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FOOD CHEMICAL CONTAMINANTS

# Interlaboratory Evaluation of Multiple LC–MS/MS Methods and a Commercial ELISA Method for Determination of Tetrodotoxin in Oysters and Mussels

Andrew D. Turner [1], Karl J. Dean [1], Monika Dhanji-Rapkova [1], Sonia Dall'Ara [2], Florella Pino [2], Claire McVey [3], Simon Haughey [3], Natasha Logan [3], Christopher Elliott [3], Ana Gago-Martinez [4], Jose Manuel Leao [4], Jorge Giraldez [4], Ryan Gibbs [5], Krista Thomas [6], Ruth Perez-Calderon [6], Dermot Faulkner [7], Hugh McEneny [7], Veronique Savar [8], Damien Reveillon [8], Philipp Hess [8], Fabiola Arevalo [9], J. Pablo Lamas [9], Eva Cagide [10], Mercedes Alvarez [10], Alvaro Antelo [10], Mirjam D. Klijnstra [11], Michalina Oplatowska-Stachowiak [12], Tim Kleintjens [12], Nermin Sajic [12], Michael J. Boundy [13], Benjamin H. Maskrey [14], D. Tim Harwood [15], Jesús M. González Jartín [16], Amparo Alfonso [16], and Luis Botana [16]

[1]Centre for Environment, Fisheries and Aquaculture Science, Barrack Rd, The Nothe, Weymouth, Dorset DT4 8UB, UK, [2]Fondazione Centro Ricerche Marine, Viale A. Vespucci, 2, 47042 Kleintjens FC, Italy, [3]Queen's University Belfast, Institute for Global Food Security, 19 Chlorine Gdns, Belfast BT9 5DL, UK, [4]University of Vigo, Department of Chemistry, Bloque E, Planta 2, Despacho 17, As Lagoas, Marcosende, 36310 Vigo, Spain, [5]Canadian Food Inspection Agency, 1992 Agency Dr, Dartmouth, NS B3B 1Y9, Canada, [6]National Research Council of Canada, Biotoxin Metrology, 1411 Oxford St, Halifax, NS B3H 3Z1, Canada, [7]Agri-Food and Biosciences Institute, Veterinary Sciences Division, Stoney Rd, Belfast BT4 3SD, UK, [8]Ifremer, PHYTOX Unit, F-44000 Nantes, France, [9]Instituto Tecnoloxico para o Control do Medio Mariño de Galicia, Peirao de Vilaxoan, s/n, 36611 Vilagarcia de Arousa, Pontevedra, Spain, [10]Laboratorio Cifga, Avenida Benigno Rivera, No. 56, 27003 Lugo, Spain, [11]Wageningen Food Safety Research, Wageningen University and Research, Akkermaalsbos 2, 6708WB Wageningen, The Netherlands, [12]R-Biopharm Nederland B.V., Beijerinckweg 18, 6827 BN Arnhem, The Netherlands, [13]Cawthron Institute, 98 Halifax St East, Nelson 7010, Private Bag 2, Nelson 7042, New Zealand, [14]CTL Ltd, Pakefield Rd, Lowestoft, Suffolk NR33 0HT, UK, [15]CNC, 3 Elms St, Stoke 7011, New Zealand, [16]Universidade De Santiago De Compostela, Deparamento de Farmacologa, Facultad de Veterinaria, 27002 Lugo, Spain

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Corresponding author's e-mail: andrew.turner@cefas.co.uk

## Abstract

**Background:** Given the recent detection of tetrodotoxin (TTX) in bivalve molluscs but the absence of a full collaborative validation study for TTX determination in a large number of shellfish samples, interlaboratory assessment of method performance was required to better understand current capabilities for accurate and reproducible TTX quantitation using chemical and immunoassay methods.

**Objective:** The aim was to conduct an interlaboratory study with multiple laboratories, using results to assess method performance and acceptability of different TTX testing methods.

**Methods:** Homogenous and stable mussel and oyster materials were assessed by participants using a range of published and in-house detection methods to determine mean TTX concentrations. Data were used to calculate recoveries, repeatability, and reproducibility, together with participant acceptability z-scores.

**Results:** Method performance characteristics were good, showing excellent sensitivity, recovery, and repeatability. Acceptable reproducibility was evidenced by HorRat values for all LC–MS/MS and ELISA methods being less than the 2.0 limit of acceptability. Method differences between the LC–MS/MS participants did not result in statistically different results. Method performance characteristics compared well with previously published single-laboratory validated methods and no statistical difference was found in results returned by ELISA in comparison with LC–MS/MS.

**Conclusion:** The results from this study demonstrate that current LC–MS/MS methods and ELISA are on the whole capable of sensitive, accurate, and reproducible TTX quantitation in shellfish. Further work is recommended to expand the number of laboratories testing ELISA and to standardize an LC–MS/MS protocol to further improve interlaboratory precision.

**Highlights:** Multiple mass spectrometric methods and a commercial ELISA have been successfully assessed through an interlaboratory study, demonstrating excellent performance.



Tetrodotoxins (TTXs) are a family of potent neurotoxins which are water-soluble, thermostable, and found in both aquatic and terrestrial environments, but most commonly associated with a range of marine organisms, notably in species of fish from the Tetraodontidae family, such as pufferfish (1–4). The parent compound, TTX, together with a suite of structurally related analogues, are thought to be produced by certain bacterial species including *Vibrio, Pseudomonas, Bacillus, Alteromonas,* and *Aeromonas* (5–9), although the biosynthetic pathway for TTX production has yet to be uncovered (7, 10–13). The toxins are sodium channel blockers, binding to receptor site 1, resulting in intoxication, including fatalities at high-dose levels, in human consumers of contaminated seafood products (14–17). In addition to TTX prevalence in pufferfish (18), TTXs have also been reported in marine invertebrates, including echinoderms, crustaceans, flatworms, ribbon worms, and molluscs (19–24). TTX research has generally been driven by regional intoxication cases in humans [reviewed in (16)] and in dogs (25). However, TTX was found recently in European seas, in both gastropods (26, 27) and bivalves (9, 28–37). Consequently, the apparent increase in occurrence of TTX-positive shellfish together with the notable absence of regular monitoring [with the exception of the Netherlands (30)] and TTX regulations for bivalve molluscs is a concern for the safety of seafood consumers globally (4, 36).

The European Food Safety Authority (EFSA) published an opinion on TTX in molluscs, proposing a concentration of 44 μg/kg TTX in edible shellfish tissue as an appropriate safety threshold, below which TTX would not impart adverse health effects in human seafood consumers (15). In addition, the opinion recommended the need for generation of more global prevalence data together with the availability of suitable, validated analytical methods for shellfish testing. To date several assay types have been published for TTX determination, including bioassays, immunoassays, cell-based assays, aptamer-based assays, biosensors, and chemical instrumentation methods [as reviewed by (4)]. Instrumental techniques include LC coupled to fluorescence detection, with detectors subsequently being replaced by single-quadrupole mass spectrometry and more recently by tandem mass spectrometry coupled to ultra high liquid chromatography (UHPLC–MS/MS). The use of UHPLC–MS/MS instruments with hydrophilic interaction liquid chromatography (HILIC) with selected reaction monitoring (SRM) acquisition of characteristic precursor–product ion transitions has ultimately become the most commonly used detection method for TTXs in marine organisms (23, 36). Variations of this approach have been subjected to single-laboratory validation studies (34, 38, 39), published as a method standard (40) and incorporated into a collaborative study for paralytic shellfish toxins (PST) and TTX combined (41). However, no official method currently exists for the determination of TTX in bivalves, so research to date incorporates a wide variety of protocols, potentially resulting in an unacceptable level of variability in performance between the quantitative determinations from different laboratories. In addition to quantitative mass spectrometric methods, TTX antibodies have been used to develop both direct and indirect immunoassays in a variety of formats, mainly in order to facilitate rapid screening methods (4). One such assay, the EuroProxima TTX assay, is available commercially in Europe and facilitates the quantitative analysis of TTX in shellfish and fish using a competitive enzyme immunoassay in 96-well microtiter plate format, with a validated LOD of 9.4 μg/kg in shellfish and a total test incubation time of 90 min (42).

This study aimed to evaluate the performance and comparability of multiple testing methods. This is especially important given the absence of a formal collaborative validation for a large number of samples, with the only exception being the recent validation of the PST LC–MS/MS method where three of the study samples also contained TTX (41). Naturally contaminated and artificially fortified shellfish materials were shipped to

participating laboratories for analysis by their preferred method. Results were assessed to determine the between-laboratory reproducibility, as well as method performance characteristics such as recovery and within-laboratory repeatability. Data were also used to generate participant z-scores as a measure of performance acceptability for each participant. Overall, the objective was to determine if the multiple LC–MS/MS methods currently employed for TTX testing around the world were suitable for routine monitoring of shellfish and do an initial assessment on whether the commercial immunoassay was capable of producing comparable results to the LC–MS/MS methods.

