

## **FOOD CHEMICAL CONTAMINANTS**

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# **Detection of Paralytic Shellfish Toxins in Mussels and Oysters Using the Qualitative Neogen Lateral-Flow Immunoassay: An Interlaboratory Study**

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**Paralytic shellfish toxins (PSTs) in bivalve molluscs represent a public health risk and are controlled via compliance with a regulatory limit of 0.8 mg saxitoxin (STX)-2HCl equivalents per kilogram of**

**shellfish meat (eq/kg). Shellfish industries would benefit from the use of rapid immunological screening tests for PSTs to be used for regulation, but to date none have been fully validated. An interlaboratory study involving 16 laboratories was performed to determine the suitability of the Neogen test to detect PSTs in mussels and oysters. Participants performed the standard protocol recommended by the manufacturer and a modified protocol with a conversion step to improve detection of gonyautoxin 1&4. The statistical analysis showed that the protocols had good homogeneity across all laboratories, with satisfactory repeatability, laboratory, and reproducibility variation near the regulatory level. The mean probability of detection (POD) at 0.8 mg STX·2HCl eq/kg using the standard protocol in mussels and oysters was 0.966 and 0.997, respectively, and 0.968 and 0.966 using the modified protocol. The estimated LOD in mussels was 0.316 mg STX·2HCl eq/kg with the standard and 0.682 mg STX·2HCl eq/kg with the modified protocol, and 0.710 and 0.734 mg STX·2HCl eq/kg for oysters, respectively. The Neogen test may be acceptable for regulatory purposes for oysters in accordance with European Commission directives in which the standard protocol provides, at the regulatory level, a probability of a negative response of 0.033 on 95% of occasions. Its use for mussels is less consistent at the regulatory level due to the wide prediction interval around the POD.**

Shellfish industries are severely impacted worldwide by the toxic dinoflagellates *Gymnodinium catenatum*, *Pyrodinium bahamense*, and species of the genus *Alexandrium* (1). These algae produce potent neurotoxins, comprising saxitoxin (STX) and its congeners, which are naturally bioaccumulated by shellfish and can cause paralytic shellfish poisonings in humans that consume contaminated shellfish. Blooms created by these dinoflagellates are increasing in recurrence and distribution (2) and cause shellfish farm closures and product recalls. Collecting wild shellfish during toxic blooms in areas where poor monitoring occurs, or no warning signage is present, has resulted in human poisonings and hospitalizations and, in extreme cases, in fatalities (3).

In many countries, shellfish industries are required to undertake biotoxin monitoring to ensure their product is both safe to eat and able to enter domestic and international markets. For regulation purposes, the recommended maximum allowable level of paralytic shellfish toxins (PSTs) is 0.8 mg STX·2HCl equivalents per kilogram of shellfish meat or 0.8 mg STX·2HCl eq/kg (4); however, this may vary in some countries. For instance, the regulatory level adopted in the Philippines is 0.6 mg STX eq/kg (5). In Australia, according to the Australia New Zealand Food and Standards Code, the regulatory level is 0.8 mg STX eq/kg (6), but not as 2HCl salt, which may vary compared with total PST in mg STX·2HCl eq/kg. The mouse bioassay (MBA; AOAC 959.08) was the official method for the determination of PST for many years, but in 2015 the Codex standard was revised to include chemical methods as well as biological and functional methods

as alternative regulatory tools (7). The United Kingdom, Ireland, Australia, and New Zealand adopted a precolumn oxidation (Pre-COX) LC method with fluorescence detection (LC-FLD; AOAC 2005.06 or the Lawrence method; 8) as the primary regulatory method. Further analytical methods have been validated and accepted by AOAC INTERNATIONAL for PST testing in shellfish, including a postcolumn oxidation (PCOX) LC-FLD method (AOAC 2011.02; 9), and a receptor-binding assay (RBA; AOAC 2011.27; 10), with improved methods currently undergoing validation [i.e., hydrophilic interaction LC (HILIC)-tandem MS (MS/MS); 11]. The U.S. Food and Drug Administration (FDA), through the National Shellfish Sanitation Program (NSSP), has recently approved the RBA (for mussels) and PCOX methods as alternatives for PST determination (12). Canada has recently moved away from the MBA and has adopted the PCOX method as the primary method for PST testing (13). However, some countries are still using the MBA as the reference method, and despite the disadvantages of being labor-intensive, having high variability, and societal concerns about animal usage, these countries do not adopt analytical tests due to the instrument expenses involved, the need for specialists to analyze the samples, and the routine use of expensive certified reference materials (1).

Due to the high cost of the biological (MBA) and analytical (e.g., LC-FLD) tests, recent studies have focused on the search for inexpensive and reliable rapid tests that could be used for screening purposes (14–18) in which negative test results would no longer require further expensive analysis for harvesting and product release. A review of available field methods for the detection of marine toxins in shellfish was recently published (19), which not only included PSTs, but also amnesic and diarrhetic shellfish toxins. The first rapid test for PSTs, the Scotia Rapid Test (SRT; formerly MIST Alert and Jellett) was introduced in the early 2000s (20, 21). This is a qualitative lateral-flow immunoassay (LFIA) that returns a positive or negative result. Quantitative ELISA rapid test kits have also been produced and are commercially available, including Abraxis, Europroxima, Beacon, Bioo Scientific, and R-Biopharm. Rapid tests are developed to target mainly STX, with varying cross-reactivity for other PST analogs, with the antibody used showing a very low reactivity for gonyautoxin 1&4 (GTX1&4), which is commonly found in contaminated shellfish from Australia and the United Kingdom (16, 17, 22). GTX1&4 is a highly potent PST analog, and when present at high levels in a contaminated sample, the result returned by the kits could be underestimated due to the low reactivity of the antibody for this analog. Thus, not all rapid test kits are suitable for all regions due to their limited cross-reactivity for some toxin analogs. The SRT was calibrated using a mixture of PSTs and possesses a low reactivity for GTX1&4. An extra conversion step for shellfish samples that are suspected to contain GTX1&4 can be performed (23), which, according to the manufacturer, increases the cross-reactivity from 1.8 to 26%, but also increases the time of analysis. The comparative performance of rapid test kits has demonstrated their limitation for analogs different from STX, as well as for highly contaminated samples due to the limited working range of their calibration curves (i.e., quantitative ELISA kits; 16, 17). Moreover, the qualitative SRT has been shown to return a large number of false-noncompliant results (i.e., positive results for samples that contained <0.8 mg STX·2HCl eq/kg; 14, 15, 17, 23, 24).

