GTX 2 and 3 were finally elucidated by Shimizu et al. [50] in 1976 using ^{13}C NMR. Ghazarossian et al. [51] hydrolysed STX with 7.5 mol L^{-1} HCl at high temperature and, again using Sephadex LH-20 column chromatography and ^{13}C NMR, purified a new toxin, decarbamoylsaxitoxin (dcSTX). Oshima et al. [52] isolated three related toxins, neoSTX, GTX4, and GTX5 from clam samples and an associated *Gonyaulax tamarensis* bloom. They used acidified ethanol to extract the toxins from both the phytoplankton and clam samples, and then used three different columns (Sephadex G-15, Bio-Gel P-2, and Bio-Rex 70 previously used by

Shimizu et al. [49]), to purify the toxins. TLC analysis with H_2O_2 spray and heating was used to induce the formation of fluorescent derivatives. This combined methodology was better able to separate the toxins, for example, use of an acetic acid concentration gradient to elute the Bo-Rex 70 column separated the STX from the neoSTX. Schimizu et al. [53] determined the structure of neoSTX and suggested that, owing to the reducibility of the *N*1-hydroxyl group, neoSTX would be converted to STX in the clam bodies. This observation led to a more intense study into the biotransformation of these molecules.

A more sensitive technique for analysis of STX was described in detail by Bates et al. [45]. They used their previous work [44] as a model, using trichloroacetic acid to extract mussel tissue and the Bio-Rex 70 column for separation, but made some modifications. The main changes to the extraction method were:

1. elute STX from the ion-exchange column with 0.25 mol L^{-1} H_2SO_4 instead of 0.5 mol L^{-1} HCl (HCl reduced fluorescence at the oxidation step); and
2. increase the NaOH concentration in the oxidation step to obtain a 0.5 mol L^{-1} final concentration.

Sullivan and Iwaoka [54] developed a gradient elution method with post-column periodate oxidation followed by fluorescence detection. The C-toxins were detected indirectly by hydrolysing them to GTXs.

In 1987, Oshima and co-authors [55] described a very detailed technique for analysis of 13 PST variants and used it to analyse the PST content of a dinoflagelate culture, a natural bloom, and extracts of mussels and oysters. They disrupted the phytoplankton cells by sonication in 0.1 mol L^{-1} acetic acid but the shellfish tissues were extracted according to the method of the Association of Official Analytical Chemists for mouse bioassays, that is by boiling 100 g of the tissue in 0.1 mol L^{-1} HCl for 5 min [34]. The most important changes from the studies of the 1970s were the use of three different isocratic mobile phases, and the use of an oxidising solution to produce fluorescent derivatives for detection. The use of three different mobile phases made possible the identification of several PST variants. However, one sample now required three different analyses, and it took a long time to prepare the solutions, calibrate the equipment three times, and analyse the samples.

Several authors made changes to the method described by Oshima et al. [55]. For example, modifications of the composition of the three eluents, such as addition of acetonitrile to better separate the STX group, changes to the pH, and use of a less expensive column, were proposed by Franco and Fernández-Vila [56]. Finally in 1995, Oshima [29] published a manual that incorporated some of these improvements to the method and also to the clean-up procedure. The main modifications were to the pH and counter-ion concentration in the mobile phases, and use of a 15 cm column that gave a good separation and sharp peaks. Overall, these changes provided better sensitivity reaching very low limits of detection. The improved clean-up method from shellfish involved using a reverse-phase cartridge column (Sep-Pak C_{18}) after extraction with 0.1 mol L^{-1} HCl as already proposed by AOAC in 1959 [34]. This removed interfering peaks and avoided toxin transformations, thus giving more accurate results and prolonging the lifetime of the column. Phytoplankton was extracted with 0.5 mol L^{-1} acetic acid using sonication to rupture the cells, but without further clean-up.

Although the analytical method developed by Oshima [29] requires a longer analysis because it takes three separate runs with three different mobile phases to detect all variants of PST, this method is still regarded as the most satisfactory because it enables analysis of individual toxins with accurate results. The Oshima method has given very consistent results for analysis of PSTs produced by Brazilian strains of cyanobacteria (Magalhaes, unpublished). However, interfering peaks with retention times identical with those of the PSTs can be encountered, leading to false-positive results. According to Onodera et al. [57], a strategy that can be used when there is doubt whether the peak obtained corresponds to a real PST is to repeat the analysis without the post-column oxidation. If the response obtained with the peak of interest changes in the same way as the standard, then this response may be used to confirm the identity of this peak.

Once the Oshima method was established, improvements to the extraction methods gained priority. Leão et al. [58], testing three different clean-up methodologies for shellfish, concluded that the one proposed by Oshima [29] produced a cleaner chromatogram and the best recovery. However, Biré et al. [59] suggested an improvement of the clean-up method for shellfish proposed by Oshima [29] including removal of the GTX4 interference with satisfactory recovery. To better extract a phytoplankton sample, Ravn et al. [60] proposed a procedure requiring three freeze–thaw cycles followed by ultrasonication in 0.5 mol L^{-1} acetic acid. Toxin profiles of dinoflagellate extracts were stable for at least six months at −20 °C using acetic acid at a concentration between 0.1 and 0.5 mol L^{-1} or in 0.01 mol L^{-1} HCl. But extraction with higher concentrations of HCl (0.03 to 1.0 mol L^{-1}) caused a decrease of C 1 and C 2 toxins and an increase of GTX2 and GTX3, that is, loss of the carbamoyl sulfate. Indrasena and Gill [61], after several storage tests with different temperatures at different pH, found the stability of all the toxins to be best when stored in acid pH 3–4 at −35 °C.

