Trends in Analytical Chemistry 75 (2016) 10–23

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

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

Proficiency testing of laboratories for paralytic shellfish poisoning toxins in shellfish by QUASIMEME: A review

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ARTICLE INFO

Keywords:
Proficiency testing
Paralytic shellfish poisoning
HPLC
Mouse bioassay
Mass spectrometry

ABSTRACT

Paralytic Shellfish Poisoning (PSP) toxins are some of the most toxic substances known to man and consumption of shellfish containing these naturally-occurring neurotoxins can lead to a range of different symptoms including death in extreme cases. It is imperative therefore, to implement robust shellfish monitoring programs to minimise the possibility of contaminated product reaching the marketplace. To improve the quality assurance of these programs, QUASIMEME, the proficiency test provider added to its scope PSP toxins in shellfish. Since 2009, six proficiency testing exercises have been delivered by QUASIMEME with a total of thirty-four different laboratories submitting data using a range of different methods. These include animal and antibody based assays, together with High Performance Liquid Chromatography (HPLC) techniques using post and pre-column oxidation and more recently LC-MS/MS methodologies.

Data from these exercises is presented and laboratory performance is assessed to determine any changes in overall performance over the six rounds, together with any potential method-related performance issues. The data showed the improvement of laboratories over the six exercises with between laboratory CV% values decreasing from an average of 39% in the first year to 22% in 2014 and the average percentage of participants receiving satisfactory z-scores increasing from 50% in 2009 to over 66% in 2014.

Contents

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http://dx.doi.org/10.1016/j.trac.2015.09.004

1. Introduction

Paralytic shellfish poisoning (PSP) is caused by a group of over 30 structurally similar analogues based on a tetrahydropurine skeleton and produced, mainly, by some marine dinoflagellates (Fig. 1) [1,2]. These potent neurotoxins can accumulate in the food chain through the filter-feeding of toxin-producing algae by bivalve mollusc shellfish. Subsequent consumption of contaminated shellfish can result in human intoxication, with symptoms including numbness in the extremities, tingling in the face, nausea, vomiting, diarrhoea and in severe cases, death by asphyxiation [3].

PSP toxin profiles in shellfish differ substantially worldwide and variations occur for a number of reasons. The toxin profile differs between algal species, between strains of the same species, between species in different geographical locations or even between isolates from the same location [4,5]. Differences also exist in the metabolic transformation of some PSP toxins into others from various shellfish species [6]. This can lead to issues for PSP proficiency test (PT) providers in supplying materials with a wide range of profiles to cater to participants from different geographical locations.

Numerous intoxications and incidences have been reported from around the world [7–12] and with global aquaculture production reaching record levels of 90.4 million tonnes in 2012 (US$144.4 billion) [13], it is vital, for human protection and as a statutory requirement in many countries, to implement robust shellfish monitoring programs [14,15].

Essential to any effective monitoring program is the proper application of a quality management system, incorporating both quality assurance and quality control [16]. PT plays a vital role in the implementation of these programs, and participation in these schemes is a requirement both legislatively in the EU [17,18] as well as by most national accreditation bodies.

QUASIMEME (Quality Assurance of Information in Marine Environmental Monitoring) was founded in 1992 and was initially funded by the European Union (EU). The aim was to establish a comprehensive network of laboratories producing demonstrably reliable chemical information while providing a holistic approach to quality assurance. The determination of PSP toxins was added to the QUASIMEME scope in 2009 as a development exercise, with one round being organised per year.

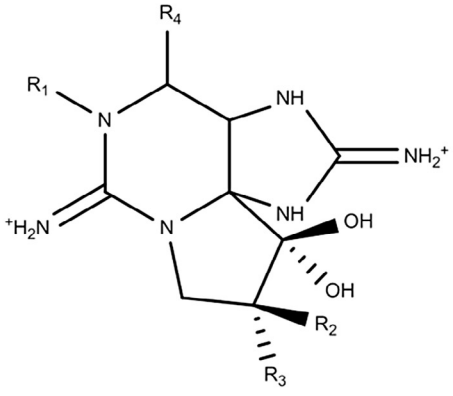
The aim of this review is to disseminate the data generated over the previous six years of PSP PT delivered by QUASIMEME and to highlight issues related to the analysis of this toxin group by the various methods used by participants. The review seeks to highlight the advantages of PT participation and shows the development of the exercises through laboratory performance.

1.1. Toxicity equivalency factor (TEF)

The PSP analogues differ substantially in their toxicity and their TEFs have been derived from MBA data. Fig. 1 lists the TEFs reported by Oshima et al. in 1995 [19] alongside values compiled by the Scientific Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) in 2009 [20]. The two sets of figures are similar with the exception of dcSTX where the TEF was increased to 1.0 from 0.5 by the CONTAM panel. This change would significantly affect reported total toxicities of samples with dcSTX as the predominant toxin present which is the case in some of the samples supplied by QUASIMEME.

1.2. Methods of analysis

There are few internationally recognised methods available for determining PSP toxins. Table 1 lists the numbers of participants



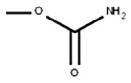
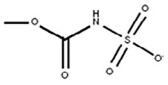
Toxin	R_1	R_2	R_3	R_4	Toxicity Equivalency Factors	
					Oshima	EFSA
STX	H	H	H		1.00	1.00
GTX2	H	H	OSO_3^-		0.36	0.40
GTX3	H	OSO_3^-	H		0.64	0.60
NEO	OH	H	H		0.92	1.00
GTX1	OH	H	OSO_3^-		0.99	1.00
GTX4	OH	OSO_3^-	H		0.73	0.70
M2	H	OH	H		-	-
M4	H	OH	OH		-	-
GTX5	H	H	H		0.06	0.10
C1	H	H	OSO_3^-		0.01	-
C2	H	OSO_3^-	H		0.10	0.10
C3	OH	H	OSO_3^-		0.01	-
C4	OH	OSO_3^-	H		0.06	0.10
GTX6	OH	H	H		0.06	0.10
M1	H	OH	H		-	-
M3	H	OH	OH		-	-
dcSTX	H	H	H		0.51	1.00
dcGTX2	H	H	OSO_3^-		0.15	0.20
dcGTX3	H	OSO_3^-	H	-OH	0.38	0.40
dcNEO	OH	H	H		-	0.40
dcGTX1	OH	H	OSO_3^-		-	-
dcGTX4	OH	OSO_3^-	H		-	-

Fig. 1. Chemical structure of the PSP toxins together with toxicity equivalence factors (TEFs) reported by Oshima and those recommended by the European Food Safety Authority (EFSA).

Table 1
Total number of participants in each round including breakdown of methods used

Year	Round	Methods Used					Total Number of Participants
		preCOX	PCOX	MBA	ELISA	LC-MS/MS	
2009	57	8	2	4	–	–	14
2010	61	9	5	2	–	–	16
2011	65	8	5	4	2*	–	19
2012	69	9	5	2	–	–	16
2013	72	13	6	2	–	1	22
2014	2014-1	13	5	2	–	3	23

* Ridascreen ELISA used by participants.

over the last six years and the methods employed are briefly described below:

1. Mouse bioassay (MBA): The MBA was first applied in the determination of PSP toxicity by Sommer and Meyer in 1937 [21]. Subsequently the procedure underwent standardisation, culminating in association of analytical communities (AOAC) official method (OMA) 959.08 [22]. This is the reference method specified in EU legislation [23] and involves the extraction of the shellfish homogenate tissue in dilute hydrochloric acid followed by intraperitoneal injection of filtered extracts into replicate mice. The time taken from injection to mouse death is recorded and the toxicity determined from a conversion table developed by Sommer and Meyer [21]. Reported drawbacks of the method include underestimations of total toxicity caused by high salt concentrations or the presence of some metals in samples [3,24]. The presence of other metals, such as zinc, particularly evidenced in oyster tissues has been reported to increase the threat of false positives [25]. The method is also controversial in its use of large numbers of mice and in stipulating death as an endpoint. A further drawback of the MBA is that it only provides a total toxicity value and no information about the specific toxin profile of a sample. However, the method has been used globally for many years and has provided an excellent preventative method for the significant reduction of PSP intoxications worldwide.
2. Liquid Chromatography-Fluorescence Detection (LC-FLD): PSP toxins do not exhibit natural ultraviolet absorption or fluorescence and must therefore be oxidised into fluorescent derivatives before detection by FLD. This oxidation process can be carried out either before or after separation by LC and has given rise to two distinct LC methods.
   a. Pre-column Oxidation (preCOX): two preCOX methods were used by participants. The first, also called the "Lawrence method" is the only named alternative to the MBA usable for official control testing in the EU and was written into EU legislation in 2006 [23]. The second closely-related method, used by one participant in 2014 is that described by the European Committee for Standardisation (CEN) and recently published as European standard DIN EN 14526 [26]. Both methods involve the oxidation of toxins into iminopurine derivatives before separation and determination by LC-FLD. The method cited in legislation was originally developed by Lawrence and Meynard [27] and underwent international validation in 2004 before being approved as an AOAC official method in 2005 [28]. The method is based on a two-step boiling acetic acid extraction followed by cleanup steps using solid phase extraction (SPE). Extracts are then oxidised using periodic acid or hydrogen peroxide depending on the toxin profile present. The main drawbacks of the method relate to the complex chromatographic output as well as the lack of separation of epimeric pairs (gonyautoxins 1&4 (GTX1 & GTX4), GTX2 & GTX3, C1 & C2, decarbamoylgonyautoxin 2&3 (dcGTX2 & dcGTX3) and C3 & C4) characteristic of this toxin group. Users of this method calculate summed toxin concentrations for each epimeric pair. Sample toxicities are estimated from the sum of toxin concentrations, which leads to a slight overestimation as the more toxic analogue for each epimer pair is used to calculate total toxicity. This is in spite of the fact that the epimer ratio in bivalve tissues typically reaches an equilibrium of 3:1 predominated by the less toxic α-epimers (GTX1, 2) compared to the more toxic β-epimers (GTX3, 4) [29]. Pre-column derivatization can produce one to three oxidation product peaks per toxin, leading to difficulties in determining toxin profiles and accurate quantitation. This is particularly pertinent for samples often used in PT schemes where two or more different toxin profiles are mixed to prepare the RMs or the analysis of toxin profiles not routinely encountered at the participants laboratory. Another drawback of using this method and all chemical based methods of analysis is the lack of certified reference standards for all the PSP toxins, most notably GTX6, C3, C4, dcGTX1 and dcGTX4. Example chromatograms, generated from preCOX LC-FLD analysis are illustrated in Fig. 2a–b which highlight the complex chromatographic output from this method and some of the potential issues in accurate identification. Although complex, the method has been refined and standardised since acceptance as a first action AOAC method, and in recent years has been implemented into routine official control testing of shellfish in a number of countries including Ireland, UK, Portugal and New Zealand.
   b. Post-column oxidation (PCOX): methods based on post-column derivatization were first utilised for shellfish monitoring by Sullivan and Wekell in 1984 [30]. Further developments were described by Oshima et al. in 1989 where separation of the full suite of known PSP toxins was achieved using three separate chromatographic conditions [31]. A method modified from that of Oshima [19] and subsequently Thomas et al. [32] was published by Rourke et al. in the journal of the AOAC in 2008 [33]. This method underwent international validation in 2010 and was accepted as AOAC OMA 2011.02 [34]. The extraction technique is based on that of the MBA, with the use of boiling hydrochloric acid. Partial hydrolysis of certain PSP toxins into more toxic analogues occurs with the PCOX/MBA extraction method which, some argue, mimics the process of digestion in the stomach thereby more accurately reflecting sample potential toxicity [26]. The milder conditions of the preCOX extraction method do not cause hydrolysis of the toxins, with toxin profile consequently remaining unaffected. A distinct advantage the PCOX method has over the preCOX method is the formers ability to separate the PSP epimers contained in this group [35]. One disadvantage with the PCOX methods is their inability to separate certain PSP toxins (dcNEO/NEO and GTX6/GTX4) unless a very long runtime is used [33]. OMA 2011.02 also requires two chromatographic runs in order to separate the full suite of PSP toxins. The method has recently been adopted into US legislation and has been implemented into official control monitoring programs within Canada, Norway and some US states. Chromatograms obtained from PCOX LC-FLD analysis of a material used in these exercises are provided in Fig. 2c–d, which clearly shows the separation of the epimer pairs contained in the sample.
3. Assays: The immunoassay technique used by QUASIMEME participants for PSP testing is that of a competitive enzyme-linked immunosorbent assay (ELISA). A number of kits are commercially available, although the one used for reporting QUASIMEME samples is available under the name Ridascreen™ (R-Biopharm, Darmstadt, Germany) and is based on the competition between free PSP toxins and PSP toxin conjugates for PSP antibody binding sites. The high number of saxitoxin (STX) congeners has posed problems for developers of these methods, with, for example, neosaxitoxin (NEO) and GTX1,4 exhibiting poor cross reactivity

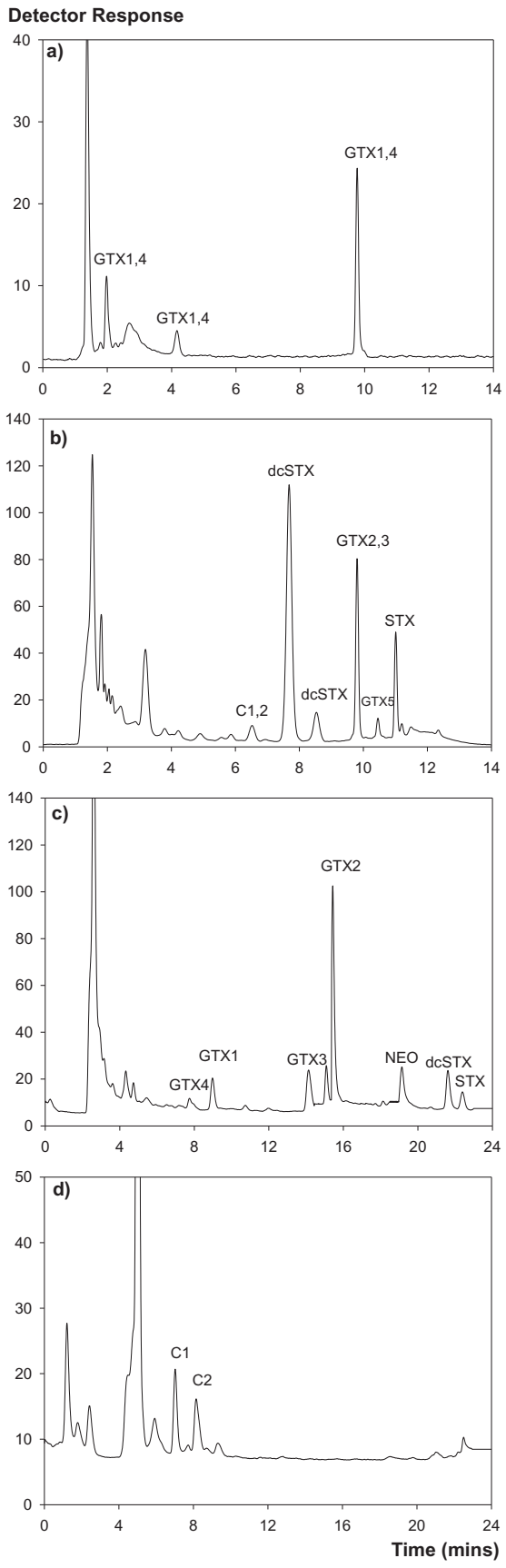


Fig. 2. Chromatograms of Tissue B (QST076BT, 095BT & 133BT) obtained by a) preCOX LC-FLD analysis of carboxylic acid solid phase extraction (SPE) fraction 2, periodate oxidised, b) preCOX LC-FLD analysis of C18 SPE cleaned, peroxide oxidised, c) PCOX LC-FLD analysis of GTX/STX toxins and d) PCOX LC-FLD analysis of C-toxins.

with the Ridascreen assay [36]. Other immunological test kits available commercially suffer the same cross reactivity issues described above apart from one test kit supplied by Neogen, where the cross reactivity of NEO was improved [37]. A hydrolysis step has also been implemented by one kit supplier, Scotia Rapid Testing Ltd., which converts GTX1,4 to NEO in order to circumvent some of the GTX1,4 cross reactivity issues [38].

