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Comparative performance of four immunological test kits for the detection of Paralytic Shellfish Toxins in Tasmanian shellfish

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ABSTRACT

Blooms of the toxic dinoflagellate *Alexandrium tamarense* (Group 1) seriously impacted the Tasmanian shellfish industry during 2012 and 2015, necessitating product recalls and intensive paralytic shellfish toxin (PST) product testing. The performance of four commercial PST test kits, Abraxis™, Europroxima™, Scotia™ and Neogen™, was compared with the official AOAC LC-FLD method for contaminated mussels and oysters. Abraxis and Europroxima kits underestimated PST in 35–100% of samples when using standard protocols but quantification improved when concentrated extracts were further diluted (underestimation ≤18%). The Scotia kit (cut off 0.2–0.7 mg STX-diHCl eq/kg) delivered 0% false negatives, but 27% false positives. Neogen produced 5% false negatives and 13% false positives when the cut off was altered to 0.5–0.6 mg STX-diHCl eq/kg, the introduction of a conversion step eliminated false negatives. Based on their sensitivity, ease of use and performance, the Neogen kit proved the most suitable kit for use with Tasmanian mussels and oysters. Once formally validated for regulatory purposes, the Neogen kit could provide shellfish growers with a rapid tool for harvesting decisions at the farm gate. Effective rapid screening preventing compliant samples undergoing testing using the more expensive and time consuming LC-FLD method will result in significant savings in analytical costs.

1. Introduction

In recent years recurrent blooms (up to 300,000 cells/L) by the Paralytic Shellfish Toxin (PST) producing dinoflagellate *Alexandrium tamarense* (Group 1) have seriously impacted the Tasmanian shellfish industry. An initially undetected bloom event in October 2012 led to product recalls with an estimated economic loss of ~US$24 million dollars (Campbell et al., 2013). The regulatory action limit or permissible concentration of PST toxins in shellfish is 0.8 mg STX-diHCl eq/kg shellfish meat (0.8 mg STX eq/kg from now on). During 2015 closures of oyster and mussel farms which lasted for up to 4 months, PST levels were instigated and reached up to 32 mg STX eq/kg, resulting in four documented hospitalizations that occurred after individuals consumed wild mussels (i.e. recreational harvesting) from an affected area with public health warning signs. The current system for shellfish testing by the Tasmanian Shellfish Quality Assurance Program (TSQAP) requires shipping samples to an accredited Sydney laboratory leading to delays (4–12 days) for shellfish growers. The AOAC Official Method AOAC.2005.06 (pre-column oxidation, Pre-COX) using liquid chromatography with fluorescence detection (LC-FLD or Lawrence method; Lawrence et al., 2005) is the designated regulatory method for PST in shellfish in Australia. The method is highly specific and sensitive, providing a complete toxin profile and concentration of each PST analogue. However, it has been claimed that the method overestimates gonyautoxin 1&4 (GTX1&4) and neosaxitoxin (NEO), and underestimates gonyautoxin 2&3 (GTX2&3) and sulfocarbamoyl C1&2 compared to AOAC Official method

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2011.02 (post-column oxidation, PCOX) (Turner et al., 2014a). Immunological PST test kits, which were first trialled in the early 2000s (Jellett et al., 2002; Laycock et al., 2000), have the advantage of being sensitive, fast, easy to use and cheaper than HPLC-based analytical methods, and ideally allow farmers to perform tests on site to guide harvesting decisions. However, due to significant variability in PST toxin profiles of different *Alexandrium* species and geographic populations, as well as widely different potency of PST analogues, the applicability of different commercial test kits for local product testing requires careful consideration. Most available kits target saxitoxin (STX), but have low cross-reactivity for GTX1&4 and GTX2&3. The latter are common in Australian shellfish products as well as shellfish in Great Britain (Turner et al., 2014b).

Enzyme-linked immunosorbent assays (ELISA), such as the Abraxis™ and Europroxima™ kits, are quantitative tests that allow the user to calculate the concentration of PST toxins (as mg STX eq/kg) using a STX standard curve. These protocols require laboratory experience to avoid high user errors. By contrast, lateral flow immunoassays (LFIA) are qualitative tests that deliver positive or negative results based on a predetermined cut off limit. Scotia Rapid Test™ (formerly Jellett Rapid Test; Jellett et al., 2002) has a detection limit of ~0.2–0.7 mg STX eq/kg, whereas Neogen™ states that it has a cut off of 0.8 mg STX eq/kg. LFIA kits are more user friendly and simpler to use than ELISA kits, while laboratory experience is not essential. Different commercial immunological tests exhibit highly variable cross-reactivity to different PST analogues (Table 1). These cross-reactivity profiles do not fully correlate with the toxicity of individual toxins as determined by the mouse bioassay and the toxicity equivalency factors applied in total toxin determination of the LC-FLD method. Therefore, commercial test kits must be shown to be fit for purpose with geographical toxin profiles prior to implementation within testing regimes.

In the present study the performance of four commercially available immunological PST test kits for shellfish testing were evaluated during a major *Alexandrium tamarense* bloom event on Tasmania's East Coast, Australia, between July and November 2015.

2. Materials and methods

2.1. Shellfish samples

Sixty nine shellfish samples, including mussel *Mytilus galloprovincialis* and Pacific oyster *Crassostrea gigas*, which originated from 12 farms along the East Coast of Tasmania, Australia were used. Samples (homogenates from whole organisms) were stored at −20 °C and analysed within 1 month after harvesting.

2.2. Liquid chromatography analysis

Advanced Analytical Australia (AAA), the certified laboratory that TSQAP uses for phycotoxin analysis, determined PST toxin concentration using the AOAC.2005.06, LC-FLD or Lawrence method. Screen and confirmation (when >0.4 mg STX eq/kg were found) analyses of the method were performed.

PST toxins were extracted from 5 g of shellfish homogenate using 3 mL of 1% acetic acid. The mixture was placed in a boiling water bath for 20 min, mixed, allowed to cool and centrifuged at 3600 × *g* for 10 min. The supernatant was recovered and the pellet resuspended in 3 mL 1% acetic acid, mixed and centrifuged again. Both supernatants were mixed and made up to 10 mL with water. A sample clean-up was performed using a SPE C18 cartridge and screen testing was performed after periodate oxidation of samples. Standards, samples and PST positive certified reference matrices were oxidised with the inclusion of a matrix modifier. The matrix chosen for the matrix modifier reflected the predominant shellfish in the run. Oxidation using the matrix modifier circumvents the need to apply recovery factors for differing shellfish matrices. A further confirmation analysis was performed after peroxide oxidation of C18 cleaned extracts. All results were converted to mg STX-diHCl eq/kg using EFSA's toxicity equivalency factors (EFSA, 2009) (mentioned as mg STX eq/kg). Subsamples analysed by AAA were returned to IMAS for use in the PST screening with the rapid test kits.

