Anal Bioanal Chem
DOI 10.1007/s00216-010-4428-7

ORIGINAL PAPER

Comparison of AOAC 2005.06 LC official method with other methodologies for the quantitation of paralytic shellfish poisoning toxins in UK shellfish species

Andrew D. Turner · Robert G. Hatfield · Monika Rapkova · Wendy Higman · Myriam Algoet · Benjamin A. Suarez-Isla · Marco Cordova · Catherine Caceres · Jeffrey van de Riet · Ryan Gibbs · Krista Thomas · Michael Quilliam · David N. Lees

Received: 17 September 2010 / Revised: 4 November 2010 / Accepted: 7 November 2010

Abstract A refined version of the pre-column oxidation liquid chromatography with fluorescence detection (ox-LC-FLD) official method AOAC 2005.06 was developed in the UK and validated for the determination of paralytic shellfish poisoning toxins in UK shellfish. Analysis was undertaken here for the comparison of PSP toxicities determined using the LC method for a range of UK bivalve shellfish species against the official European reference method, the PSP mouse bioassay (MBA, AOAC 959.08). Comparative results indicated a good correlation in results for some species (mussels, cockles and clams) but a poor correlation for two species of oysters (Pacific oysters and native oysters), where the LC results in terms of total saxitoxin equivalents were found to be on average more than double the values determined by MBA. With the potential for either LC over-estimation or MBA under-estimation, additional oyster and mussel samples were analysed using MBA and ox-LC-FLD together with further analytical and functional methodologies: a post-column oxidation LC method (LC-ox-FLD), an electrophysiological assay and hydrophilic interaction liquid chromatography with tandem mass spectrometric detection. Results highlighted a good correlation among non-bioassay results, indicating a likely cause of difference was the under-estimation in the MBA, rather than an over-estimation in the LC results.

Keywords Paralytic shellfish poisoning · LC · AOAC 2005.06 · Mouse bioassay · Shellfish · Saxitoxin · Oysters

A. D. Turner (✉) · R. G. Hatfield · M. Rapkova · W. Higman · M. Algoet · D. N. Lees
Centre for Environment, Fisheries and Aquaculture Science (Cefas),
Barrack Road, The Nothe,
Weymouth, Dorset DT4 8UB, UK
e-mail: andrew.turner@cefas.co.uk

B. A. Suarez-Isla · M. Cordova · C. Caceres
Laboratory of Marine Toxins, Institute of Biomedical Sciences,
Faculty of Medicine, University of Chile,
Santiago, Chile

J. van de Riet · R. Gibbs
Canadian Food Inspection Agency (CFIA),
1992 Agency Drive,
Dartmouth, NS B3B 1Y9, Canada

K. Thomas · M. Quilliam
Institute for Marine Biosciences,
National Research Council of Canada (NRCC),
1411 Oxford Street,
Halifax, NS B3H 3Z1, Canada

Introduction

Paralytic shellfish poisoning toxins (PSTs) are a group of more than 20 structurally related chemical compounds of the saxitoxin family produced naturally by certain species of algae in both marine and freshwater environments [1, 2]. PSP toxins are found to accumulate in filter-feeding bivalve shellfish, which poses a significant risk of human illness resulting from consumption of contaminated food products [3, 4]. As such, the requirement to monitor for occurrences of PSP in shellfish is stipulated in law [5, 6]. The current European official reference method for the analysis of PSTs in bivalve shellfish is the mouse bioassay (MBA), based on the AOAC 959.08 official method (OM) [7], involving the extraction of shellfish homogenates with hydrochloric acid (HCl) prior to subsequent bioassay in replicate (three) mice. In 2006, an alternative non-animal method, known as the