A receptor binding assay (RBA) was first developed in the early 90's by Vieytes et al. [39] and after extensive method refinement and further development was accepted as an official AOAC method of analysis in 2011 (OMA AOAC 2011.27) [40]. Although the method has performed well in comparison with the MBA and HPLC [41], its use in routine monitoring programmes has been limited in part by the reliable availability of tritiated saxitoxin and from the use and handling of radioisotopes used in the method. The RBA has not been used for the analysis of any Quasimeme samples to date.

4. Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS): The use of mass spectrometry as a detection system for determining PSP toxins is desirable, based on the high sensitivity and selectivity this technique potentially gives the user. Early LC-MS/MS methods required the use of ion-pairing reagents in the mobile phase in order to ensure adequate retention of the charged PSP species [42,43]. This, along with the aqueous mobile phase these methods employed, leads to poor ionization and signal suppression. Hydrophilic interaction liquid chromatography (HILIC), which does not require the use of ion pairing reagents, was first utilised for PSP determination by Dell'Aversano et al. in 2005 [44]. The technique is ideally suited to the separation of polar compounds and for electrospray ionisation (ESI) MS detection owing to the high organic content of mobile phases used [44]. Despite these advantages the technique still suffers from significant matrix effects issues caused by salts and co-extractives present. These issues coupled with the high detection limits reported have limited this methods use in monitoring programmes, despite improved sensitivity compared to previously developed LC-MS/MS methods [44]. Recent developments in the field have focused on sample pre-treatment in order to overcome the issues highlighted above. Boundy et al. reported on an effective desalting pre-treatment cleanup step using inexpensive graphitised carbon SPE cartridges prior to HILIC separation and MS detection [45]. The procedure reduced matrix effects substantially and the method was subjected to an extensive single-laboratory validation study on various shellfish species as reported by Turner et al. [46]. Method sensitivity was significantly increased and detection limits were found to be similar or below those reported for both preCOX and PCOX LC-FLD methods. This represents a major breakthrough in overcoming the technical issues that have traditionally affected LC-MS/MS determination of PSP toxins in complex matrices. The use of LC-MS/MS methods for PSP testing by QUASIMEME participants has been limited to date with results submitted by three participants in 2013 and 2014 only. The LC-MS/MS methods used by these participants were based on HILIC-MS/MS with one participant (Lab 28) using the method of Boundy et al. [45].
5. Others: alternative methods for PSP determination include capillary electrophoresis coupled to ultraviolet (UV) or MS detection, although very little research has been carried out in this area [47–52] and no QUASIMEME participants have submitted data based on these technologies to date.

1.3. *QUASIMEME's Cofino model for data assessment*

The data assessment carried out by QUASIMEME is based on ISO guide 13528 concerning the proficiency testing of analytical chemistry laboratories [53] with some slight modifications. The assigned

value and z-scores are calculated using a model developed by Cofino et al. [54] which was specifically designed for use in the determination of population characteristics [55]. Robust statistics form the basis to the ISO13528 guide to data assessment but this model can be limited where a high percentage of extreme values are submitted by participants, as the model can typically deal with ~7–10% tailing data. The Cofino model is unique as it can be used directly with a whole range of datasets including tailing or skewed data, datasets containing extreme outliers and bimodal or multimodal distributions.

The model works by identifying clusters of data within a dataset exhibiting a high level of agreement. From this the mean, standard deviation (s.d.) and percentage of data associated with each cluster is calculated. A distinct advantage this model has over the standard robust model is that no preparation of the dataset is necessary, either by using subjective boundaries or outlier testing before entering it into the database for assessment.

The Cofino model uses probability density functions for each observation weighting values centred around the mean more heavily than those further away from the mean.

The detailed, graphical information provided by the model can be seen in Fig. 3 and the derivation of each is described in the QUASIMEME handbook [55]. The graphical information includes:

- A plot of the population density functions (Fig. 3a).
- Matrix overlap (Kilt) plot which is a colour density plot very sensitive to identifying the structure of data, especially modality (Fig. 3b).
- A ranked overview of the means and standard deviations of each data set (Fig. 3c).
- Z-score plot for reviewing performance against targets (Fig. 3d).

1.4. Proficiency testing for PSP toxins

QUASIMEME currently provide the only commercially available, open access PT schemes for marine biotoxins in shellfish worldwide. Previous interlaboratory and PT schemes for PSP toxins have focused on the MBA [56], on the preCOX LC-FLD method [57] or on a combination of the most widely used methods including MBA, preCOX and PCOX LC-FLD, ELISA and LC-MS/MS [58–62].

A pilot study organised by the Food Analysis Performance Assessment Scheme FAPAS® in 2003 highlighted issues related to the choice of extraction method used by participants [63]. Under boiling HCl conditions, partial hydrolysis of the N-sulfocarbamoyl toxins, GTX5 and C1,2, present in the sample, were transformed into their

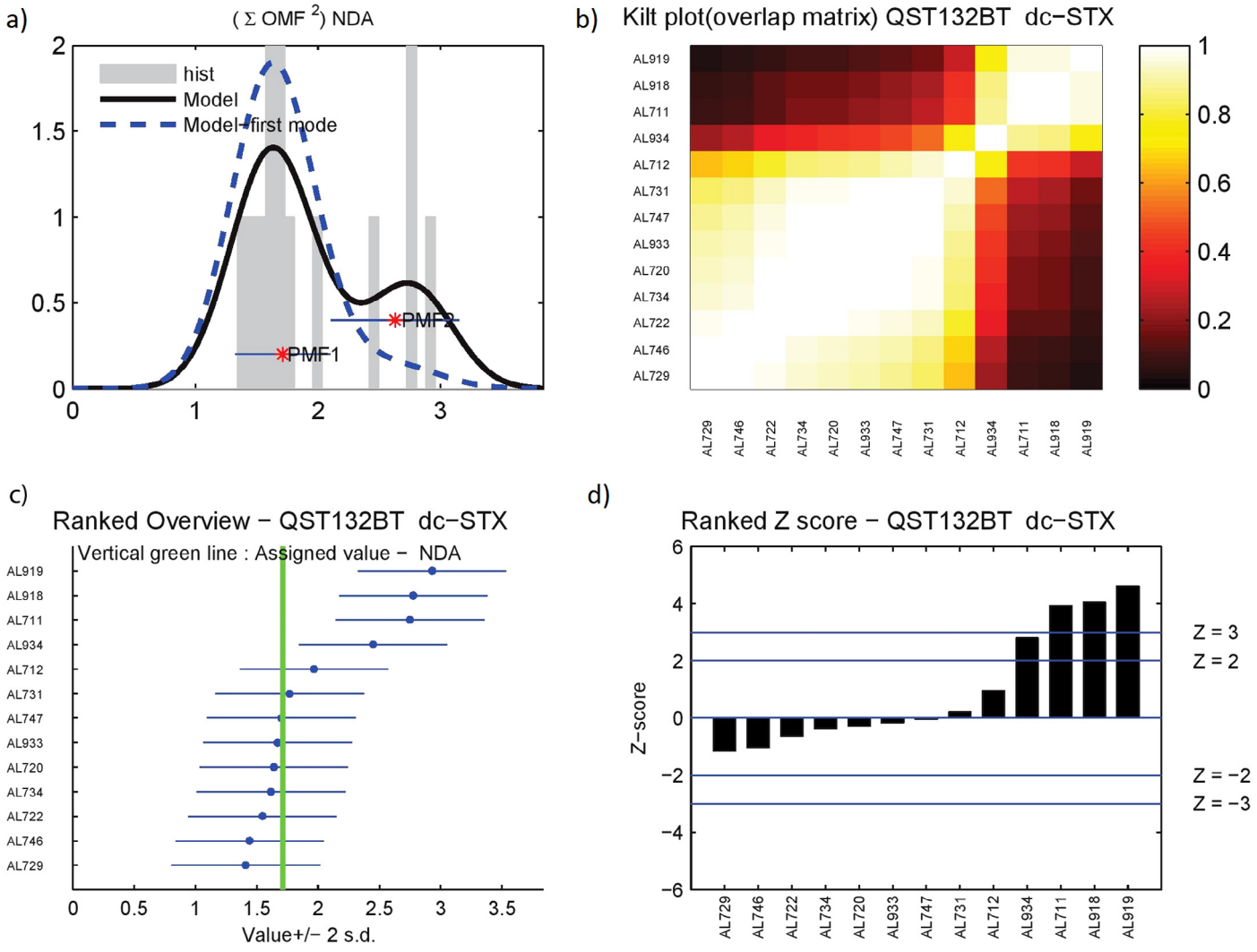


Fig. 3. Data plots for the toxin dcSTX found in sample QST132BT in 2012. a) Summed probability density functions (PDFs) for all data (black line) and for the first mode, PMF1, (blue line) with histogram of individual measurements in grey. Each observation from a participant is described by a PDF and is not regarded as a value using the Cofino model. b) The Kilt plot (Overlap matrix) showing degree of overlap of each pair of data. Areas of the map coloured white indicate complete overlap (agreement) for the observations concerned while black indicates no overlap. c) Ranked overview of all data with error bars of ±2 s.d. d) Ranked z-score plot for all data.

