Talanta 99 (2012) 668–676

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies

Betsy Jean Yakes [a,*], Samantha M. Prezioso [b,1], Stacey L. DeGrasse [a]

[a] U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5100 Paint Branch Parkway, College Park, MD 20740, USA
[b] Joint Institute for Food Safety and Applied Nutrition (JIFSAN), University of Maryland, College Park, MD 20742, USA

ARTICLE INFO

Article history:
Received 17 April 2012
Accepted 25 June 2012
Available online 25 July 2012

Keywords:
Surface plasmon resonance
Paralytic shellfish poisoning
Saxitoxin
Lateral flow immuno-chromatography
Immunoassay
Cross-reactivity

ABSTRACT

Paralytic shellfish toxins (PSTs) are a risk to humans upon consumption of contaminated seafood. The PST family is comprised of more than twenty congeners, with each form having a different potency. In order to adequately protect consumers yet reduce unnecessary closures of non-contaminated harvesting areas, a rapid method that allows for analysis of sample toxicity is needed. While a number of PST immunoassays exist, the outstanding challenge is linking quantitative response to sample toxicity, as no single antibody reacts to the PST congeners in a manner that correlates with potency. A novel approach, then, is to combine multiple antibodies of varying reactivity to create a screening assay. This research details our investigation of three currently available antibodies for their reactivity profiles determined using a surface plasmon resonance biosensor assay. While our study shows challenges with detection of the R1-hydroxylated PSTs, results indicate that using multiple antibodies may provide more confidence in determining overall toxicity and the toxin profile. A multiplexed approach would not only improve biosensor assays but could also be applied to lateral flow immuno-chromatographic platforms, and such a theoretical device incorporating the three antibodies is presented. These improved assays could reduce the number of animal bioassays and confirmatory analyses (e.g., LC/MS), thereby improving food safety and economic use of shellfish resources.

Published by Elsevier B.V.

1. Introduction

Paralytic shellfish poisoning (PSP) is caused by a suite of toxins, known collectively as paralytic shellfish toxins (PSTs) [1]. Saxitoxin (STX) and its congeners originate from certain dinoflagellates and some cyanobacteria [2]. Filter feeding bivalves (e.g., mussels, clams, cockles, scallops and oysters), as well as other seafood species, can accumulate and metabolize these toxins which can then lead to potentially dangerous seafood [3,4]. Human consumption of toxic seafood can result in tingling, numbness, respiratory paralysis and potentially death [5], as the PSTs bind to site 1 and block the opening of voltage gated sodium channels [6]. These small molecule toxins are also quite robust, and typical preventative food safety measures (i.e., use of heat or acid during cooking) do not destroy the PSTs [1].

Proper monitoring and implementation of harvesting bans when toxin concentrations exceed safe levels (typically 80 μg STX equivalents per 100 g tissue) have minimized PSP illnesses [1]. However, outbreaks still occur, especially in developing countries [7] and with an estimated worldwide mortality of 6% [8]. For example, a major PSP epidemic occurred in Guatemala in 1987 that claimed the lives of 26 people out of the 187 affected [7,9]. A review of PSP cases and outbreaks has been compiled by FAO, which reports PSP prevalence along coastal European nations, parts of Africa, the West Coast and Northeast region of North America, South America, and parts of Asia [10]. Within the US, the majority of illnesses and outbreaks are reported from recreational harvests among fishermen and tribal communities. For example, during May and June of 2011, 21 cases of PSP illness were reported in Southeast Alaska due to unprecedented high levels of PSTs in surrounding waters [11].

Recent reviews on PST detection have focused on improved analysis of both coastal waters and seafood [1,2,12,13]. The mouse bioassay (MBA) is one of the AOAC approved and most commonly used testing methods for PSTs [14]. While simple, this bioassay suffers performance related challenges (e.g., poor quantitation and low dynamic range, interferences to detection, low sample throughput, and lack of determination of the specific toxin associated with death) as well as ethical concerns.

A second AOAC approved method for determining PSTs is high performance liquid chromatography (HPLC) with fluorescence detection (FD) [15,16]. This method is quite effective at identifying and quantifying the toxins in a seafood sample. However, it requires a lengthy sample clean-up and pre-column oxidation procedure to

* Corresponding author. Tel.: +1 240 402 2720, fax: +1 301 436 2624.
E-mail address: betsy.yakes@fda.hhs.gov (B.J. Yakes).
[1] Present address: Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA.

0039-9140/$ - see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.talanta.2012.06.073

create fluorescent derivatives of the toxins for detection as well as multiple analytical runs for complete PST determination. The post-column HPLC-FD method created by Oshima [17] was refined [18] and is also now AOAC approved [19]. This post-column oxidation method has a simpler sample preparation procedure than pre-column HPLC-FD; however, multiple analytical runs under different chromatographic conditions must be conducted in order to analyze all potential PST congeners. Furthermore, both HPLC-FD approaches can be hindered by sample materials that have native fluorescence, requiring additional steps to ensure the presence of toxins [20].

Other analytical techniques that are advancing include liquid chromatography (LC) coupled with mass spectrometry (MS) [21,22], some in tandem with biosensors [23]. The major limitation of this analytical approach is matrix interference and ionization suppression, which restricts its ability to serve as a reliable, quantitative monitoring tool. Limited availability of internal reference standards (e.g., isotopically labeled toxins) currently hinders wider-spread implementation of monitoring by LC/MS.

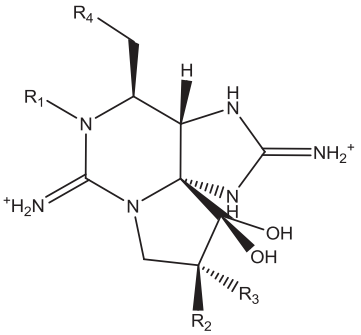
In order to overcome the challenges associated with MBA and LC methods, rapid screening techniques have been explored. These methods can be simple, cost-effective, sensitive, and accurate for high-throughput detection needs. Such methods include receptor binding assays (RBA) [24–27], lateral flow immuno-chromatography [28,29], enzyme-linked immunosorbent assays (ELISA) [30–32], and cell bioassays [33,34]. While these methods allow for high throughput and ease of use, they suffer from the use of difficult to procure radiolabeled materials for RBA, high probability of false-positive and potential for false-negative results with current immuno-chromatographic PSP tests, large amounts of manual labor and limited antibody cross-reactivity for ELISA, and nonspecific toxin recognition for the cell bioassays.