Table 1
Cross-reactivity (mole %) of four immunological test kits as specified by the manufacturers.

PST analogue	Quantitative		Qualitative	
	Abraxis	Europroxima	Neogen[a]	Scotia[b]
STX	100	100	100	100
NEO	1.3	1.4	129	26
GTX2&3	23	5.6	23	100
GTX1&4	<0.2	<0.1	6	1.8[c]
C1&2	nd	0.2	3	nd
GTX5	23	26.2	23	62
dcSTX	29	19.2	56	100
dcNEO	0.6	0.5	28	nd
dcGTX2&3	1.4	0.2	8	15

nd = not determined.
[a] Jawaid et al. (2015).
[b] Formerly Mist Alert and Jellett (Jellett et al., 2002; Laycock et al., 2000).
[c] If an extra step involving hydrolysis conversion of GTX1&4 to NEO is performed, this cross-reactivity can be increased to 26%.

2.3. Test kits

2.3.1. Quantitative tests

2.3.1.1. Abraxis™. Abraxis test kits (52255B, lot number 15B5951) were stored at 4 °C until analysis. PST toxin quantification was performed according to the manufacturer's protocol.

2.3.1.1.1. Extract preparation. A subsample of 10 g of shellfish homogenate was mixed with 10 mL of 0.1 M HCl (modified version of the AOAC.959.08 method, extraction protocol as per the mouse bioassay) and placed in a boiling water bath for 5 min, allowed to cool down and centrifuged at 3500 × *g* for 10 min. Supernatants were recovered and pH adjusted to 3.0, and diluted in 1× sample diluent (1:1000). Initially, all 69 samples were considered as blind samples and analysed as per the standard test protocol (i.e. 1:1000 dilution). For a second analysis, 15 of these samples were further diluted (i.e. 1:10 or 1:100) based on the known toxin concentration (LC-FLD by AAA) in order to bring them within the working range of the calibration curve.

The Abraxis kit can operate with an alternative extraction method using 80% methanol (MeOH). For this purpose the 15 samples mentioned above (i.e. with extra dilution performed) were tested. Shellfish homogenate (1 g) was mixed with 6 mL of MeOH for 1 min using a Vortex mixer, centrifuged at 3000 × *g* for 10 min and the supernatant transferred into a clean tube. MeOH (2 mL) was added to the pellet, mixed and centrifuged. Both supernatants were combined and made up to 10 mL with MeOH. Similar to the HCl extracts, the MeOH extracts were analysed using the standard test protocol dilution (1:100) and also with an extra dilution (i.e. 1:10 or 1:100) as required.

2.3.1.1.2. Test protocol. A volume of 50 μL of STX standards (provided at 0, 0.02, 0.05, 0.10, 0.20 and 0.40 ng mL^{-1}) and samples (in HCl or MeOH) was transferred into the 96-well coated plate in duplicate, followed by 50 μL of enzyme conjugate and 50 μL of antibody. The microplate was mixed and incubated for 30 min at

room temperature. Solutions were decanted and wells rinsed four times with 1× washing buffer solution. Substrate solution was added to all wells (100 μL), mixed and incubated for 30 min in the dark. Stop solution was added to the wells (100 μL) and mixed, with the absorbance read immediately at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG Labtech 413–3350). For data analysis, $\%B/B_0$ values (i.e. average absorbance of STX standards divided by average absorbance of blank standard) were obtained and toxin quantification in samples determined by interpolating response values in the standard curve.

2.3.1.2. Europroxima™

Europroxima test kits (5191SAXI, lot number QN5327) were stored at 4 °C until analysis. Manufacturer's protocol was followed to perform the test. Similar to the Abraxis test kits, all samples were considered as blind samples, with an extra set of 15 samples further diluted (i.e. 1:10, 1:100 or 1:500) based on the known toxin concentration.

2.3.1.2.1. Extract preparation. In brief, 1 g of shellfish homogenate was mixed with 5 mL of 0.2 M sodium acetate buffer (freshly prepared), centrifuged at 3000 × *g* for 10 min and the supernatant recovered for experiments. The extract was diluted in dilution buffer (1:50).

2.3.1.2.2. Test protocol. STX standards (0.0125, 0.025, 0.05, 0.1, 0.2 and 0.3 ng mL^{-1}) and samples (50 μL) were transferred into the 96-well plate in duplicate, followed by 25 μL of conjugate and 25 μL of antibody. The plate was shaken for 1 min and incubated for 30 min at room temperature. The solutions were discarded and all wells rinsed three times with rinsing buffer. Substrate was added to all wells (100 μL), the plate was shaken and incubated for 15 min at room temperature in the dark. Stop solution was added (100 μL) and absorbance was read immediately at 450 nm. % maximal OD (optical density) was calculated for all standards and samples using the absorbance readings from the standards with no STX (standard zero, provided by supplier). The concentration of PST in samples was calculated using the calibration curve.

2.3.2. Qualitative tests

2.3.2.1. Scotia™. Scotia test kits (PSP Rapid Test, lot number 40,000) were stored at 5 °C until analysis. These kits use the modified AOAC.959.08 extraction method boiling the shellfish sample in 0.1 M HCl (or mini-AOAC, Jellett et al., 2002), and thus the same extracts prepared for the Abraxis tests were used for the Scotia kits. Manufacturer's protocol was followed for the test. A volume of 400 μL of PSP Scotia rapid buffer was mixed with 100 μL of the shellfish extract, 100 μL of this mix was placed into the test kit sample slot and allowed to develop for 35 min. The test kits were scanned using the Scotia Skannex system, which scans the strip and analyses the bands to give a positive or negative result with a numeric value based on the intensity comparison of both the C (control) and T (test) bands. Values ≥ 0.5 indicate negative samples, or positive if < 0.5 (Turner et al., 2015).

2.3.2.2. Neogen™. Neogen test kits (NEO9562, lot number 9562-11) were stored at room temperature until analysis. The certificate analysis report accompanying the kits showed that STX-diHCl standards at two concentrations equivalent to 0.16 mg/kg and 0.80 mg/kg returned negative and positive results, respectively, with no further information in performance within that concentration range. Neogen's standard protocol uses 1 g (±0.05 g) of shellfish homogenate mixed with 30 mL (±0.5 mL) of distilled water in a plastic extraction bag with an inner mesh filter (280 μM pore size, provided with kits) to perform the toxin extraction manually using the metal roller. The filtered liquid (extract) is recovered and 100 μL of this is transferred into a container with buffer saline solution (as provided) and mixed for 30 s. A volume of 100 μL of this mix is then transferred into a microwell and a LFIA test strip inserted and allowed to develop for exactly 5 min, after which it is placed into the AccuScan Pro Reader to obtain the positive or negative result based on the intensity of the test band that appears in the strip (see Jawaid et al., 2015).