## Experimental

### *Reagents and Chemicals*

Certified reference material (CRM) standards for TTX used by participants were obtained either from Cifga (Lugo, Spain) or the National Research Council Canada (NRCC, Halifax, NS, Canada). Solvents used by study participants for chromatographic mobile phases were LC–MS grade or better, with other solvents used for sample preparation and solid-phase extraction being HPLC grade. Participants were required to source their own calibration solution CRM and prepare working standards to enable quantitation by external calibration.

### *Materials*

The two shellfish species incorporated into the study were oysters and mussels. Naturally contaminated oysters (*Crassostrea gigas*) were collected on a weekly basis between June and August 2020 from Dorset, UK. The samples were transported under temperature-controlled conditions to the Cefas laboratory and shucked to remove the entire flesh. A minimum of ten oysters were combined for each weekly sample, thoroughly homogenized using a Waring high-speed blender and used for preliminary analysis of TTX concentrations. The study materials were prepared by combining different weekly homogenized tissue samples, using TTX-free oyster tissue homogenates for dilution. In total, eight separate oyster materials were prepared for the study, with at least 150 g of tissue for each, and 250 g for three of the materials that were to be provided as duplicate samples.

Toxin-free mussels (*Mytilus edulis*) were collected from western Scotland, UK and whole-flesh homogenate was spiked with TTX standard (Enzo, Exeter, UK) to prepare an additional three study materials. The TTX spike solution was calibrated against the certified reference standard solution obtained from the National Research Council of Canada (NRCC CRM-TTX; https://doi.org/10.4224/crm.2017.ttx.20170328). The fortified TTX concentrations were 44, 160, and 400 μg/kg, equating to the EFSA-recommended safe concentration threshold (15) and the shellfish monitoring program action limit in the Netherlands (30), as well as 20 and 50% of the maximum permitted limit (MPL) for total saxitoxin (STX) equivalents, respectively, noting the reported similarity between oral toxicity of TTX and STX (43). For each separate material, enough homogenate was prepared to provide aliquots for analysis by study participants and aliquots for assessment of both homogeneity and stability. All study samples were stored at −80°C until ready for shipment, whereupon they were shipped frozen under temperature-controlled conditions to participants. A summary of the materials prepared for shipment and collaborative assessment is shown in Table 1.

For homogeneity assessment, ten aliquots of each sample (excluding duplicate study samples) were randomly selected from the fill series, including the first and last aliquots dispensed. The ten selected samples were extracted using the acetic acid TTX extraction method and subjected to analysis using HILIC–MS/MS following the method of (39) under repeatability conditions. Analysis on the instrument was conducted in duplicate with the first batch in numerical order and the second randomly ordered.

The stability of TTX in the tissue samples was tested at two temperatures: freezer (−18°C) and fridge (+4°C), over a 32 day time period, using a reverse isochronous experimental design. Triplicate tissue aliquots were taken for each temperature regime at five different time points, specifically 0, 4, 7, 17 and 32 days exposure at each temperature. Once the samples had been exposed to the temperatures, all samples were extracted and analyzed in the same batch under repeatability conditions.

### *Analysis Methods*

Ten different LC–MS/MS methods were used, including those published as single-laboratory validations (35, 39, 44, 45), a collaborative validation (41), four different in-house methods, and the European Union Reference Laboratory for Marine Biotoxins (EURLMB) protocol as published on the EURLMB website (40). Table 2 summarizes the methods used. As expected, all participants used 1% acetic acid as the extraction solvent, with one exception (Laboratory 7) using acidified aqueous methanol. Eight labs used 5.0 g sample weights with the majority employing 1:1 sample-to-solvent extraction ratios (39, 41, 44). Six laboratories applied methods with lower sample weights, notably with Laboratory 9 employing an extraction method utilizing 0.2 g tissue, a single extraction step using grinding beads, and a sample to solvent ratio of 2:5. All participants used a single-step dispersive extraction, with the exception of Laboratory 15 who used a double-step extraction. A variety of cleanup approaches were taken resulting in a range of dilution factors prior to analysis (Table 2). All LC–MS/MS instruments used were modern systems, mostly Agilent, Waters and Sciex, capable of conducting highly sensitive, targeted MRM acquisition. The majority of participants used a Waters Acquity BEH Amide UHPLC analytical column (or Glycan equivalent; 44) with Laboratory 9 using instead a ZIC HILIC column from SeQuant. Method recovery data were made available by 11 participants, which they generated through either method validation experiments or sample/blank spiking during the study sample analysis. Despite the variety of methods, recoveries were quite similar overall (83 ± 11% across all participants, using mean recoveries from each laboratory), with the notable exception of Laboratory 3, which reported recoveries ranging from 9–94%. Whilst its mean recoveries were generally acceptable, occasional recovery spikes were found to be very low, as a result of high concentrations of arginine interfering with the TTX response, even though previous work has shown these effects can be minimized through chromatography modifications (39). Five participants corrected TTX concentrations against recovery, while eight did not. Laboratory 7, used standard addition for quantitation purposes, showing a variation of approaches in relation to recovery correction across the laboratories.

### *Immunoassays*

Two laboratories used a commercial TTX ELISA, specifically the EuroProxima kit (5191TTX). The method protocol was followed

**Table 1.** Summary of study materials prepared showing sample numbers, shellfish species, mean (± SD), and TTX concentration (μg/kg) determined during homogeneity assessment by study organizer, and comments on sample content

| Sample number | Species | Mean TTX concentration, μg/kg | RSD, % | Comments |
|---|---|---|---|---|
| RT-1 | Oysters | 15.9 ± 0.3 | 1.8 | |
| RT-2 | Oysters | 32.9 ± 1.1 | 3.2 | |
| RT-3 | Oysters | 53.7 ± 1.6 | 3.0 | |
| RT-4 | Oysters | 58.4 ± 1.1 | 1.9 | |
| RT-5 | Oysters | 40.7 ± 1.1 | 2.8 | |
| RT-6 | Oysters | 39.0 ± 1.1 | 3.0 | |
| RT-7 | Oysters | 40.7 ± 1.1 | 2.8 | Duplicate of RT-5 |
| RT-8 | Oysters | 28.7 ± 1.7 | 5.9 | |
| RT-9 | Oysters | 24.9 ± 2.0 | 8.0 | |
| RT-10 | Oysters | 15.9 ± 0.3 | 1.8 | Duplicate of RT-1 |
| RT-11 | Oysters | 53.7 ± 1.6 | 3.0 | Duplicate of RT-3 |
| RT-12 | Mussels | 49.0 ± 1.6 | 3.2 | 44 μg/kg recovery spike |
| RT-13 | Mussels | 150 ± 3.1 | 2.1 | 160 μg/kg recovery spike |
| RT-14 | Mussels | 400 ± 5.9 | 1.5 | 400 μg/kg recovery spike |

exactly by both participants. The assay uses an anti-mouse-horseradish peroxidase (HRP) conjugate, employs a TTX standard range of 0 to 20 ng/mL, and has a reported LOD of 7 and 9.4 μg/kg in fish and shellfish, respectively, with a detection capability CCβ of 20 μg/kg. Validated recoveries range from 104 to 110% when measured between 20 and 40 μg/kg in shellfish (46) with associated intra- and inter-assay RSDs of 6.6 and 7.3%, respectively. The protocol for the assay involved pipetting antibody with standards and samples into the well plate, incubating for 30 mins at 20–25°C before washing, adding conjugate solution, and incubating for a further 30 mins. After a second washing, substrate was added and a third 30 min incubation step was conducted prior to stopping the reaction and measuring spectrophotometric absorbance at 450 nm. Optical densities measured by the plate reader were used to calculate TTX concentrations against the responses generated from TTX standards.

### *Method Performance and Statistical Evaluation*

Quantitative results were sent to the study organizer from each participant using a blank results template, ensuring all data was received in the same format. Toxin concentrations were calculated as μg/kg shellfish tissue for each toxin analogue measured by the participant's detection methods. Data was summarized and initially used to calculate mean toxin concentrations for each material and each detected analogue, together with associated SDs and percentage RSD. These data were initially screened visually to determine toxin profiles within each sample and to decide which analogues would be appropriate for further statistical assessment.

Three TTX-spiked mussel samples (RT-12, RT-13, and RT-14) were evaluated for participant recovery by comparing participant results against expected concentrations. Recoveries at the three concentration levels were calculated for all laboratories and averaged to assess mean recovery across all methods applied.