An immunological technique that has been shown to provide high throughput detection of PSTs is surface plasmon resonance (SPR) biosensors [35–38], though this method faces the same challenges with respect to antibody reactivity. SPR immunoassays are based on specific biosensor platforms that bind the molecule of interest at the surface. The change in mass due to binding is detected as a change in refractive index (RI) at the dielectric interface (i.e., gold immunoassay substrate and solution in the flow cell). This RI change causes a shift in the SPR band position that can be tracked in real-time using standard spectroscopy optics [39]. This automated technique allows for real-time analysis of PST-containing samples, requires minimal sample cleanup, no labeling of the analytes, and yields sub-ppb limits of detection in less than ten min [40,41].

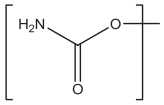
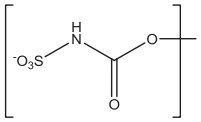
The SPR assay for the determination of PSTs currently implemented in our laboratory is robust and shows good repeatability and reproducibility; however, quantitative results do not always correlate with overall sample toxicity due to the many PST congeners having widely varying potency. The toxicities for common PSTs are shown in Table 1, and the inability to correlate results with sample toxicity when using immunological assays could lead to unsafe seafood harvested for consumers (false-negative) or destruction of safe seafood and closure of non-contaminated harvesting areas (false-positive). Clearly, there is a need for improved assays to not only protect the public but also to improve the economic viability of the industry and utilization of seafood resources. Unfortunately, a single antibody that reacts to the congeners with respect to their potency has yet to be produced. An advantage to the SPR assay is that while the response may not always correlate with toxicity, the cross-reactivity of individual congeners with an antibody can be calculated. A novel approach, then, would be to combine multiple antibodies of varying reactivity to the congeners, as screened via the SPR assay, to create a multiplexed immunoassay.

One disadvantage to SPR biosensors is the size of instrumentation and cost of materials which could prohibit routine testing in the field or dockside. Lateral flow immuno-chromatographic tests (LFIs) have been used for PST testing and could fulfill the requirements of an easy-to-use and cost-effective technique for monitoring potential toxicity of seafood when the quantitation and automation of the SPR instrumentation is not necessary.

Table 1
PST structure, congener forms, and relative toxicities [17]. Toxins used in this study are listed in bold.



Toxin	Relative Toxicity
STX	1.00
dcSTX	0.51
GTX2,3	0.36, 0.64
B1 (GTX5)	0.06
C1,2	0.01, 0.10
dcGTX2,3	0.15, 0.38
NEO	0.92
dcNEO	–
GTX1,4	0.99, 0.73

R1	R2	R3	Carbamate	Decarbamoyl	*N*-sulfocarbamoyl
H	H	H	**STX**	**dcSTX**	**B1 (GTX 5)**
OH	H	H	**NEO**	**dcNEO**	B2 (GTX 6)
OH	H	OSO_3^-	**GTX1**	dcGTX1	C3
H	H	OSO_3^-	**GTX2**	**dcGTX2**	**C1**
H	OSO_3^-	H	**GTX3**	**dcGTX3**	**C2**
OH	OSO_3^-	H	**GTX4**	dcGTX4	C4
		R4:			

The challenge with these rapid tests also lies in the inability to accurately measure sample toxicity, and the performance of such devices has been extensively studied [28,29,42]. To potentially enhance the reliability of the LFIs, multiple antibodies that have distinct reactivity patterns as determined in the SPR assay could be employed.

This manuscript details our work that evaluated three antibodies for their reactivity to nine commonly occurring PSTs. The data indicate that a multiplexed approach may not only improve SPR biosensor assays but could also be incorporated into LFI platforms for more reliable, rapid, inexpensive screening options. Such approaches could then allow for more successful assessment of overall sample toxicity and better use of confirmatory (e.g., LC-MS or MBA) techniques. The research introduced herein sets the stage for these multi-antibody devices and discusses the potential challenges when using the antibodies profiled in this study.

2. Materials and methods

2.1. Reagents

Saxitoxin (STX) dihydrochloride used in this research is the FDA reference standard, now available from the National Institute of Standards and Technology (81.0 μg/mL free base in 20% ethanol/80% water). *N*-sulfocarbamoyl-gonyautoxin-2 and -3 (C1,2, 70.8 μg/mL), decarbamoylgonyautoxin-2 and -3 (dcGTX2,3, 51.4 μg/mL), decarbamoylneosaxitoxin dihydrochloride (dcNEO, 7.93 μg/mL free base), decarbamoylsaxitoxin dihydrochloride (dcSTX, 16.0 μg/mL free base), gonyautoxin-1 and -4 (GTX1,4, 58.0 μg/mL), gonyautoxin-2 and -3 (GTX2,3, 62.1 μg/mL), gonyautoxin-5 (B1, 24.7 μg/mL), and neosaxitoxin dihydrochloride (NEO, 20.6 μg/mL free base) were purchased from NRC Certified Reference Materials Program, Institute for Marine Biosciences (Halifax, Nova Scotia, Canada).

Standard laboratory reagents were procured from Sigma-Aldrich (St. Louis, MO), Pharmaco AAPER (Shelbyville, KY), and J.T. Baker (Phillipsburg, NJ). Millipore Milli-Q 18.2 MΩ cm water (Billerica, MA) was used to prepare buffers. Sensor chips, amine coupling kit (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine), and buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20 buffer (HBS-EP+)) were obtained from GE Healthcare, Biacore (Piscataway, NJ).

2.2. Clam matrix

Control clam extract was prepared following a standard shellfish extraction procedure for PSTs (see [15] for procedure). A total of 100 g of clam was homogenized, from which a 5.0 g ($\pm$0.1 g) aliquot was thoroughly mixed with 3.0 mL of 1% acetic acid in water. This was heated at 95 °C for 5 min. The sample was then removed and placed on ice until cool enough to handle. The sample was vortexed and then centrifuged at 3600*g* for 10 min. The supernatant was collected into a 15 mL glass centrifuge tube. Another 3.0 mL, 1% acetic acid aliquot was added to the homogenate; the solution was vortexed and centrifuged at 3600*g* for 10 min. Following collection of the supernatant in the same 15 mL centrifuge tube, the extract volume was brought up to 10 mL with 0.1 N HCl with a final pH of 4.0. The clam extract was then filtered through a Supelco Supelclean LC-18, 3 mL solid phase extraction (SPE) cartridge (Sigma-Aldrich). The cartridge was conditioned with 6 mL methanol followed by 6 mL of water. Clam extract (1 mL) was added to the cartridge followed by 2 mL of water, and the cartridge was run dry. This extraction procedure produced 5 g of clam tissue per 40 mL and at the action level (i.e., 80 μg STX equivalents per 100 g tissue), equates to 100 ng STX eq./mL in the SPR biosensor assay. Standards were prepared by spiking the control clam matrix with the PSTs followed by serial dilution of these stock concentrations using the control clam matrix. Blank solutions containing no PSTs (0 ng/mL) were performed with control clam matrix for each PST calibration curve.