Published online: 27 November 2010

Table 1 Sources of oyster and cockle samples used in study

Sample no.	Samples	Species	Harvesting date	Harvesting location	Source/algal strain
1–2	BTX2008/1604 and PO 200	PO	May 2008	Northumberland, England	Natural toxic event
3	Cefas RM1	PO	May 2008	Portland, England	*A. tamarense* CCMP 1598
4	Cefas RM2	PO	June 2008	Portland, England	*A. fundyense* CCMP 1846
5	Cefas RM3	PO	Oct 2008	Portland, England	*A. fundyense* CCMP 1719
6–15	PO 1–19	PO	Jan 2009	Loch Creran, Scotland	*A. tamarense* CCAP 1119/17
16–18	PO 197–199	PO	Oct 2008–Mar 2009	Scotland and Weymouth, England	*A. fundyense* CCMP 1719 and *A. tamarense* CCAP 1119
19	Cefas RM4	NO	Oct 2008	Poole, England	*A. fundyense* CCMP 1719
20–24	NO 49–60	NO	Feb 2009	Loch Ryan, Scotland	*A. tamarense* CCAP 1119/17
25	Cefas RM9 131	NO	July 2009	Poole, England	*A. fundyense* CCMP 1846
26–34	NO 169–177	NO	Feb–May 2009	Scotland	*A. fundyense* CCMP 1719 and *A. tamarense* CCAP 1119
35–36	BTX2009/3063 and 3160	NO	Sept 2009	Cornwall, England	Natural toxic event
37	BTX2010/1605	NO	June 2010	Cornwall, England	Natural toxic event
38–42	CoA 21–35	Co	Feb 2009	Isle of Barra, Scotland	*A. minutum* AM89
43–47	CoB 61–66	Co	March 2009	Isle of Barra, Scotland	*A. tamarense* CCAP 1119/17
48–52	Cefas RM6a-e	Co	Jan 2009	Portland, England	*A. fundyense* CCMP 1846
53–55	Cefas RM7a-c	Co	Jan 2009	Portland, England	*A. fundyense* CCMP 1846
56–59	Co 202–205	Co	March 2009	Weymouth, England and Scotland	*A. minutum* AM89 and *A. fundyense* CCMP 1846

PO Pacific oysters, *NO* native oysters, *Co* cockles

'Lawrence method' [8–11] and formally validated as AOAC 2005.06 OM [12], was also adopted into European Commission law [6]. This method involves the extraction of shellfish homogenates in acetic acid, followed by various stages of clean up, a sample derivatisation step involving the oxidation of extracts to create fluorescent toxin oxidation products and subsequent quantitation of individual PSP toxins using liquid chromatography with fluorescence detection (LC-FLD). Total PSP toxicity is calculated by summing individual toxin concentrations and applying toxicity equivalents factors (TEF) determined for each toxin. The AOAC 2005.06 OM has been refined in the UK by Cefas and extended to include additional toxins for mussels. Validation characteristics were acceptable [13] and additionally showed a good correlation between the performance of the MBA and LC methods for both non-contaminated and PSP-contaminated mussels collected over a 3-year period. The method has been used for routine official control monitoring of UK mussels since May 2008. In addition, single laboratory validation has been conducted for other bivalve shellfish species of interest to the UK shellfish industry, specifically Pacific oysters (*Crassostrea gigas*), native oysters (*Ostrea edulis*), common cockles (*Cerastoderma edule*), hard clams (*Mercenaria mercenaria*) and razors (*Ensis* sp.) [14]. However, the low availability of naturally contaminated non-mussel bivalves prevented any thorough assessment of the relative performance of the MBA and LC methodologies [14].

The aims of this work were therefore to produce and test a range of suitable PSP-contaminated non-mussel shellfish materials to enable the comparison of methods for the determination of PSP toxins in Pacific oysters, native oysters and cockles. Data generated would demonstrate equivalence or otherwise of the LC method as compared with the reference MBA. With single laboratory validation data shown previously to be acceptable for these species [14], evidence of equivalence in method performance between the MBA and LC methods would give a good indication that the LC method would be safe as a replacement method for MBA in the UK official control monitoring programme.

Experimental

Reagents and chemicals

LC-grade solvents and analytical grade chemicals were used throughout for sample preparation and LC analysis. Certified reference materials (GTX1&4, NEO, dcSTX,