In contrast with the Oshima post-column oxidation method, liquid chromatography using pre-column oxidation

to produce fluorescent derivatives of the PST has been described by Lawrence and Ménard [62] and Lawrence et al. [63–65]. They demonstrated better sensitivity using hydrogen peroxide oxidation to analyse the nonhydroxylated toxins and periodate oxidation to analyse the hydroxylated ones. During this period they made improvements to the techniques, such as the addition of ammonium formate to the periodate oxidant, which improved results for neoSTX, GTX1, B2, and C3, and addition of this compound to the mobile phase, leading to better chromatography of neoSTX and B2 (GTX6). They proposed the use of solid-phase extraction (SPE) C_{18} cartridge and SPE-COOH ion-exchange chromatography for shellfish extract clean-up, mainly to separate neoSTX from B2 (GTX6).

Another important issue is the pH used to oxidize the samples. Gago-Martinez et al. [66], using the pre-column methodology, showed different yields of the fluorescent oxidation products from the periodate and peroxide oxidation processes by adjusting the pH between 7.2 to 12 and 8.2 to 12.8 respectively. Greatest yield of neoSTX and GTX1/4 was achieved using periodate oxidation at pH 8.2, and of STX, GTX2/3, dcSTX and GTX5 at pH 10 to pH 11.5. But most toxins produce more than one fluorescent product. Lawrence et al. [67] state that the pH is easier to control with pre-column oxidation and hence the results are more reproducible because small changes in pH of the post-column oxidant can have marked effects on the yield of fluorescent products. However, although the pre-column methodology is relatively simple, some PSTs form the same oxidation product and others form more than one fluorescent product.

This methodology was proposed as a screening method for monitoring programmes [65]. The initial proposal was to first use periodate oxidation, when the most toxic PSTs are detected, and, if the concentration exceeds the regulatory limit of 80 μg STXeq/100 g^{-1}, follow this with the peroxide oxidation, that is, the full method. Where toxins co-elute, it should be assumed to be the more toxic analogue. Almost a decade later, Lawrence et al. [67] reported the results of a collaborative study in which 16 laboratories from 12 different countries participated. Naturally contaminated and spiked samples of mussels, clams, oysters, and scallops, plus a blank, were sent to the collaborators to quantify STX, neoSTX, GTX2/3, GTX1/4, dcSTX, B1 (GTX5), C1, C2, C3 and C4. At the end of the study, as the results were considered satisfactory with very good correlation with the MBA, they recommended that the method for quantitative determination of PSP toxins in shellfish using prechromatographic oxidation should be accepted by the AOAC International as an official method, and it was accepted in the same year [36].

This method has been used for shellfish monitoring [68] and also for studying PST distribution in different regions of rat brains [69]. These authors considered it a suitable and sensitive method. In addition it was established as an alternative method for detection of PSTs in shellfish for official control of PSP toxins in Europe (Regulation 1664/2006). However, Ben-Gigirey et al. [70] conducted an interlaboratory study which concluded that although the method is suitable for monitoring studies, analysis of samples with more complicated toxin profiles did not produce satisfactory results, and also noted that the method is not validated for all the PSTs. On the other hand, satisfactory results were achieved when the more toxic analogues, for example STX, GTX2/3, dcSTX and dcGTX2/3, were analysed. Turner et al. [71] further validated the Lawrence method using such criteria as selectivity, linearity, limit of detection, accuracy, recovery, precision, ruggedness, applicability, repeatability, and reproducibility, and included additional PSTs such as dcNEO and dcGTX2/3. They made some modifications to increase the stability of the oxidation product and to improve the solid-phase ion-exchange clean-up, but concluded that the method produced satisfactory results.

Some other methods have been proposed during the last decade. He et al. [72], analysing marine microalgal samples, made some adjustments to the pre-column derivatization process, in which the periodate oxidation and mobile phase were modified, but the main change was the use of an analytical μBondapak NH_2 column which enables the use of eluents ranging from pH 2 to pH 13 and a wide variety of buffers. They analysed STX, neoSTX, GTX1/4, and GTX2/3, achieving good recoveries with low limits of detection, and without the use of organic solvents (LOD at S/N = 3 for neoSTX, STX, GTX1/4, and GTX2/3 were 1.10, 0.32, 1.26, and 0.041 ng mL^{-1}, respectively). Diener et al. [73] proposed a post-column derivatization method, similar to the Oshima [29] method but using only one gradient run with different mobile phases containing ion-pair reagents. With this methodology they separated the most toxic PSTs and demonstrated low LODs for most toxins (LOD at S/N = 5 for GTX1, GTX2, GTX3, GTX4, neoSTX, dcSTX, and STX were 0.820, 0.014, 0.015, 0.96, 0.306, 0.06, and 0.035 ng, respectively), using various matrices (algal extracts and *Macrocallista* spp.).

Rourke et al. [74] developed a new method of post-column derivatization based on the Oshima [29] method. In this study, the new method was compared with the MBA, the pre-column oxidation method from Lawrence [67], and the post-column method from Oshima [29], taking into account criteria such as practicality, equivalence with the bioassay method and/or the Lawrence method, applicability, cost, reliability, and time. The method comprises important modifications of the Oshima method, for example the combination of two mobile phases in order to

analyse STXs and GTXs together in a single run using a gradient, different oxidation conditions, a different column, and a different clean-up procedure. The advantage of this method is the need for only two runs that provide good separation, with only a single artefact peak that did not compromise the method. Compared with the MBA, the post-column method had a correlation coefficient of 0.86 whereas the Lawrence screening method had a correlation coefficient of 0.36 only, suggesting that the full Lawrence method is required. When the full Lawrence method was compared with the post-column method, the slope was 2.06 indicating values were significantly higher in the post-column method. This increase was related to losses in the Lawrence method during the clean-up process with the COOH and C_{18} cartridges, and during pH adjustment after use of C_{18}. After extensive work, the authors concluded that the greatest benefits of their proposed new method were higher throughput and faster turnaround of positive samples.