Geographical areas where shellfish are contaminated mainly with STXs have benefited from the use of rapid test kits due to their specific selectivity for this particular analog. Such is the

case with Georges Bank in the United States in which the use of the SRT and a modified version of the Abraxis ELISA kit led to their limited-use approval by the NSSP for regulatory purposes. The SRT was approved in 2004 to be used specifically to (1) determine when to perform an MBA in a previously closed area, (2) maintain an area in the open status, and (3) instigate a precautionary closure. The Abraxis Shipboard ELISA was approved in 2011, offering a few advantages over the SRT because it is a quantitative tool and does not return many false-noncompliant results (12, 15). The more recent LFIA Neogen kit (25) has proven to offer important advantages over other rapid test kits, such as time of analysis, ease of use, suitability for field use, low cost, and use of a reader that allows an objective assessment of the test result. It is a qualitative tool that returns less false-noncompliant results than the SRT, although the recent work by Harrison et al. (16) and Dorantes-Aranda et al. (17) found a small number of false-compliant results (i.e., negative results for samples that contained  $\geq 0.8$  mg STX $\cdot$ 2HCl eq/kg). Dorantes-Aranda et al. (17) successfully introduced a conversion step that eliminated the false-compliant results, and thus recommended the Neogen LFIA as an appropriate rapid tool for shellfish contaminated with GTX1&4, as is the case for Tasmanian (Australian) shellfish. Additionally, the single-laboratory validation (SLV) of this rapid kit, which followed AOAC guidelines for validation of qualitative binary chemistry tests, fulfilled the selectivity for PSTs and returned a satisfactory matrix and probability of detection (POD) of 1.0 at the regulatory limit for oysters and mussels (26).

In this study, we present the outcomes of an interlaboratory study using the Neogen kit to detect PSTs in mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) using AOAC procedures for the validation of binary qualitative tests (27). The methodology included both the standard protocol recommended by the manufacturer and a modified protocol proposed by Dorantes-Aranda et al. (17), which involves a conversion step to increase the cross-reactivity of GTX1&4 and gonyautoxin 2&3 (GTX) to the antibody used in the test kits. If the results of the study prove satisfactory (i.e., positive responses at the regulatory limit and above, with a false-compliant error of  $<5\%$ ), the test kit will provide international shellfish industries and potentially regulators with a rapid tool to detect PSTs in mussels and oysters, creating significant savings in costs and time when negative test results will no longer require further testing with more expensive and time-consuming analytical methods.

## METHODS

### Interlaboratory Study

The study was structured and performed following AOAC's *Guidelines for Validation of Qualitative Binary Chemistry Methods* (27). Eighteen laboratories were invited to participate in this study, of which 17 agreed. These laboratories were distributed in eight countries: Australia, Belgium, China, the Netherlands, New Zealand, Spain, the United Kingdom, and the United States. Participants included government regulatory laboratories as well as research institutes and centers, university laboratories, independent/commercial laboratories, and a shellfish hatchery QA laboratory. Some laboratories were experienced with analytical tests, but not with rapid tests; others with both;

and others did not have any experience with any type of tests. All laboratories carried out the tests with Neogen rapid kits, and three laboratories from Australia, New Zealand, and the United Kingdom also performed the Pre-COX analytical chemistry method (8) on the samples.

All laboratories were provided with consumables to carry out the rapid Neogen tests, including sample cups, disposable pipets (supplied with kits), L-cysteine to perform the extra conversion step, and a reader to obtain the final positive/negative result.

### Preparation of Shellfish Homogenates

Mediterranean mussel (*M. galloprovincialis*) and Pacific oyster (*C. gigas*) homogenates were sourced from the Tasmanian Shellfish Quality Assurance Program (TSQAP) from samples that were part of the routine biotoxin-monitoring program analyzed using the Pre-COX method. PST-free mussels and oysters were collected from areas free of toxins where blooms had not occurred (confirmed with the Pre-COX method). Eight naturally contaminated shellfish homogenates were used for this study, four of each matrix, which were diluted using the PST-free shellfish homogenates to achieve concentration levels targeted at 0, 0.2, 0.6, and 0.8 mg STX $\cdot$ 2HCl eq/kg. PST-contaminated homogenates originated from blooms of *Alexandrium tamarense* (group 1) that occurred on the east coast of Tasmania during 2012, 2015, and 2016, and from a bloom of *G. catenatum* in the Derwent River, Tasmania, in 2015. One naturally contaminated sample was spiked with STX standard to ensure there was a sample with a broader toxin profile, and another sample was spiked with GTX1&4 standard. Both standards were purchased from the National Research Council (NRC)-Canada. Shellfish homogenates were mixed for at least 20 min using an electric blender to achieve sample homogeneity.

Each laboratory received two sets of six replicates of all eight shellfish homogenates (96 samples in total), which were randomly labeled using numbers generated from [Random.org](http://Random.org) (28). The participants tested the samples using one set for (1) the standard protocol by the manufacturer and (2) a modified protocol with an extra conversion step, as per Dorantes-Aranda et al. (17). Each sample tube contained 1.5 g shellfish homogenate.

### Shipment of Material

(a) *Samples*.—Samples were separated into two sets per package, corresponding to those to be analyzed with the standard or modified protocol. An extra set of six blind shellfish homogenates was included so that laboratories were able to practice the protocols before testing the experimental samples. Samples that were sent to laboratories outside of Tasmania (i.e., mainland Australia or overseas) were dispatched in dry ice to keep samples frozen, except for those sent to New Zealand, which contained ice packs and were placed in a freezer at each connection point (dry ice is prohibited for import). Each parcel was accompanied by paper work for export and import of samples by the laboratories, including declaration letters and permits. A list of the contents, test protocols, result sheets, and instructions for AccuScan Pro reader setup were also sent with the samples.

(b) *AccuScan Pro reader*.—The endpoint of the test is analyzing the lateral-flow test strip with the AccuScan Pro reader in the recommended timeframe, which gives a positive or negative result. Readers were sent to all laboratories from the

Neogen headquarters in the United States; Neogen distributors in destination countries were used, when available, to import and deliver the readers to the laboratories.

(c) *Neogen kits*.—Neogen test kits were sent to all laboratories from the Neogen European headquarters in Scotland. All laboratories received the same lot number of Neogen kits (Lot No. 9562-20). According to production, one box with kits contained 24 lateral-flow test strips, 24 buffer containers, 24 microwells, 25 extraction bags, and 48 disposable 100  $\mu$ L pipets. Four of these boxes were provided to each laboratory, for a total of 96 tests, plus a mini kit with 6 tests (same lot, No. 9562-20) in case any samples needed to be retested; another mini kit with 6 tests (Lot No. 9562-07) was also provided for laboratories to run practice tests. Marine biotoxin starter kits (Neogen 9563) were also provided to laboratories. Each starter kit contained a microwell holder, roller, and bag clip to perform shellfish toxin extractions.

#### *Protocols for the Neogen Rapid Test*

(a) *Standard protocol*.—Laboratories were instructed to weigh out 1 g ( $\pm 0.05$  g) homogenate in a sample cup, add 30 mL ( $\pm 0.05$  mL) distilled or Milli-Q water, and mix vigorously for 30 s. This mix was poured into one side of the extraction bag, sealed with the bag clip, and then mixed for 30 s using the roller. The filtered solution from the other side of the extraction bag was returned to the sample cup and mixed for another 30 s; 100  $\mu$ L of this extract was transferred into a buffer container and mixed for 30 s. One hundred microliters were transferred into a microwell and a lateral-flow test strip placed in the microwell to incubate for 5 min, after which the strip was read using the AccuScan Pro reader (25).