2.3.2.2.1. Standard curves with the Neogen test kit. The analysis of standard curves of different PST analogues was performed to test the cut off levels for each analogue using the same dilutions as the Neogen standard protocol. Standards of STX, NEO, GTX2&3, GTX1&4 and C1&2 were purchased from the National Research Council Canada (NRC). Saxitoxin standard was tested at concentrations equivalent to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mg/kg following the standard Neogen protocol (i.e. 1:31 sample dilution). STX, NEO, GTX2&3, GTX1&4 and C1&2 standards were tested again but now using the adjusted dilution at concentrations of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1.2 mg STX eq/kg. Additionally, two mixtures of these standards recreating what has been observed in natural contaminated samples were tested. Mix A: 1, 60, 30 and 9% of STX, GTX1&4, GTX2&3 and C1&2, respectively, and mix B: 0.5, 90, 4.5 and 5.0%, respectively. A concentration range of 0.2, 0.4, 0.6, 0.8 and 1.2 mg STX eq/kg were tested. For toxins that were combined, only the epimer with greatest toxicity was used for calculative purposes, using the EFSA toxicity equivalency factors (EFSA, 2009). In addition, because of the low reactivity (6%) of Neogen test towards GTX1&4, two extra batches of GTX1&4 were treated with L-cysteine (Sigma W326305), to explore the possibility of PST analogue conversion (Asakawa et al., 1987) and thus increase the reactivity of the Neogen kit. The second batch of GTX1&4 (at 0.2–1.2 mg STX eq/kg) was incubated with cysteine (2 M) in a water bath at 70 °C for 30 min, and the third batch was treated under the same conditions but for 60 min.

2.3.2.2.2. Test of shellfish samples. The first dilution step was modified since we observed that STX standard at 0.4 mg/kg returned a positive result when mixing 1 g of shellfish sample (or equivalent standard) with 30 mL of type 1 water, the new dilution was using 45.5 mL of type 1, 18.2 MΩ·cm, water. Sixty nine shellfish samples were tested using 1 g (±0.05 g) of homogenate and extracted with 45.5 mL of type 1 water; the rest of the protocol was as mentioned previously. The Neogen standard protocol (i.e. dilution of 1:31 during extraction) was used only for 11 shellfish samples that included false negatives; a cysteine treatment was also applied to these 11 samples but that were extracted at a dilution of 1:46.5 (extract incubated with 2 M cysteine at 70 °C for 30 min). Once all the tests were completed and positive/negative results registered, the data recorded by the Neogen AccuScan Pro Reader was exported to Excel (Microsoft Office™), which includes peak and area values for both the control and test bands, plus the positive or negative result corresponding to the test band.

3. Results

Using the LC-FLD analytical method, 23 of the 69 samples had PST levels same as or above the action limit of 0.8 mg STX eq/kg (non-compliant), 33 samples had levels ranging between 0.10 and 0.77 mg STX eq/kg, and 13 samples levels of <0.10 mg STX eq/kg (compliant) (Table S1, Supplementary material). Most contaminated samples contained high proportions of the PST analogues GTX1&4 (25–88%) and GTX2&3 (8–70%), followed by C1&2 (4–21%) and STX (0–3%) (Fig. 1). Due to the varying nature of the shellfish matrices, toxin profiles and concentrations, multiple SPE steps, and pH variances in the oxidation steps, the standard error is fairly high at lower concentrations.

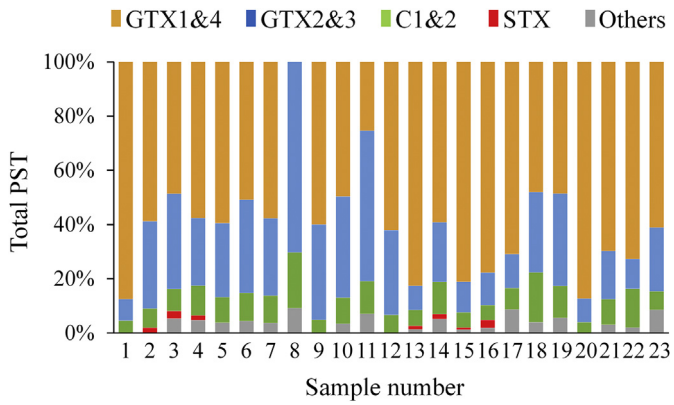

Fig. 1. Proportion of PST analogues in contaminated shellfish during an *Alexandrium tamarense* (group 1) bloom in Tasmania, Australia, as per Table S1 from Supplementary material. Only samples with ≥0.80 mg STX eq/kg of LC-FLD confirmation analysis are shown.

3.1. Quantitative ELISA kits

3.1.1. Abraxis

The Abraxis kit showed poor performance on the 69 shellfish samples when the standard protocol was followed; 8 of the 23 non-compliant samples (34.8%) were underestimated, delivering values below the regulatory action limit (i.e. between 0.24 and 0.77 mg STX eq/kg) in samples containing 0.80–3.29 mg STX eq/kg (as per LC-FLD). Correlation between Abraxis and LC-FLD was poor ($r^2 = 0.33$ for linear adjustment) (Fig. 2A). Performance of the Abraxis kit increased when shellfish extracts were diluted (up to 1:100000) based on the LC-FLD toxin concentrations. Such tests were performed on 15 samples, of which 11 had PST levels >0.8 mg STX eq/kg. Shellfish samples extracted with 80% MeOH showed very similar results as the HCl extracts. Abraxis quantification of HCl and MeOH extracts with extra dilutions improved considerably showing higher correlations with the LC-FLD method, increasing from $r^2 = 0.38–0.39$ to $r^2 = 0.82–0.91$ (Fig. 2B). Despite the improvement in the performance with the extra dilution step (performed in 15 samples, of which 11 were non-compliant), 2 samples (18%) with 0.92–0.97 mg STX eq/kg (LC-FLD) were underestimated by Abraxis, generating values below the action limit (Table 2).

3.1.2. Europroxima

The Europroxima kit showed poor performance on the 69 shellfish extracts. All 23 samples with ≥0.8 mg STX eq/kg were underestimated, delivering values of 0.10–0.19 mg STX eq/kg when manufacturer's protocol was followed (using sample dilution of 1:50 prior). Correlation between Europroxima and LC-FLD quantification was poor ($r^2 = 0.13$, linear adjustment) (Fig. 3A). Quantification was improved by performing an extra dilution to the samples based on their known PST concentrations. Due to the high PST levels in some samples, extra dilutions of up to 1:500 were required (final dilution of 1:150000). The correlation between Europroxima and LC-FLD improved from $r^2 = 0.0004$ to 0.91 in the 15 samples that were diluted, which included 11 non-compliant (Fig. 3B). However, one sample with 0.92 STX eq/kg (LC-FLD) was still underestimated by Europroxima, as having 0.44 mg STX eq/kg (Table 2).