Liquid chromatography–mass spectrometry (LC–MS) is a powerful analytical technique used to identify unknown compounds, quantify known materials, and elucidate the chemical and structural properties of new molecules, providing high sensitivity, selectivity, and accurate quantification and also with high throughput. However, LC–MS systems are expensive and require a skilled professional with detailed technical knowledge, not only to operate but also to maintain the equipment. Nevertheless, Quilliam [75] proposed the LC–MS analysis method as universal for all marine toxins, including the PST. One of the challenges for MS detection of PSTs, however, is the ion-pairing agents that are required for efficient reverse phase chromatography of the PSTs, because they interfere with the ionisation of the target compounds [76]. Therefore, Jaime et al. (2001) described a chromatographic method with anion and cation-exchange columns in series using non-ion-pair eluents and post-column electrochemical oxidation. With this technique it was possible to combine LC–FLD and LC–MS detection [77]. Dell'Aversano et al. [76, 78] explored the usefulness of hydrophilic interaction liquid chromatography (HILIC) for separation of not only the major PSTs but also the other cyanobacterial toxins anatoxin-a, cylindrospermopsin, and deoxycylindrospermopsin [76, 78]. Excellent detection was achieved for 15 PSTs (LODs in the sub-nmol L^{-1} range) plus the other toxins (LODs in the nmol L^{-1} range) when the MS was run in selective reaction monitoring mode (in which characteristic fragment ion "transitions" are monitored). With the use of this method, new analogues are still being identified [79]. Diener and co-authors [80] used zwitterionic hydrophilic interaction chromatography with both MS and fluorescence detection of PSTs in a single gradient run. They concluded that both methods achieved a reliable quantification, enabling good separation of the more relevant PSTs. The detection limit varied slightly between the two detectors used but, in general, the fluorescence detector provided greater sensitivity (LOD $S/N = 3$ variation between 0.04 ng for GTX2, GTX3, and dcGTX3 to 0.8 ng for neoSTX), and the MS detector enabled greater selectivity with a shorter run time. Although technically demanding, developments continue to be published [81] and it seems that MS-based methods most likely represent the future of chromatographic analysis of PSTs for those laboratories with the capability to implement them.

A limitation of chromatographic methods for quantification of such a diverse range of toxins is the lack of standards, and, particularly, certified standards. Currently 13 of the approximately 30 known variants are available as certified reference standards from the Institute for Marine Biosciences in Canada. Another factor that underlies all chromatographic methods for determination of PSTs for regulatory purposes is the need to convert the observed quantities of individual analogues into a single STXequivalent number. This is done by calculating the sum of the observed quantities of the analogues multiplied by their respective MBA toxicities relative to STX. However, there is significant variation (up to threefold) in MBA-based estimates of the toxicity of individual PSTs [82], meaning that true toxicity differs somewhat from that calculated from the analytical quantification. Reasonably large discrepancies between sodium channel receptor binding assay and MBA relative potencies have been reported for dcSTX, GTX5, and neoSTX. This led the authors to suggest that the published MBA relative potencies for these analogues may have been over-estimated by 500%, 200%, and 30%, respectively [83]. These variations may have occurred because of:

1. the inherent variability of the MBA; and
2. lack of purity and/or accurate quantification of the analogues being tested.

Selected retesting with certified reference standards may be required to address these concerns.

Toxicity-based methods

The major advantage of the MBA is that it detects all relevant PSTs, whether or not they have been identified and characterised, and so the results reflect actual toxicity. The results are therefore directly relevant to human health risk assessment. A number of alternative methods have been designed to preserve this advantage but to also improve sensitivity and precision, whilst also minimising the use of live animal testing. To do this, these assays have isolated

the functional site of action of the PSTs, either the nerve cell or the sodium channel receptor.

Nerve-cell-based bioassays

A cell-based bioassay for PST detection has been developed as an alternative to the mouse bioassay for toxicity testing. The method specifically detects sodium channel-blocking toxins such as PST. In the nerve cell, the voltage-gated sodium channels play an important role in initiating action potentials (nerve impulses). Under resting conditions the inside of the cell is negatively charged relative to the positive charge outside. This is because of the high concentration of Na^+ ions outside the cell compared with K^+ ions inside the cell. The ionic gradient is maintained by a Na^+/K^+ pump in the cell membrane. When a nerve cell is stimulated, the membrane near the synapse becomes depolarised. The voltage-gated sodium channels in this region respond to this change in membrane potential by opening, thus allowing further inflow of sodium and activation of more distal sodium channels. By this mechanism, the action potential is propagated along the nerve axon to the next synapse [84]. PSTs cause paralysis by blocking sodium ion inflow through the channel.

The neuroblastoma assay was designed to detect PSTs at the level of the sodium channel in the nerve cell in culture. The action of PSTs as sodium channel-blockers can be detected by their antagonism of the effect of another toxin, veratridine, which opens the sodium channel. This cell bioassay was first established by Kogure et al. [85] who used murine neuroblastoma cell morphology as the endpoint to assess the assay outcome. The procedure was then modified by Jellet et al. [86] and Manger et al. [87, 88] to be more amenable to automation, using colorimetric endpoints to measure cell viability. For the assay, neuroblastoma cells are grown in a 96-well microplate and treated with the sodium channel-activator veratridine to enhance sodium ion influx into the cell along with the Na^+/K^+-ATPase (sodium pump) inhibitor ouabain to block sodium re-export. The effect of these toxins is that the cells swell and lyse. The presence of PST blocks the sodium channel, protecting the cells from the lethal influx of sodium ions. Cell viability in the presence of PST (or analogues) is determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay [89]. By using a standard amount of veratridine and oubain, the extent of cellular protection can be used to quantify the amount of PST toxin present in comparison with the PST standards. The results are expressed as STXequivalents. Both mouse (Neuro-2A) and human (SK-N-SH) neuroblastoma cells have been used for the assay [90].