Table 2
List of materials used in each exercise, assigned codes, predominant toxins present, matrices studied and homogeneity results determined using OMA AOAC2005.06

Tissue	Rounds Used	Year	Codes Used	Predominant Toxins Present	Matrix	Homogeneity Results (n = 15) µgSTXdiHCl eq./kg
A	57	2009	QST075BT	dcSTX, STX, GTX-5	*Mytilus galloprovincialis*	533 ± 24
	61	2010	QST093BT			
	69	2012	QST132BT			
	2014-1	2014	Sample 1			
B	57	2009	QST076BT	dcSTX, GTX-2,3, GTX-1,4, STX, GTX-5 & C-1,2	*Mytilus edulis & Mytilus galloprovincialis*	1174 ± 45
	61	2010	QST095BT			
	69	2012	QST133BT			
C	61	2010	QST094BT	dcGTX-2,3, dcSTX & dcNEO	*Spisula solida*	2829 ± 62
	65	2011	QST111BT			
	69	2012	QST134BT			
	2014-1	2014	Sample 2			
D	65	2011	QST113BT	GTX-2,3 & STX	*Mytilus edulis*	1400 ± 44
	72	2013	QST152BT			
	2014-1	2014	Sample 3			
E	65	2011	QST114BT	GTX-2,3, STX & dcSTX	*Mytilus edulis*	1249 ± 55
	72	2013	QST154BT			
F	69	2012	QST135BT	GTX-2,3, STX, GTX-1,4 & NEO	*Crassostrea gigas*	803 ± 22
G	72	2013	QST155BT	GTX-2,3, STX, GTX-1,4 & dcSTX	*Mytilus edulis*	829 ± 29
H	2014-1	2014	Sample 4	GTX-2,3, STX, GTX-1,4 & dcSTX	*Crassostrea gigas*	1715 ± 51

carbamoyl counterparts, STX and GTX-2,3 respectively [36], leading to significant differences in the results supplied by users of both extraction methods.

A ring trial organised by CEFAS in 2011/2012 found statistical differences between the preCOX and PCOX methods, most notably for the toxins GTX1,4 and NEO, with significantly higher results on average submitted by preCOX LC-FLD users compared to PCOX LC-FLD. Conversely results returned for the other toxins covered in that study, C1,2, GTX2,3 and STX were found to be lower on average when determined by preCOX LC-FLD compared to PCOX LC-FLD. The organisers surmised that the higher levels of inter-laboratory method variability associated with preCOX LC-FLD analysis, as evidenced by the higher standard deviations submitted by participants contributed to the differences observed [59].

Other studies have highlighted issues relating to the analysis of the toxins dcNEO and GTX6 by both LC-FLD methods. The toxin dcNEO co-elutes with another toxin, NEO, when analysed using the PCOX method, leading to potentially significant over-estimations of total toxicity due to the differences in TEF values for both toxins, (Fig. 1). The lack of certified reference materials for all the known PSP toxins but in particular GTX6 and C3,4, highlights another issue between both LC-FLD methods. An hydrolysis step, converting GTX6 into NEO and C3,4 into GTX1,4, can be applied by users of the preCOX method, allowing indirect quantitation of these toxins, which is not possible when using the PCOX method.

2. Materials and methods

All materials were prepared by the lead author and designed to test the performance of QUASIMEME participant methods with the analysis of a wide variety of analytes, whilst minimising the number of samples to be tested. Materials incorporated a range of toxin concentrations and different complexities in toxin profile composition. The shellfish tissues used were naturally contaminated with a range of PSP toxins and prepared following in-house procedures to ensure homogeneity. Stabilisation of the toxins and matrices was achieved using a combination of heat treatment and the addition of antibiotics and an antioxidant [64,65] with short-term stability and homogeneity studies performed on all materials before distribution to participants [66].

A total of eight materials have been used over the six exercises 2009–2014, with Tissues A & C being incorporated into four rounds each, Tissues B & D used in three rounds, Tissue E used in two rounds and Tissues F, G & H used in one round only. Table 2 lists each of the materials and the rounds in which they were used, the assigned codes, predominant toxins present, matrices studied and the homogeneity results determined. The coefficients of variation were calculated from the homogeneity data contained in Table 2 and compared to expected levels of method variability determined through validation of the test method, below which the RM was deemed sufficiently homogenous.

The exercises are true proficiency tests so no standardised method protocol was provided by QUASIMEME and participants were requested to use the analytical method routinely employed at their laboratories. The only stipulation in the protocol was in the use of TEFs supplied and these were requested to be used in total toxicity calculations. In 2009 (Round (R) 57) and 2010 (R61), the TEFs supplied in the protocol were those reported by Oshima [19], while in 2011 (R65), 2012 (R69), 2013 (R72) and 2014 (R 2014-1) TEFs recommended by the EFSA [20] were prescribed in the protocol (Fig. 1).

The format of the data submitted by participants is dependent upon the method employed for material testing. All participants are required to submit a total toxicity result for each sample, enabling assessment and comparison of all methodologies including participants using MBA. Participants using either the preCOX, PCOX or LC-MS/MS methods of analysis, where individual analogue concentrations can be determined, were able to submit results such that a data assessment and therefore z-scores can be calculated for each individual toxin, or epimeric pair, in addition to total toxicity. This allows participants receiving less than satisfactory z-scores to pinpoint potential causes of method failure or operator error, if they relate to the determination of a particular analogue.

3. Intercomparison results

Table 3 summarises participants' data taken over the six exercises and contains assigned values, number of observations per determinant, between-laboratory CV (%) and percentage of participants receiving satisfactory z-scores. The performance of laboratories was not assessed in cases where an assigned value could not be calculated. In these cases an indicative value was generated and no z-scores were calculated. Only materials where assigned values could be calculated are listed in table 3. The criteria set out in calculating an assigned value and z-scores are contained in the QUASIMEME manual [55].

Table 3
Summary data from all methods 2009–2014, showing assigned values, number of observations, coefficient of variation and % of z-scores satisfactory