2.3. Mixed PST standards

Stock solutions of 90% STX with 10% NEO, 77% B1 with 23% STX, and 80% GTX1,4 with 20% STX were prepared and then serially diluted in clam matrix. The stock solutions were designed to have overall toxicity of 160 μg STX eq./100 g tissue for those standards containing NEO and GTX1,4, while the B1 standard had a stock concentration of 80 μg STX eq./100 g.

2.4. Antibodies

Two antibodies used in this research are commercially available in ELISA kits. The first antibody (Ab1) was used as received from the kit (Ridascreen Fast PSP SC, R-Biopharm AG, Darmstadt, Germany). Standard antibody dilutions were run on the STX chip; the 1:5 dilution in HBS-EP+ used throughout these studies had a response similar to that obtained from the 8 μg/mL burro anti-STX also used in this study as described below (see [41]).

The second antibody (polyclonal anti-STX, Ab2) was generously supplied in a purified form (ammonium sulfate precipitation followed by dialysis against 10 mM PBS, pH 7.3) from Beacon Analytical Systems (Saco, ME). The concentration that gave the same signal ($\sim$150 RU) as 8 μg/mL burro anti-STX was 25 μg/mL, and this concentration was used for all PST immunoassays.

The third antibody (polyclonal, protein G purified burro anti-STX, Ab3) was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD). Antibody dilutions were run to determine the appropriate concentration for immunoassay, and 8 μg/mL was used for all studies herein.

2.5. Instrumentation

A Biacore T100 (GE Healthcare) surface plasmon resonance biosensor was used for all SPR immunoassays. The instrument was run via the Biacore T100 Controller Software v. 2.0, and data evaluation was performed with the Biacore T100 Evaluation Software v. 2.0. The instrument and sensor chips were normalized following the manufacturer procedures prior to performing the PST immunoassays. The SPR response (Resonance Unit, RU) is a measure of the angle of minimum reflected intensity that occurs upon changes in refractive index where 1 RU corresponds to a 10^{-6} change in refractive index ($\sim 10^{-4}$ degree angle shift).

2.6. Sensor chip

The STX biosensor surface was prepared on a Series S CM5 sensor chip and has been previously described [41]. Briefly, all flow cells were activated to succinimidyl esters using the instrument amine immobilization wizard and EDC/NHS from the amine coupling kit. Flow cell one was then deactivated with ethanolamine to create a reference surface, while flow cells two through four were activated with jeffamine, and unreacted sites were blocked with ethanolamine. The chip was removed from the instrument, and STX was conjugated to the chip surface via 15 h, 37 °C reaction with formaldehyde in 100 mM phosphate buffer. The chip was then rinsed with water, dried with $N_{2(g)}$, and docked into the SPR instrument. The fluidics and sensor chip were primed with HBS-EP+, and three startup cycles with 50 mM NaOH were performed prior to running the standards.

2.7. Immunoassay

Immunoassays were performed using the Biacore T100 optical biosensor with HBS-EP+ as the running buffer. The sample compartment temperature was set at 10 °C while the analysis temperature was held constant at 25 °C. The antibodies were diluted as noted above and then mixed in the instrument autosampler (90% Ab to 10% standard, 600 s mix time) prior to injection. This mixture was injected over the STX sensor chip at a flow rate of 20 μL/min for 120 s followed by a 60 s dissociation period and then a 240 s regeneration with 50 mM NaOH. STX controls (0.3, 3, 30, and 300 ng/mL in HBS-EP+ with 8 μg/mL Ab3) were run with every cycle to ensure chip stability.

2.8. Data processing

The response (RU_{PST}) for each sample was obtained by subtracting the baseline (10 s prior to injection) from the stability point (15 s after sample injection completion). These values were then normalized to the blank (0 ng/mL PST for associated antibody, RU_0), and the results multiplied by 100 to achieve percent binding for each antibody/PST combination: % binding$=(RU_{PST}/RU_0)\times 100$.

Each flow cell was normalized separately, and the data from flow cells 2, 3, and 4 were averaged together. Due to nonspecific binding and bulk effects for Ab1, the background from the blank measurement (0.45 normalized response) was subtracted from each data point for this antibody. The average response was then plotted versus the original solution concentration for each PST in ng/mL. Curve fitting was performed using a variable slope, four parameter model for log(inhibitor) vs. response in GraphPad Prism (v. 5.02, La Jolla, CA). GraphPad Prism was then used to calculate the values at inhibitory concentrations (IC): IC_{20} (80% binding), IC_{50} (50% binding), and IC_{80} (20% binding). Additionally, the response at 100 ng/mL for each PST based on the generated curves was determined. For concentrations where full inhibition was not seen (e.g., C1,2 with Ab3 where the highest standard gave 49.8% binding without flattening of the curve), the software extrapolates the curve fit to determine the 20% binding point.

Cross-reactivities (CR) for each PST were calculated from the IC_{50} values of each toxin with respect to the IC_{50} of STX: % CR$=(IC_{50,\ STX}/IC_{50,\ PST})\times 100$. The 100 ng/mL level is defined as the response at the action level (RAL) and is equivalent to a sample containing only STX at 80 μg/100 g tissue based on extraction dilution.

For the theoretical lateral flow immuno-chromatography, the reactivity for each antibody-PST congener was translated to a band in the device. For Fig. 3, the RALs (100 ng/mL from Table 3) were used, while in Fig. 4 the response at 80 μg STX eq./100 g tissue were translated to band patterns. To account for differing sensor chips and nonspecific binding in Fig. 3, the values were corrected for each antibody by subtracting the background (Ab1=12.45, Ab2=8.60, Ab3=3.43) from each response. Using PowerPoint, the color of the band was defined by the RGB parameters 128 red, 0 green, 0 blue with the transparency of the band equal to (100—Response).