The repeatability (or within-laboratory precision) was determined through comparison of concentrations determined for each blind duplicate, subsequently allowing calculation of the RSD under repeatability conditions ($RSD_r$). The variabilities in quantitative data were also assessed to determine between-laboratory reproducibility of the methods utilized. RSDs were calculated for each individual sample based upon the SD of the mean concentrations, allowing calculation of the RSD under reproducibility conditions ($RSD_R$). The acceptability of the reproducibility data across all participants was determined through calculation of the Horwitz ratio (HorRat), specifically comparing the calculated $RSD_R$ values against predicted $RSD_R$ values determined from the Horwitz curve (47). For analyte concentrations ≤100 μg/kg, Thompson's theory was used, giving a Predicted Relative Standard Deviation ($PRSD_R$) of 22% (48). Data acceptability was calculated following international published guidance (49–51). Assigned values were determined from participant consensus results using an iterative robust statistical approach, allowing calculation of z-scores for each participant result. A score of zero implies a perfect result and values between −2 and +2 deemed acceptable. Values lower than −2 or higher than +2 indicate questionable results, with scores below −3 and above +3 deemed unacceptable (51). z-scores were replaced by with zeta (z') scores, if the standard uncertainty was greater than 0.3 times the target SD of the proficiency assessment (49, 50). In addition, the participant data was assessed to determine any statistical differences between the results generated by ELISA versus LC–MS/MS as well as between participating laboratories and different method protocols (using RStudio, version 1.3.1056).

Full details of calculations performed to assess repeatability, reproducibility, and acceptability are provided in the Supplemental Information.

## Results and Discussion

### *Study Sample Suitability*

Assessment of toxin concentrations in the study materials prior to the study showed the main toxin analogue present in the naturally contaminated oyster samples was TTX. Only small chromatographic peaks were present for other TTX analogues, but at levels below method LOD. Consequently, all sample assessment was conducted with a focus on TTX only, noting also the lack of individual calibrants for other TTX analogues.

Homogeneity was assessed for each of the 14 study materials using ten samples, each analyzed in duplicate. Mean, SD and percentage RSDs were calculated for both the numerically ordered group and the randomly ordered group, as well as the

**Table 2.** Summary of UHPLC–MS/MS methods utilized by participants (concentrations in μg/kg)

| | Laboratory (participant) identifier number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | 1 | 2 | 3 | 4 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Reference | Turner et al., 2017 (39) | Turner et al., 2017 (39) | Boundy et al., 2015 (44) | In-house | Rodriguez et al., 2018 (45) | In-house | Turner et al., 2020 (41) | Réveillon et al., 2021 (35) | Turner et al., 2017 (39) | Turner et al., 2020 (41) | In-house | EURLMB, 2017 (40) | Turner et al., 2017 (39) | In-house |
| Mass extracted, g | 5.0 | 5.0 | 5.0 | 5.0 | 1.0 | 2.0 | 3.0 | 0.2 | 5.0 | 5.0 | 1.0 | 5.0 | 5.0 | 4.0 |
| Extraction type | Single dispersive | Single dispersive | Single dispersive | Single dispersive | Single dispersive (n = 3) | Single dispersive + ultrasonication | Single dispersive | Single dispersive with bead grinding | Single dispersive | Single dispersive | Single dispersive | Single, dilute to 10 mL | Single dispersive | Double, dilute to 10 mL |
| Sample: solvent ratio | 1:1 | 1:1 | 1:1 | 1:1 | 1:1 | 2:3 | 1:1 | 2:5 | 1:1 | 1:1 | 1:1 | 1:1 | 1:1 | 2:5 |
| Solvent | 1% HAC[a] | 1% HAC | 1% HAC | 1% HAC | 1% HAC | methanol/0.3M HAC in water (1:1) | 1% HAC | 1% HAC | 1% HAC | 1% HAC | 1% HAC | 1% HAC | 1% HAC | 1% HAC |
| Cleanup | Yes | Yes | Yes | Yes | No | No | Yes | No | Yes | Yes | Yes | Yes | Yes | Yes |
| Cleanup used | Supelco Envi-carb | Supelco Envi-carb | Supelco Envi-carb | Merck Hybrid Phospholipid Ultra | — | — | Supelco Envi-carb | —- | Supelco Envi-carb | Supelco Envi-carb | Supelco Envi-carb | Supelco Envi-carb | Supelco Envi-carb | 1:1 precipitation with MeCN[b] |
| Dilution factor (total method) | 40 | 40 | 40 | 40 | 6 | 2 | 40 | 2.5 | 40 | 40 | 40 | 40 | 40 | 5 |
| UHPLC used | Agilent 1290 Infinity II | Waters Acquity I-Class | Sciex Exion LC | Sciex Exion LC | Agilent 1290 Infinity | Waters Acquity I-Class | Waters Acquity | Shimadzu Nexera UFLC | Waters Acquity I-Class | Waters Acquity H-Class | Waters Acquity I-Class | Agilent 1290 Infinity II | Waters Acquity H-Class | Agilent 1290 Infinity II |
| MS/MS used | Agilent 6495B | Waters Xevo TQ-S | Sciex 6500+ | Sciex 6500+ | Agilent 6460 | Waters Xevo TQ-S | Waters Xevo TQ | Sciex 5500 | Waters Xevo TQ-XS | Waters Xevo TQ-S | Waters Xevo TQ-S Micro | Agilent 6495 | Waters Xevo TQ-XS | Sciex 5500 |

(continued)

**Table 2.** (continued)

| Parameter | Laboratory (participant) identifier number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Column used | Waters BEH Amide 1 | Waters Glycan Amide 2 | Waters BEH Amide 3 | Waters BEH Amide 4 | Waters BEH Amide 6 | Waters BEH Amide 7 | Waters BEH Amide 8 | SeQuant ZIC HILIC 9 | Waters BEH Amide 10 | Waters Glycan Amide 11 | Waters BEH Amide 12 | Waters BEH Amide 13 | Waters BEH Amide 14 | Waters BEH Amide 15 |
| Column dimensions | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 3.5 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 150 mm × 2.1 mm; 1.7 μm | 100mm × 2.1 mm; 1.7 μm |
| Guard used | Waters BEH Amide | Waters Glycan Amide | None | None | Waters in-line filter | Waters BEH Amide | Waters BEH Amide | SeQuant ZIC HILIC | Waters BEH Amide | Waters BEH Amide | Waters BEH Amide | Waters BEH Amide | Waters BEH Amide | None |
| Standards used | Cifga | Cifga | Cifga | Cifga | Cifga | Latoxan and Cifga | Cifga | Cifga | Cifga | Cifga | NRCC | Cifga | NRCC | NRCC |
| Calibration matrix | Solvent[c] | Oyster extract | Solvent | Solvent | Oyster extract | Mussel extract | Sample RT-01 | Pacific oyster extract | Oyster extract | Mussel extract | Pacific oyster extract | Oyster extract | Mussel extract | NRC CRM-Zero-Mus extract |
| Concentration range, μg/kg | 1 to 2000 | 2 to 2400 | 2 to 1000 | 2 to 1000 | 2.3 to 600 | 0 to 250 | 11 to 2790 | 12.5 to 628 | 12.6 to 1004 | 12.8 to 559 | 0 to 600 | 10 to 160 | 0.2 to 313 | 8.8 to 610 |
| Method LOD, μg/kg | 1 | 2 | 1–10 | 1.4 | 2.3 | 5 | 3.2 | 5 | 3 | 7 | 8 | 0.9 | 8 | 9 |
| Method recovery | 80–90% mussels and oysters | 70–85% | 9–94% | 76–81% | 76% | — | 77% | 88% | 92% | 85–90% | 89% | 72–92% | 80–120% | — |
| Results recovery corrected | No | No | Yes, per sample | Yes, average for batch | Yes | Standard addition quant | Yes | No | Yes | No | No | No | No | No |

[a] HAC = Acetic acid.
[b] MeCN = Acetonitrile.
[c] Solvent = 80% MeCN, 0.25% HAC.
*Note*: ELISA methods not detailed in this table.

two duplicate groups combined. The sum of squares was calculated for both between groups (samples) and within groups (analytical) which was then used to calculate *F*-value and compared against *F*-critical values. Table 1 summarizes mean TTX concentrations quantified ± SD, with RSDs ranging from 1.5 to 8.0%. All *F*-test values were lower than *F*-critical, evidencing overall acceptable homogeneity of aliquots prepared for each of the study samples.