3.2. Qualitative LFIA kits

3.2.1. Scotia

The Scotia kit showed good performance on high PST shellfish. However, considering that the sensitivity is 0.2–0.7 mg STX eq/kg

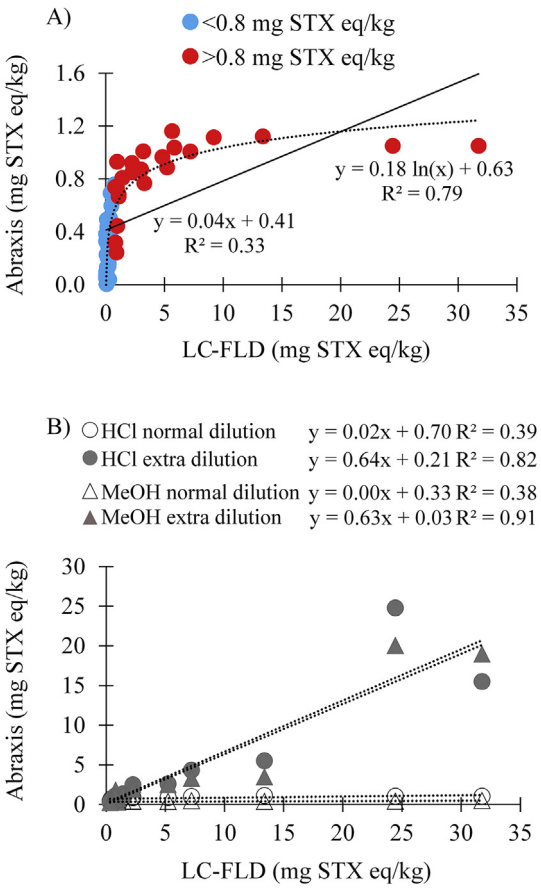

Fig. 2. Quantification of PST in Tasmanian shellfish samples using the Abraxis™ ELISA kit as compared with the AOAC.2005.06 (LC-FLD) official method. Values are average from duplicate samples (see Table 2). A) All 69 samples with equations from linear and logarithmic regressions. Linear regression is the desired adjustment; however the logarithmic adjustment showed a better correlation, showing the limitation of the Abraxis test for an accurate quantification of concentrated PST extracts. B) Extra analysis of 15 samples using two extraction techniques (HCl and MeOH) with an extra sample dilution step.

(Scotia Rapid Testing, pers. comm.), the test kits delivered 16 false positives (27%). Among these, 9 contained 0.10–0.19 mg STX eq/kg and the other 7 were reported as <0.10 mg STX eq/kg by the LC-FLD method (Fig. 4). No false negatives were detected using the Scotia test but only 9 of the 18 shellfish extracts with ≤0.2 mg STX eq/kg were negative (Table 2). The PST concentration in shellfish extracts and numerical values recorded by the Scotia scanner were not well correlated ($r^2 = 0.26–0.45$) (Fig. 4).

3.2.2. Neogen

Performance of the Neogen kit using PST toxin standards is shown in supplementary material (Figs. S1 and S2).

3.2.2.1. Performance on contaminated shellfish samples. Control bands showed an average peak and area of 8192 (±555) and 11,755 (±696), respectively (data not shown). Both values for peak and area for the test bands of the 69 samples were highly correlated (Table S2 and Fig. S3, Supplementary material). Although peak and area values generally depended on toxin concentration, their quantitative values may not always be indicative due to varying cross-reactivity for different PST analogues.

Neogen performed well on most of the 69 shellfish samples. Considering that the dilution in the extraction step was modified from 1:31 (cut off observed to be 0.4 mg STX eq/kg) to 1:46.5 to set

Table 2
Summary of the performance of the four PST kits on 69 shellfish samples. The confirmation results determined by LC-FLD are included. PST quantification on 15 samples was further investigated with the Abraxis kit by using the two recommended extraction methods (HCl and MeOH), following the standard protocol and by performing an extra dilution step. This was also done for Europroxima. Results shaded in gray indicate false positives (Neogen and Scotia) and in black false negatives (Neogen) based on the sensitivity of each kit (0.2–0.7 mg STX eq/kg for the Scotia test, and altered to 0.5–0.6 mg STX eq/kg for Neogen). NT = not tested.