(b) *Modified protocol with conversion step*.—This protocol is presented in detail in Dorantes-Aranda et al. (17). Participants were requested to weigh out 1 g ( $\pm 0.05$  g) homogenate in a sample cup, add 45.5 mL ( $\pm 0.05$  mL) distilled or Milli-Q water, and mix vigorously for 30 s. This suspension was poured into the extraction bag, sealed with the bag clip, and then mixed for 30 s using the roller. The extract was returned to the sample cup and mixed for another 30 s; 300  $\mu$ L were transferred into a vial containing L-cysteine (final concentration of 2 M), mixed well for 30 s, and incubated in a water bath at 70°C for 30 min. The extract was allowed to cool down for approximately 5 min in ice, removed and mixed for 30 s, and 100  $\mu$ L transferred into a buffer container and mixed for 30 s. One hundred microliters of the extract diluted in buffer were transferred into a microwell, and a test strip placed in the microwell with the sample, which was incubated for 5 min, after which the strip was read using the AccuScan Pro reader.

#### *LC Analysis*

Three laboratories were asked to analyze the eight shellfish homogenates with the Pre-COX method. Eight extra samples with approximately 6 g each were provided to the three laboratories for this purpose. These laboratories performed the Pre-COX method with their own minor refinement versions, as described below. All laboratories used PST-certified standards from NRC-Canada for toxin quantification by comparing the samples' peak areas with those of the standards. Analytical results returned by the three laboratories were adjusted to

STX·2HCl eq with total toxicity recalculated using toxicity equivalency factors (TEFs) recommended by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO; 29) to allow for the unification of the data and comparability.

(a) *Laboratory A*.—PSTs were extracted from  $5.0 \pm 0.1$  g shellfish homogenate. A volume of 3 mL of 1% acetic acid was added and the mixture mixed on a vortex mixer for 90 s. The samples were placed in a boiling water bath for 5 min and cooled in running cold water for another 5 min before mixing on the vortex mixer for 90 s and centrifugation at  $3600 \times g$  for 10 min. The supernatant was recovered, the pellet was resuspended in 3 mL of 1% acetic acid, and the solution was mixed on the vortex mixer and centrifuged again. Both supernatants were combined and diluted to a 10 mL volume with deionized water. Sample extracts were cleaned up using solid-phase extraction (SPE) cartridges (Phenomenex, C18-T). HPLC analysis was performed on an Agilent 1200 HPLC system with FLD (excitation, 340 nm; emission, 395 nm). Chromatographic analysis was carried out with a Phenomenex Kinetex column ( $150 \times 4.6$  mm id, 5.0  $\mu$ m particle size) in conjunction with a Security Guard (Part No. KJO-4282 and AJO-7597). Mobile phases A and B consisted of 0.1 M aqueous ammonium formate and 0.1 M ammonium formate with 5% acetonitrile, respectively; the pH was adjusted to  $6.0 \pm 0.1$  with 0.1 M acetic acid, and a flow rate of 2 mL/min was used. The solvent gradient was 100% solvent A, increasing to 80% solvent B at 4 min and maintained through to 5.5 min, and then returning to 100% solvent A at 5.51 min, with column re-equilibration through to 7 min. Total toxicity was reported as STX·2HCl eq using the TEFs recommended by the European Food Safety Authority (EFSA; 30).

(b) *Laboratory B*.—PSTs were extracted from  $5.0 \pm 0.1$  g shellfish homogenate. A volume of 3 mL of 1% acetic acid was added and the mixture mixed on the vortex mixer for 90 s. The samples were placed in a boiling water bath for 5 min and cooled in running cold water for another 5 min before mixing on the vortex mixer for 90 s and centrifugation at  $3200 \times g$  for 10 min. The supernatant was recovered, the pellet was resuspended in 3 mL of 1% acetic acid, and the solution mixed on the vortex mixer and centrifuged again. Both supernatants were combined and diluted to a 10 mL volume with deionized water. Sample extracts were cleaned up using Phenomenex polymeric Strata-X SPE cartridges. The ultra-performance LC (UPLC) method was carried out on a Waters Acquity instrument coupled with FLD (excitation, 340 nm; emission, 395 nm). Chromatographic separation was carried out with a Phenomenex Kinetex C18 reversed-phase column ( $100 \times 2.1$  mm id, 1.7  $\mu$ m particle size) using gradient elution at a flow rate of 0.35 mL/min. Mobile phase A consisted of 0.1 M ammonium formate (adjusted to pH 6.0 with 1% acetic acid), and mobile phase B consisted of 90% solvent A with 10% methanol. The solvent gradient was 100% solvent A increasing to 5% solvent B at 2 min, then to 60% solvent B at 4.5 min before returning to 100% solvent A at 4.55 min with column re-equilibration through to 6 min. Total toxicity was reported as STX·2HCl eq using TEFs based on Oshima data using averaged toxicities of combined epimers, neosaxitoxin (NEO) oral toxicity (31), and several other exceptions.

(c) *Laboratory C*.—PSTs were extracted from  $5.0 \pm 0.1$  g shellfish homogenate. A volume of 3 mL of 1% acetic acid was added and the mixture mixed on the vortex mixer for 90 s. The samples were placed in a boiling water bath for 20 min and

cooled in running cold water for 5 min before mixing on the vortex mixer for 90 s and centrifugation at  $3600 \times g$  for 5 min. The supernatant was recovered, the pellet was resuspended in 3 mL of 1% acetic acid, and the solution was mixed on the vortex mixer and centrifuged again. Both supernatants were combined and diluted to a 10 mL volume with deionized water. Sample extracts were cleaned up using SPE cartridges (Phenomenex Strata-X, 200 mg/3 mL). The UPLC method was carried out on a Waters Acquity UPLC instrument coupled with a Waters Acquity FLD (excitation, 340 nm; emission, 395 nm). Chromatographic separation was carried out on an Agilent Polaris 3 C18 ether column (100  $\times$  2.0 mm id) using gradient elution at a flow rate of 0.5 mL/min. Mobile phase A consisted of 10 mM ammonium formate with 0.04% acetic acid, and mobile phase B was 10 mM ammonium formate with 5% acetonitrile and 0.04% acetic acid. The solvent gradient was 100% solvent A for 1 min, increasing to 5% solvent B at 3.5 min, and then to 30% solvent B at 5 min before returning to 100% solvent A at 5.6 min with column re-equilibration through to 8 min. Total toxicity was reported as STX equivalents (not as 2HCl salt) and calculated using TEFs derived from Oshima (31), except for decarbamoylgonyautoxin 2&3 (dcGTx2&3), which was subsequently re-examined by Quilliam (33). TEFs of the analogs with higher toxicity were used for those epimers that were combined.