No degradation was observed for TTX over the 32 day stability study period in any of the materials stored at −20°C. For samples stored at +4°C, the majority showed good stability up to 16 days, after which there was evidence for slight TTX degradation at the 32 day time point. Consequently, the assessment confirmed that samples should be stored frozen, and that transportation under chilled, non-frozen conditions, should not take longer than 2 weeks. As such, and given that all study materials were transported frozen and received by study participants in less than 1 week, there was good evidence for study sample stability in all samples analyzed. Results are summarized graphically in Supplemental Figure S1.

### *TTX Results*

Participant data confirmed the dominance of the parent TTX in the TTX profiles, with the near-absence of other TTX analogues. All laboratories reported the detection of TTX in all study samples, with all of the reported concentrations above the method LOQ with the exception of two samples for Laboratory16, utilizing the immunoassay. Samples RT-7 and RT-6 were not analyzed by one and two laboratories, respectively, due to a shipping error.

Table 3 summarizes the TTX concentrations quantified in each study material by all participants, together with the calculated mean concentrations. TTX analogues 6,11-dideoxy-TTX, 4,9-anhydro-TTX, 5,6,11-trideoxy TTX, and 11-deoxy TTX were reported by 3, 5, 2, and 3 participants, respectively. Mean TTX analogue concentrations determined from these participants are summarized in Table 4.

### *Recovery*

TTX recoveries calculated from all participant data are illustrated in Figure 1. The mean overall recoveries were acceptable at 115 ± 22, 102 ± 18, and 101 ± 17% for the each of the three mussel samples, spiked at 44, 160, and 400 μg/kg respectively. For study material RT-12 spiked at the low concentration, equivalent to the EFSA-recommended health threshold (15), 13 out of 16 (81%) laboratories achieved recoveries between 70 and 130%, with the three remaining laboratories achieving recoveries around the 150% level (Laboratories 7, 13, and 14). Recoveries were more acceptable at the two higher concentrations of 160 and 400 μg/kg, with just one recovery result higher than 130% (Laboratory 14) at 160 μg/kg, and two recoveries outside of the 70 to 130% for the 400 μg/kg spiked materials. A full summary of the TTX percentage recovery results for each participant is provided in Supplemental Table S1. Overall, the results highlighted acceptable recoveries for the three mussel samples, noting that future work should incorporate recovery determination in a wider range of shellfish species.

### *Repeatability*

Repeatability standard deviations ($RSD_r$) values were 23, 14 and 12% in each of the three blind duplicate samples (Table 5). The highest variability of 23% was measured in the first duplicate containing the lowest toxin concentrations (mean = 17.1 μg/kg), equivalent to approximately one-third of the EFSA threshold limit of 44 μg/kg (15). Variability was notably lower in the other duplicates, containing higher concentrations of TTX (55.7 and 42.1 μg/kg mean values), with $RSD_r$ values of 14 and 12%, respectively. Consequently, the data show acceptable levels of within-laboratory precision for the determination of TTX concentrations in oysters and mussels using the variety of methods employed.

### *Reproducibility*

Table 6 summarizes the median concentrations and associated $RSD_R$ values calculated from the SD of values in single oyster and mussel samples across all laboratories, as well as the $RSD_R$ values determined from duplicate samples. Between-laboratory reproducibility varied from 0.16 to 0.35, with the lowest and highest values associated with samples containing TTX at the highest and lowest TTX concentration, respectively. $PRSD_R$ values calculated using the Horwitz equation (47) at concentrations >100 μg/kg and using (48) for lower levels enabled determination of HorRat values, which ranged from 0.73 to 1.60. Consequently, in comparison to the expected reproducibility estimated from the

**Table 3.** Summary of TTX/epi-TTX concentrations (μg/kg) quantified in each material across all 16 participating laboratories[a], together with associated mean concentration across all labs

| Material | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RT-1 | 13.2 | 16.0 | 14.9 | 19.4 | 17.3 | 15.8 | 11.8 | 12.8 | 22.1 | 17.0 | 13.0 | 17.5 | 33.0 | 9.5 | 24 | < 12.5 | 17.1 |
| RT-2 | 24.5 | 30.0 | 28.6 | 46.4 | 27.8 | 25.6 | 24.8 | 20.8 | 45.6 | 39.5 | 36.0 | 36.5 | 47.5 | 36.2 | 35 | 33.9 | 34.1 |
| RT-3 | 39.8 | 47.6 | 57.6 | 73.1 | 61.0 | 39.1 | 72.7 | 41.8 | 69.6 | 65.4 | 34.5 | 58.5 | 90.7 | 57.0 | 63 | 41.2 | 57.9 |
| RT-4 | 42.6 | 50.4 | 46.4 | 74.0 | 49.4 | 48.4 | 62.3 | 66.2 | 71.0 | 65.7 | 45.0 | 60.5 | 81.7 | 88.6 | 57 | 51.1 | 61.5 |
| RT-5 | 31.3 | 38.0 | 32.3 | 60.3 | 43.1 | 32.5 | 51.7 | 40.0 | 57.6 | 46.3 | 29.7 | 35.9 | 61.5 | 44.2 | 44 | 34.1 | 43.2 |
| RT-6 | 29.3 | 35.2 | 38.5 | 51.8 | 42.1 | NA[b] | 44.6 | 29.8 | 57.9 | 43.6 | 40.5 | 38.9 | NA | 41.0 | 45 | 38.2 | 41.4 |
| RT-7 | 26.1 | 33.2 | 34.9 | 54.4 | NA | 34.1 | 45.0 | 42.0 | 45.7 | 40.9 | 33.6 | 50.9 | 52.9 | 53.3 | 48 | 28.6 | 42.5 |
| RT-8 | 27.4 | 31.2 | 27.3 | 32.9 | 30.0 | 29.3 | 34.3 | 25.2 | 39.3 | 30.1 | 22.1 | 27.1 | 44.0 | 24.4 | 40 | 24.8 | 31.1 |
| RT-9 | 18.4 | 18.8 | 30.6 | 22.4 | 34.1 | 19.7 | 37.6 | 22.2 | 32.8 | 30.4 | 10.8 | 24.0 | 25.8 | 22.4 | 31 | 23.8 | 24.8 |
| RT-10 | 13.3 | 13.6 | 12.1 | 12.1 | 21.9 | 15.1 | 20.1 | 6.2 | 20.0 | 18.0 | 18.8 | 15.5 | 22.5 | 18.9 | 29 | < 12.5 | 16.8 |
| RT-11 | 46.8 | 49.2 | 45.0 | 55.4 | 54.6 | 41.3 | 66.7 | 52.4 | 70.3 | 61.4 | 56.5 | 46.3 | 66.1 | 59.5 | 57 | 41.8 | 55.3 |
| RT-12 | 41.7 | 42.8 | 49.1 | 53.4 | 53.7 | 41.7 | 62.8 | 43.9 | 40.3 | 54.0 | 43.6 | 44.0 | 68.7 | 70.0 | 53 | 42.8 | 50.7 |
| RT-13 | 143.1 | 136.0 | 166.5 | 176.6 | 180.0 | 142.2 | 168.1 | 144.0 | 159.9 | 202.2 | 176.5 | 121.1 | 133.3 | 243.0 | 169 | 142.7 | 162.9 |
| RT-14 | 403.0 | 367.2 | 467.4 | 489.3 | 383.6 | 474.2 | 402.0 | 532.5 | 365.9 | 454.2 | 324.3 | 341.3 | 394.7 | 276.4 | 424 | 332.5 | 408.3 |

[a] Laboratories 5 and 16 using ELISA.
[b] NA = Not analyzed.