Sample	LC-FLD (mg STX eq/kg)	Neogen	Scotia	Abraxis HCl				Abraxis MeOH				Europroxima			
				Normal		Extra		Normal		Extra		Normal		Extra	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	9.20	+	+	1.11	0.02	NT		NT		NT		0.15	0.12	NT	
2	5.85	+	+	1.04	0.01	NT		NT		NT		0.12	0.05	NT	
3	4.83	+	+	0.96	0.01	NT		NT		NT		0.14	0.16	NT	
4	5.65	+	+	1.16	0.02	NT		NT		NT		0.13	0.08	NT	
5	3.20	+	+	1.01	0.06	NT		NT		NT		0.13	0.02	NT	
6	3.02	+	+	0.87	0.00	NT		NT		NT		0.12	0.01	NT	
7	3.29	+	+	0.93	0.03	NT		NT		NT		0.13	0.04	NT	
8	1.10	+	+	0.68	0.00	NT		NT		NT		0.10	0.00	NT	
9	0.96	+	+	0.77	0.05	NT		NT		NT		0.13	0.01	NT	
10	0.98	+	+	0.44	0.02	NT		NT		NT		0.12	0.04	NT	
11	1.10	+	+	0.66	0.02	NT		NT		NT		0.15	0.06	NT	
12	0.82	-	+	0.32	0.01	NT		NT		NT		0.11	0.01	NT	
13	31.75	+	+	1.05	0.01	15.51	0.24	0.48	0.02	18.94	3.17	0.15	0.00	31.70	4.81
14	24.44	+	+	1.05	0.01	24.78	0.02	0.41	0.02	20.04	1.58	0.15	0.03	41.22	11.16
15	13.38	+	+	1.12	0.01	5.51	0.15	0.38	0.01	3.48	0.04	0.16	0.04	14.49	0.17
16	7.21	+	+	1.01	0.01	4.32	0.02	0.48	0.00	3.30	0.02	0.16	0.06	8.95	0.75
17	5.23	+	+	0.89	0.00	2.59	0.02	0.37	0.00	2.51	0.00	0.16	0.10	5.07	0.47
18	2.26	+	+	0.85	0.02	2.47	0.04	0.39	0.02	1.90	0.03	0.14	0.03	4.78	0.07
19	0.80	+	+	0.74	0.01	1.09	0.01	0.38	0.01	1.79	0.34	0.18	0.10	1.29	0.41
20	0.97	+	+	0.73	0.02	0.75	0.00	0.31	0.00	0.70	0.01	0.16	0.06	1.93	0.08
21	1.43	+	+	0.81	0.03	1.34	0.03	0.38	0.01	1.23	0.01	0.19	0.05	2.63	0.07
22	2.24	+	+	0.92	0.04	1.94	0.01	0.42	0.01	2.50	0.44	0.15	0.03	3.61	0.37
23	0.92	-	+	0.24	0.00	0.34	0.00	0.31	0.03	0.27	0.00	0.12	0.02	0.44	0.05
24	0.75	+	+	0.69	0.01	0.52	0.00	0.30	0.00	0.61	0.04	0.15	0.08	0.57	0.05
25	0.77	+	+	0.76	0.02	1.08	0.01	0.28	0.00	1.13	0.02	0.15	0.06	1.07	0.35
26	0.42	-	+	0.69	0.01	0.51	0.02	0.28	0.01	0.49	0.01	0.16	0.06	0.65	0.01
27	0.33	-	+	0.43	0.01	0.29	0.00	0.22	0.00	0.23	0.00	0.10	0.03	0.41	0.01
28	0.70	+	+	0.65	0.02	NT		NT		NT		0.15	0.03	NT	
29	0.49	+	+	0.60	0.01	NT		NT		NT		0.12	0.02	NT	
30	0.77	+	+	0.68	0.01	NT		NT		NT		0.12	0.04	NT	
31	0.48	-	+	0.44	0.01	NT		NT		NT		0.11	0.01	NT	
32	0.41	-	+	0.30	0.00	NT		NT		NT		0.10	0.00	NT	
33	0.22	+	+	0.46	0.00	NT		NT		NT		0.12	0.01	NT	
34	0.29	-	+	0.40	0.01	NT		NT		NT		0.12	0.05	NT	
35	0.34	-	+	0.40	0.01	NT		NT		NT		0.13	0.07	NT	
36	0.31	-	+	0.31	0.00	NT		NT		NT		0.12	0.06	NT	
37	0.21	-	+	0.47	0.01	NT		NT		NT		0.12	0.04	NT	
38	0.20	-	+	0.36	0.01	NT		NT		NT		0.11	0.01	NT	
39	0.34	-	+	0.34	0.00	NT		NT		NT		0.12	0.02	NT	
40	0.36	-	+	0.34	0.00	NT		NT		NT		0.12	0.05	NT	

41	0.23	-	+	0.22	0.00	NT	NT	NT	0.10	0.01	NT
42	0.15	-	+	0.43	0.00	NT	NT	NT	0.09	0.03	NT
43	0.10	+	+	0.49	0.01	NT	NT	NT	0.11	0.02	NT
44	0.11	-	+	0.39	0.01	NT	NT	NT	0.11	0.01	NT
45	0.04	-	+	0.33	0.01	NT	NT	NT	0.12	0.00	NT
46	0.08	-	+	0.23	0.00	NT	NT	NT	0.10	0.02	NT
47	0.03	+	+	0.38	0.00	NT	NT	NT	0.11	0.04	NT
48	<0.35	+	+	0.35	0.01	NT	NT	NT	0.11	0.01	NT
49	<0.20	-	+	0.38	0.00	NT	NT	NT	0.09	0.02	NT
50	<0.20	-	+	0.33	0.00	NT	NT	NT	0.09	0.01	NT
51	<0.10	-	+	0.13	0.00	NT	NT	NT	0.07	0.01	NT
52	<0.20	-	+	0.28	0.00	NT	NT	NT	0.11	0.02	NT
53	<0.03	-	-	0.05	0.00	NT	NT	NT	0.04	0.00	NT
54	<0.03	-	-	0.07	0.00	NT	NT	NT	0.05	0.00	NT
55	<0.15	-	-	0.20	0.01	NT	NT	NT	0.10	0.00	NT
56	<0.21	-	-	0.21	0.00	NT	NT	NT	0.09	0.01	NT
57	<0.20	-	+	0.16	0.00	NT	NT	NT	0.07	0.01	NT
58	<0.15	-	-	0.05	0.00	NT	NT	NT	0.08	0.01	NT
59	<0.03	-	-	0.06	0.00	NT	NT	NT	0.09	0.00	NT
60	<0.20	-	+	0.14	0.00	NT	NT	NT	0.10	0.03	NT
61	<0.10	-	-	0.04	0.00	NT	NT	NT	0.07	0.01	NT
62	<0.20	-	+	0.28	0.00	NT	NT	NT	0.11	0.03	NT
63	<0.05	-	-	0.10	0.00	NT	NT	NT	0.10	0.01	NT
64	<0.07	-	-	0.00	0.00	NT	NT	NT	0.16	0.04	NT
65	<0.05	-	+	0.04	0.00	NT	NT	NT	0.10	0.01	NT
66	<0.33	-	+	0.50	0.01	NT	NT	NT	0.12	0.02	NT
67	<0.08	-	+	0.10	0.00	NT	NT	NT	0.09	0.01	NT
68	<0.26	-	+	0.04	0.00	NT	NT	NT	0.09	0.01	NT
69	<0.08	-	+	0.06	0.00	NT	NT	NT	0.08	0.01	NT

the cut off to 0.5–0.6 mg STX eq/kg, Neogen delivered false positives in only 4 samples (13%), three of which had 0.10 to <0.35 mg STX eq/kg and the other contained 0.03 mg STX eq/kg (as per the LC-FLD method). Two false negatives (5%) were observed in samples with 0.82 and 0.92 mg STX eq/kg (Table 2). These samples were reanalysed following the Neogen standard protocol (dilution 1:31): the sample with 0.92 mg STX eq/kg turned positive, but the sample with 0.82 mg STX eq/kg was still negative (false negative). To overcome this, cysteine treatment (2 M at 70 °C for 30 min) was applied to the two false negative samples (1:46.5 dilution), together with 9 other samples of varying toxin concentrations. Cysteine treatment effectively eliminated the false negatives, although one other false positive was generated (at 0.20 mg STX eq/kg) (Table 3).