### Statistical Analysis

All statistical analyses were performed using R software (R Core Development Team, Version 3.1.3; 2015). In accordance with AOAC's *Guidelines for Validation of Qualitative Binary Chemistry Methods*, the statistical analysis of this interlaboratory study was based on methods outlined in Wehling et al., Labudde and Harnly, and Macarthur and von Holst (34–36). Laboratory POD (LPOD) is the composite POD pooled across laboratories and includes between-laboratory variation in addition to variation inherent in the binomial nature of the binary probabilities. In addition to an estimate of the average POD across laboratories, 95% confidence intervals were also calculated for the LPOD (34).

To assess the variability in the interlaboratory results, the reproducibility SD (within-laboratory variation pooled across all laboratories), laboratory SD (between-laboratory variation), and  $s_R$  (variability between single test results obtained in different laboratories) were calculated. The homogeneity test of LPODs was also applied to assess whether there was any detected intercollaborator effect (35).

For each matrix and protocol combination, linear interpolation was used to estimate the LPOD and 95% prediction interval at the regulatory level of 0.8 mg STX·2HCl eq/kg, as well as the LOD at a 95% POD. A logistic regression model was also fitted to the data in order to relate observed LPODs across experimental concentration levels, and this model was interpolated for specified concentration levels and to estimate LPODs and LODs.

### Results and Discussion

The toxin profile of the shellfish homogenates used in this study comprised a range of STX analogs. The three oyster homogenates originated from Tasmanian *A. tamarensis* (group 1) blooms occurring in 2015 and 2016. One homogenate was not modified and was dominated by the PST analog GTX2&3 (67%

of total mg STX·2HCl eq/kg; all percentages reported herein represent the contribution of each PST analog to total toxicity). The two other oyster homogenates were spiked with PST-certified standards to mimic profiles observed in other parts of the world. One sample was spiked with STX to contain similar levels of GTX2&3, GTX1&4, and STX; the other homogenate was spiked with GTX1&4 to ensure dominance by this analog. These toxin profiles resemble those commonly found in the Gulf of Maine (United States) and the United Kingdom, respectively (15, 16, 22). Mussel homogenates also contained variable toxin profiles, and they were not modified or spiked because they originated from different blooms. One mussel homogenate originated from a *G. catenatum* bloom dominated by dcSTX (50%), another originated from the same *A. tamarensis* bloom as the oyster homogenates (2015; dominated by GTX2&3 at 50%), and the third mussel homogenate originated from an *A. tamarensis* bloom that occurred in Tasmania in 2012, which was dominated by GTX2&3 and STX (Table 1).

### LC Analysis

This study highlights the variability in reporting total PST levels in shellfish for monitoring programs because each laboratory used different calculations to obtain STX equivalency. One laboratory reported in mg STX eq/kg (not as 2HCl salt) using TEFs derived from Oshima and Quilliam (31, 33), following the requirements of the Australian New Zealand Food Standards Code (6) and using the TEFs of analogs with greater toxicity for those that were combined. The two other laboratories reported in mg STX·2HCl eq/kg; one laboratory used TEFs recommended by the European Scientific Panel on Contaminants in the Food Chain (30), and the other laboratory used TEFs based on Oshima and Quilliam with averaged toxicities of combined epimers, and a higher TEF for neosaxitoxin based on oral toxicity (32). Results reported by the three laboratories were harmonized to mg STX·2HCl eq/kg using the TEFs recommended jointly by the FAO/WHO (29) in order to make all results comparable (Table 1; refer to the SLV study of the Neogen kit for differences in TEFs by Oshima, EFSA, and FAO/WHO; 26).

Results obtained from most shellfish homogenates were higher than expected for each targeted concentration (initial PST concentration based on data from the routine monitoring program using the Pre-COX method). The total toxicity returned by the three laboratories showed variability (Table 1), particularly at high PST concentrations (targeted at 0.8 mg STX·2HCl eq/kg), ranging from 0.758 to 1.217 mg STX·2HCl eq/kg for oysters (average of  $0.970 \pm 0.231$  mg STX·2HCl eq/kg) and 0.888 to 1.362 mg STX·2HCl eq/kg for mussels (average of  $1.126 \pm 0.237$  mg STX·2HCl eq/kg). Additionally, there were some differences observed in the toxin profile reported for four of the six contaminated homogenates. For the oyster homogenate targeted at 0.2 mg STX·2HCl eq/kg, Laboratories A and B found 34 and 24% of GTX1&4 and 54 and 60% of GTX2&3, respectively; however, Laboratory C did not find any GTX1&4, but reported 86% of GTX2&3. For the oyster homogenate at 0.8 mg STX·2HCl eq/kg, the three laboratories detected GTX1&4, GTX2&3, and N-sulfocarbamoylgonyautoxin 2&3 (C1&2), but Laboratory A also reported STX, although at low levels (1%). The highest variability was observed on mussel homogenates. For the mussel homogenate targeted at 0.2 mg STX·2HCl eq/kg,

**Table 1. Total PST and average toxin profile of naturally contaminated mixes used in the present study determined by Official Method<sup>SM</sup> 2005.06 performed by three participant laboratories, calculated using FAO/WHO TEFs**

Matrix	Bloom source (all from Tasmania, Australia)	Target,mg STX·2HCl eq/kg	Total PST, mg STX·2HCl eq/kg					Avg. toxin profile, % of total; mg STX·2HCl eq/kg
			Lab A	Lab B	Lab C	Mean <sup>a</sup>	SD	
Oyster	Nil	0.0	<0.025	<0.025	<0.025	<0.025	NA <sup>b</sup>	NA
	<i>Alexandrium tamarense</i> complex 2015 <sup>c</sup>	0.2	0.248	0.271	0.239	0.253	0.017	67% GTX2&3 29% GTX1&4 13% C1&2
	<i>A. tamarense</i> complex 2015 & 2016 + spiked with STX standard	0.6	0.721	0.752	0.744	0.739	0.016	33% GTX2&3 32% GTX1&4 26% STX 9% C1&2
	<i>A. tamarense</i> complex 2015 + spiked with GTX1&4 standard <sup>d</sup>	0.8	0.935	0.758	1.217	0.970	0.231	58% GTX1&4 31% GTX2&3 10% C1&2
Mussel	Nil	0.0	<0.025	<0.025	<0.025	<0.025	NA	NA
	<i>Gymnodinium catenatum</i> 2015 <sup>e</sup>	0.2	0.130	0.231	0.296	0.219	0.084	50% dcSTX 24% dcGTX2&3 13% C1&2
	<i>A. tamarense</i> complex 2015 <sup>d</sup>	0.6	0.705	0.785	0.603	0.698	0.091	50% GTX2&3 41% GTX1&4 8% C1&2
	<i>A. tamarense</i> complex 2012 <sup>f</sup>	0.8	0.888	1.362	1.130	1.126	0.237	48% GTX2&3 37% STX 10% C1&2 2% GTX5 2% dcGTX2&3

<sup>a</sup> Only PST analogs found in common by two or three laboratories were used for the average calculations.

<sup>b</sup> NA = Not available.

<sup>c</sup> Lab C did not detect GTX1&4, but returned higher levels of GTX2&3.

<sup>d</sup> Lab A also found STX at 1 and 4% in oyster and mussel mixes, respectively.