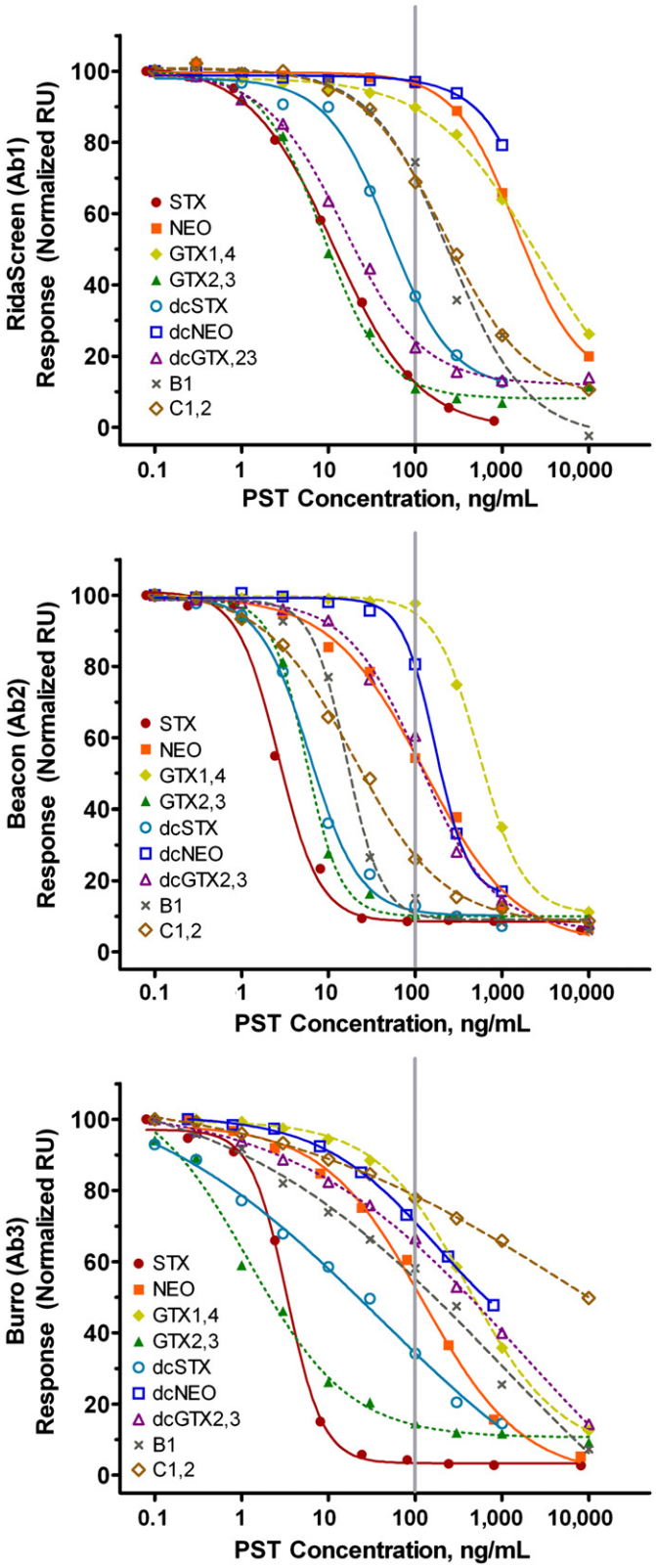


Fig. 1. Antibody reactivity with nine common PSTs. $N=3$ with error bars smaller than the data points. The vertical grey line at 100 ng/mL represents the action level for a sample containing only STX (80 μg STX/100 g tissue).

3. Results and discussion

3.1. Cross-reactivity (CR) of antibodies

Previous research has focused on developing rapid, sensitive SPR assays that are capable of detecting PSTs in buffer [41] and common shellfish matrices [36]. The challenge to correctly estimating sample potency lies in the current inability of antibodies to react with the congeners according to their toxicities (Table 1). The PSTs can be classified into three major categories with their toxicities generally following the R4 substituent groupings: carbamate > decarbamoyl > *N*-sulfocarbamoyl. It has been shown that

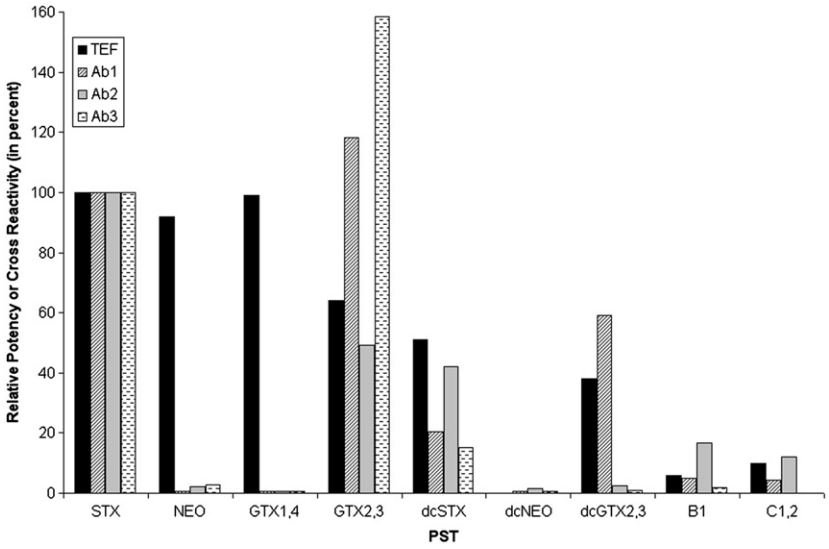


Fig. 2. Cross-reactivity for each antibody (from Table 3) in comparison to the toxin equivalency factors (from [17]) for each PST. For PST congeners where epimers are reported in pairs (e.g., GTX1,4) the value of the epimer with the higher TEF is used. No TEF value for dcNEO is reported by Oshima.

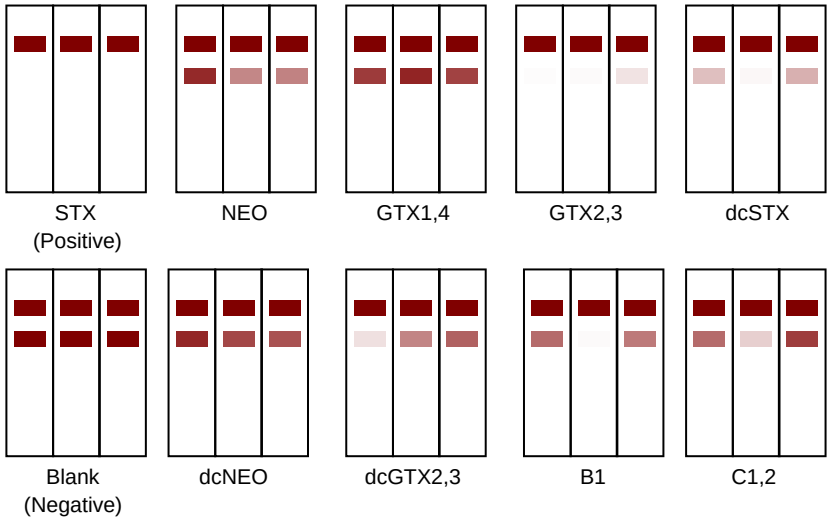


Fig. 3. Conceptual lateral flow immunochromatographic devices for samples containing a single PST at 100 ng/mL. Each strip corresponds to an individual antibody (left to right: Ab1, Ab2, Ab3) in which a competition assay is performed with the analyte.