**Table 4.** Summary of means of individual TTX analogue concentrations (μg/kg) quantified and reported by participants who reported using LC–MS/MS, together with mean total TTX values reported from all participants including ELISA

| Material | TTX/ epiTTX | 11-nor TTX-6-ol | 4,9-anhydro-5,6,11-trideoxyTTX | 4,9-anhydro TTX | 5,6,11-trideoxy TTX | 5-deoxy TTX | 11-deoxy TTX | 6,11-dideoxy TTX | 11-oxoTTX, 4-epi-11-oxoTTX | Total TTXs |
|---|---|---|---|---|---|---|---|---|---|---|
| RT-1 | 17.1 | <0.1 | <0.1 | 0.9 | 6.0 | <0.1 | <0.1 | 0.6 | <0.1 | 18.3 |
| RT-2 | 34.1 | <0.1 | <0.1 | 0.8 | 11.2 | 0.3 | <0.1 | 2.5 | <0.1 | 36.1 |
| RT-3 | 57.9 | <0.1 | <0.1 | 1.1 | 22.9 | 2.1 | 2.0 | 5.1 | <0.1 | 62.3 |
| RT-4 | 61.5 | <0.1 | <0.1 | 1.3 | 19.7 | 1.0 | 2.1 | 5.7 | <0.1 | 65.0 |
| RT-5 | 43.2 | <0.1 | <0.1 | 0.8 | 8.5 | 0.5 | 2.0 | 3.8 | <0.1 | 45.4 |
| RT-6 | 41.4 | <0.1 | <0.1 | 1.4 | 10.1 | 0.8 | 1.0 | 1.9 | <0.1 | 43.7 |
| RT-7 | 42.5 | <0.1 | <0.1 | 1.5 | 6.1 | <0.1 | <0.1 | 2.2 | <0.1 | 43.5 |
| RT-8 | 31.1 | <0.1 | <0.1 | 0.9 | 4.6 | <0.1 | 3.1 | 1.9 | <0.1 | 32.4 |
| RT-9 | 24.8 | <0.1 | <0.1 | 0.6 | 12.1 | <0.1 | 0.1 | 1.6 | <0.1 | 27.5 |
| RT-10 | 16.8 | <0.1 | 0.3 | 0.5 | 7.5 | <0.1 | 2.7 | 0.7 | <0.1 | 18.9 |
| RT-11 | 55.3 | <0.1 | <0.1 | 0.6 | 8.4 | 1.2 | 1.4 | 5.0 | <0.1 | 58.1 |
| RT-12 | 50.7 | <0.1 | <0.1 | 0.7 | 3.4 | <0.1 | <0.1 | <0.1 | <0.1 | 51.0 |
| RT-13 | 162.9 | <0.1 | <0.1 | 2.1 | 3.4 | <0.1 | <0.1 | <0.1 | <0.1 | 163.7 |
| RT-14 | 408.3 | <0.1 | <0.1 | 5.7 | 2.2 | <0.1 | <0.1 | <0.1 | <0.1 | 404.1 |

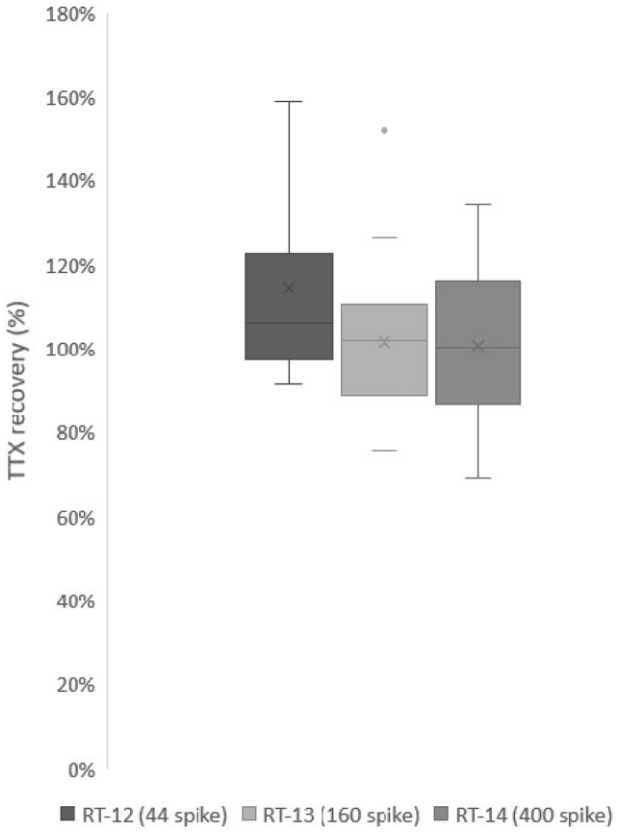


**Figure 1.** Box and whisker plot illustrating variability of TTX recoveries determined across all participants from spiked mussel samples, RT12 (44 μg/kg), RT13 (160 μg/kg), and RT14 (400 μg/kg).

Horwitz/Thompson curve, all reproducibility values were shown to be acceptable for both oyster and mussel samples.

### *Sensitivity*

Participants' method LODs ranged from 1 to 10 μg/kg, with a mean LOD of 5 μg/kg (Table 2). The study material containing the lowest TTX concentration (approximately15 μg/kg; samples RT-1 and RT-10) was detected and reported by all participants utilizing LC–MS/MS analysis. As such, there was good evidence for all instrumental methods being capable of quantifying TTX at concentrations ≤35% of the EFSA-recommended threshold (44 μg/kg; 15). From the two participants using ELISA, all samples were quantified, with the exception of samples 1 and 10 from Laboratory 16, where <12.5 μg/kg was reported, with the other ELISA user returning concentrations of 17.3 and 21.9 μg/kg for sample 1 and 10, respectively. Overall, both LC–MS and ELISA methods provided acceptable levels of method sensitivity for the determination of TTX in oysters and mussels in relation to the proposed EFSA threshold level (15).

### *Acceptability*

Participant TTX study data showed normal distributions for each sample and the absence of high proportions of outliers. There were no apparent gross errors such as those resulting from the incorrect units or concentration calculations. A one-way analysis of variance (ANOVA) with repeated measures (for each sample) was conducted on log-transformed data with results showing results from different participants had a statistical interaction with toxin concentrations ($F = 7.38$; $P = 1.04 \times 10^{-11}$), indicating there were statistically significant differences between the results returned between different participants. Subsequently a linear, mixed effect model utilizing a repeated measure approach (for each sample) was produced and post-hoc analysis using a Tukey multiple comparison of means analysis was performed. Supplemental Table S2 summarizes the calculated *P*-values and highlights which participant combinations were significantly different ($P < 0.05$). Although there were statistically significant differences between some laboratory pairs, no obvious patterns were identified. Interestingly, Laboratories 5 and 12 both showed very little statistical difference with any other participant. Figure 2 shows the robust average concentrations calculated for each sample across all participants, together with the associated robust SDs.

Acceptability was determined through z-scores as the standard uncertainty, $u_x$, was insignificant for all samples. Table 7 tabulates the z-score values calculated for each sample across all participants, with individual results shown graphically in

**Table 5.** Summary of TTX concentrations (μg/kg) determined for each blind duplicate across all laboratories, together with calculated within-laboratory precision $S_r$ and $RSD_r$ for each of the three samples

| Material | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | $S_r$ | $RSD_r$, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RT-1 | 13.2 | 16.0 | 14.9 | 19.4 | 17.3 | 15.8 | 11.8 | 12.8 | 22.1 | 17.0 | 13.0 | 17.5 | 33.0 | 9.5 | 23.8 | < 12.5 | 3.96 | 23 |
| RT-10 | 13.3 | 13.6 | 12.1 | 12.1 | 21.9 | 15.1 | 20.1 | 6.2 | 20.0 | 18.0 | 18.8 | 15.5 | 22.5 | 18.9 | 29.1 | < 12.5 | –[a] | – |
| RT-3 | 39.8 | 47.6 | 57.6 | 73.1 | 61.0 | 39.1 | 72.7 | 41.8 | 69.6 | 65.4 | 34.5 | 58.5 | 90.7 | 57.0 | 63.1 | 41.2 | 7.92 | 14 |
| RT-11 | 46.8 | 49.2 | 45.0 | 55.4 | 54.6 | 41.3 | 66.7 | 52.4 | 70.3 | 61.4 | 56.5 | 46.3 | 66.1 | 59.5 | 57.4 | 41.8 | – | – |
| RT-5 | 31.3 | 38.0 | 32.3 | 60.3 | NA[b] | 32.5 | 51.7 | 40.0 | 57.6 | 46.3 | 29.7 | 35.9 | 61.5 | 44.2 | 44.0 | 34.1 | 5.01 | 12 |
| RT-7 | 26.1 | 33.2 | 34.9 | 54.4 | NA | 34.1 | 45.0 | 42.0 | 45.7 | 40.9 | 33.6 | 50.9 | 52.9 | 53.3 | 47.8 | 28.6 | – | – |

[a]– = Not available.
[b]NA = Not analysed (missing sample).