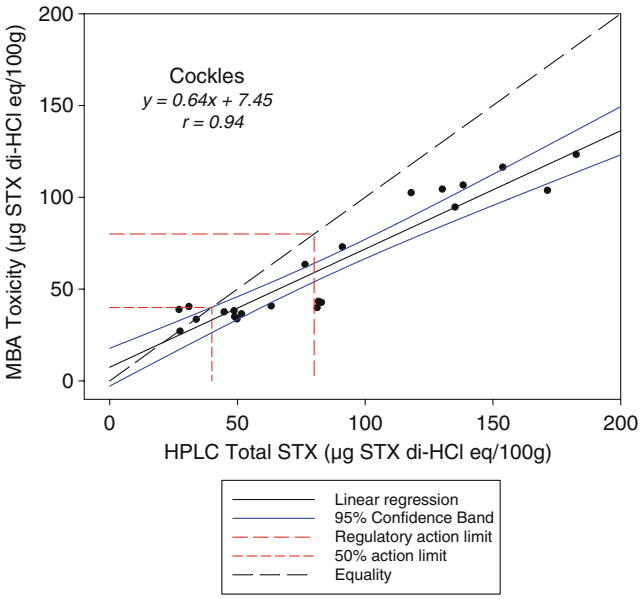

Fig. 1 Comparison of total saxitoxin equivalents quantified by pre-column HPLC-FLD (ox-LC-FLD) with the MBA reference method for UK cockles, showing 95% confidence, regulatory limits and equality

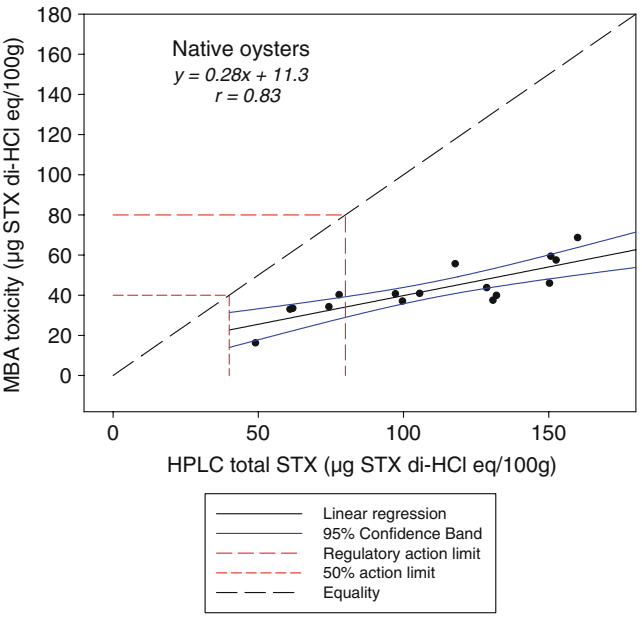

Fig. 3 Comparison of total saxitoxin equivalents quantified by pre-column HPLC-FLD (ox-LC-FLD) with the MBA reference method for UK native oysters, showing 95% confidence, regulatory limits and equality

GTX2&3, GTX5, C1&2, STX di-hydrochloride (di-HCl), dcNEO and dcGTX2&3) were obtained from the Institute for Marine Biosciences, National Research Council Canada (IMB, NRCC, Halifax, NS, Canada). Primary toxin standards were diluted in ~4.5 mL water to form concentrated stock standard solutions prior to dilution in 0.1 mM acetic acid to produce instrument calibration standards for use in

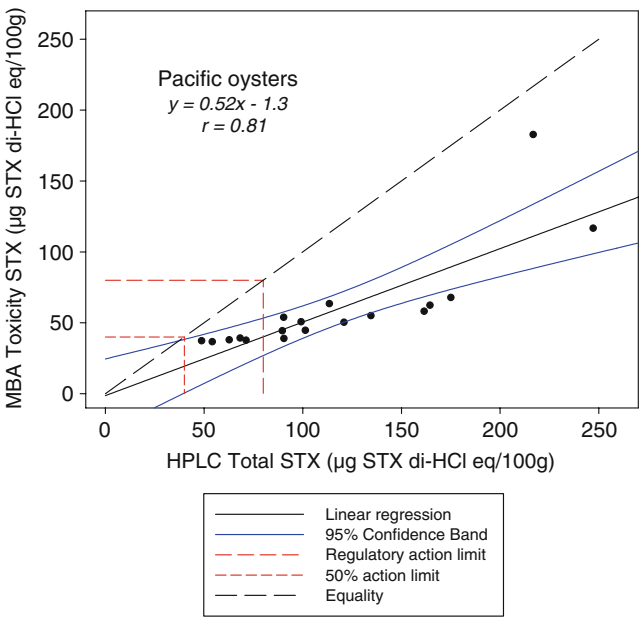

Fig. 2 Comparison of total saxitoxin equivalents quantified by pre-column HPLC-FLD (ox-LC-FLD) with the MBA reference method for UK Pacific oysters, showing 95% confidence, regulatory limits and equality