<sup>e</sup> Lab A also detected gonyautoxin 5 (GTX5) (1.5%); Lab B also found N-sulfocarbamoylgonyautoxin 1&4 (C3&4) (11.5%); and Lab C detected GTX1&4 (39%), but not C1&2, and lesser levels of decarbamoylsaxitoxin (dcSTX) (37%) compared with the other two laboratories.

<sup>f</sup> Lab A also detected dcSTX (0.6%); Lab B found GTX1&4 (6.7%), but not dcGTX2&3; and Lab C did not detect GTX5.

Laboratories A and B reported similar levels of dcSTX, dcGTX2&3, and C1&2, but laboratory A also detected GTX5 (1.5%) and Laboratory B reported C3&4 (11.5%). Further, Laboratory C detected 39% of GTX1&4 (the other two laboratories did not detect this PST analog) and did not detect C1&2. For the mussel homogenate at 0.8 mg STX·2HCl eq/kg, the three laboratories similarly reported GTX2&3, STX, and C1&2; however, Laboratory A also reported dcSTX (0.6%), dcGTX2&3 (1%; together with Laboratory C at 4%), and GTX5 (together with Laboratory B at 2%), whereas Laboratory B was the only laboratory to detect GTX1&4 (6.7%), but not dcGTX2&3. These results confirm the limitation of the Pre-COX method in quantifying GTX1&4, especially when other PST analogs are present in the sample. Previous work reports an overestimation of this PST analog compared with the PCOX or HILIC-MS/MS methods (37, 38).

Oyster and mussel homogenates from the *A. tamarense* 2015 bloom showed slightly different toxin profiles. Oysters contained predominantly GTX2&3 (67%), followed by GTX1&4 (29%) and C1&2 (13%); in contrast, mussels had GTX2&3 (50%), GTX1&4 (41%), and C1&2 (8%). The toxin

profile of an algal net sample from this bloom was dominated by GTX1&4 (70%), with lesser amounts of GTX2&3 (15%) and C1&2 (12%). This suggests a higher capacity of oysters to convert more toxic congeners, such as GTX1&4, to the less toxic GTX2&3, and thus leading to a decrease in total toxicity. Similar observations were found in PST-contaminated *C. gigas* oysters and *M. galloprovincialis* mussels from Korea and the Mediterranean (Bizerte Lagoon). Korean mussels contained higher proportions of GTX1&4 (65–71%) and less GTX2&3 (4%), with higher total toxicity than oysters (GTX1&4 = 41–42%; GTX2&3 = 17–22%; 39). In contrast, oysters from the Mediterranean did not contain GTX1&4 (dominated by C1&2 = 72% and GTX5 = 27%), but mussels contained GTX1&4 (11%), with lesser levels of C1&2 (47%; GTX5 = 28%), accounting for higher total toxicities that were also maintained for longer periods (40). Biotransformation of PSTs has been previously reported and varies among species of molluscs; for instance, *M. edulis* mussels showed a lower capacity for PST transformation than clams (41), but king scallops showed a much higher biotransformation capacity than Pacific oysters (42). The role of enzymes and bacteria in the transformation of PST underpins detoxification processes

by shellfish (43, 44). In this study, the use of the amino acid L-cysteine in the modified protocol allowed conversion of GTX1&4 and GTX2&3 congeners to neosaxitoxin (NEO) and STX, respectively, to increase the cross-reactivity of the Neogen test.

**Rapid Test Kit Results**

Although samples were sent to 17 laboratories, 1 laboratory had issues with import certifications and only 16 laboratories were able to perform the tests. Laboratories returned results for six replicates of each sample, with the exception of one laboratory that spilled one replicate. Thus, each concentration in each matrix measured by each protocol had 96 replicate results, except the 0.74 mg STX·2HCl eq/kg oyster sample measured via the modified protocol, which had 95 replicates (Table 2).

**Standard Protocol**

All laboratories returned negative results for all the samples in both matrixes when PST levels were <0.025 mg STX·2HCl eq/kg. Almost all laboratories reported six out of six positive results in all oyster replicates when the analytical method indicated the PST level was ≥0.74 mg STX·2HCl eq/kg; only one laboratory reported five out of six positive results at 0.97 mg STX·2HCl eq/kg. With mussels, laboratories reported six out of six positives at PST concentrations ≥0.70 mg STX·2HCl eq/kg, with the exception of one laboratory reporting two out of six positives at 0.70 mg STX·2HCl eq/kg and another reporting five out of six positives at 1.13 mg STX·2HCl eq/kg (Table 2). The strong homogeneity of results from the different laboratories in each matrix at low and high PST levels (below detection and above the regulatory limit by the analytical method) is shown by the low *s<sub>p</sub>*, laboratory SDs, and *s<sub>R</sub>* (<0.5; Table 3).

The LPOD of PST in each matrix at each concentration is summarized in Table 3, along with 95% confidence limits. The LPOD in oysters using the standard protocol was relatively low (0.18) at the 0.25 mg STX·2HCl eq/kg concentration. LPOD increased as the PST level increased and was ≥0.99 at PST concentrations above 0.70 mg STX·2HCl eq/kg. In contrast, the test was shown to be highly sensitive for mussels with high POD at relatively low PST levels. The LPOD was ≥0.95 for all PST concentrations above 0.20 mg STX·2HCl eq/kg (Table 3 and Figure 1). These results indicate positive detections will occur at PST concentrations well below the regulatory limit when using the LFIA Neogen kit with the standard protocol in mussels, similar to the SRT kit, although not at very low PST concentrations (i.e., <0.20 mg STX·2HCl eq/kg) (14, 15, 23). The homogeneity test at these PST levels showed no significant difference between laboratories (*P*>0.05), indicating the method is reproducible at these levels. For oysters, the homogeneity test showed the method was also reproducible at the 0.74 mg STX·2HCl eq/kg level; however, this was not the case for mussels at 0.70 mg STX·2HCl eq/kg due to Laboratory 8 only returning two out of six positives (Table 2).

Within-laboratory reproducibility was used to estimate prediction intervals within which 95% of laboratories are expected to give a positive result. The prediction intervals are shown in Figure 2 alongside the individual POD for each laboratory for each concentration. As expected from Figure 1, the LPOD obtained for mussels was higher than for oysters using the standard protocol, with an estimated LPOD of 0.95 at 0.22 mg STX·2HCl eq/kg in mussels, and an estimated LPOD of 0.18 at 0.25 mg STX·2HCl eq/kg in oysters.