4. Discussion

Dinoflagellate blooms of *Alexandrium tamarense* (Group 1) in 2012 and 2015 generated closure of Tasmanian shellfish farms for up to 4 months, causing major economic losses. Mussels and oysters contained gonyautoxins as the major PST analogues. In 2012 GTX2&3 was the major analogue (51–100%), followed by STX (14–18%), C1&2 (10–24%) and dcGTX2&3 (5–16%). In contrast, in 2015 GTX1&4 was the major analogue (25–88%), followed by GTX2&3 (8–70%), C1&2 (4–21%) and STX (0–3%). Due to the low cross-reactivity of commercially available kits for GTX1&4, it was necessary to determine which kit would be the most suitable for shellfish growers to potentially incorporate in their monitoring program. To date few studies have critically compared PST immunological test kit results against AOAC official methods. Most studies used Scotia (formerly Jellett) and Abraxis (Costa et al., 2009; DeGrasse et al., 2014a; Turner et al., 2015; Wong et al., 2010), whereas others used ELISA kits with low or no reactivity to GTX1&4 (Burrell et al., 2016; Sato et al., 2014). The results of the present study are summarised in Table 4, which compares the main characteristics of the four immunological PST test kits, ease of use and their performance on Tasmanian mussels and oysters.

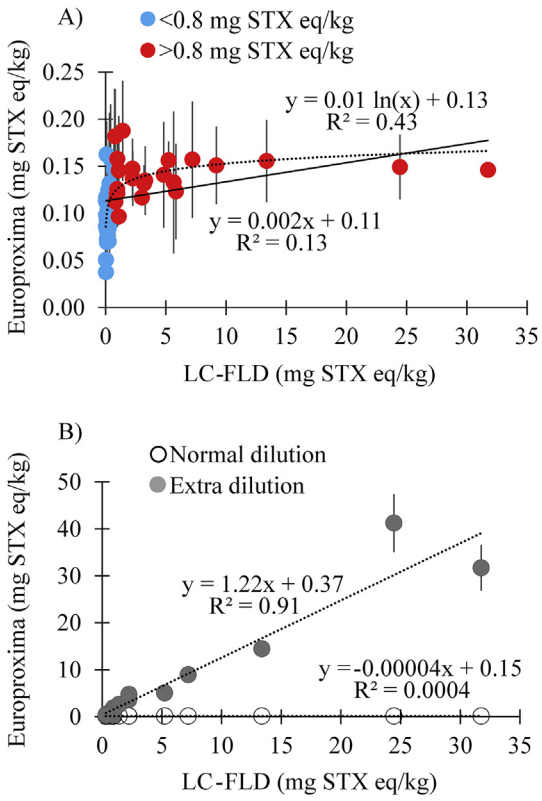

Fig. 3. Quantification of PST shellfish samples using the Europroxima™ ELISA kit compared to the AOAC.2005.06 (LC-FLD) official method. Values correspond to average of duplicate samples, and bars represent their standard deviations (see Table 2). A) 69 samples with equations from linear and logarithmic regressions are shown for each protocol. Similarly to Abraxis, Europroxima showed limitations in quantification of concentrated samples. B) Extra analysis in 15 samples following the standard protocol and an extra sample dilution.

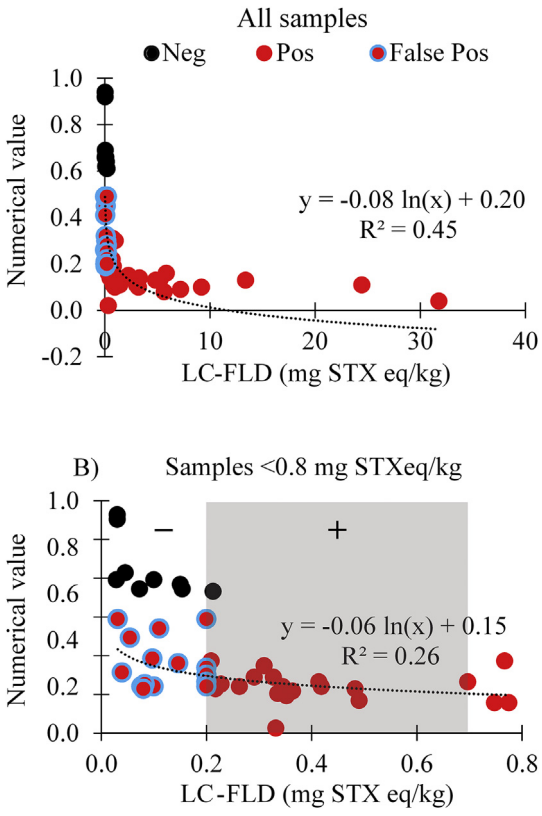

Fig. 4. PST toxin tests using the Scotia kit in 69 shellfish samples (A) with PST concentration as per the LC-FLD method (mg STX eq/kg) on the x-axis, showing those with <0.8 mg STX eq/kg in (B), where the sensitivity of the Scotia test is shaded (0.2–0.7 mg STX eq/kg) and positive results were expected. The y-axis indicates the numerical value generated by the Scotia Skannex system based on the intensity comparison of the control and test bands of the test. Values ≥ 0.5 indicate negative samples (●), or positive if < 0.5 (●). False positives (●) are those samples with ≤0.20 mg STX eq/kg (LC-FLD analysis) but generated a positive result. See Table S2 (Supplementary material) for complete list of numerical values.

To date, PST determination in shellfish samples using AOAC official methods is still subject to considerable variability (Burrell et al., 2016; Costa et al., 2009; Turner et al., 2014a). A recent study did not conclusively select a method of choice but rather concluded that the method to choose should be based on practicality, including access and cost of equipment, and skills of the analyst (Burrell et al., 2016). A continuous input in methodological improvements and innovations is needed to overcome current method limitations, efficiency and sample turnaround time (Boundy et al., 2015; Campbell et al., 2011; Yakes et al., 2012), as well as their application to other matrices, such as human urine and blood serum (DeGrasse et al., 2014b).

4.1. Ease of use

The advantage of the Abraxis and Europroxima kits is that they deliver a quantitative PST result. A disadvantage is that both kits require laboratory skills. Abraxis requires extracting the toxin by boiling the shellfish sample in HCl, but this can be avoided by using MeOH and not boiling. The extraction step with Europroxima requires preparing the extraction buffer (0.2 M sodium acetate buffer), while the remaining extraction steps are easy to follow. Both the Abraxis and Europroxima call for pipetting small volumes (10–100 μL and 25–100 μL, respectively) for dilution and perform the assays using 96-well microplates. Stock solutions (i.e. dilution buffer, wash solution, conjugate or antibody) need to be diluted, and different incubation periods are required. Total test times amount to 60 and 45 min for Abraxis and Europroxima, respectively, but including sample extraction and solutions preparation, the protocol can take up to 3 h depending on the number of samples to be tested. Data analysis requires a calibration curve to be constructed but no specialised software is required. An adaptation of the 96-well plate Abraxis kit into a compact and easy-to-use shipboard version was successfully used by fishermen during a pilot study in Georges Bank, USA, with good correlations between Abraxis, mouse bioassay and LC-FLD (DeGrasse et al., 2014a). It is noted that the surf clams tested contained mostly STX, for which the Abraxis kit is well suited. However, the Abraxis and the Europroxima test kits had limited applicability to Tasmanian shellfish containing mostly GTX1&4.