Cefas LC analysis. For Cefas MBA, toxicity was determined against NRCC STX di-HCl CRM calibration standards. For the CFIA LC-ox-FLD analysis, individual stock standards were prepared gravimetrically as per NRCC instructions. Solutions were diluted to 2 mL with 0.003 M HCl for the GTXs and STXs and with de-ionised water (DIW) at pH 5.0 for C toxins. GTX–STX matrix matched calibration solutions were prepared by diluting using a toxin-free mussel extract. C-Toxin working solutions were prepared by diluting with DIW (pH 5.0). For the electrophysiological method, analytical grade reagents as described in methods were used. For calibration, standard STX di-HCl provided by the US Food and Drug Administration, Office of Seafood, was used. All sample extracts were diluted in the external recording solution, and dilutions of USFDA STX di-HCl were prepared from fresh vials as indicated in AOAC 959.08 official method, using the external recording solution. For the purpose of method performance comparison in this study, all total STX equivalents results generated from analyses conducted using FDA STX di-HCl calibration standard were multiplied by a factor of 0.86 to account for the difference between the actual and stated concentration of STX di-HCl present in the FDA standard [15, 16]. For liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis, matrix matched calibration solutions were prepared by spiking three different levels of saxitoxin calibration solution CRMs (NRCC) into acetic acid or HCl extracts of negative control samples of native oysters

Table 2 Summary of results from HPLC (ox-LC-FLD) and MBA analysis of PSP-contaminated oysters and cockles (total toxicity in microgramme STX di-HCl eq./100 g flesh)

	Cockles	Pacific oysters	Native oysters
Number of samples	22	18	16
Mean HPLC concentration	85	117	110
Mean MBA concentration	62	60	43
Mean HPLC/MBA (RSD%)	136% (25%)	203% (23%)	259% (20%)
Pearson correlation coefficient (r)	0.94	0.81	0.83
Linear regression slope equation	$y=0.64x+7.45$	$y=0.52x-0.3$	$y=0.28x+11.3$
HPLC>AL; MBA<AL	4 (18%)	11 (61%)	11 (69%)
HPLC<AL; MBA>AL	0	0	0
HPLC and MBA both < or > AL	17 (77%)	7 (39%)	5 (31%)

AL action limit (80 µg STX di-HCl eq./100 g)

and Pacific oysters (provided by Cefas), NRCC CRM-Zero-Mus (a new zero-level mussel tissue reference material) or a sample of UK cockles that had been previously determined to be free of PSP toxins. Only a single-level spike was used for GTX5 due to an error.

Generation of PSP-contaminated shellfish

For the purpose of testing method performance, naturally contaminated shellfish samples were obtained wherever possible through the official control monitoring programmes of Great Britain. However, due to the low availability of naturally contaminated oysters and cockles, additional contaminated material was generated within the Cefas laboratory through the mass culturing of toxic algae and shellfish feeding experiments [17]. A range of toxic *Alexandrium* strains were cultured and fed to PSP-free cockles, Pacific oysters and native oysters, obtained from a variety of locations over a period of 2 years (Table 1). The sample set was supplemented by additional cockle samples provided by VeroMara and samples of cockles and oysters provided by the Scottish Association of Marine Science (SAMS), both generated through mass culturing of toxic algae and shellfish feeding experiments [18]. Fifty-six oyster and cockle samples in total were generated, with shellfish sourced from a number of different locations over a period of more than 2 years. In addition, the samples created through shellfish feeding experiments were contaminated through a number of different feeding techniques and with a variety of strains of *Alexandrium*, the toxin-producing dinoflagellate.

Shellfish sample preparation and testing regime

Bulk shellfish samples of each batch of contaminated shellfish were shucked, homogenised and stored frozen (<−20 °C) until required. Total saxitoxin equivalents were determined for each bulk sample, and sub-samples of homogenates were subsequently combined to create additional samples with a range of PSP toxin concentrations in each shellfish species. Each sample was extracted using both the AOAC 2005.06 acetic acid extraction [12] and the AOAC 959.08 [7] hydrochloric acid extraction methods. Sub-samples forwarded to LC-FLD analysis were additionally cleaned up using C18 solid phase extraction as detailed in [12, 13].