**Table 6.** Summary of median concentrations (μg/kg), between-laboratory reproducibility, $RSD_R$, predicted relative SDs ($PRSD_R$), and HorRat values for TTX in study samples, showing results for all single samples, together with values calculated for three duplicates

| Sample | Median concentration | $RSD_R$ | $PRSD_R$ | HorRat |
|---|---|---|---|---|
| RT-1 | 16.0 | 0.35 | 0.22 | 1.60 |
| RT-2 | 34.5 | 0.25 | 0.22 | 1.16 |
| RT-3 | 58.1 | 0.28 | 0.22 | 1.26 |
| RT-4 | 59.0 | 0.23 | 0.22 | 1.05 |
| RT-5 | 41.6 | 0.25 | 0.22 | 1.15 |
| RT-6 | 40.8 | 0.20 | 0.22 | 0.90 |
| RT-7 | 42.0 | 0.21 | 0.22 | 0.96 |
| RT-8 | 29.7 | 0.21 | 0.22 | 0.94 |
| RT-9 | 23.9 | 0.28 | 0.22 | 1.30 |
| RT-10 | 18.0 | 0.33 | 0.22 | 1.50 |
| RT-11 | 55.0 | 0.16 | 0.22 | 0.73 |
| RT-12 | 46.6 | 0.20 | 0.22 | 0.91 |
| RT-13 | 163.2 | 0.19 | 0.21 | 0.92 |
| RT-14 | 398.3 | 0.17 | 0.18 | 0.94 |
| RT1&10 | 16.5 | 0.33 | 0.22 | 1.49 |
| RT3&11 | 56.7 | 0.23 | 0.22 | 1.03 |
| RT5&7 | 42.0 | 0.35 | 0.22 | 1.60 |

Supplemental Figure S2. Out of the 219 data points generated, 208 (95%) of results were deemed acceptable, with z-scores between −2 and +2. Two data points (0.9%) had z-scores less than −2 or greater than +2 (questionable) and nine data points (4.1%) had z-scores less than −3 or greater than +3 (unacceptable). Interestingly, all questionable or unacceptable results were associated with study materials RT-13 and RT-14 only (two and nine samples, respectively), which contained the highest concentrations of TTX, and were both fortified mussel tissues rather than naturally contaminated oysters. The absence of any questionable or unacceptable results for RT-12, the mussel spiked at the lowest concentration of 44 μg/kg, indicated the issues were not associated with the mussel tissue or the spiking procedure. A visual examination of the raw TTX concentration data (Table 3) shows that in RT-14 notably low concentrations and associated toxin recoveries were reported by Laboratory 14. These data, together with the higher than expected recoveries for RT-14 reported by Laboratories 4, 6, and 8, have resulted in the higher z-score results determined for this sample. The reasons for this higher variability in the higher concentration spiked samples remains unknown, however, it is noted that z-score data was 100% acceptable for all naturally contaminated oyster samples, indicating the methods used were successful in quantifying TTX concentrations in this matrix.

### *LC–MS/MS Performance*

As summarized in Table 2, there were notable differences between some of the ten LC–MS/MS methods used by the 14 different participants employing the instrumental approach. A total of eight participants used the validated method described by (39, 41) based on (44) and written as an EURL method (40). These participants (Laboratories 1, 2, 3, 8, 10, 11, 13, and 14) all employed the single-step dispersive 1:1 sample-to-solvent extraction ratio with 1% acetic acid with centrifugation, carbon solid phase extraction (SPE) clean-up and acetonitrile (MeCN) dilution prior to analysis. From the data returned using this method, 93% of results were acceptable with just seven z-scores either less than −2 or greater than +2. As with the other methods used, higher z-scores were found in the two high-spike mussel samples (RT-13, RT-14), with Laboratories 3, 8, and 10 showing higher than consensus concentrations for RT-14, and Laboratories 11 and 14 showing evidence for under-estimation of concentrations for RT-14 (Table 7). Interestingly, Laboratory 14 over-estimated the TTX concentration in the 160 μg/kg mussel spike (RT-13; z = +4.0) whilst under-estimating the concentration at the 400 μg/kg level in mussels (z = -4.3). Out of the participants using these published validated methods, there were notable differences in approaches taken for calibration standard preparation (Table 2), with calibration matrixes including solvent (80% MeCN, 0.25% acetic acid (HAC)), SPE-cleaned and MeCN-diluted mussel or oyster extract, as well as one laboratory using a commercial toxin-free mussel extract (NRC CRM-Zero-Mus; Laboratory 15). Such differences in calibration approaches did not appear to have any significant effect on the acceptability of the performance of the participants.

Some of the alternative LC–MS/MS methods employed notably different protocols in comparison to the 5 g + 5 mL extraction protocol described by (44). An ANOVA analysis assessing the statistical significance of using different LC–MS/MS method parameters showed no statistical differences between any of the method parameters described in Table 2. Laboratory 9 used a single dispersive extraction of 0.2 g tissue using a bead grinding method and a sample-to-solvent ratio of 2:5, without any SPE clean-up, and showed 100% acceptable results in terms of z-scores and no statistically significant differences between the robust average of other participant data, providing good evidence that scaling down the extraction and omitting the carbon SPE clean-up step resulted in acceptable method performance for analysis of oysters and mussels, even without recovery correction. Other participants who omitted the carbon SPE clean-up step for extract desalting were Laboratories 6, 7, 9, and 15. Out of these, three scored 100% acceptable z-scores with no apparent bias in performance, with Laboratory 6 showing one

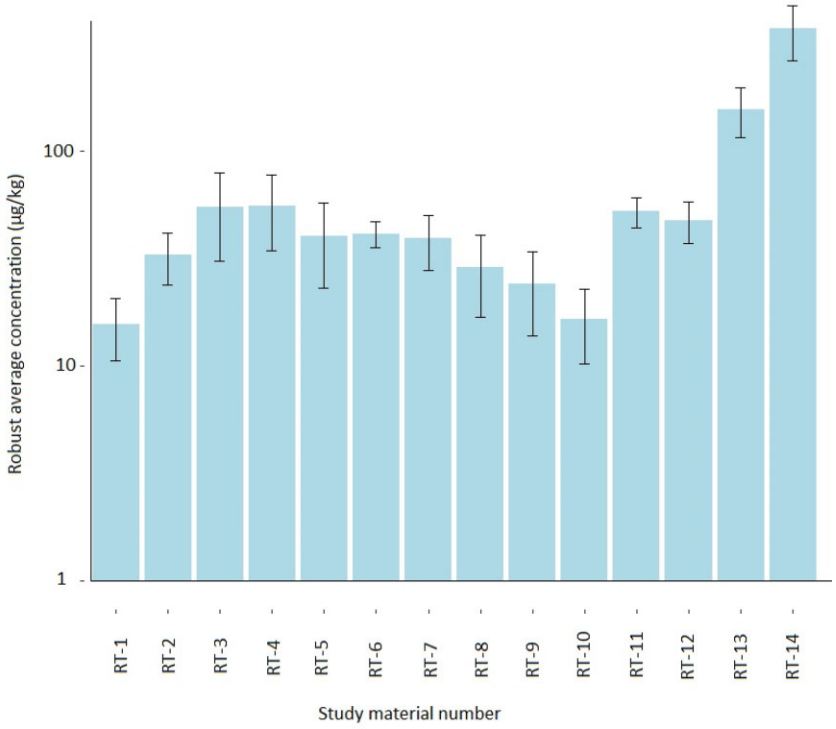


**Figure 2.** Bar chart displaying robust average TTX concentrations (µg/kg) on a log scale for each study material across all participants, with error bars indicating associated robust SDs.

**Table 7.** Summary of z-scores (samples RT-6, 8, 11–14) and z'-scores (samples RT-1–5, 7, 9–10) for each participant (laboratory number shown) based on acceptability of analysis of TTX in shellfish tissues