Linear interpolation of the LPOD curves was used to estimate the LOD, the LPOD at the regulatory limit of 0.8 mg STX·2HCl eq/kg, and the 95% prediction interval at this value (Table 4). The estimated LOD for mussels was 0.316 mg STX·2HCl eq/kg, less than half the regulatory limit,

**Table 2. Number of positive test results from six replicates recorded by all the laboratories<sup>a</sup>**

Protocol	Matrix	Total PST, mg STX·2HCl eq/kg <sup>b</sup>	No. of participant labs															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Standard	Oyster	<0.025	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0.25	2	3	0	0	2	2	0	0	3	0	3	0	0	0	2	0
		0.74	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
		0.97	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	Mussel	<0.025	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0.22	6	5	6	6	6	6	6	4	6	6	6	6	4	6	6	6
		0.70	6	6	6	6	6	6	6	2	6	6	6	6	6	6	6	6
1.13	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6		
Modified	Oyster	<0.025	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0.25	3	2	2	0	2	2	0	1	3	0	2	0	2	1	6	0
		0.74	6	6	6	5	6	5 <sup>c</sup>	6	6	5	6	6	4	6	6	6	6
		0.97	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	5
	Mussel	<0.025	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
		0.22	5	6	6	1	6	2	5	4	5	4	6	5	5	4	5	1
		0.70	6	6	6	5	6	6	6	6	6	6	6	6	6	6	6	3
1.13	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6		

<sup>a</sup> Participant numbers were randomly assigned and are not linked to the author list affiliation.

<sup>b</sup> Total PST shown is the average obtained from three laboratories performing the analytical method (as per Table 1).

<sup>c</sup> Five replicates only due to a spillage of one replicate.

**Table 3. Estimated parameters summarizing the results obtained by the 16 laboratories**

Protocol	Matrix	Total PST,mg		No. of positives	LPOD (95% CI) <sup>a</sup>	s <sub>r</sub>	Lab SDs	s <sub>R</sub>	Homogeneity test of LPODs
		STX·2HCl eq/kg	No. of replicates						
<b>Standard</b>	Oyster	<0.025	96	0	0 (0, 0.038)	0	0	0	NA <sup>b</sup>
		0.25	96	17	0.18 (0.069, 0.285)	0.351	0.160	0.386	0.019
		0.74	96	96	1 (0.962, 1.0)	0	0	0	NA
		0.97	96	95	0.99 (0.943, 0.998)	0.102	0	0.102	0.44
	Mussel	<0.025	96	0	0 (0.00, 0.038)	0	0	0	NA
		0.22	96	91	0.95 (0.884, 0.978)	0.209	0.081	0.224	0.049
		0.70	96	92	0.96 (0.898, 0.984)	0.129	0.158	0.204	<0.0001
		1.13	96	95	0.99 (0.943, 0.998)	0.102	0	0.102	0.44
<b>Modified</b>	Oyster	<0.025	96	0	0 (0, 0.038)	0	0	0	NA
		0.25	96	26	0.27 (0.137, 0.405)	0.398	0.209	0.449	0.007
		0.74	95	91	0.96 (0.897, 0.984)	0.195	0.054	0.202	0.149
		0.97	96	95	0.99 (0.943, 0.998)	0.102	0	0.102	0.44
	Mussel	<0.025	96	1	0.01 (0.002, 0.057)	0.102	0	0.102	0.44
		0.22	96	70	0.73 (0.587, 0.871)	0.387	0.229	0.450	0.002
		0.70	96	92	0.96 (0.90, 0.984)	0.171	0.109	0.202	0.001
		1.13	96	96	1.0 (0.962, 1.0)	0	0	0	NA

<sup>a</sup> CI = Confidence interval.

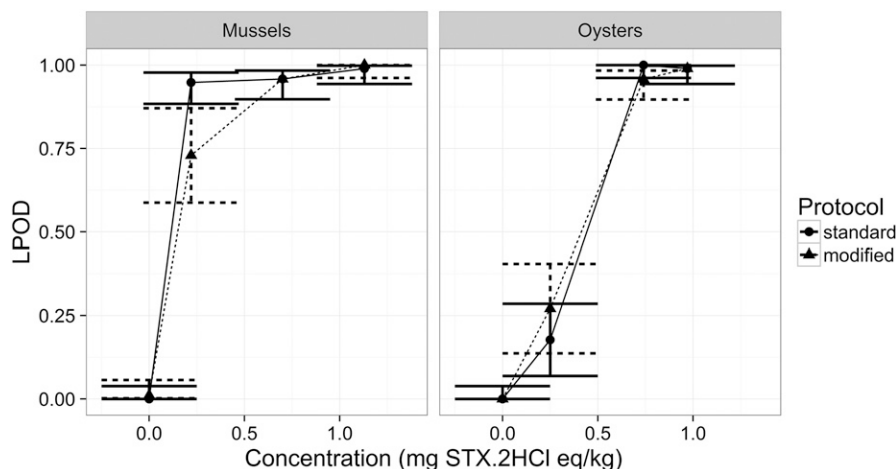
<sup>b</sup> NA = Not applied.

although 95% of laboratories were predicted to have an estimated LOD in mussels of less than 1.113 mg STX·2HCl eq/kg. The estimated LOD for oysters displayed a narrower range, with an average LOD of 0.710 and with 95% of laboratories predicted to have an estimated LOD of less than 0.731 mg STX·2HCl eq/kg. For both oysters and mussels, the LPOD at the regulatory level was high using the standard protocol (>0.96).

The LPOD is a measure of the probability of a positive response at a given concentration, but for regulatory purposes, it is the probability of a negative response at the regulatory limit that is more relevant to public safety. Defined as the β-error, the laboratory probability of a negative response can be obtained by subtracting the LPOD from 1. European Commission (EC) Decision 2002/657/EC, section 2.2, requires that screening methods must be validated in a documented manner, and the

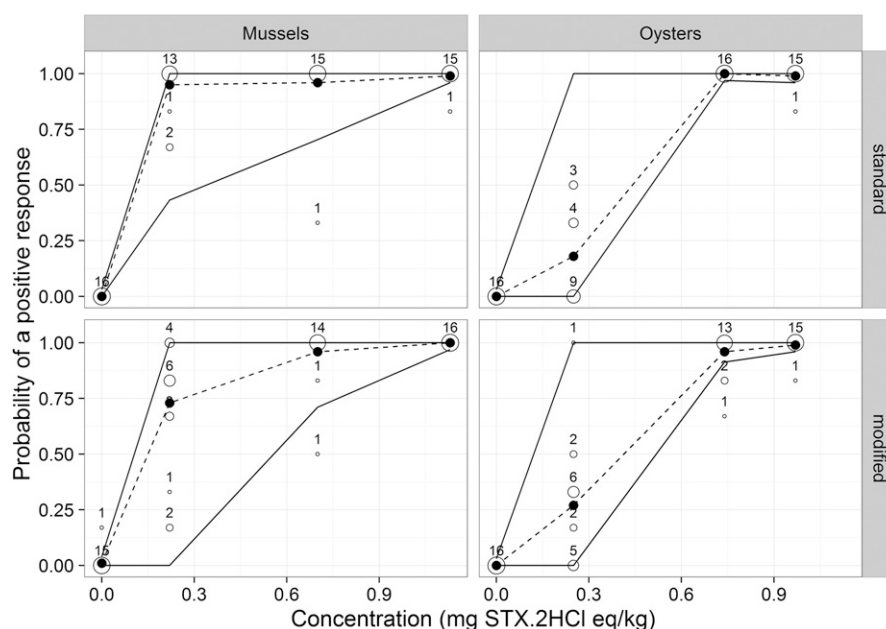
β-error at the regulatory limit must be <0.05 if they are to be used in conformity with Directive 96/23/EC (45). The estimated β-error at the 0.8 mg STX·2HCl eq/kg level complied with this requirement for mussels and oysters using the standard protocol; however, the prediction interval for 95% of tests in mussels to record a negative response at this level covered a wide range (0.000–0.237). Oysters showed a narrower 95% prediction interval, ranging from 0.000 to 0.033.