Acetic acid extracts of shellfish samples were screened by LC-FLD and fully quantified using the refined AOAC 2005.06 method [13] at Cefas. MBA was conducted on the HCl extracts of all samples, following internal standard operating procedures based on AOAC 959.08. A small number of samples were analysed by MBA at Agri-Food and Biosciences Institute, Northern Ireland (AFBINI). PSP toxin concentration data derived from LC-FLD analysis were compared with MBA results, and the correlation between the two data sets was determined. Subsequently, acetic acid

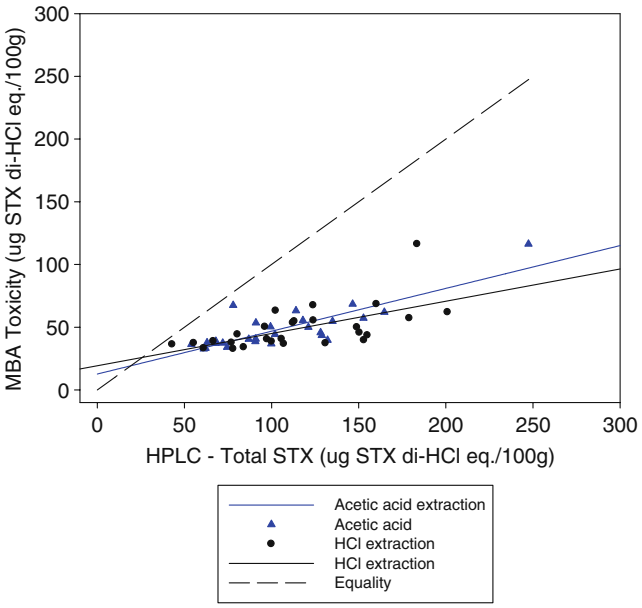

Fig. 4 Comparison of total saxitoxin equivalents quantified by pre-column HPLC-FLD (ox-LC-FLD) and the MBA reference method for UK oysters in both hydrochloric and acetic acid extracts

and HCl extracts of samples were analysed at CFIA for determination of PSP toxin concentrations using post-column oxidation (LC-ox-FLD) LC-FLD [19, 20]. Results obtained following AOAC 2005.06 LC-FLD analysis at Cefas from both HCl and acetic acid extracts were compared to determine the effects of extraction method on the comparative results. Additional sub-samples of HCl extracts were provided to the NRCC for LC-MS/MS analysis [21] and to the University of Chile for testing using electrophysiological assay (EA) [22], as well as repeat MBA following AOAC 959.08, including additional mussel samples previous analysed at Cefas as part of the UK routine monitoring programme. PSP toxicity results and total saxitoxin equivalents quantified were summarised, and correlations were determined between the various methods.

Non-bioassay methods employed for quantifying PSP toxins

Cefas pre-column oxidation LC-FLD method

An Agilent (Stockport, UK) fluorescence detector (1200 model FLD) was used for the detection of the PSP toxin oxidation products. Fluorescence excitation was set to 340 nm and emission to 395 nm. Mobile phase A consists of 0.1 M ammonium formate, adjusted to pH 6±0.1 with 0.1 M acetic acid; mobile phase B consists of 0.1 M ammonium formate with 5% acetonitrile, also adjusted to pH 6±0.1 with 0.1 M acetic acid. The mobile phase was delivered by an Agilent 1200 series LC at a flow rate of 2 mL/min. Chromatographic separation was performed using a Gemini C18 reversed-phase column (150× 4.6 mm, 5 μm; Phenomenex, Manchester, UK) with a Gemini C18 guard column (both set at 35 °C). The LC gradient was as follows: 0% to 5% mobile phase B in the first 5 min, 5% to 70% B for the next 4 min, hold at 70% B for 1 min and back to 100% A over the next 2 min. One hundred percent A was held for a further 2 min to allow for column equilibration prior to subsequent sample injections.

Samples were oxidised using both periodate and peroxide oxidation, and chromatographic results were reviewed to determine toxin oxidation peak retention times and peak area responses. Toxin concentrations were quantified against five-point calibration standards, with TEFs taken from Oshima [23]. In the case of isomeric pairs (GTX1&4, GTX2&3, C1&2 and dcGTX2&3), the highest TEF was used for each pair. Individual toxin concentrations were reported as microgramme STX dihydrochloride eq./100 g, and the total PSP toxicity was estimated by summing the individual concentration contributions from all quantified toxins and is quoted in terms of microgramme STX di-HCl eq./100 g.