Table 2
IC_{50} and dynamic range (DR = IC_{20} to IC_{80}) for each antibody. All values in ng/mL.

PST	Ab1: RidaScreen		Ab2: Beacon		Ab3: Burro	
	IC_{50}	DR	IC_{50}	DR	IC_{50}	DR
STX	11.9	2.7–54.3	3.0	1.4–7.7	3.4	1.6–6.9
NEO	1834.9	530.5–9986.0	131.7	23.1–793.1	116.0	17.5–726.4
GTX1,4	2346.7	324.9–16197.1	630.3	260.9–1955.7	470.3	79.2–3438.4
GTX2,3	10.1	3.1–41.4	6.0	3.0–14.3	2.1	0.4–24.6
dcSTX	58.6	16.2–290.7	7.1	2.6–24.7	22.0	0.8–442.4
dcNEO	2369.6	964.5–3724.2	199.3	100.5–593.0	642.5	43.2–39711.6
dcGTX2,3	20.1	4.2–165.9	126.9	30.7–606.1	426.6	15.9–5851.5
B1	236.4	58.1–920.7	17.7	8.8–40.8	167.4	5.5–2673.5
C1,2	266.4	56.1–1716.3	24.8	4.6–190.7	9983.5	75.1–2091542

different antibodies can have different cross-reactivities to the PST congeners [43]. To take advantage of this characteristic, the cross-reactivity of three available antibodies are examined herein using SPR biosensor evaluation to determine the feasibility and merits of using a multi-antibody approach for both SPR biosensors and LFIs.

Fig. 1 shows the cross-reactivity curves of the binding of each antibody with STX, NEO, GTX1,4, GTX2,3, dcSTX, dcNEO, dcGTX2,3, B1 and C1,2. These assays were designed to have nearly-complete inhibition with STX at the action level (indicated by a vertical grey line in the graphs). Qualitatively, it is clear that

Table 3
SPR-determined antibody cross-reactivities (CR in %), reported ELISA cross-reactivities, and responses at the action level (RAL in normalized RU, at 100 ng STX/mL).

PST	Ab1			Ab2			Ab3	
	CR	ELISA[a]	RAL	CR	ELISA[a]	RAL	CR	RAL
STX	100.0	100	12.5	100.0	100.0	8.6	100.0	3.4
NEO	0.7	12	96.5	2.3	0.8	55.4	2.9	52.8
GTX1,4	0.5		89.6	0.5	< 0.1	94.9	0.7	76.9
GTX2,3	118.3	70	12.5	49.4	12.0	10.2	158.5	14.1
dcSTX	20.4	20	37.0	42.0	18.0	11.5	15.2	34.4
dcNEO	0.5		97.1	1.5	0.7	80.2	0.5	71.1
dcGTX2,3	59.2		24.3	2.3	0.4	55.7	0.8	65.0
B1	5.0		70.1	16.8		10.8	2.0	55.3
C1,2	4.5		70.2	12.0		26.9	0.03	78.5

[a] Values for the ELISA cross-reactivities obtained from pamphlet information contained in the kits.

the antibodies have distinct reactivity with the nine PST congeners, as the calibration curve patterns for each antibody are dissimilar. This can be further seen in the individual congener plots (Supplementary materials A) where it is visually easy to compare the similarities (e.g., the three antibody curves for STX are similar with Ab2 and Ab3 responses nearly overlapping) and differences (e.g., the curve shapes for the three antibodies reacting with B1 are quite dissimilar). The data can also be quantitatively evaluated via IC_{50} and dynamic range (Table 2) as well as percent cross-reactivities and responses at the action level (RAL) (Table 3). When these results are considered together, it is clear that no antibody profile accurately reflects potency; however, each antibody has a distinct profile with advantages and disadvantages as discussed below.

For RidaScreen (Ab1), high reactivity (> 100% CR) with STX and GTX2,3 is seen while low reactivity (< 5% CR) with many congeners is observed. When used in an assay alone, Ab1 would be expected to yield false-negative results when the highly toxic NEO and GTX1,4 are present. Beacon (Ab2) has high reactivity with STX but low reactivity with NEO, GTX1,4, dcNEO, and dcGTX2,3. In this case, samples high in GTX1,4 and/or NEO may also result in false-negatives. Additionally, the moderate reactivity (~ 15% CR) with low potency B1 and C1,2 may result in false-positive results if these toxins dominated the profile. Finally, the Burro (Ab3) has high reactivity with STX and GTX2,3 and to a lesser extent with all congeners tested. In this scenario, a sample with a toxin profile dominated by GTX1,4, and/or NEO may be screened as negative when toxin levels may be above the action level, whereas a false-positive may occur if GTX2,3 dominated the sample.

When comparing the profiles based on substitution groups (R1 to R4 as shown in Table 1, Supplementary materials B1–B3), all antibodies show limited reactivity with OH modification at the R1 group potentially due to steric hindrance, charge, or hydrophilicity imparted by this group. Indeed, weak reactivity with the R1-hydroxylated PSTs is frequently found with antibodies [43]. Antibody cross-reactivity for the non-hydroxylated compounds is mainly driven by the R4 functionality with highest reactivity seen in the carbamate modified PSTs followed by decarbamoyl PST forms and *N*-sulfocarbamoyl conjugations.