| Material | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RT-1 | −0.1 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | −0.1 | −0.1 | 0.2 | 0.0 | −0.1 | 0.0 | 0.6 | −0.2 | 0.3 | <[a] |
| RT-2 | −0.3 | −0.1 | −0.2 | 0.5 | −0.2 | −0.3 | −0.3 | −0.5 | 0.5 | 0.2 | 0.1 | 0.1 | 0.5 | 0.1 | 0.1 | 0.0 |
| RT-3 | −0.7 | −0.4 | 0.0 | 0.7 | 0.2 | −0.7 | 0.7 | −0.6 | 0.5 | 0.4 | −0.9 | 0.1 | 1.4 | 0.0 | 0.3 | −0.6 |
| RT-4 | −0.7 | −0.4 | −0.5 | 0.6 | −0.4 | −0.5 | 0.1 | 0.3 | 0.5 | 0.3 | −0.6 | 0.0 | 0.9 | 1.2 | −0.1 | −0.3 |
| RT-5 | −0.4 | −0.2 | −0.4 | 0.7 | 0.0 | −0.4 | 0.4 | −0.1 | 0.6 | 0.1 | −0.5 | −0.3 | 0.7 | 0.1 | 0.1 | −0.3 |
| RT-6 | −0.4 | −0.2 | −0.1 | 0.4 | 0.1 | NA[b] | 0.1 | −0.4 | 0.7 | 0.1 | 0.0 | −0.1 | NA | 0.0 | 0.2 | −0.1 |
| RT-7 | −0.6 | −0.3 | −0.3 | 0.5 | NA | −0.3 | 0.1 | 0.0 | 0.2 | 0.0 | −0.3 | 0.4 | 0.4 | 0.5 | 0.2 | −0.5 |
| RT-8 | −0.1 | 0.0 | −0.1 | 0.1 | 0.0 | 0.0 | 0.1 | −0.2 | 0.3 | 0.0 | −0.3 | −0.1 | 0.5 | −0.2 | 0.4 | −0.2 |
| RT-9 | −0.3 | −0.2 | 0.2 | −0.1 | 0.3 | −0.2 | 0.4 | −0.1 | 0.3 | 0.2 | −0.5 | −0.1 | 0.0 | −0.1 | 0.2 | −0.1 |
| RT-10 | −0.1 | −0.1 | −0.2 | −0.2 | 0.2 | −0.1 | 0.1 | −0.4 | 0.1 | 0.0 | 0.1 | 0.0 | 0.2 | 0.1 | 0.4 | < |
| RT-11 | −0.3 | −0.2 | −0.4 | 0.0 | 0.0 | −0.5 | 0.5 | −0.1 | 0.6 | 0.3 | 0.1 | −0.3 | 0.5 | 0.2 | 0.1 | −0.5 |
| RT-12 | −0.3 | −0.3 | 0.0 | 0.1 | 0.2 | −0.3 | 0.5 | −0.2 | −0.4 | 0.2 | −0.2 | −0.2 | 0.8 | 0.8 | 0.1 | −0.3 |
| RT-13 | −0.8 | −1.1 | 0.3 | 0.8 | 1.0 | −0.8 | 0.4 | −0.8 | 0.0 | 2.0 | 0.8 | −1.9 | −1.3 | 4.0 | 0.4 | −0.8 |
| RT-14 | 0.1 | −1.9 | 3.6 | 4.8 | −1.0 | 4.0 | 0.0 | 7.2 | −2.0 | 2.9 | −4.2 | −3.3 | −0.4 | −6.9 | 1.2 | −3.8 |

[a] < = Below reporting limit.
[b] NA = Not analyzed.

unacceptable result for RT-14 (z = 4.0). As such there appeared to be no clear bias in the data generated by participants whether or not SPE was used. Laboratory 4 conducted the published extraction (44) but utilized a novel clean-up approach incorporating the Merck Hybrid phospholipid ultra SPE cartridges, specifically for removal of arginine that is known to interfere with TTX quantitative accuracy (39). The results obtained using this method were all acceptable, with the exception of RT-14 (400 µg/kg mussel spike sample), where z = 3.0. Laboratory 7 utilized an in-house method with differences including an extraction in acetic acid and methanol using ultrasonication, with a sample-to-solvent ratio of 2:3 and no SPE clean-up, prior to quantitation using standard addition (Table 2). Results for Laboratory 7 were all acceptable as evidenced by z-scores between −0.3 and +0.7 (Table 7). Laboratory 6 used a published method incorporating the multi-toxin UPLC–MS/MS analysis of both hydrophilic and lipophilic marine toxins, including 15 TTX analogues (45), all in one single run. Z-scores were acceptable for all samples, with the exception of the high-level spiked mussel sample RT-14, (z = 4.0). Overall, therefore, the ten LC–MS/MS methods utilized for the determination of TTX in oyster and mussel tissues have demonstrated the acceptability of the method performance for the majority of approaches taken. Future work should ideally focus on both the interlaboratory

validation of a standardized LC–MS/MS method for TTX determination, and the extension of the method to a wide range of shellfish species, including at least scallops and clams.

### *Immunoassay Performance*

In this study, two participants (Laboratories 5 and 16) analyzed oyster and mussel samples using the commercial EuroProxima ELISA kit, which provided quantitative data on total TTX concentrations. Table 3 summarizes the total TTX results determined by ELISA with similar results obtained in comparison to LC–MS/MS data. Figure 3 illustrates the comparison between normalized ELISA and LC–MS/MS data and shows very similar average results, with a lower variance associated with ELISA (n = 2) in comparison to the variability determined by the larger number of laboratories with LC–MS/MS (n = 14). ANOVA analysis on normalized data (normalized against the robust average) with method (ELISA or LC–MS/MS), laboratory, and sample as variables was conducted, with results showing the method not having a statistical interaction with toxin concentrations ($P = 0.827$). The absence of any statistical difference between the ELISA and LC–MS/MS data was further confirmed using a Welch two-sample *t*-test on normalized data ($P = 0.834$). Mean concentrations were 73.4 and 75.16 μg/kg for the ELISA and LC–MS/MS, respectively. TTX recoveries determined from the three spiked samples ranged from 83 to 122% within the two laboratories using ELISA, with a mean recovery of 100 ± 14.8% across all ELISA recovery results. These data therefore provide further evidence for acceptable method recovery of the assay in oysters and mussels, as previously reported in the EuroProxima validation study, where recoveries ranged from 104.6 to 110.0% at spiked concentrations of TTX between 20 and 40 μg/kg (46). The repeatability of the assay was also good, with an intra-sample repeatability across the two laboratories and all duplicate samples of 8.4%, very similar to the intra-assay and inter-assay variability of 6.6 and 7.3%, respectively, determined for a 2.5 ng/mL TTX standard during the EuroProxima validation (45). ELISA results acceptability was also found to be good, with no unacceptable results returned by either participant. Overall, therefore, with results showing excellent method performance characteristics, a statistical equivalence to the LC–MS/MS data and the absence of any unacceptable z-scores, the results indicated the ELISA method is effective and was fully capable of providing acceptable quantitative data for TTX determination in the oyster and mussel samples incorporated into this study. The work here also consequently extends the range of concentrations assessed (16 to 400 μg/kg) in comparison to the range previously studied and reported by the kit manufacturer (20 to 40 μg/kg). Noting that there is no performance data on related TTX analogues, and very low levels of these analogues were present in these samples, further work is required with a larger number of laboratories to assess the full interlaboratory performance characteristics of the assay in a wider range of shellfish tissue samples and containing varying TTX analogue profiles.

### *Comparison with Previous Validation Studies*

Whilst numerous reports of TTX in seafood products have been published in recent years using LC–MS/MS, a relatively low number of these methods have been subjected to full validation studies. Table 8 summarizes the main method performance characteristics from this study in comparison with those determined in published LC–MS/MS validation studies to date (23, 34, 36, 39–41, 45) together with the performance characteristics for the ELISA method (46) and a comparative biosensor method (52). Previous LC–MS/MS single-laboratory validations (SLVs) have demonstrated recoveries which are generally good, ranging from 61 to 121% on average. Both within- and between-batch precision has also been shown to be acceptable; 4 to 11% and 3 to 20%, respectively. Method sensitivities, as quantified by LOD and LOQ, varied enormously, but such data is hard to standardize for MS/MS detection methods, given the notable variation in the algorithms utilized by instrument software for calculating S/N. Notably, LODs quoted by participants in this study were higher than those reported by four previous SLV studies (36, 39, 40, 45). However, other than the initial validation which was targeting quantitation of TTX at a higher order of magnitude in sea slugs [mg/kg (23)], all methods published

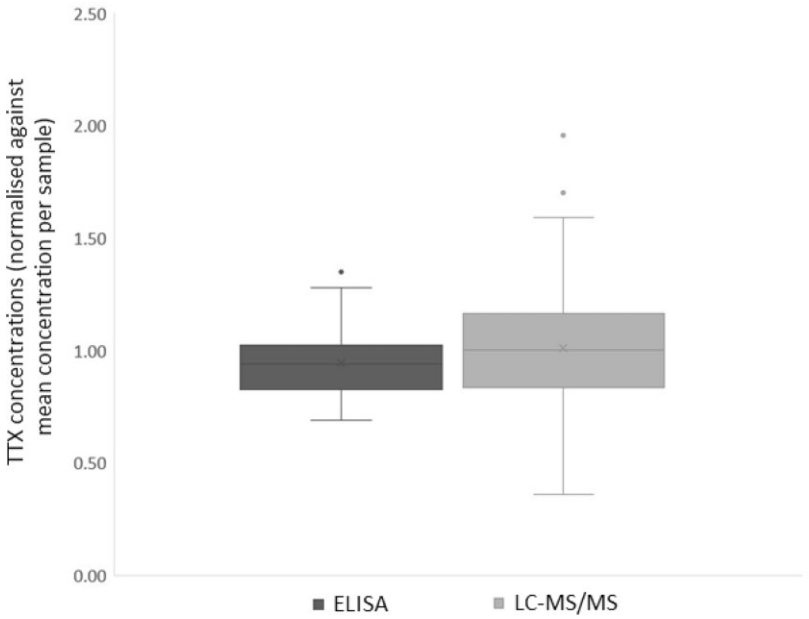


**Figure 3.** Box and whisker plot comparing TTX concentrations, normalized against mean TTX concentration for each sample, between ELISA and LC–MS/MS.