Year	Test Material and Code	STX	dcSTX	dcGTX-2	dcGTX-3	dcGTX-2,3	C-1,2	GTX-2	GTX-3	GTX-2,3	GTX-5	GTX-1	GTX-4	GTX-1,4	neoSTX	dcNEO	Total Toxicity
		µmol/kg															µgSTXdiHCleq./kg
2009	Mussel QST075BT Tissue A																461, 14 35, 50
	Mussel QST076BT Tissue B																977, 14 44, 50
2010	Mussel QST093BT Tissue A	0.07, 6 31, 42	1.69, 13 34, 85								0.20, 10 39, 69						400, 16 44, 50
	Clam QST094BT Tissue C		3.47,14 41, 50													1.37, 7 36, 57	1886, 16 45, 44
	Mussel QST095BT Tissue B	0.37, 11 23, 77	0.93, 13 28, 71				0.48, 8 43, 50			1.36, 11 13, 75	0.13, 7 16, 46			1.14, 8 22, 55	0.17, 5 58, 33		950, 15 36, 44
2011	Clam QST111BT Tissue C		3.53, 12 14, 83			9.32, 7 29, 71										1.40, 8 20, 63	2670, 19 19, 89
	Mussel QST113BT Tissue D	2.56, 12 15, 83						1.01, 5 22, 80		1.15, 7 29, 86							1139, 19 30, 58
	Mussel QST114BT Tissue E	1.92, 12 16, 75	0.58, 12 27, 92							1.54, 7 18, 71							1187, 19 27, 63
2012	Mussel QST132BT Tissue A	0.08, 10 63, 69	1.71, 13 23, 69								0.21, 10 20, 75						629, 15 18, 60
	Mussel QST133BT Tissue B	0.35, 14 23, 86	0.97, 13 29, 69				0.35, 6 26, 71			1.09, 8 21, 75	0.12, 7 4, 64	0.83, 5 14, 80		0.90, 7 19, 86	0.14, 6 35, 56		986, 15 32, 53
	Clam QST134BT Tissue C		3.34, 13 18, 62			9.00, 8 24, 63					0.10, 4 44, 44					1.32, 9 24, 67	2734, 15 19, 67
	Oyster QST135BT Tissue F	0.60, 14 28, 86								1.62, 7 21, 71				0.69, 7 39, 50	0.12, 7 80, 50		789, 15 38, 47
2013	Mussel QST152BT Tissue D	2.59, 19 13, 68						1.06, 6 5, 100	0.45, 6 2, 100	1.31, 12 27, 92							1245, 18 11, 72
	Mussel QST154BT Tissue E	1.98, 19 20, 68	0.56, 17 25, 76					1.36, 6 17, 100	0.54, 6 5, 83	1.53, 12 18, 83							1246, 18 17, 78
	Mussel QST155BT Tissue G	0.32, 18 27, 78	0.54, 17 26, 76					0.84, 6 25, 100	0.30, 6 7, 83	0.95, 12 17, 83			0.17, 5 48, 83	0.54, 10 38, 64			742, 17 19, 67
2014	Mussel Sample 1 Tissue A	0.10, 13 77, 65	1.96, 21 31, 57								0.20, 13 45, 75						750, 22 33, 55
	Clam Sample 2 Tissue C		3.46, 20 24, 65	11.20, 6 4, 100	3.07, 6 9, 67	10.20, 14 37, 50										1.46, 13 53, 46	3045, 22 22, 68
	Mussel Sample 3 Tissue D	2.61, 20 18, 80						0.94, 6 9, 83	0.42, 6 12, 100	1.21, 14 32, 79							1289, 21 17, 71
	Mussel Sample 4 Tissue H	1.94, 21 9, 90	0.09, 13 55, 63					2.24, 7 8, 86	0.74, 7 7, 100	2.41, 14 25, 64			0.17, 6 27, 86	0.74, 9 45, 55	0.24, 11 47, 57		1569, 22 15, 68

Codes: assigned value, number of observations (Nobs) / coefficient of variation % (CV%), % z-scores satisfactory (|z| <2).

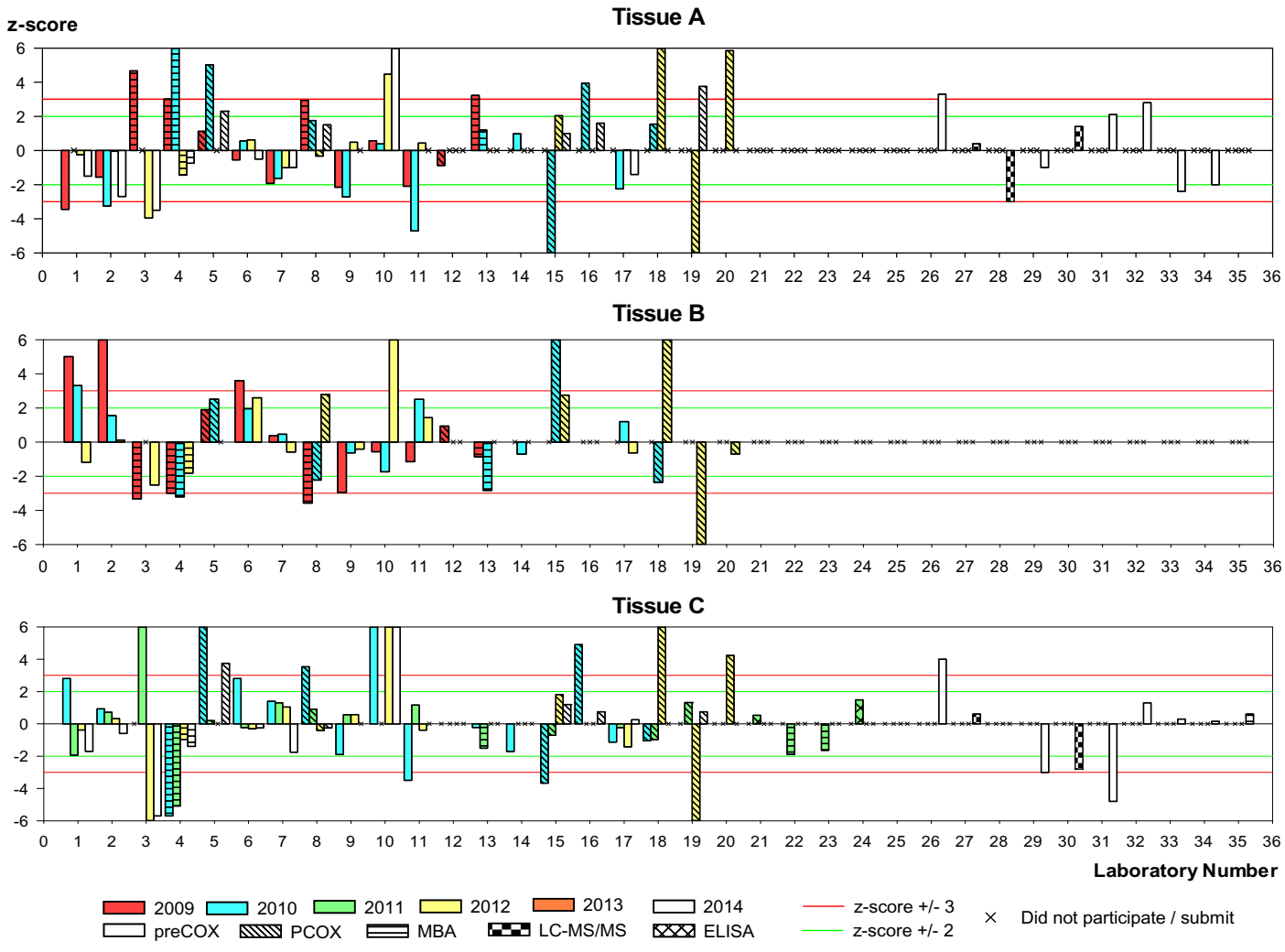


Fig. 4. Distribution of participants' z-scores for Tissues A–C used in various rounds from 2009–2014.

Figs 4 and 5 graphically represent z-scores generated over the last six exercises with calculated upper and lower z-score limits for satisfactory ($z \leq |2|$), questionable ($|2| < z \leq |3|$) and unsatisfactory ($z \geq |3|$) data.

A list of the methods used by participants since 2009, including references where possible, is detailed in Table 4. Please note that labs 3 and 8 changed from using the MBA as their method of analysis in R57 (2009) to using preCOX and PCOX methods respectively, when they next participated in 2010 and 2011.

All materials were prepared from naturally contaminated tissues at levels ranging from approximately half to three and a half times the current EU regulatory limit of 800 micrograms per kilogram [15]. The materials were chosen to test participant performance using the most common shellfish species of global commercial importance where possible, with toxin profiles of varying complexity and over a range of concentrations. The results summarised in Table 3 show the improvement of participants over the six exercises. The coefficients of variation have decreased from an average of 39% in 2009 to 22% in 2014 and the percentage of participants receiving satisfactory z-scores have increased from an average of 50% in 2009 to 65% in 2014.

Although substantial improvements have been made during these development exercises certain issues have been highlighted which may have contributed to some of the variability seen in the datasets.