The two antibodies used for this work that were obtained from commercial ELISA kits have been previously evaluated for cross-reactivities (Table 3). For Ab1, the published cross-reactivity order (STX > GTX2,3 > dcSTX > NEO) is similar to the SPR results (GTX2,3 > STX > dcGTX2,3 > dcSTX > B1 > C1,2 > NEO > GTX1,4 = dcNEO) except for the exchange of STX with GTX2,3. This discrepancy may not be significant due to the very similar reactivity of the antibody to these PSTs as seen in the SPR curves in Fig. 1. For Ab2, the published results are STX > dcSTX > GTX2,3 > NEO > dcNEO > dcGTX2,3 > GTX1,4; however, the SPR analysis showed STX > GTX2,3 > dcSTX > B1 > C1,2 > NEO = dcGTX2,3 > dcNEO > GTX1,4. The order differences of GTX2,3/dcSTX as well as NEO/dcNEO/dcGT2,3 could be expected due to the very similar curve shapes and, with the error in measurements, these values may not be significantly different.

One further and important consideration is how cross-reactivity corresponds to toxin-equivalency factors (TEFs) for each PST (Fig. 2). The values for STX are set at 100%, based on the definitions for TEF and CR, and therefore show no differences between potency and cross-reactivity with each antibody. Ideally, the best antibody profile would have CRs to each congener that match their TEFs. However, it is clear that antibodies perform poorly at matching the TEF values, especially for R1-hydroxylated toxins (e.g., NEO and GTX1,4) and with varied success for the non-hydroxylated congeners. For example, Ab2 cross-reactivities correspond reasonably well with the TEF for GTX2,3, dcSTX and C1,2, while Ab1 and Ab3 cross-reactivities correlate closely with the TEF for B1. These distinctions in reactivity between the antibodies could be exploited for use in a multiplexed format to create an assay that would yield more information regarding the toxin profile and thus more confidence in sample potency.

3.2. Theoretical lateral flow immuno-chromatography

In general, the format of the LFI used to detect PSTs is a competitive displacement assay. In this assay, sample extract is added to the sample well of the pad and is drawn up through the membrane. The toxin first interacts with the conjugate pad containing antitoxin-coated gold particles. The antibody and toxin interact and remaining antitoxin-coated gold particles bind to the test line coated with toxin conjugated protein. The higher the toxin concentration in solution the fainter the red line at the test line position. A control line is also present and should always yield a strong red response. In this way, two red bands indicate that a sample contains little to no toxin, whereas a single band at the control position indicates the sample contains toxin.

By using the antibodies screened via SPR, combinations of antibodies that create a unique pattern could be incorporated into an LFI for higher confidence in sample toxicity. Prior to undertaking extensive studies in incorporating multiple antibodies into an LFI, a theoretical model was designed and is shown in Fig. 3 with each strip in the three-strip system containing a single antibody-gold colloid. In envisioning the LFI functionality, only single toxin solutions at 100 ng/mL (e.g., equivalent to the action level for a toxin containing only STX, per extraction procedure used) are applied to each three-strip system. As can be seen in the STX LFI and Blank LFI, a positive sample would have only the three control bands while a negative result would show control bands as well as three strong bands at the test line position below each control band.

The theoretical LFIs for the other PST congeners show that, indeed, more confidence in sample toxicity could be gained by using multiple antibodies. For example, the pattern for B1 (which has low toxicity) with only Ab2 indicates an unsafe sample (a false-positive result), but when used in combination with the information from the Ab1 and Ab3 strips the pattern and strength of the bands could allow a user to realize the sample is safe for harvest and consumption. Unfortunately, false-negative results were still not eliminated. This is demonstrated in the NEO and GTX1,4 conceptual tests in which these two LFIs demonstrate very little discernible difference from the blank (negative), yet have concentrations equivalent to toxicity near the action level.