Because the assay was initially established for monitoring shellfish and fish extracts for marine toxins, it has been well-validated for detection of marine neurotoxins in these samples [86, 87, 91, 92]. Other researchers have also validated the assay for use with freshwater cyanobacteria, which are known to be dominated by PST analogues different from those in marine-derived samples [11, 93]. Studies have shown that the neuroblastoma bioassay gives results comparable with those from mouse bioassay (r^2 = 0.96) and has the advantage of having greater sensitivity. The detection limit has been reported to be 10 ng STXequivalents mL^{-1} extract (2.0 μg STXeq/100 g shellfish tissue) [86]. PST results obtained by bioassay are also reported to correlate well with those from chromatographic methods. However, the bioassay has the distinct advantage being sensitive to any toxin that can inhibit the sodium channel, whether it is a known PST analogue or an undescribed variant. As described by Humpage et al. [11], the assay detected as yet uncharacterised toxins that could not be detected by chromatographic methods. Because bioassays do not differentiate among related toxins, but only provide a value for the cumulative effects of all the toxins it is difficult to determine the relative toxic responses of analogues in the cell culture assay compared with the MBA unless each of the analogues are analysed individually. However, Llewellyn et al. [43] provided data showing that the cell culture bioassay is a good predictor of toxicity compared with the mouse bioassay, when using complex PST mixtures. A total of 21 shellfish meat extracts with complex PST profiles (up to 10 different PSTs) were analysed by MBA and cell culture assay, along with HPLC and radio-receptor assays. Of all the techniques used in this study, the cell culture correlation with the mouse bioassay was nearest to unity in terms of predicting toxicity.

There are however some disadvantages of the cell bioassay format. First, because the assay relies on antagonism of the effects of veratridine and ouabain, appropriate concentrations of each must be used to allow a sensitive response to PST. As discussed by Jellet et al. (1992) [86], changes to either of these toxin concentrations can reduce the sensitivity of the assay to PSTs. This is compounded by:

1. the toxins, particularly ouabain, being only sparingly soluble at the concentrations recommended for the preparation of stock solutions; and
2. their steep concentration–response curves (Humpage et al. unpublished).

Further, the standard assay set-up is slow, requiring long cell-incubation times (24–48 h) for cytotoxicity to develop in the neuroblastoma cells. Recent work has focussed on modification of the neuroblastoma assay to enable more rapid detection of the toxins. This can be achieved by addition of yet another toxin in combination with veratridine and oubain. Manger et al. [90] demonstrated a dose-dependent antagonistic effect of purified PST on

brevetoxin-induced cytotoxicity in Neuro-2A cells that enabled detection of PST (0.1–10 ng/well) within 7 h. In this study, brevetoxin was added to neuroblastoma cells immediately after addition of veratridine and ouabain. Samples containing PST were then applied. While this enables more rapid detection of PSTs, the addition of another toxin to the cells introduces a further level of complexity. All toxin concentrations need to be appropriately balanced to retain sensitivity to PST. An alternative method uses maitotoxin (a Ca^{2+} agonist) to intensify the toxic response of veratridine and oubain. In this case, the maitotoxin is added at the end of the incubation period, 15 min before the end of a 6-h PST exposure [94]. The maitotoxin enhances the influx of Ca^{2+} ion across the cell membrane, working synergistically with the veratridine and ouabain to induce toxic effects.

Another approach to developing a more rapid assay is the detection of toxin-induced membrane depolarisation by use of voltage-sensitive fluorescent dyes [95–97]. PST antagonism of veratridine-induced depolarization can be detected by a decrease in fluorescence of $DiSBAC_2(3)$, which has previously been loaded into the plasma membrane of the nerve cells. Analysis of the cells by flow cytometry has been reported to provide a sensitive measure, enabling detection of PST in a sample within minutes [97]. Similarly, Louzao et al. [96] used bisoxinol to detect PST-induced changes in membrane potential, with quantification of the result with a microplate reader within minutes of exposure to PST. The authors reported that this simple microplate assay was sensitive to PSP toxins at <1 ng STXeq mL^{-1}, over 100-fold more sensitive than the mouse bioassay (400 ng mL^{-1}). These functional assays using fluorescent probes provide promise for sensitive and rapid detection of PSTs in the future. However, further validation of the rapid cell-based endpoints will be required for acceptance of these techniques in the future.

Assays using rat brain sodium channel preparations

Vieytes et al. [98] first published a rapid and sensitive method that used isolated rat brain sodium-channel preparation. Fifteen to forty rat brains were used to produce a solution containing 5.8±0.06 pmol sodium channels mL^{-1} which could be frozen at −80°C for at least 30 days. Sodium channels were adsorbed by the bottom of wells in a microtitre plate and used to bind ^{3}H-STX from a control solution added to the wells. After washing to remove unbound ^{3}H-STX, the bound toxin was quantified by scintillation counting. Despite use of bovine serum albumin to block non-specific binding to the plates, the authors noted that 21% of radioactivity detected was non-specifically bound. However, despite this relatively high background, competition experiments using unlabelled and ^{3}H-STX produced an IC_{50} value of 1.7 ng STX mL^{-1}. Experiments with crude mussel extracts containing PST mixtures showed good correlation with MBA and HPLC results in terms of STXeq toxicity [98]. Precision (10–15%) was reasonable for a prototype method and a result could be obtained within a few hours.