The application of TEFs to estimate the total toxicity of a sample was necessary in order to compare all methods together. Two different sets of TEFs have been prescribed in the protocols over the last 6 years as described in the previous section. The differences between both sets of TEFs are minimal apart from one toxin, dcSTX, which was increased from 0.5 to 1.0 by the EFSA. These changes account for the variability in assigned values of materials used in multiple rounds where dcSTX was a predominant toxin present. This is clearly evident in tissues A and C where assigned total toxicity values of 461 and 400 μg STX diHCleq./kg (tissue A) and 1886 μg STX diHCleq./kg (tissue C) in early rounds increased to 629 and 750 μg STX diHCleq./kg (tissue A) and 2670, 2734 and 3045 μg STX diHCleq./kg (tissue C) in latter exercises. Fig. 6 illustrates this point and graphically presents the assigned values for all tissues used in these exercises. Recent studies carried out by Munday et al. determined the median lethal doses of some PSP analogues through interperitoneal injection, gavage and feeding experiments [67]. They concluded that there was a lower risk with dcSTX than that proposed by the EFSA with their figures being more in line with earlier data such as those determined by Oshima et al. [19]. This may explain some of the differences observed between instrument based methods of analysis and the MBA in samples with dcSTX as a predominant toxin present.

Another interesting observation from the data contained in Table 3 relates to the comparison of methods capable of epimer separation, such as the PCOX and LC-MS/MS methods to the preCOX method, where epimer separation is not possible. In tissues where values were assigned to both individual epimers as well as the sum of the epimers, significant differences can be observed. In the

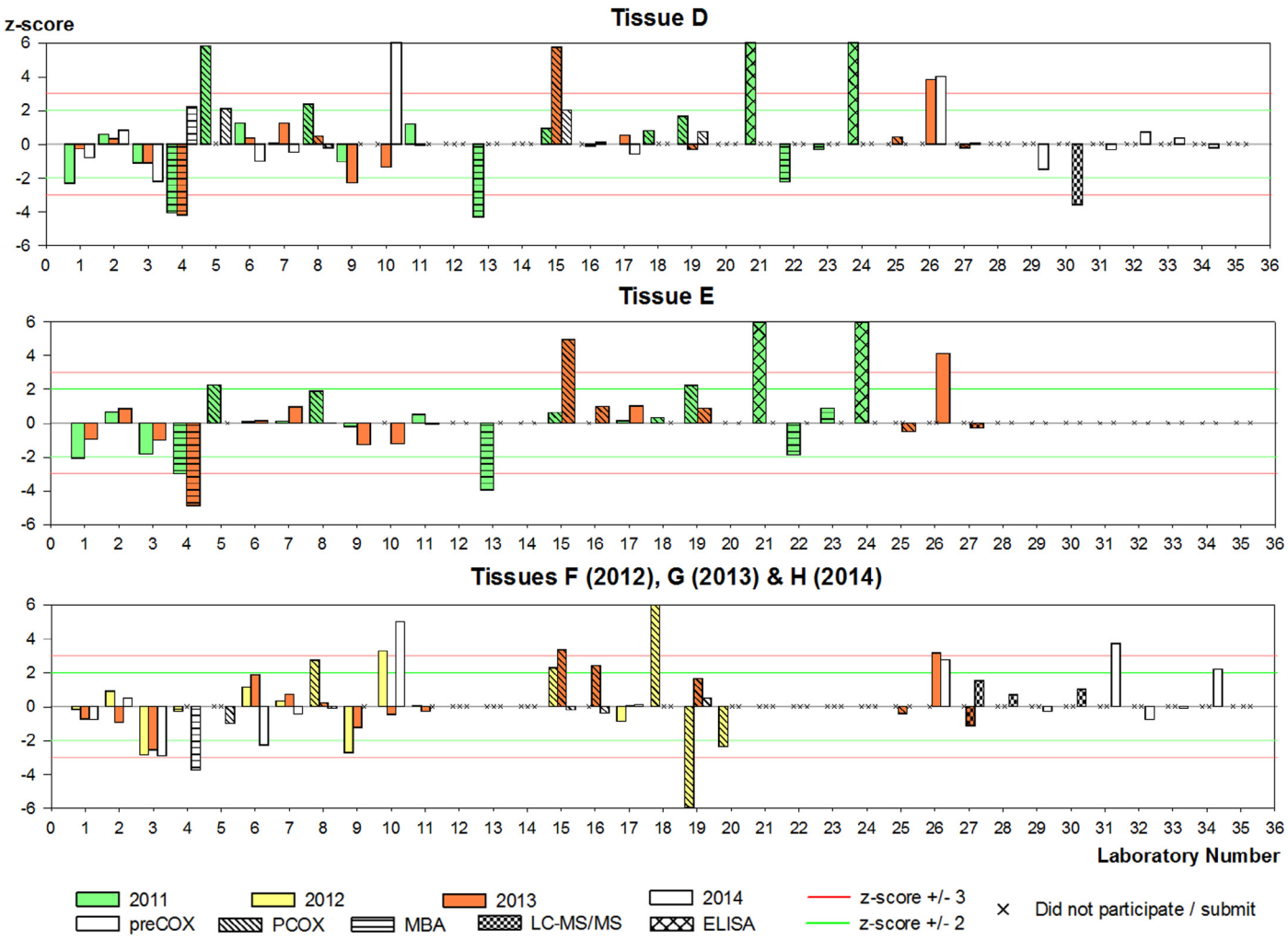


Fig. 5. Distribution of participants' z-scores for Tissues D–F used in various rounds from 2011–2014.

determination of GTX2 and GTX3, present in all three tissues used in 2013, the addition of both assigned values for each individual epimer, determined by PCOX LC-FLD and LC-MS/MS users only, was substantially higher than the assigned value of the epimers determined together by preCOX LC-FLD users only. This was also observed in tissues D and H used in 2014 for GTX 2 and GTX3 but also for the toxins dcGTX2 and dcGTX3 in tissue C in 2014. This observation is surprising given the accepted overestimation of epimer concentrations determined when using the preCOX LC-FLD method as the more toxic analogue of each epimer pair is used to calculate total toxicity. As in the case of the CEFAS ring trial discussed previously, the higher inter-laboratory method variability of the preCOX LC-FLD method may explain some of the differences observed.

Where individual analogues could be determined by a method such as preCOX and PCOX LC-FLD and LC-MS/MS, assigned values could be calculated from the datasets if certain conditions, as highlighted in the Quasimeme handbook were met [55]. The analysis of one analogue, NEO has been problematic for participants as evidenced by the high %CVs, with an average value of 55% (n = 4) calculated from the four datasets where assigned values could be determined for this toxin (Table 3). This value is high when compared to other individual analogues determined in QUASIMEME tissues with average %CVs determined for dcNEO (33%, n = 4) and GTX5, dcSTX and STX (28% each, n = 13). This observation is not surprising given the fact that NEO co-elutes with dcSTX, if also present in a sample, when determined by the preCOX LC-FLD method. In this instance the NEO concentration in a sample is estimated through a back calculation which takes into account the dcSTX contribution to the overall response. PCOX LC-FLD and LC-MS/MS methods can directly determine this toxin so no back calculation is required.

4. Discussion

4.1. Identification of poor performance

The source or sources of poor performance in PT schemes can be difficult to pinpoint for both the participant and organiser. Finding correlations between method choice and analytical performance is very difficult even with the large amount of data submitted by participants over the last six years. This is mainly due to the large number of variabilities potentially influencing PSP analysis and results, for example changes to staff and associated training, reagents used, slight variations in the oxidation of toxins or batch to batch variability of consumables such as SPE cartridges.

The majority of extreme z-scores ($z \geq |6|$) received by participants can be traced back to either gross calculation errors (as high as 2–3 orders of magnitude difference) made during the conversion of concentration data into total sample toxicities or the misidentification of toxins present. Adequate training of personnel, particularly new employees, in-house validation of methods and a comprehensive review process prior to submission is therefore

Table 4
Overview of methods used from 2009–2014 with references where applicable

Lab Number	Method Used					
	2009	2010	2011	2012	2013	2014
1	1	1	1	1	1	1
2	1	1	1	1	1	1
3	2	–	1	1	1	1
4	2	2	2	2	2	2
5	3	3	3	–	–	4
6	1	1	1	1	1	1
7	1	1	1	1	1	1
8	2	4	4	4	4	4
9	1	1	1	1	1	–
10	1	1	–	1	1	1
11	1	1	1	1	1	–
12	4	–	–	–	–	–
13	2	2	2	–	–	–
14	–	1	–	–	–	–
15	4	4	4	4	4	4
16	–	4	–	–	4	4
17	–	1	1	1	1	1
18	–	4	4	4	–	–
19	–	–	4	4	4	4
20	–	–	–	4	–	–
21	–	–	5	–	–	–
22	–	–	2	2	–	–
23	–	–	2	–	–	–
24	–	–	5	–	–	–
25	–	–	–	–	4	–
26	–	–	–	–	1	1
27	–	–	–	–	6	6
28	–	–	–	–	–	6
29	–	–	–	–	–	1
30	–	–	–	–	–	6
31	–	–	–	–	–	1
32	–	–	–	–	–	7
33	–	–	–	–	–	1
34	–	–	–	–	–	1
35	–	–	–	–	–	2

Codes:
1. OMA AOAC 2005.06 [28]
2. OMA AOAC 959.08 [22]
3. Oshima (PCOX) [19]
4. OMA AOAC 2011.02 [34]
5. ELISA (Ridascreen)
6. HILIC-MS-MS
   Internal method
7. DIN EN 14526 [26]

of huge importance and would minimise or eliminate these extreme results. Experienced laboratories can also suffer from variable performance levels due to the recruitment of new staff requiring training or the implementation of new methodologies and instrumentation.