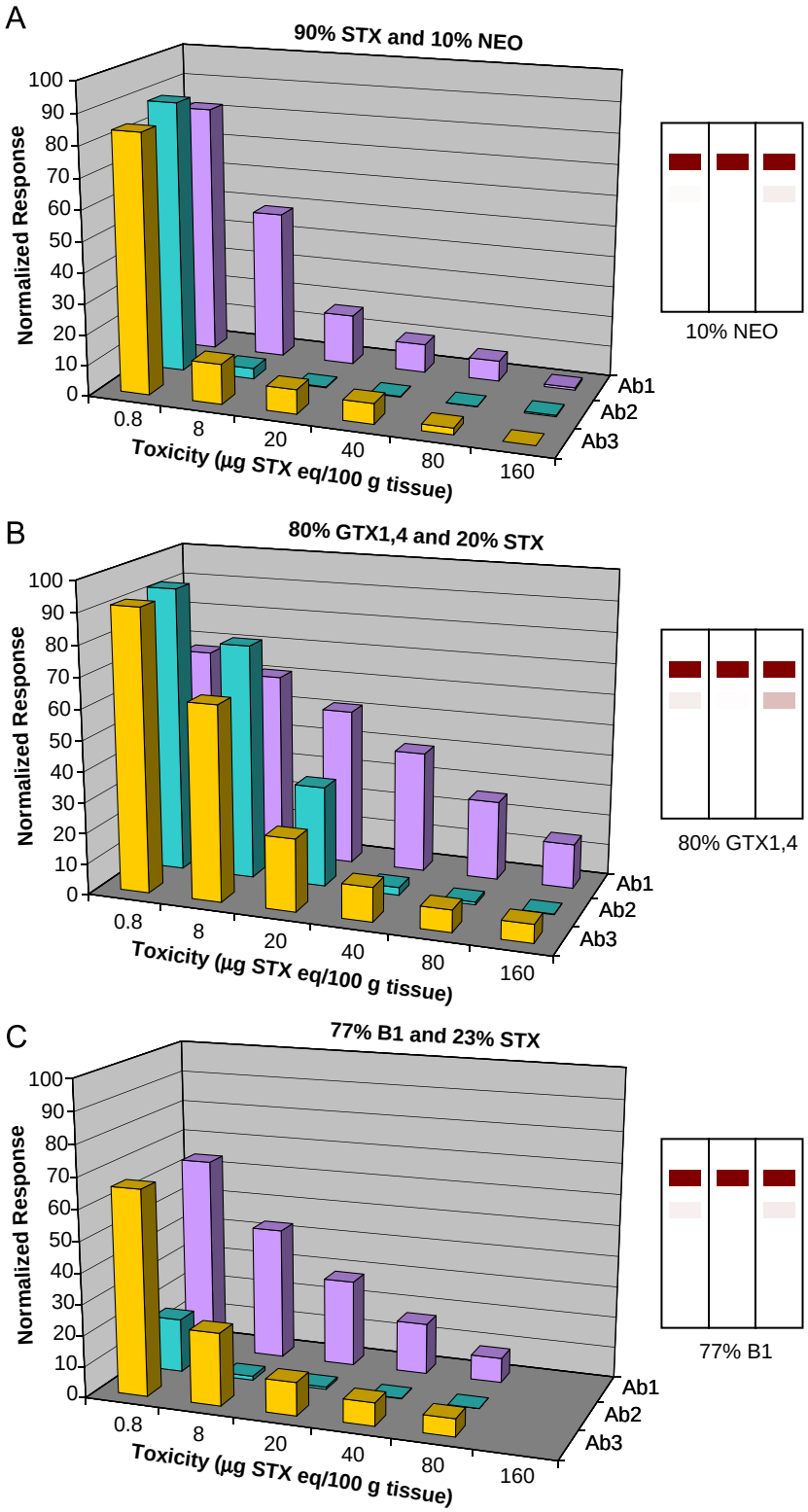


Fig. 4. Reactivity of antibodies to PST mixtures using the SPR biosensor and corresponding theoretical LFI device for mixtures containing 80 µg STX eq. per 100 g tissue for (A) 90% STX and 10% NEO, (B) 80% GTX1,4 and 20% STX, and (C) 77% B1 and 23% STX.

3.3. *PST mixtures*

PST congeners commonly exist as mixtures in naturally contaminated shellfish which could complicate such a simplistic "pattern matching" approach of the LFIs. To investigate this, experiments were performed with the antibodies and mixtures of PST compounds. Differing toxicity PST mixtures were tested to determine if the antibodies would perform well in situations that challenged high and low cross-reactors that do not correlate with toxicity (i.e., the potential for false-positive or false-negative results, respectively). The selected mixtures represented examples encountered in natural waters: 90% STX with 10% NEO

(e.g., *Alexandrium* sp. and shellfish in Alaska [anticipated accurate test performance]), 80% GTX1,4 with 20% STX (e.g., shellfish in Scotland [anticipated potential false-negative]), and 77% B1 with 23% STX (e.g., *Pyrodinium* sp. in Florida [anticipated potential false-positive]). Results from these SPR assays, and corresponding theoretical LFIs, are shown in Fig. 4 where the toxicity at 80 μg STX eq. per 100 g tissue is the action level in the United States.

For all mixtures, the antibodies respond well for samples in which there is high toxicity, with all SPR biosensor data showing the desired inhibition at the 80 μg STX eq./100 g tissue action level. If these were naturally contaminated samples, the SPR screening technique would indicate that further, confirmatory testing is required. With the mixture of 90% STX with 10% NEO, the curves nearly follow that predicted from a sample of STX alone, thus yielding a representative, accurate test. From this, an arbitrary cut-off level of all antibodies having an SPR response below 50 could be set for the level at which further confirmatory testing would be required. For the 90% STX with 10% NEO, this level corresponds to samples of approx. 8 μg STX eq./100 g tissue and would allow for adequate screening without over burdening confirmatory testing techniques. As expected, the potential false-negative system (80% GTX1,4 with 20% STX) would only have further testing indicated for samples containing more than 20 μg STX eq./100 g tissue. While this is below the action level, any potential systematic errors could lead to a false-negative result. Finally, for the potential false-positive mixture of 77% B1 with 23% STX, samples very low in toxicity (<8 μg STX eq./100 g tissue) would be indicated for further testing. In this case, false-positive results could still be common.

With regard to the theoretical LFI devices for these mixtures, all conceptual LFIs with the three antibody system show patterns which indicate further testing would be required based on the faint-red response of the test lines for all three antibodies. In the case of the 80% GTX1,4 with 20% STX, the red line from Ab3 is clearly visible and a faint line from Ab1 can be seen. If a pattern matching approach was used, the pattern for this toxic sample could mimic that of the nontoxic B1 sample in Fig. 3. Thus, while the pattern matching and intensity approach could enhance reliability, the current antibody combination does not fully alleviate the challenges associated with false-negative and false-positive results.

While this three antibody system could increase the knowledge of a sample composition and potential toxicity, the antibodies and LFI format used herein do not allow for full resolution of the false-negative and false-positive challenges that currently plague PST immunoassays. Furthermore, the SPR results will also suffer from similar challenges but could allow for more confidence in results due to the quantitative nature of the immunoassay versus reliance on visual readout of minor hue variations from the LFI device. Our research studies continue to focus on the generation and screening of antibodies with differing cross-reactivities, especially with improved reactivity to the hydroxylated toxins, and to determine if a mixture of multiple antibodies [44] or spatially separate antibodies (as shown in the conceptual LFIs) will be best for rapid tests.

4. Conclusions

Rapidly screening seafood samples for potential contamination by PSTs remains an analytical challenge. Sensitive, real-time SPR assays for PSTs have been developed and were used to evaluate the reactivity of three antibodies. The results show that each antibody has a unique reactivity for the PST congeners. This highlights the potential for developing antibodies that could have a higher correlation of response with sample potency. While the production of such antibodies continues, techniques employing the current antibodies for higher-confidence screening were evaluated and a conceptual model was created. This model indicated that while false-positive and false-negative results were not completely eliminated, there is potential to improve immunoassays and reduce the use of MBA and confirmatory analytical tests. Current research is focusing on the development of PST binders (i.e., antibodies, aptamers, receptors) that have better cross-reactivity with the congeners. Once candidate binders are evaluated by the SPR biosensor, they will be incorporated into a multi-binder, rapid test to fully realize the potential of a potency-based screening technique.

Acknowledgments

The authors express their gratitude to Mark Poli and his colleagues at USAMRIID for providing the burro anti-STX used in this work. We also thank Titan Fan from Beacon Analytical for providing purified anti-STX. Thank you to Katrina Campbell, Christopher Elliott, and Simon Haughey for valuable discussions and their contributions to the field of PST biosensors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.06.073.