The qualitative Scotia and Neogen kits comprise easier steps and laboratory experience is not essential. Scotia, similar to Abraxis, recommends extracting the toxin by boiling shellfish in HCl, but it also has an alternate rapid method using a mix of 2.5 parts of 70% isopropyl with 1 part of 5% acetic acid but this protocol was not performed in the present study. The remainder of the Scotia protocol involves mixing the shellfish extract with Scotia buffer and transfer an aliquot of this mix into the test strip followed by 35–60 min incubation period. The strip then can be scanned using the Scotia Skannex system which delivers an immediate positive or negative result. However, if GTX1&4 is suspected to be the dominant PST analogue, an extra step is recommended which increases the duration of the test by an extra 60 min. The Neogen kit offers the greatest ease of use since the extraction step is achieved using

Table 3
Comparison of the Neogen standard protocol (dilution 1:31) against two modifications (dilution 1:46.5 with or without cysteine 2 M, 70 °C, 30 min). The four main PST analogues in the samples are indicated together with the total concentration (confirmation result). Shaded results indicate false negatives (eliminated when treated with cysteine). Sample numbers are the same as in Table 2. All these samples tested positive with the Scotia kit.

Sample No.	Total PST mg STX eq/kg	STX	GTX1&4	GTX2&3	C1&2	1:31	1:46.5	1:46.5 + cysteine
12	0.82	*	62%	31%	7%	-	-	+
23	0.92	*	61%	24%	7%	+	-	+
30	0.77	*	*	64%	18%	+	+	+
31	0.48	*	*	80%	13%	-	-	+
32	0.41	*	28%	46%	15%	-	-	-
36	0.31	*	47%	46%	7%	-	-	-
38	0.20	*	*	80%	20%	-	-	+
44	0.11	*	41%	*	28%	-	-	-
50	<0.20	NA	NA	NA	NA	-	-	-
67	<0.08	NA	NA	NA	NA	-	-	-
69	<0.08	NA	NA	NA	NA	-	-	-

*Reported as <0.05 mg STX eq/kg.
NA = Not available.

Table 4
Summary of the characteristics and performance of four immunological commercially available test kits on the detection of PST toxins on Tasmanian mussels and oysters, which contained mostly GTX1&4.

	Neogen™	Scotia™	Abraxis™	Europroxima™
Characteristics				
Cut off or working range (mg STX eq/kg)	0.5–0.6	0.2–0.7	0.04–0.8[a]	0.0038–0.09[a]
Cross-reactivity for GTX1&4 (mole %)	6	1.8	<0.2	<0.1
Conversion step for GTX1&4	Yes (introduced here)	Yes	No	No
Improved cross-reactivity for GTX1&4 (mole %)	129	26	–	–
Cost per test[b] (~US$)	22	22	13[c] to 84[d]	12[c] to 96[d]
Extraction	Distilled or type 1 water, manually with roller	0.1 M HCl, boil & centrifuge	0.1 M HCl, boil & centrifuge	0.2 M sodium acetate buffer, mix & centrifuge
Dilution for extraction	1:31 (standard) 1:46.5 (present study)	1:2	1:2	1:6
Time for analysis[e]	20-35 min	35-95 min	Up to 3 h	Up to 3 h
Result	Positive or negative, immediate with reader	Positive or negative, immediate with scanner	mg STX eq/kg, prior data analysis required	mg STX eq/kg, prior data analysis required
Performance in Tasmanian shellfish (n = 69)				
% False positives	13	27	25[f]	25[f]
% False negatives	5→0[g]	0	18[h]	9[h]

[a] Working range of shellfish samples calculated from lowest and highest STX standard concentrations provided with kits for calibration curve.
[b] Price does not include taxes and reader, scanner or software for data analysis.
[c] Considering that the whole 96-well plate is used, 41 or 40 samples can be tested in a single plate with Abraxis or Europroxima, respectively.
[d] Considering only one sample is run at a time (unused wells can be stored and used within the expiry date. Used wells must not be re-used).
[e] It does not include preparation of shellfish homogenate.
[f] Reported as non-compliant when they in fact contained <0.80 mg STX eq/kg (as per LC-FLD), calculated from the 15 samples with extra dilutions to fit in the working range of the calibration curve. This corresponds to only one sample (of four) that was reported as 0.77 mg STX eq/kg with the LC-FLD method.
[g] False negatives eliminated with the cysteine conversion step introduced in the present study.
[h] Reported as compliant when they in fact contained >0.80 mg STX eq/kg (as per LC-FLD), calculated from the 15 samples with extra dilutions to fit in the working range of the calibration curve.

distilled water (or type 1 water, as in this study), and no boiling is required. Extraction is performed using a plastic filter bag which is homogenised mechanically with a roller (both provided). An aliquot of this mix is directly poured into a bottle containing Neogen buffer, which is mixed manually and a subsample used to perform the test. The Neogen strip is incubated only for 5 min and immediately analysed by the Accuscan Pro reader, which delivers a positive or negative result.

A drawback of Abraxis and Europroxima is that a microplate reader with absorbance detection is necessary. Similarly, a scanner and computer are recommended for Scotia to remove subjectivity of visual interpretation of the bands (Turner et al., 2015). Neogen has also developed their own dedicated reader (Accuscan Pro reader). Both readers have to be purchased from the companies or brand suppliers since they use a specific software or have been calibrated in-house. The advantage of these readers is that they deliver an immediate result. Both Scotia and Neogen kits can be stored at room temperature (Scotia: 4–25 °C, Neogen: 18–30 °C), whereas the Europroxima and Abraxis kits need to be stored at 2–8 °C and 4–8 °C, respectively.

4.2. Sensitivity and performance

Both Abraxis and Europroxima tended to underestimate the toxin concentrations in shellfish extracts when manufacturers' recommended protocols were followed. Abraxis underestimated in all 23 samples with ≥0.80 mg STX eq/kg, with 15 of these samples (65%) estimating at 0.8–1.2 mg STX eq/kg (e.g. LC-FLD: 1.43, 9.20, 31.75 mg STX eq/kg = Abraxis: 0.81, 1.11, 1.05 mg STX eq/kg, respectively). Critically, 8 samples (34.8%) were reported below the recommended regulatory action limit, within a concentration range of 0.80–3.29 mg STX eq/kg (LC-FLD), which showed 0.24–0.77 mg STX eq/kg with the Abraxis test. All samples extracted with MeOH were underestimated below the action limit. Europroxima underestimated 100% of samples, including those with up to 31.75 mg STX eq/kg. In some cases, Abraxis and Europroxima overestimated toxin concentration, especially those with ≤0.05 mg STX eq/kg, but none of them above 0.38 mg STX eq/kg.