While the causes of extreme z-scores are in general easier for participants to trace and remedy, unsatisfactory z-scores ($|3| < z < |6|$) can be a lot more difficult to investigate. New participants to these schemes typically take two to three exercises before receiving satisfactory z-scores, the exact causes of which are unknown but are most likely because of their unfamiliarity with the submission process, reporting units required or analysing toxin profiles atypical of their geographical location. This can be seen in Fig. 4, particularly for laboratories 1, 2 and 4 where initial unsatisfactory z-scores received in the first round were improved in subsequent exercises. The opposite of this can also be the case however (see laboratory 5 in Fig. 4) so participants have to be continually vigilant to ensure satisfactory performances are maintained.

4.2. Factors affecting poor performance

Previous PT schemes, ring trials and interlaboratory studies for PSP toxins have highlighted issues related to each of the methods used [56,57,59,63]. The MBA is known to suffer from recovery issues caused by "salt" effects [56] and the presence of some metals, particularly zinc can have a large suppressive effect on the bioassay [24] with underestimation of total toxicity particularly in samples close to the EU regulatory limit. These observations could explain some of the MBA results returned by participants in particular tissues B-E where results were consistently below the assigned value (24 of 27 in total) with some results ~60% below the assigned value.

The choice of extraction method can also have a fundamental influence on results, with previous studies showing that under boiling HCl conditions (MBA & PCOX methods), partial hydrolysis of the N-sulfocarbamoyl toxins, GTX-5 and C-1,2 transformed them into their carbamoyl counterparts, STX and GTX-2,3 respectively [63]. It was noted however that the N-sulfocarbamoyl toxins were not present at very high concentrations in the tissues used in these exercises.

4.3. CRMs

The lack of certified reference materials (CRMs), both solvent and matrix for all the PSP toxins is a problem consistently highlighted in the area, although a matrix CRM has recently been produced by CEFAS [68]. This lack of CRMs, particularly for the toxins GTX6 and C3,4, although not present in any QUASIMEME samples to date, has highlighted problems in the PT schemes delivered by the EU Reference Laboratory for Marine Biotoxins (EURL-MB) [60,61]. Participants using preCOX methods can perform an hydrolysis step converting GTX6 into NEO and C3,4 into GTX1,4 which allows an indirect means of quantification for those toxins. Although the toxicity of GTX6 is relatively low it can still contribute significantly to the overall toxicity of some samples [35].

4.4. Misidentification of toxins

The misidentification of toxins, particularly dcNEO has been an issue for some participants and has been a source of poor performance in these exercises. The dcNEO misidentification was particularly highlighted in the analysis of Tissue C. Users of PCOX methods of analysis misidentify this toxin as NEO due to the methods inability to separate both toxins unless a very long run time is utilised (>60 mins). This is an accepted limitation of the PCOX method and presents no consumer risk in a real sample scenario owing to the 10-fold difference in TEF values between the two toxins. It can lead to a significant over estimation of total toxicity owing to the differences in TEF however, and for the most part resulted in participants receiving unsatisfactory or extreme z-scores. This was not always the case however as some laboratories received satisfactory z-scores even with dcNEO misidentified, (see laboratories 8 and 15 in 2011, 2012 and 2014 for Tissue C, Fig. 4). The analysis of dcNEO can also pose problems to preCOX participants as this toxin co-elutes with dcSTX, and requires a back calculation in order to estimate the dcNEO concentration in the sample. Only two preCOX participants (Lab 11 & 14) have failed to correctly identify dcNEO as being present in Tissue C in 2010. Lab 11 received an unsatisfactory z-score for this sample while Lab 14 received a satisfactory z-score as they significantly overestimated the dcSTX content in the sample, negating the fact that dcNEO was not quantified and used in the total toxicity calculation. In subsequent rounds Lab 11 correctly identified dcNEO in this sample and received satisfactory z-scores again highlighting participant improvements as experience is gained. Lab 14 did not participate in subsequent rounds. As no data is removed by QUASIMEME before assigned values and z-scores are calculated, the onus is on participants to have a comprehensive review process of final reports and z-score results to ensure correct identification of all toxins present in the materials has been achieved. Personalised feedback to participants, particularly those with less than satisfactory performance is not provided

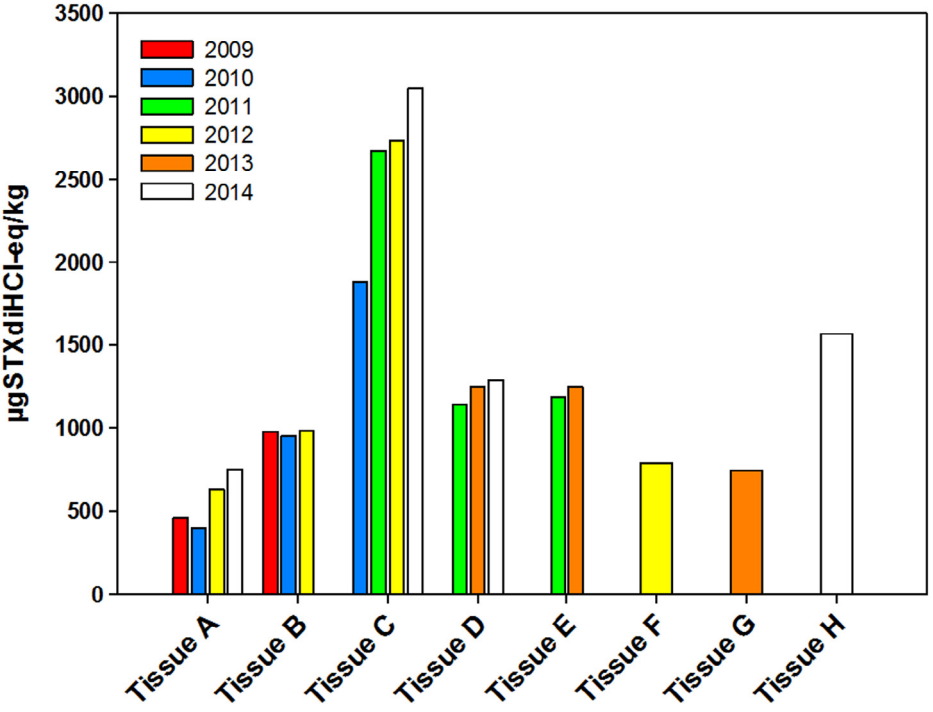


Fig. 6. Assigned values determined in multiple rounds for Tissues A–E and in single rounds for Tissues F–H.

by QUASIMEME after each round due to the extensive scope of their program, the number of determinants in each exercise coupled with the logistical problems this approach would entail. The relevant authorities in each participant's country, such as national accreditation bodies should comprehensively review PT results to ensure that the accurate identification of toxins present has been adequately demonstrated.

4.5. Method recovery correction factors

The application of method recovery correction factors to results generated from preCOX analysis is an important issue and has been high on the agenda of the EURL-MB Working Group for PSP toxins over the last number of years. Only one QUASIMEME participant has submitted both non-recovery corrected (lab 10) and recovery corrected (Lab 26) results in 2013 and 2014 (Figs 4 and 5), with none of the recovery corrected results receiving satisfactory z-scores in either round. This observation is not surprising given the fact that the assigned values (from which z-scores are calculated) generated for each tissue are calculated from the group which is weighted more heavily by non-recovery correcting participants. In order to assess all data together the application of recovery correction factors would need to be stipulated in future protocols.