Doucette et al. [99] extended this concept by implementing it in a relatively high-throughput format, and validated it using a larger range of samples. They showed that the sodium channel preparation was stable at −80°C for at least six months. They also provided more extensive QA/QC criteria for interpretation of assay results. They reported a K_i for competitive binding of ^{3}H-STX and unlabelled STX of 3.66 nmol L^{-1} and the LOD in the sample extract was estimated to be 5 ng STX mL^{-1}. Twenty-six shellfish and twenty phytoplankton samples were analysed to demonstrate reasonable correlations with HPLC and MBA determinations of molar STXeq toxicity. The sodium channel-binding assay (SCBA)-determined toxicity was somewhat higher in phytoplankton samples and shellfish extracts compared with HPLC determinations and MBA, respectively (regression slope ~1.8 in each case). The authors also commented that this level of correspondence is exceptional given the uncertainty of MBA relative toxicities [82].

Ruberu et al. [100] then showed that this method could be modified to provide accurate and precise results in a different laboratory using a different scintillation counter and a procedure optimised for that instrument. In an inter-laboratory comparison between the laboratories of Doucette et al. and Ruberu et al. using 12 shellfish samples, the modified method produced results that were on average 0.89 times those of the Doucette method (95% CI = 0.78–0.99). In comparison, the SCBA result (average from the two laboratories) was 1.7 times higher (95% CI = 1.1–2.2) than the MBA result for each sample. The authors cite a reasonably high sample throughput of 18 samples per analyst per day, with the major time limitation being the AOAC shellfish-extraction procedure rather than the SCBA detection method. Usup et al. [83] reported extensive assessment of the SCBA using a suite of certified PST reference standards to determine its sensitivity to the various analogues relative to STX in comparison with their potency in the MBA. All analogues produced reproducible concentration–response curves and thus reliable results in terms of STXeq potencies. The EC_{50} for STX in the assay was 4.4 nmol L^{-1}, which is very similar to results from other laboratories that used slightly different procedures [99, 101].

Methods employing macromolecular sensors or chemosensors

These methods are characterised by detection of occupancy of PSTs at a molecular or macromolecular binding site that

has high affinity for compounds having the structural characteristics of PSTs. A range of sensor molecules have been used including antibodies, the transferrin-related saxiphilins, and chemical constructs such as crown ethers. Although assays based on binding to sodium channel preparations at least theoretically retain a functional relatedness to in-vivo toxicity, those based on antibodies or crown ethers usually do not detect the PSTs in a way that is directly interpretable in terms of toxicity.

Saxiphilin-based assay

Saxiphilin is a protein, related in structure to transferrins, that has been shown to have very high affinity for PSTs (the binding constant, K_d, for STX is approximately 0.2 nmol L^{-1} [102]). This has made saxiphilin an attractive option for development of a molecular sensor-type PST assay [103, 104]. However, different saxiphilins have different affinities for the various analogues, with that from the tropical centipede *Ethmostigmus rubripes* having the least selectivity of those tested so far. An assay based on this isoform has been found to be quite robust to potentially interfering matrix components and pH [103]. The limit of detection was estimated to be 6.3 μg STXeq L^{-1}, or 1.3 μg STXeq/100 g shellfish, but the authors state that this could be reduced by increasing the volume of sample, without additional matrix interference. The extraction procedure to produce crude saxiphilin from this centipede is very simple, and the preparation is stable to freeze–thawing and storage for more than a year at −80 °C [104]. Further work has demonstrated the utility of using both the SCBA and saxiphilin assays for detecting non-PST sodium channel activity (putatively TTX) in crustaceans and molluscs (because saxiphilin does not bind to TTX [105]) and good correlation of results between HPLC, RBA, and saxiphilin for PST detection in *A. circinalis* [106].

Antibody-based assays

Antibodies have been used as the detection mechanism for a wide range of assays, particularly in the medical and food-testing fields. The enzyme-linked immunosorbent assay (ELISA) is now a widely used platform for sensitive and accurate detection of thousands of analytes. Because of their sensitivity, rapidity, and ease of use, these assays have proved particularly successful when a single analyte is the target. The challenge for their use for detection of mixtures of related compounds such as the PSTs is to retain the ability to recognise the range of structural variants of the target (called "cross-reactivity") whilst ignoring other compounds in the complex matrices to be tested. Usleber et al. [107] summarised progress towards the creation of antibody-based detection techniques to that date. They reported that antibodies raised against STX had low cross-reactivity to neoSTX, and visa versa, and that this selectivity applied to all analogues in each of these lineages. Thus, Chu et al. [108] found poor correlation between assays based on either anti-STX antibodies or anti-neoSTX antibodies, but combining the results from the two assays markedly improved the detection rate, to the extent that these authors concluded that their combined use could screen out 80–85% of MBAs that produce negative or low positive results. Continuing the theme of multiple assays to improve specificity, Kawatsu et al. [109] raised monoclonal antibodies (designated GT-13A) to GTX2/3 to complement those previously raised against STX and neoSTX. These antibodies retained the STX/neoSTX lineage discrimination found in other antibodies, but did have a near equal affinity for GTX2/3, dcGTX2/3, and C1/2. The multiple assay approach was taken even further by Garthwaite et al. [110], who recommended the use of a suite of ELISAs to screen shellfish samples for not only PSTs, but also amnesic, diarrhoeic, and neurotoxic shellfish poisons.