References

[1] S.M. Etheridge, Toxicon 56 (2010) 108–122.
[2] A.R. Humpage, V.F. Magalhaes, S.M. Froscio, Anal. Bioanal. Chem. 397 (2010) 1655–1671.
[3] M. Kodama, S. Sato, in: L.M. Botana (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, CRC Press, Taylor and Francis Group, Boca Raton, FL, 2008, pp. 165–175.
[4] S.E. Shumway, Rev. Fish. Sci. 3 (1995) 1–31.
[5] C.Y. Kao, in: I.R. Falconer (Ed.), Algal Toxins in Seafood and Drinking Water, Academic Press, San Diego, CA, 1993, pp. 75–86.
[6] G.M. Lipkind, H.A. Fozzard, Biophys. J. 66 (1994) 1–13.
[7] J.A. Kalaitzis, R. Chau, G.S. Kohli, S.A. Murray, B.A. Neilan, Toxicon 56 (2009) 244–258.
[8] G.K. Isbister, M.C. Kiernan, Lancet Neurol. 4 (2005) 219–228.
[9] D.C. Rodrigue, R.A. Etzel, S. Hall, E. Deporras, O.H. Velasquez, R.V. Tauxe, E.M. Kilbourne, P.A. Blake, Am. J. Trop. Med. Hyg. 42 (1990) 267–271.
[10] FAO, in, Food and Agriculture Organization of the United Nations (FAO) Food and Nutrition Paper 80, Rome, 2004.
[11] Bulletin, in, June 23, 2011.
[12] N. Vilarino, E.S. Fonfria, M.C. Louzao, L.M. Botana, Sensors 9 (2009) 9414–9443.
[13] B. Ben-Gigirey, A. Villar-Gonzalez, in: L.M. Botana (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, CRC Press, Taylor and Francis Group, Boca Raton, FL, 2008, pp. 177–196.
[14] AOAC, in: K. Helrich (Ed.), Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Arlington, VA, 1990, pp. 881–882.
[15] AOAC, Paralytic shellfish poisoning toxins in shellfish: Prechromatographic oxidation and liquid chromatography with fluorescence detection. Method 2005.06, in: Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Arlington, VA, 2005.
[16] J.F. Lawrence, B. Niedzwiadek, C. Menard, J. AOAC Int. 88 (2005) 1714–1732.
[17] Y. Oshima, J. AOAC Int. 78 (1995) 528–532.
[18] J.M. van de Riet, R.S. Gibbs, F.W. Chou, P.M. Muggah, W.A. Rourke, G. Burns, K. Thomas, M.A. Quilliam, J. AOAC Int. 92 (2009) 1690–1704.
[19] AOAC, Paralytic shellfish toxins in mussels, clams, oysters, and scallops post-column oxidation (PCOX). First Action, Method 2011.02, in: Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Arlington, VA, 2011.
[20] S. Etheridge, J. Deeds, S. Hall, K. White, L. Flewelling, J. Abbott, J. Landsberg, S. Conrad, D. Bodager, G. Jackow, Afr. J. Mar. Sci. 28 (2006) 383–387.
[21] C. Dell'Aversano, P. Hess, M.A. Quilliam, J. Chromatogr. A 1081 (2005) 190–201.
[22] E. Turrell, L. Stobo, J.P. Lacaze, S. Piletsky, E. Piletska, J. AOAC Int. 91 (2008) 1372–1386.

[23] G.R. Marchesini, H. Hooijerink, W. Haasnoot, J. Buijs, K. Campbell, C.T. Elliott, M.W.F. Nielen, TrAC, Trends Anal. Chem. 28 (2009) 792–803.
[24] G.J. Doucette, M.M. Logan, J.S. Ramsdell, F.M. VanDolah, Toxicon 35 (1997) 625–636.
[25] C.L. Powell, G.J. Doucette, Nat. Toxins 7 (1999) 393–400.
[26] G. Usup, C.P. Leaw, M.Y. Cheah, A. Ahmad, B.K. Ng, Toxicon 44 (2004) 37–43.
[27] F.M. van Dolah, T.A. Leighfield, G.J. Doucette, L. Bean, B. Niedzwiadek, D.F.K. Rawn, J. AOAC Int. 92 (2009) 1705–1713.
[28] M.V. Laycock, M.A. Donovan, D.J. Easy, Toxicon 55 (2010) 597–605.
[29] C.-K. Wong, P. Hung, E.A.L. Ng, K.L.H. Lee, G.T.C. Wong, K.-M. Kam, Harmful Algae 9 (2010) 636–646.
[30] E. Garet, A. Gonzalez-Fernandez, J. Lago, J.M. Vieites, A.G. Cabado, J. Agric. Food Chem. 58 (2010) 1410–1415.
[31] F. Kasuga, Y. HaraKudo, K. Machii, J. Food Hyg. Soc. Jpn. 37 (1996) 407–410.
[32] E. Usleber, R. Dietrich, C. Burk, E. Schneider, E. Martlbauer, J. AOAC Int. 84 (2001) 1649–1656.
[33] R.L. Manger, L.S. Leja, S.Y. Lee, J.M. Hungerford, M.A. Kirkpatrick, T. Yasumoto, M.M. Wekell, J. AOAC Int. 86 (2003) 540–543.
[34] M. Okumura, H. Tsuzuki, B. Tomita, Toxicon 46 (2005) 93–98.
[35] H.X. Chen, Y.S. Kim, S.R. Keum, S.H. Kim, H.J. Choi, J.B. Lee, W.G. An, K. Koh, Sensors 7 (2007) 1216–1223.
[36] K. Campbell, S.A. Haughey, H. van den Top, H. van Egmond, N. Vilarino, L.M. Botana, C.T. Elliott, Anal. Chem. 82 (2010) 2977–2988.
[37] E.S. Fonfria, N. Vilarino, K. Campbell, C. Elliott, S.A. Haughey, B. Ben-Gigirey, J.M. Vieites, K. Kawatsu, L.M. Botana, Anal. Chem. 79 (2007) 6303–6311.
[38] S.A. Haughey, K. Campbell, B.J. Yakes, S.M. Prezioso, S.L. DeGrasse, K. Kawatsu, C.T. Elliott, Talanta 85 (2011) 519–526.
[39] B.J. Yakes, M.M. Mossoba, in: S. Carnazza (Ed.), New Research Trends in Protein Chip Technology, Transworld Research Network, Kerala, India, 2010, pp. 55–73.
[40] H.J. van den Top, C.T. Elliott, S.A. Haughey, N. Vilarinì *f* o, H.P. van Egmond, L.M. Botana, K. Campbell, Anal. Chem. 83 (2011) 4206–4213.
[41] B.J. Yakes, S. Prezioso, S.A. Haughey, K. Campbell, C.T. Elliott, S.L. DeGrasse, Sens. Actuators, B 156 (2011) 805–811.
[42] A. Komano, H. Maruko, H. Sekiguchi, Y. Seto, Forensic Toxicol. 29 (2011) 38–43.
[43] K. Campbell, L.D. Stewart, G.J. Doucette, T.L. Fodey, S.A. Haughey, N. Vilarino, K. Kawatsu, C.T. Elliott, Anal. Chem. 79 (2007) 5906–5914.
[44] K. Campbell, D.F.K. Rawn, B. Niedzwiadek, C. Elliott, Food Addit. Contam., Part A 28 (2011) 711–725.