CFIA post-column oxidation LC-FLD method

LC-ox-FLD was conducted following the protocol described in [19]. Hydrochloric acid extracts were deproteinised with trichloroacetic acid, and the pH was

Table 3 Linear regression gradients and coefficients calculated for PSP toxin calibration standards prepared in extracts of Pacific oysters and solvent over the working calibration range (0 to 1.0 μg STX di-HCl eq./gramme per toxin)

Toxin	Matrix	Calibration gradient	Correlation (r^2)	Percentage difference in matrix-spiked gradient compared to solvent calibration
GTX1,4	Solvent	0.973	1.000	
	P. Oysters	0.865	0.993	89%
NEO	Solvent	3.316	0.995	
	P. Oysters	2.509	0.996	76%
dcSTX	Solvent	93.138	1.000	
	P. Oysters	91.516	0.998	98%
GTX2,3	Solvent	18.250	0.999	
	P. Oysters	18.516	0.998	101%
GTX5	Solvent	12.884	0.999	
	P. Oysters	12.679	0.999	98%
STX	Solvent	22.943	0.999	
	P. Oysters	22.304	1.000	97%
C1,2	Solvent	55.000	0.999	
	P. Oysters	55.444	0.999	101%
dcGTX2,3	Solvent	15.561	0.999	
	P. Oysters	15.057	0.998	97%

Table 4 Comparison of total saxitoxin equivalence (microgramme STX di-HCl eq./100 g) in cockles (n=22) and mussels (n=24) generated following MBA, pre-column oxidation HPLC-FLD (ox-LC-FLD), LC-ox-FLD, LC-MS/MS and electrophysiological assay

Sample	Species	MBA Cefas	ox-LC-FLD Cefas	LC-ox-FLD CFIA	LCMS/MS NRCC	EA UChile
BTX2007/1434	M	84	105	37		
BTX2007/1535	M	43	28		95	32
BTX2007/1780	M	46	65	69	100	65
BTX2007/2316	M	37	37			
BTX2007/2319	M	40	47		69	63
BTX2007/2357	M	81	84			
BTX2007/2360	M	37	30		49	39
BTX2007/2419	M	38	25			
BTX2007/2432	M	42	66		70	40
BTX2007/2444	M	38	38			
BTX2007/2445	M	165	250		269	102
BTX2007/2451	M	44	50		98	102
790	M	41	43	38		
791	M	37	33	17		
868	M	113	118	78		
924	M	54	57	52		
960	M	nd	nd	nd		
998	M	39	29	35		
1007	M	52	51	51		
1047	M	76	57	60		
1075	M	53	65	48		
1109	M	48	57	64		
1286	M	nd	8	4		
2306	M	nd	11	24		
Co 202	Co	123	183	188	252	
Co 203	Co	104	172	153	188	230
Co 204	Co	94	135	146	218	194
Co 205	Co	106	139	156	206	170
CoA 21	Co	27	28	16		
CoA 22	Co	33	34	24		
CoA 24	Co	39	27	23		
CoA 29	Co	40	31	19		
CoA 35	Co	37	45	30		
CoB 61	Co	63	77	58		
CoB 62	Co	73	91	87		
CoB 64	Co	104	130	101		
CoB 65	Co	102	118	92		
CoB 66	Co	116	154	140		
CO RM6a	Co	43[a]	82	42		
CO RM6b	Co	40[a]	81	50		
CO RM6c	Co	36[a]	52	36		
CO RM6d	Co	34[a]	50	31		
CO RM6e	Co	38[a]	49	33		
CO RM7a	Co	43[a]	83	65		
CO RM7b	Co	41[a]	63	50		
CO RM7c	Co	35[a]	49	39		