The β-error was also obtained through the use of a logistic regression model. The model fit for results from the mussel matrix was poor due to the large number of positives at low PST concentrations and, therefore, not included in the analysis. The logistic regression model for the data obtained from the oyster matrixes (shown in Figure 3) was used to determine the LPOD expected at various PST concentrations (Table 5). The model estimates that the LFIA Neogen kit used in oysters with the



**Figure 1. POD of PSTs in mussels and oysters at various PST concentrations by the Neogen kit using two protocols.**





**Figure 2.** POD for all laboratories (black dots) for each matrix using the standard and modified protocols over a range of PST concentrations. Estimates of the average POD (dashed line) and the POD for the 5th and 95th percentile of laboratories (solid lines) are shown. Open circles indicate the number of laboratories with the same POD, and their size is proportional to the number of laboratories with number of each listed above each circle.

standard protocol will return a positive result at 0.8 mg STX·2HCl eq/kg 100% of the time, and at 0.6 mg STX·2HCl eq/kg will detect a positive response 94% of the time (i.e., a  $\beta$ -error of 0.00 and 0.06, respectively). The LPOD at lower PST concentrations decreased, meaning the probability of a positive result occurring at half the regulatory limit is estimated to be 0.57. The  $\beta$ -error at 0.8 mg STX·2HCl eq/kg estimated via modeling was similar but slightly lower than that estimated via linear interpolation of the POD curves (0.003).

#### Modified Protocol

Similar to the standard protocol, laboratories returned zero out of six positive results for the samples at <0.025 mg STX·2HCl eq/kg in both matrixes, except for Laboratory 5, which returned one positive result for the mussel homogenate. One laboratory reported five out of six positive results in the oyster matrix when the analytical method indicated the total PST level was 0.97 mg STX·2HCl eq/kg; all other laboratories reported six out of six positives in both matrixes at this PST concentration. Results were more variable for both the oyster and mussel matrixes at the 0.74 and 0.70 mg STX·2HCl eq/kg concentrations, returning a total of 91 and 92 positive tests (out of 95 and 96), respectively. The strong homogeneity of results

from all laboratories at negligible PST levels and PST levels above the regulatory limit is shown by the low  $s_r$ , laboratory SDs, and  $s_R$ , leading to the homogeneity test showing no significant difference between laboratories ( $P > 0.05$ ). Thus, this protocol also resulted in reproducible results at these PST levels in both matrixes. As with the standard protocol, the homogeneity test showed the method was reproducible in oysters at 0.74 mg STX·2HCl eq/kg, but not in mussels at a similar concentration (Table 3).

The LPOD and 95% confidence limits of PST showed relatively high detection rates at low PST concentrations in mussels (0.73 LPOD at 0.22 mg STX·2HCl eq/kg; Table 3). Thus, a high number of positive results at PST concentrations below the regulatory limit are expected using this protocol on mussels, but less than with the standard protocol (0.95 LPOD at 0.22 mg STX·2HCl eq/kg). The LPOD of the oyster homogenates was relatively low (0.27) at the 0.25 mg STX·2HCl eq/kg concentration, and it also increased as PST levels increased ( $\geq 0.96$  for samples with PST concentrations  $\geq 0.74$  mg STX·2HCl eq/kg).

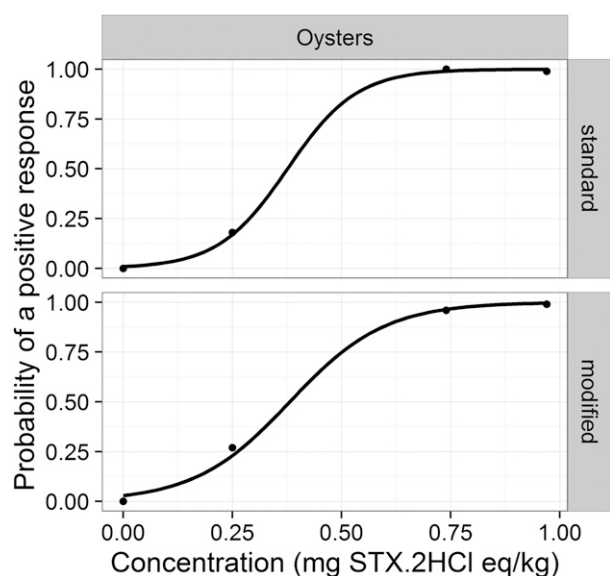
As with the standard protocol, the estimated LPOD in both matrixes complies with the requirement of EC Decision 2002/675/EC of having a  $\beta$ -error of <0.05 at the regulatory level. Again, the prediction interval for negative responses in 95% of tests in mussels covered a wide range (0.000–0.229); in contrast,

**Table 4.** LPODs at 0.8 mg STX·2HCl eq/kg (with 95% prediction intervals) and LODs calculated by linear interpolation

Protocol	Mussels		Oysters	
	Estimated LPOD (95% PI) <sup>a</sup>	Estimated LOD <sup>b</sup>	Estimated LPOD (95% PI)	Estimated LOD
Standard	0.966 (0.763, 1.00)	0.316 (1.113)	0.997 (0.967, 1.00)	0.710 (0.731)
Modified	0.968 (0.771, 1.00)	0.682 (1.098)	0.966 (0.925, 1.00)	0.734 (0.921)

<sup>a</sup> PI = Prediction interval.

<sup>b</sup> In parentheses: 95% of laboratories are predicted to have an LOD lower than the concentration shown.



**Figure 3.** LPOD modeled by logistic regression across a range of PST concentrations in oysters using the LFIA Neogen kit with both the standard and modified protocols.

oysters showed a narrower 95% prediction interval (0.000–0.075; Table 4). The estimated LOD for both matrixes using the modified protocol was lower than the regulatory limit; however, 95% of laboratories were predicted to have an LOD of less than 1.098 mg STX·2HCl eq/kg for mussels and 0.921 mg STX·2HCl eq/kg for oysters. LPOD calculations by logistic regression modeling for oysters using the modified protocol were 0.98 at 0.8 mg STX·2HCl eq/kg. This translates to a  $\beta$ -error of 0.02 at 0.8 mg STX·2HCl eq/kg, similar but slightly lower than that obtained via linear interpolation of the LPOD curves (0.034 at 0.8 mg STX·2HCl eq/kg; Figure 3; the fit for mussels was poor and was not included). The LOD values estimated through modeling for oysters using both the standard and modified protocols were 0.60 and 0.70 mg STX·2HCl eq/kg, respectively, which were lower than those estimated via interpolation and below the regulatory limit.