**Table 8.** Summary of TTX determination in shellfish method performance characteristics determined in this study in comparison to those confirmed through single-laboratory and interlaboratory validation studies.

| Method | Description | Matrix | Recovery, % | Within-batch repeatability, % | Between-batch repeatability, % | Between-laboratory reproducibility, % | LOD/LOQ, μg/kg[a] |
|---|---|---|---|---|---|---|---|
| McNabb et al., 2014 | LC–MS/MS | Bivalves | 107 ± 7 | 9 | 13 ± 4 | NA[b] | 100/500 |
| Bane et al., 2016 (38) | LC–MS/MS | Fish, gastropod | 61.2 ± 5.4 | 4.2 ± 2.3 | 3.1 ± 2.8 | NA | 0.00041/0.001 |
| Turner et al., 2017 (28) | LC–MS/MS | Bivalves | 77 ± 8 | 11 ± 4 | 12 ± 1 | NA | 0.24/0.79 |
| EURLMB, 2017 (40) | LC–MS/MS | Bivalves | 89 ± 13.7 | NA | 6.1 ± 4.8 | 0.36/1.03 | |
| Rodriguez et al., 2018 (45) | LC–MS/MS | Bivalves | NA | 4.3 | 7.7 | NA | 0.47/1.56 |
| Turner et al., 2020 (41) | LC–MS/MS | Bivalves | 121 ± 52[c] | NA | 20 ± 2 [b] | 32 ± 11.5[c] | NA |
| Hort et al., 2020 (34) | LC–MS/MS | Bivalves, gastropod | 92.8 ± 11.7 | 8.9 ± 4.1 | 10.7 ± 3.9 | NA | 3.8/15.0 |
| R-Biopharm, 2018 (46) | ELISA | Fish, bivalves | 107 ± 8.9 | 6.6 | 7.3 | NA | 9.4/20 |
| Campbell et al., 2013 (52) | Biosensor | Gastropod | 106 ± 18 | 5.8 ± 2.6 | 6.6 ± 1.8 | NA | 100/200[c] |
| This study | LC–MS/MS | Bivalves | 107 ± 21[d] | 16.7 ± 6.1[d] | NA | 25.8 ± 6.0[d] | NA |
| This study | ELISA | Bivalves | 100 ± 15[e] | 11.8 ± 5.7[e] | NA | 15.2 ± 7.3[e] | NA |

Number of laboratories n = 1 unless otherwise specified in footnote for collaborative studies.
[a] For ELISA/biosensor assays, LOQ taken as detection capability (CCβ). LOD taken as decision limit (CCα).
[b] NA = Not applicable/no data provided.
[c] n = 21.
[d] n = 14.
[e] n = 2.

and/or utilized in this study are capable of quantifying at concentrations well below the EFSA recommended threshold of 44 μg/kg (Table 8) and consequently are appropriate for regulatory control purposes. In terms of previous interlaboratory validation studies, the only one reported to date focussed primarily on PSTs and incorporated just three TTX-positive shellfish tissue samples, specifically an oyster, mussel and clam sample (41). Therefore, the recoveries and associated precision data were skewed and unlikely to give a reliable indication of actual interlaboratory performance. The characteristics determined in this study from LC–MS/MS data show excellent performance in terms of recovery and repeatability, with the between-laboratory reproducibilities (25.8 ± 6.0%; n = 14) likely to provide a more accurate assessment of laboratory performance using a variety of extraction, cleanup and instrumental approaches. In comparison, although fewer ELISA or biosensor methods have been validated, and are used by a lower number of laboratories, evidence from this study shows acceptable method performance. More data would be required, however, utilizing a greater number of participating laboratories and a wider number and range of samples, in order to determine a more reliable assessment of interlaboratory method performance. In particular, a standardized method would ideally be subjected to a full collaborative validation study, with samples comprising a wide range of shellfish species.

## Conclusions

The interlaboratory study used 14 different shellfish study materials, involving 16 participating laboratories, with 14 using multiple LC–MS/MS methods and the remaining two employing a commercial ELISA test. Homogenous and stable oyster and mussel tissues were shipped to all participants who used either published, validated, or in-house developed methods for sample analysis. Samples were analyzed in one batch and consisted of three blind duplicates, as well as three mussel tissues spiked with known concentrations of TTX. Analysis showed that TTX was the dominant analogue present, so all statistical assessment was conducted on this analogue only. Results confirmed acceptable sensitivity and TTX recoveries in the majority of laboratories and the within-batch repeatability was excellent across all participants. The between-laboratory reproducibility was also deemed acceptable in terms of all HorRat values being >0.5 and ≤1.6. Performance acceptability, as described by z-scores, showed acceptable results for 95% of all sample/laboratory combinations, with notable lower success with the mussel samples spiked at higher toxin concentrations. Data from naturally contaminated oyster samples, however, showed 100% acceptable results from all participants. No statistical differences were found between the commercial ELISA and LC–MS/MS method results, nor between different sample preparation or LC–MS/MS protocols. Overall, this interlaboratory assessment has provided further evidence that the multiple LC–MS/MS methods currently employed by monitoring laboratories around the world are generally appropriate for the accurate and reproducible determination of TTX concentrations in mussels and oysters but would ultimately benefit from a more formalized standardization, as well as assessment in a larger number of shellfish species. Furthermore, the results have also shown the EuroProxima TTX ELISA to return quantitative results which are equivalent to those quantified by LC–MS/MS, although more assessment is required in a larger number of laboratories and shellfish species.

## CRediT Author Statement

Andrew David Turner (Conceptualization-Lead, Data curation-Lead, Formal analysis-Lead, Funding acquisition-Lead, Investigation-Lead, Methodology-Lead, Project administration-Lead, Resources-Lead, Software-Lead, Supervision-Lead, Validation-Lead, Visualization-Lead, Writing—original draft-Lead, Writing—review & editing-Lead), Karl James Dean (Formal analysis-Supporting, Software-Supporting, Writing—review & editing-Supporting), Monika Dhanji-Rapkova (Formal analysis-

Equal, Writing—review & editing-Supporting), Sonia Dall'Ara (Formal analysis-Equal), Florella Pino (Formal analysis-Equal, Writing—review & editing-Supporting), Claire McVey (Formal analysis-Equal), Simon Haughey (Formal analysis-Supporting), Natasha Logan (Formal analysis-Supporting), Chris Elliott (Investigation-Supporting, Resources-Supporting), Ana Gago-Martinez (Formal analysis-Equal, Writing—review & editing-Supporting), Jose Manuel Manuel Leao Martins (Formal analysis-Supporting, Writing—review & editing-Supporting), Jorge Giraldez (Formal analysis-Supporting), Ryan S. Gibbs (Formal analysis-Equal, Writing—review & editing-Supporting), Krista M. Thomas (Formal analysis-Equal, Writing—review & editing-Equal), Ruth Perez-Calderon (Formal analysis-Equal, Writing—review & editing-Equal), Dermot Faulkner (Formal analysis-Equal, Writing—review & editing-Supporting), Hugh McEneny (Formal analysis-Equal), Veronique Savar (Formal analysis-Equal), Damien Réveillon (Formal analysis-Equal), Philipp Hess (Formal analysis-Supporting, Writing—review & editing-Supporting), Fabiola Arevalo (Formal analysis-Equal), Juan Pablo Lamas Castro (Formal analysis-Equal), Eva Cagide (Formal analysis-Equal), Mercedes Alvarez (Formal analysis-Equal), Álvaro Antelo (Formal analysis-Equal), Mirjam D. Klijnstra (Formal analysis-Equal), Michalina Oplatowska-Stachowiak (Formal analysis-Equal, Investigation-Supporting, Writing—review & editing-Supporting), Tim Kleintjens (Formal analysis-Equal), Nermin Sajic (Formal analysis-Equal), Michael Boundy (Formal analysis-Equal), Benjamin H. Maskrey (Formal analysis-Equal, Writing—review & editing-Supporting), David Tim Harwood (Formal analysis-Supporting, Writing—review & editing-Supporting), Jesús M. González (Formal analysis-Equal), Amparo Alfonso (Formal analysis-Supporting, Writing—review & editing-Supporting), and Luis M. Botana (Conceptualization-Supporting, Funding acquisition-Supporting, Methodology-Supporting, Writing—review & editing-Supporting).

## Supplemental Information

Supplemental information is available on the *J. AOAC Int.* website.

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## Conflict of Interest

The authors declare no conflict of interest.

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