Shellfish extracts whose PST toxin content were outside the constructed calibration curve were under or overestimated, 30% (HCl extraction) to 60% (MeOH extraction) and 91% of samples were outside the calibration curve for the Abraxis and Europroxima tests, respectively. This could be overcome by performing extra dilutions to make them fit the standard curve; however, re-testing those samples falling outside the calibration curve doubles the cost and time of analysis. Abraxis does recommend a further dilution of 1:10 in highly contaminated samples, however this dilution is not sufficient to obtain a satisfactory quantification since some samples contained up to 31.75 mg STX eq/kg. Extra dilutions of 1:100 or 1:500 were necessary for Abraxis and Europroxima, respectively, which improved toxin quantification significantly, although some samples were still underestimated: 2 with Abraxis and 1 with Europroxima, reporting compliant toxin levels when in fact they contained >0.80 mg STX eq/kg (as per LC-FLD). Costa et al. (2009) also performed extra dilutions to bring mussel and clam extracts within the working range of Abraxis. These authors obtained a correlation (r^2 = 0.87) comparable to the one we observed (r^2 = 0.82–0.91) and similarly reported underestimation in some samples above the regulatory action limit, thus recommending that this ELISA kit was not suitable for samples containing multiple PST analogues. DeGrasse et al. (2014a) compared Scotia with a modified Abraxis kit on surf clams, and reported that Abraxis accurately detected high PST in shellfish contaminated with mostly STX (82%). The difference was claimed due to Scotia having been calibrated against a mixture of PST toxins, whereas Abraxis uses STX for the standard curve. Abraxis proved to be unreliable for samples contaminated with analogues other than STX, especially GTX1&4 for which reactivity is <0.2%.

Although Neogen standard protocol claims to return positive results for samples ≥0.8 mg STX eq/kg, Jawaid et al. (2015) reported a cut off of 0.68 mg STX eq/kg. In our work we observed a cut off of 0.4 mg STX eq/kg, and hence increased the dilution in the extraction step to increase the cut off to 0.5–0.6 mg STX eq/kg as suggested by Tasmanian shellfish growers. Jawaid et al. (2015) observed 38% false positives (samples with 0.265–0.408 mg STX eq/kg), whereas we observed 13% false positives in our samples (samples with 0.03 to <0.35 mg STX eq/kg). While Jawaid et al. (2015) did not observe false negatives, we found 5% false negatives in our Tasmanian studies (two samples containing 0.82 and 0.92 mg STX eq/kg). This difference might be due to the fact that (i) we included more samples in the range 0.5–1.3 mg STX eq/kg, (ii) our samples contained a higher proportion of GTX1&4 (low cross-reactivity by the Neogen and all kits), or (iii) because of the greater dilution we performed. It should be noted that the screen by LC-FLD for one of the false negative samples (with 0.82 mg STX eq/kg, confirmation result) was less than the regulatory limit (0.59 mg STX eq/kg screen result), and homogeneity could play a role in the differentiation of this sample; however, considering the screen result of that particular sample, a positive result was expected. The novel introduction in our work of cysteine treatment effectively overcame the false negatives due to conversion of GTX1&4 to NEO, and GTX2&3 to STX, as it has been shown by this and other thiol compounds (Asakawa et al., 1987; Sakamoto et al., 2000). This conversion step was more convenient with the Neogen kit since it possess the highest reactivity for NEO (129%, Table 1), and while it could generate false positives, this would not be as serious as for the Scotia kit (Table 3). Costa et al. (2009) recommended extra sample dilutions for Scotia to avoid false positives, but Turner et al. (2015) concluded that attempting to fine-tune the sensitivity of Scotia through extra dilutions is potentially unsafe. In our work we observed that by adjusting the dilution of the Neogen protocol and introducing a cysteine conversion step (increasing test time by 30 min), it eliminated false negatives. Furthermore, the Neogen kit also proved to perform well for contaminated samples from the 2012 Tasmanian bloom, which were dominated by GTX2&3 (samples used for early work, data not shown).

Turner et al. (2015) reported that Scotia returned negative results in samples up to 0.35 mg STX eq/kg. In their work the use of an extra hydrolysis step increased variability since samples with 0.10–0.70 mg STX eq/kg delivered positive results (improvement in performance), even though some (i.e. 0.10–0.23 mg STX eq/kg) were reported as negative. These authors observed many false positives without the extra step, 50% in samples with <0.1 mg STX eq/kg (including three samples with 0.01–0.08 mg STX eq/kg), and >95% in samples between 0.1 and 0.2 mg STX eq/kg. In the present Tasmanian study we observed 53.8% false positives in samples with <0.1 mg STX eq/kg, and 75% in samples between 0.1 and 0.2 mg STX eq/kg. Costa et al. (2009), DeGrasse et al. (2014a), and Wong et al. (2010) all previously reported a high percentage of false positives using Scotia (>58%). The kits these authors used were claimed to have a detection limit of 0.4 mg STX eq/kg (Jellett et al., 2002), but DeGrasse et al. (2014a) found that the practical detection limit was 0.1–0.2 mg STX eq/kg. The introduction of the Scotia Skannex system improved problems with subjective visual comparison of band intensities (Turner et al., 2015). We did not observe any false negatives with the Scotia kits, but numerical values returned by the Scotia scanner and the toxin concentration determined by LC-FLD were not well correlated (r^2 = 0.45) which might be due to the higher concentration of GTX1&4 in our samples. The correlation

could have been improved by using the extra hydrolysis step, but was not necessary for our samples since we did not obtain any false negatives. The introduction of a conversion step for the Neogen kit presented in this study, by incubating GTX1&4 standards and shellfish samples with cysteine, successfully eliminated false negatives. More work is being performed to fine-tune this extra step.

The Neogen kit was elected as the most suitable tool for our purposes since it offers advantages over other kits: (i) it is more user friendly and laboratory experience is not required, (ii) it returns faster results (~20–35 min), and (iii) is more practical for field use; (iv) it returned a lower number of false positives, and although 5% of false negatives were obtained, the introduction of a conversion step to increase the sensitivity of GTX1&4 successfully eliminated these false negatives. An international validation including Neogen standard protocol and the modification proposed in this study (i.e. altered dilution to change cut off and introduction of cysteine conversion step) is in process to facilitate approval of the Neogen kit for regulatory purposes as a screening tool. Significant savings in analytical costs will result when Neogen negative screen samples will no longer need to be tested using the more expensive and time consuming LC-FLD method.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2016.11.262.

Transparency document

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