Jawaid et al. (25), who developed the Neogen kit (as per the standard protocol), observed that STX·2HCl (as an individual toxin standard) generated a positive response at a mean concentration ( $n = 3$ ) of 0.68 mg/kg, but they obtained

positive results in mussels and oysters from 0.34 and 0.39 mg STX·2HCl eq/kg, respectively (values recalculated using TEFs recommended by the FAO/WHO). In contrast, Harrison et al. (16) obtained positive results from concentrations of 0.43 and 0.49 mg STX·2HCl eq/kg in oysters and mussels, respectively, using the Neogen test with the standard protocol (values calculated from their positive results showing the percentages of individual PST analogs and total toxicity using FAO/WHO TEFs). Dorantes-Aranda et al. (17) observed positive results from concentrations of 0.40 mg/kg for STX·2HCl individual toxin standard, which correlates better with the findings of Harrison et al. compared with Jawaid et al. when they used individual STX·2HCl toxin standard, but correlates well with Jawaid et al.'s oyster sample positive results (from 0.39 mg STX·2HCl eq/kg). Dorantes-Aranda et al. modified the dilution for the toxin extraction step to alter the Neogen test to obtain positive results from 0.50 to 0.60 mg STX·2HCl eq/kg and observed positive results in mussels and oysters from 0.53 and 0.73 mg STX·2HCl eq/kg, respectively (modified Neogen protocol, but without the conversion step). However, four positive results (13%) were also obtained at 0.04–0.35 mg STX·2HCl eq/kg in oysters (false-noncompliant). These observations confirm the findings of the single-laboratory study of the Neogen test (26) in which 100% of positive results were obtained for mussels and oysters at  $>0.4$  and  $>0.7$  mg STX·2HCl eq/kg, respectively. The low LOD calculated for mussels by linear interpolation using the standard protocol in the present interlaboratory study (0.316 mg STX·2HCl eq/kg) is close to the concentration from which Jawaid et al. (25) obtained positive results in mussels (0.34 mg STX·2HCl eq/kg; but not described as an LOD). This was also supported by the single-laboratory study in which a POD of  $\geq 0.9$  was obtained for mussels with  $\geq 0.25$  mg STX·2HCl eq/kg.

The toxin profile variability of the shellfish homogenates used in this study may have influenced obtaining a lower LOD for mussels compared with oysters when using the standard protocol. At the lowest PST level (0.2 mg STX·2HCl eq/kg), mussels were dominated with dcSTX (by 50%, originating from a *G. catenatum* bloom), whereas oysters were predominantly contaminated with GTX2&3 (by 67%, originating from an *A. tamarense* bloom). Given that the cross-reactivity of the Neogen test for these two PST analogs were 56 and 23%, respectively, a higher number of positives in mussels at low PST levels was observed, which may have led to an estimation of a lower LOD using the standard protocol. In the SLV of the Neogen test, which used the modified protocol in dilution series of varying toxin profiles in mussels and oysters, it was observed, however, that the difference in POD was due to matrix differences, as opposed to the toxin profile (26). This suggests that the toxin profile had an impact only when using the standard protocol in which no conversion of PST occurred, and dcSTX and GTX2&3 were present compared with the modified protocol in which GTX2&3 was hydrolyzed to STX (46) in order to increase reactivity from 23 to 100% (supported by oyster LPOD values of 0.18 and 0.27 for standard and modified protocols, respectively, at 0.25 mg STX·2HCl eq/kg; Table 3). (Note: GTX1&4 is converted to NEO using the modified protocol, but this analog was not present in the mussel matrix containing dcSTX and GTX2&3.) Conversion of GTX1&4 and GTX2&3 to NEO and STX, respectively, involves the reduction of the *O*-sulfate group (46); it is unclear, however, what the conversion products of dcSTX are, although the analog *Gymnodinium catenatum* toxin 3 (GC3) has

**Table 5.** LPODs for nominal PST concentrations in oysters calculated by binomial logistic regression modeling

Protocol	PST, mg STX·2HCl eq/kg	LPOD
<b>Standard</b>	0.0	0.01
	0.2	0.10
	0.4	0.57
	0.6	0.94
	0.8	1.00
<b>Modified</b>	0.0	0.03
	0.2	0.16
	0.4	0.54
	0.6	0.88
	0.8	0.98

been found as a hydroxybenzoate derivate of dcSTX, whereas *Gymnodinium catenatum* toxin 1 and 2 (GC1 and GC2) are hydroxysulfate derivatives of *Gymnodinium catenatum* toxin 3 (47). Despite the fact that the toxicity of these new derivatives is still to be determined, it is possible that they are less reactive with the Neogen test. Additionally, the change in response may be due to the higher dilution with the modified protocol because the LPOD for the mussel homogenate at 0.22 mg STX·2HCl eq/kg was lower using the modified protocol compared with the standard protocol (0.73 versus 0.95 respectively; Table 3), and the LOD values calculated by linear interpolation were 0.682 and 0.316, respectively.

## Conclusions

This study highlights the challenge of the different ways of reporting total PST toxicity by different countries that use different TEFs. New TEFs were recommended by FAO/WHO in 2016, but may need to be updated after more recent TEFs based on oral toxicities were recently published (48). If adopted internationally, these TEFs will facilitate standard toxicity calculations and enable comparisons among countries. Furthermore, this will create a higher degree of accuracy and confidence for the international trade of shellfish.

The Neogen test showed good homogeneity across all 16 laboratories using both standard and modified protocols. The Neogen test may be acceptable for screening purposes in accordance with EC directives for oysters, especially using the standard protocol. The standard protocol showed a more conservative approach for oysters, with a probability of a negative response ( $\beta$ -error) at the regulatory limit of less than 0.033 on 95% of occasions and an LOD of 0.710 mg STX·2HCl eq/kg and 0.734 mg STX·2HCl eq/kg for the standard and modified protocols respectively, both of which were below the regulatory limit applied in most countries (0.8 mg STX·2HCl eq/kg). Using the standard protocol, 95% of laboratories were expected to have an LOD less than the regulatory level (0.731 mg STX·2HCl eq/kg). However, using the modified protocol for oysters, the LOD below which 95% of laboratories fell was 0.921 mg STX·2HCl eq/kg. The test may not be acceptable for use with mussels in the European Union due to the high variability in the LPOD at the regulatory level of 0.8 mg STX·2HCl eq/kg and high variation in the LOD. For the toxin profiles used in the present study, the estimated LOD for mussels was 0.316 and 0.682 using standard and modified protocols, respectively. However, the LOD estimated with a 95% prediction interval was 1.1 mg STX·2HCl eq/kg using both protocols, which is above the regulatory limit. The test may still be useful for mussels for nonregulatory purposes as part of a business risk mitigation program. The modified protocol with the conversion step could be further explored to be applied in shellfish samples that are highly contaminated with GTX1&4. This study has proven the efficacy and applicability of the Neogen kit for oysters using the standard protocol. The authors recommend this rapid kit for use in industry testing, with parallel testing by PST-regulation laboratories for potential future official control.

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