The ELISA platform technology is relatively mature, and this has facilitated commercialisation of antibody-based PST assays. Two commercial kits based on the ELISA format are available—the Ridascreen fast saxitoxin test (R-BioPharm) and the Abraxis ELISA for PSP (Abraxis). The Ridascreen assay is tailored specifically for shellfish testing, having a much higher LOD than the Abraxis test (50 and 0.02 μg L^{-1}, respectively). However, cross-reactivity is highly variable as shown in Table 1.

This need not be an impediment if cross-reactivity correlates with toxicity. For example, the C-toxins have low cross-reactivity in these assays but because they also have low toxicity, the assay outcome can indicate actual toxicity. Of more concern are the highly toxic decarbamoyl and *N*1-hydroxylated variants, which the assays do not detect. Hence, to use these ELISAs with confidence, it is necessary that the PSTs that commonly occur in the

Table 1 Relative potency of PSTs in the mouse bioassay (MBA) and their reported cross-reactivity in commercially available antibody-based assays

	MBA [29]	Ridascreen	Abraxis	Jellett
STX	100	100	100	100
neoSTX	92	12	1.3	21
GTX2/3	36/64	70	23	93
GTX1/4	99/73		<0.2	3
dcSTX	51	20	29	40
dcneoSTX			0.6	
dcGTX2/3	65/75		1.4	5
C1/2	0.6/9.6		2.0	7
GTX5	6.4		23	40

samples to be tested have been identified and the mixture of analogues shown to be a relatively stable. Because of these concerns, antibody-based tests for PSTs should not be thought of as quantitative assays but more as screening tools. But if this is the approach, then the ELISA format is not necessarily the best platform. As an alternative to the standard ELISA, Cordova et al. [111] published a novel latex agglutination assay in order to make a field-usable test. The assay was based on the principle that STX in shellfish extracts compete with STX bound to latex beads for the binding sites of monoclonal anti-STX antibodies added to a test solution. Thus, the more STX in the extract, the less agglutination that was observed. The assay showed good correlation with MBA for a range of shellfish extracts tested but its LOD was similar to that of the MBA (1.25–2.5 μg STX mL^{-1}). In addition, cross-reactivity with other PSTs was poor, with the LODs for neoSTX, GTX2/3, and GTX1/4 being 10 to 20-fold higher than for STX (LOD for GTX5 was ~100-fold higher).

The most successful field-usable format so far has been the Jellett rapid testing lateral flow immunochromatographic (LFI) strips. The test works by setting up a competition for binding of labelled anti-STX antibodies between PSTs in the sample and toxin bound to a test line in the device. The test line disappears as the PST concentration in the sample increases, with the amount of antibody added being tailored to produce the required level of sensitivity. To screen for PSTs in shellfish the standard shellfish test is calibrated to 25 μg STX/100 g tissue so that for naturally occurring mixtures of analogues the average "break-point" is similar to the mouse bioassay at 30 to 40 μg STXeq/100 g tissue [112]. There has been some confusion resulting from the first publication describing characteristics of an early version of the test [113]. Although the same batch of antibodies has been used since the PSP test was first produced under the name MIST Alert by Jellett Biotek, quality control has been refined and the increased availability of PST standards has enabled the characteristics of the test to be better defined. For example, it was previously reported that the test had good cross-reactivity with C-toxins, but this was an error because of an impure standard. Also, cross-reactivities to NEO and GTX1/4 were unusually high (M. Laycock, personal communication). The assay has been assessed against alternative assays by a number of groups [11, 112, 114–116], and results from the assay have generally been found to have good correlation with the MBA for testing shellfish. The only group that assessed the assay for use with cyanobacteria (Jellett rapid test version [11]) found some problems with accuracy (compared with LC–MS–MS) and reproducibility, but further work in our laboratory has found the improved tests to be suitable for its intended purpose of screening samples for PSTs in the field. The PSP test requires no sample clean-up, because matrix effects are rare and crude extracts can be tested after dilution in the running buffer. However, a suitable sample-preparation procedure is advisable, involving acid hydrolysis, to minimise the chance of false negatives because of the relatively high C-toxin content of Australian *Anabaena circinalis* samples (data not shown). Since the earlier versions of the MIST Alert, sensitivity of the test to STX has been fairly consistent at around 25 μg/100 g. However, the sensitivity depends on the toxin composition of the sample and an LOD can only be determined with reference to a given toxin composition [112]. Setting the sensitivity at 25 μg STX/100 g enables the test to still give positive results below the 80 μg STXeq/100 g shellfish regulatory limit when mixtures of PSTs are present in the sample. Thus, the "false positive" rates reported by Oshiro et al. [116] compared with those found by Inami et al. [115] indicate differences in toxin profiles and are not a result of differences in test sensitivity. It is important to understand that the test is not quantitative and should be used solely for screening. A version with, perhaps, 100-fold greater sensitivity is under development (Laycock, personal communication).

Biacore sensors

A number of groups have attempted to construct biosensors by adsorbing the rat brain sodium channel [117], anti-PST antibodies [117, 118], or saxiphilin (Llewellyn personal communication) on the Biacore surface plasmon resonance detector chip. This technology works by detecting changes in molecular conformation associated with ligand binding to a macromolecule immobilised on the sensor chip. Although still in its infancy, this method is showing promising results, particularly when antibodies such as GT-13A are used as the sensing molecule [117, 118]. However, development has concentrated on testing of shellfish and detection limits are still not adequate for detection of PSTs in drinking water. Moreover, when antibodies are used as the sensor molecule, the same concerns regarding cross-reactivity that limit the usefulness of ELISAs will also apply to this technology.