4.6. Method dependency and individual analogues

Finding correlations between proficiency test performance and method choice or between performance and the presence of specific analogues in test samples is difficult. For instance the plot of the population density functions in Fig. 3a clearly shows a bimodal distribution of the data in the determination of dcSTX in sample QST132BT with the smaller mode (PMF2) resulting from PCOX users (3 labs). The main mode of data (PMF1) was shown to arise from the remaining preCOX users, although one PCOX participant is also contained in this main mode of data. Another sample, QST133BT used in the same round also showed a bimodal distribution in the determination of dcSTX (data not shown). It might be easy to deduce from this and the Kilt plot (Fig. 3b) that there is a method dependency issue between both LC-FLD methods relating to the analysis of dcSTX. This trend however, is not consistent, as the analysis of the same two samples in 2009 and 2010 showed no bi-modality for this toxin with the entire data set fitting a Gaussian distribution and results from PCOX users randomly spread throughout the entire series. Furthermore other samples used in these exercises that contained dcSTX showed no bi-modality in their distributions.

ELISA results showed variable performance levels with the only analysis of Tissue C (lab 24, Fig. 4) by this method producing a satisfactory z-score, whereas ELISA analysis carried out on Tissues D and E (lab 21 and 24, Fig. 5) produced all extreme z-scores (>6). Tissues D and E contain the toxins GTX2,3 and STX, with Tissue E also containing dcSTX. This profile is relatively simple and would represent a fairly standard North European profile so the ELISA results if not pertaining to analyst or submission errors should be further investigated.

Although LC-MS/MS has not been used extensively in PSP PT schemes to date, results generated by this method are encouraging with only one unsatisfactory result submitted to date with most receiving satisfactory z-scores. LC-MS/MS participants have also demonstrated the applicability of their methods to cover a variety of different toxin profiles with satisfactory results received from a range of different samples [46].

Although we have attempted to look for correlations between method choice and performance, no obvious or consistent patterns could be discerned however.

4.7. Method choice

Choosing which method to use in determining PSP toxins depends on the final requirements of the laboratory with each method having

its own advantages and limitations [24,35]. In a routine monitoring scenario laboratories in the EU or exporting into the EU are legally bound to use designated methods of analysis such as the MBA or OMA AOAC 2005.06. The MBA is still the reference method in the EU however. Each of the methods used over the previous six exercises have demonstrated their applicability for these purposes but have also highlighted the potential pitfalls, with each method receiving the entire range of possible z-scores, satisfactory, questionable, unsatisfactory and extreme. No one method stands out as providing a clear advantage over the others so the final choice may be based on practical issues associated with the method, access and cost of equipment or the training and skills of the analyst, rather than any perceived shortcomings of each method.

MS determination of PSP toxins is desirable primarily based on the specificity the method provides and the significant reduction of matrix related problems recently reported have eliminated critical issues affecting method performance.

The LC-MS/MS results highlight the applicability of using HILIC-MS/MS technology for the separation and determination of PSP toxins with only one of thirteen results submitted to date receiving an unsatisfactory z-score. With sensitivity issues greatly improved through the application of an SPE clean-up step, detection limits are comparable or below those of the more established AOAC official methods. Each of the official AOAC methods based on LC-FLD have sufficient sensitivity to enable effective monitoring at current regulatory levels however.

Sample turnaround times using the method of Boundy et al. [45] are increased through the application of an SPE clean-up step compared to the original HILIC method of Dell'Aversano et al. [44]. Sample turnaround times are however similar when compared to both AOAC LC-FLD methods where two SPE steps and two oxidation reactions may need to be performed on a single sample (AOAC2005.06), or two chromatographic runs in the case of AOAC 2011.02. SPE can be fully automated and the application of UPLC technology greatly reduces analysis times which are critical in its application to a high throughput statutory monitoring scenario.

4.8. *Performance improvements*

Overall the development of this exercise and the performance of participating laboratories have improved since its inception in 2009. Participant numbers have increased which highlights the strengthening of the exercise and its economic sustainability going forward. The performance of laboratories has mostly improved and this is clearly evidenced from Fig. 7 which displays the averages of both the coefficient of variation for all samples in each round and the percentage of participants receiving satisfactory z-scores. The percentage of participants receiving satisfactory z-scores has increased most years as laboratories gain experience in these exercises and in determining potentially atypical toxin profiles. The precision of the laboratories as expressed by the coefficient of variation has also showed signs of significant improvement from the early exercises in 2009 and 2010.

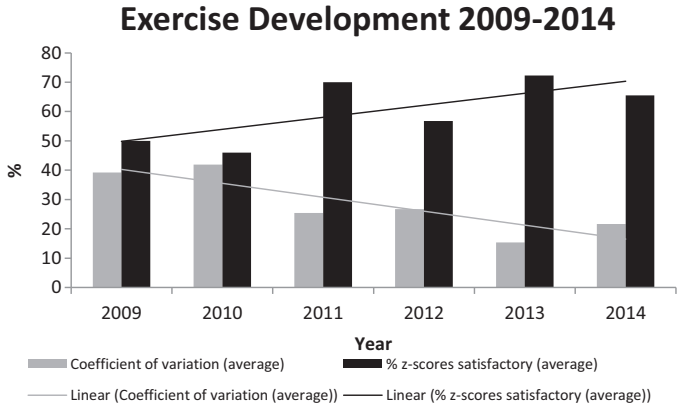


Fig. 7. Improvements made over the duration of the PSP development exercise.

The datasets also compare favourably with the PSP PT scheme operated by the EURL-MB and the lipophilic biotoxin exercise (BT-11) operated by QUASIMEME. In 2012 and 2013 where data from all methods was assessed together by the EURL-MB, between laboratory %CVs ranged from 33–52% in 2012 and 25–44% in 2013. In 2014 the EURL-MB did not assess the entire dataset from all participants together so comparison to the 2014 QUASIMEME PSP data was not possible. The QUASIMEME lipophilic biotoxin exercise in 2014 generated between laboratory %CVs in the range 20–38%, which equated to between 45–80% of participants receiving satisfactory z-scores. These exercises compare favourably with the QUASIMEME PSP exercise over the same period with average %CVs of 27, 15 and 22% in the years 2012–2014 equating to 56, 72 and 66% of participants receiving satisfactory z-scores respectively.

Through adding PSP toxins to their scope, QUASIMEME have established a comprehensive PT scheme for all EU regulated shellfish biotoxins.

5. Conclusions

Six development exercises for PSP toxins have been delivered since 2009 by QUASIMEME, with participation from laboratories in the EU, the Americas, Asia and Oceania using a range of different methods of analysis based on LC-FLD, LC-MS/MS, animal toxicity and immuno-based assays. Whilst no specific and consistent method dependency issues could be detected from the datasets, factors affecting poor performance were highlighted with suggestions made on how improvements could be made by participants. Monitoring the trends in z-scores from consecutive rounds has allowed QUASIMEME to determine which participants have improved, declined or maintained performance in these exercises.

The range of different methods used by QUASIMEME participants has highlighted the choice available for determining PSP toxins in shellfish. All the methods used over the previous six exercises have received the full range of z-scores from satisfactory to extreme. The choice of which method to use is therefore dependent on legislative requirements in the participants region, access and cost of equipment and the training and skills of the analyst rather than any perceived limitations of a particular method.

The use of HILIC-MS/MS technology in determining PSP toxins has shown the comparability this technique has compared to the other more established methods in routine monitoring programmes worldwide. Recent advances that have overcome issues affecting method performance have, for the first time provided a viable MS alternative for routine monitoring purposes. It is recommended that this modified HILIC-MS/MS method should be considered for further validation through a full collaborative study under the auspices of the AOAC or other relevant bodies.

QUASIMEME will continue to deliver a PSP exercise annually with data continually monitored by the Scientific Advisory Board (SAB) to determine issues contributing to poor performance and any method related issues.

Acknowledgements

We wish to thank all the participants over the last six years for their time and effort and for providing method specific information. Particular thanks to Wade Rourke (Canadian Food Inspection Agency) for helpful discussions on the data sets and the Biotoxin

Chemistry Team (BCT) at the Marine Institute for assistance in the preparation of materials.

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