Chemical sensors

The Gawley group has published a number of papers tracing the development of the use of crown ethers, attached to various chromophores, for detection of saxitoxin [119–122]. Progress has been made in lowering the limit of detection for STX, and discrimination between STX and TTX has been demonstrated. However, to date, no other PSTs have been tested using this approach so cross-reactivity cannot be assessed.

Comparative studies

A number of studies have compared different assay formats and reported on their relative merits. Doucette et al. [99] compared their SCBA method with HPLC-derived PST quantifications for 21 algae samples, and with MBA and HPLC results for 26 shellfish samples. Correlation coefficients for these comparisons were 0.88, 0.95, and 0.97, respectively, with all correlations being linear ($p = 0.0001$) over a wide range of toxin concentrations. However, the SCBA tended to give different toxicity estimates than other methods. SCBA results were approximately 1.8-fold higher than HPLC for algal samples and MBA for shellfish samples. However, SCBA gave lower toxicity than the HPLC-determined shellfish results (regression slope 0.8). Llewellyn et al. [43] conducted parallel analyses of 32 shellfish extracts by MBA [36], HPLC [29], MIST cell-based assay [86], sodium channel binding assay (SCBA) [99], and saxiphilin assay [103]. They found reasonably good correlations between the various assays (Spearman's correlation coefficients between 0.71 and 0.96, all being 0.81 or better when compared with the MBA), however the slopes of log–log correlation plots varied much more markedly (overall range 0.3–6.5, with variation from the MBA slope ranging from 0.4–2.7). In this study, the SCBA and saxiphilin assays tended to be 50% lower than the MBA result whereas HPLC was almost 3-fold higher. The MIST cell-based assay most closely estimated the MBA result (regression slope = 1.1). Negri and Llewellyn [105] compared HPLC [15, 29], SCBA [123] and saxiphilin [123] quantifications of PSTs in 190 crustacean and mollusc samples. Correlation between the saxiphilin and HPLC results was reasonable ($r^2 = 0.81$). The correlation between SCBA and HPLC was much lower ($r^2 = 0.33$) but this improved when samples thought to contain TTX were removed from the analysis. Azanza et al. [124] used the MBA (AOAC 1990), SCBA [99], and MIST Alert [113] to assess the toxicity of green mussels from three locations in the Philippines [124]. The SCBA detected averages ($n = 3$) of 8.57, 5.44, and 15.98 μg STXeq/100 g mussel meat in the three samples, the MIST Alert gave a positive result with all three samples, and the MBA showed non-fatal neurotoxic symptoms with the first two samples and 28.98 μg STXeq/100 g with the last sample, although it was noted that this concentration is still below that for which the MBA can provide a reliable quantification [124]. Inami et al. [115] compared the MBA with the Ridascreen saxitoxin ELISA, the MIST Alert, and the 5-h neuroblastoma assay (with brevetoxin addition; [90]) for at least 76 shellfish samples (106 samples for all but the neuroblastoma assay). Because the intention was to select a cost-effective screening tool, to reduce the use of the MBA for testing negative samples, quantitative outcomes from the Ridascreen and neuroblastoma assays were converted to positive or negative results based on selected negative cut-off values. Interestingly, better correlations were found between these assay results and the MBA when the cut-off values were optimised for the assay rather than simply using 37 μg STXeq/100 g, the LOD of the MBA. The Pearson correlation coefficients for comparison of each of the optimised assays with the MBA were similar at approximately 0.8, and false positive and false negative rates were low.

All of these assays have adequate sensitivities for use in shellfish PST regulatory compliance testing, but only HPLC/LC–MS, SCBA, saxiphilin assay, neuroblastoma assay, and Abraxis ELISA for PSP have LODs that may be low enough for drinking water testing. Only two studies have compared methods for use with extracts of cyanobacteria [11, 106]. Llewellyn et al. [106] compared a sodium channel-binding assay [99] and a saxiphilin-binding assay [103] with HPLC [29] for detection of PSTs in 14 Australian *A. circinalis* samples. HPLC results showed that all but one of the toxic samples were dominated by C1/2 toxins (approximately 80% molar ratio), with lesser amounts of GTX2/3, STX, dcGTX2/3, and dcSTX. Interestingly, the saxiphilin assay responded much more strongly than the other assays to one sample in particular, a finding that was confirmed by MBA. Excluding this sample, correlation coefficients for both binding assays with HPLC were high ($r^2>0.9$), but the HPLC data consistently indicated higher concentrations of PSTs than were found by the binding assays (4-fold for the SCBA and 1.5-fold for the saxiphilin assay). Humpage et al. [11] assessed the neuroblastoma cell-based method [88] for use with cyanobacteria using eight Australian *A. circinalis* samples and three Brazilian *C. raciborskii* samples. The results were qualitatively compared with those from an LC–MS–MS method [76], an HPLC–FD method [29] and the Jellett rapid test. The neuroblastoma assay was conducted in parallel in two laboratories. The neuroblastoma assay was found to respond to a range of pure PSTs relative to their MBA toxicities. There was no significant difference between neuroblastoma assay results for cyanobacterial samples obtained in the two laboratories. Again, chromatographic methods indicated that all but one of the toxic Australian samples were dominated by C-toxins (C1/2), whereas the Brazilian toxic samples contained STX, neoSTX, dcSTX, and dcneoSTX. However, because of variations in methods and standards used at different times, there was substantial variation between different chromatographic quantitative results. The neuroblastoma assay also exhibited some lack of precision, but no more than the various HPLC estimates had done. Anomalous results were also seen in this cyanobacterial study, with two of the Brazilian samples being approximately 35-times more

potent in the cell assay than could be explained by the chromatographic or Jellett rapid test results. These samples have previously been shown to exhibit a higher than expected MBA potency also [10].

The results of these studies indicate that most of the alternative assays have reasonable correlation with the MBA, although the regression slopes are often significantly either below or above unity. The lack of consistency in the quantitative relationship between assays suggests that the observed variability is because of factors other than systematic differences between the assays. The reasons for this variability have been discussed by many authors, and include:

- MBA-related factors, for example the need for mouse colony standardisation, inter-individual variation, the subjective endpoint (the "last gasp"), salt interference, high LOD, 20% precision;
- HPLC-related factors, for example detection limits that vary for different toxins so that some may be missed and not included in STXeq calculation, inaccuracy of toxicity equivalent values used for conversion to STXeq, ability to only detect and quantify known toxins, and limited availability of toxin standards;
- SCBA-related factors, for example the requirement for the sodium channel to remain functionally normal in isolated membranes;
- saxiphilin and antibody-related factors, for example binding affinities for toxins that do not correlate with mouse or sodium channel sensitivities;
- cell-based assay-related factors, for example their relatively complex procedures; and
- toxin inter-conversions that may occur in archived regulatory samples that may change their toxicity after the initial MBA was performed.

Thus, method selection must take into account other factors, such as turn-around-time, toxicity versus toxin detection, expense, and availability of trained staff.

Conclusions

The compounds known as the paralytic shellfish poisoning toxins (PSTs) have a long history of causing death and illness in human consumers of contaminated seafood. More recently they have also been discovered in freshwater cyanobacteria, and thus present an additional potential risk through contaminated drinking water. Since the isolation of saxitoxin in 1957, the number of PSTs characterised has increased steadily until today about 30 are recognised, with evidence for others that are yet to be fully characterised. They vary substantially in both chemical nature and toxicity, and this presents a major analytical challenge for those charged with determining compliance of seafood or drinking water safety regulations.

The method that has been used for most of the history of PST testing is the mouse bioassay (MBA), and this is still the method of choice throughout much of the world. It has many advantages. Murine nerve cells respond to the PSTs in the same way that human nerves do, so the assay results are directly relevant to human health risk assessment. The effects of the different PST analogues are, by definition, coincident with their toxicity. The MBA was also the first assay developed, even before the toxins had been identified, and so all other detection methods must be assessed in relation to the MBA. However, the MBA also has some major disadvantages that have already been enumerated in this review, not least being its very high limit of detection which makes it useless for drinking water testing. Substantial effort has thus been devoted to finding alternatives.

Chromatographic techniques have been developed over the years both to quantify the known toxins and to identify new ones. The ever-expanding number of PSTs has led to a concomitant increase in the complexity of the analytical methods. The chromatographic method of choice for many years has been the HPLC/post-column oxidation/fluorescence detection method proposed by Oshima in 1995, which requires three separate runs to quantify all of the most important PSTs. This complexity makes it less than ideal as a routine compliance testing method, because it takes much too long to perform. The new Lawrence method reduces this complexity somewhat and was recently accepted by the AOAC as the only officially sanctioned alternative to the MBA. However, LC–MS detection has many advantages in terms of selectivity, relatively simple chromatography without the need for derivatisation, and capacity for high sample throughput, so it seems likely that this technology will eventually supersede the oxidation/fluorescence detection methods.

However, a weakness of all chromatographic methods is the need to convert quantities of individual PSTs into a saxitoxin toxicity equivalent (STXeq). This relies on the accuracy of the toxicity equivalent conversion factors, and there is much variation in the published estimates of these for some PSTs. This contributes statistical uncertainty to any STXeq derived from a chromatographic method, no matter how precise the results produced by that method. These toxicity equivalents are also derived purely from acute toxicity experiments, and there is at least a theoretical, though untested, possibility that even episodic exposures could have long-term consequences, particularly in the developing nervous system.

A means of avoiding this problem is to base assay detection on the target of the toxins, the sodium channel. Two approaches have been taken, either by using cell-lines that express the functional sodium channel or by isolating

the sodium channel itself in a membrane suspension. Both methods have been shown to produce results that correlate reasonably well with those from both the MBA and chromatographic methods. The isolated sodium channel technique has the advantages of greater sensitivity and rapidity, but unfortunately relies on the use of radio-labelled STX and non-standardised sodium channel preparations. Recent advances to speed up the cell-based method to provide almost instantaneously detectable responses mean that this approach may yet provide a high-throughput toxicity-based method to replace the MBA.

The third non-MBA approach to PST detection has been the use of sensor molecules with high affinity for these toxins, that is antibodies or saxiphilin. Very low limits of detection are achievable, but usually only for a small proportion of the known PSTs. A way around this limitation might be to use multiple sensor molecules with affinities specific for the different PST sub-classes (STX, neoSTX, GTX, C-toxin), but to date this has only been done using separate assays, whereas the ideal would be to combine them all in a single assay. Nevertheless, the antibody-based methods, in particular, have gained acceptance as screening tools to eliminate negatives from the MBA testing programme.

Each method has advantages and disadvantages, and so the technique to be adopted will also depend on the availability of resources and capable personnel. Sophisticated LC–MS methods may become the new gold standard in a relatively few specialised centres in the West, but given the continued discovery of new PSTs, toxicity-based techniques will remain essential for both confirmation that the chromatography is detecting all PSTs of concern, and also for determining the toxic potency of new analogues discovered by LC–MS. However, in many parts of the world where both toxic shellfish and toxic cyanobacteria are found in abundance, expensive analytical instruments and technical expertise are not available. There is also difficulty and considerable cost in shipping standards to some countries, a situation exacerbated by current security concerns. Hence there is still a great need to develop simple, sensitive and inexpensive methods that can help protect lives in these regions.

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