M mussels, *Co* cockles, *nd* not detected

[a] Samples analysed by MBA at AFBINI

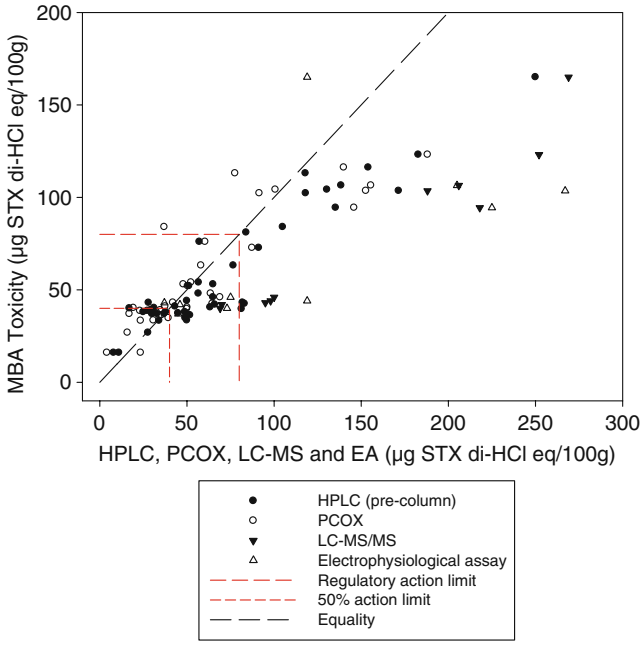

Fig. 5 Total saxitoxin equivalents in non-oysters (cockles and mussels) quantified by pre-column HPLC-FLD ox-LC-FLD, post-column HPLC-FLD (LC-ox-FLD), HPLC-MS/MS and electrophysiological assay as compared with the MBA PSP toxicity reference method

adjusted to 3. A portion of filtered extract was chromatographed, using an Agilent (Kirkland, PQ, Canada) LC-FLD 1200 system, on a C-18 column with a step gradient using a heptane sulfonic acid/phosphoric acid buffer system for the analysis of gonyautoxins and saxitoxins. The extract was also chromatographed on a C-8 isocratic tetrabutylammonium phosphate buffer system for the *N*-sulfocarbamoyl gonyautoxins, C1, C2, C3 and C4. The toxins were derivatised by post-column oxidation of the analytes at 85 °C with a phosphoric acid, periodic acid buffer solution and detected by fluorescence (excitation, 330 nm; emission, 390 nm).

NRCC hydrophilic-interaction liquid chromatography tandem mass spectrometry method

HILIC-MS/MS analyses were performed on an Agilent 1200 LC coupled with an API4000-QTRAP mass spectrometer equipped with an electrospray ionization source (AB-Sciex, Concord, ON, Canada) with MRM monitoring [21]. The column was 2.1×250 mm packed with 5-µm TSKgel Amide-80 and fitted with a guard cartridge (Tosoh Bioscience, Grove City, OH, USA). The injection volume was 5 µL, and the column was maintained at 40 °C. Two mobile phase solvents were used: A=water with 50 mM HCOOH and 2 mM NH_4COOH; B=acetonitrile (MeCN). A gradient elution was used with A programmed linearly from 90% B to 55% B at 25 min, then to 30% B at 27 min and a hold to 40 min. An equilibration time of 15 min was used between samples. The flow rate was 0.2 mL/min. Portions of all extracts were passed through a 60-mg OASIS-HLB cartridge (Waters, Milford, MA, USA) and then a 0.45-µm spin filter prior to analysis.

University of Chile electrophysiological assay

Cultured HEK 293 cells stably expressing a STX-sensitive rat skeletal muscle Na channels (Nav1.4) [24] were patch clamped in the whole-cell configuration [22]. Na currents (1–4 nA) were recorded under control conditions, after perfusion with dilutions of extracts of shellfish samples or saxitoxin (STX) dihydrochloride calibration solutions, and again under control conditions. Extracts of toxic shellfish samples blocked peak sodium currents in a concentration-dependent manner. Calibration curves were generated with increasing concentrations (0.01–100 nM) of standard STX-dihydrochloride (US FDA) with IC_{50}=1.45±0.10 nM; n=26. The recording external solution was (in millimolar) 70 NaCl, 70 tetraethylammonium chloride (or 70 tetramethylammonium chloride), 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10

Table 5 Summary of results in cockles and mussels comparing ox-LC-FLD, LC-ox-FLD, LC-MS/MS and electrophysiological assay against the reference MBA

	ox-LC-FLD	LC-ox-FLD	LC-MS/MS	EA
Number of samples	46	36	11	10
Mean ratio to MBA (RSD%)	117% (37%)	100% (35%)	191% (31%)	146% (61%)
Pearson correlation coefficient (r)	0.940	0.875	0.963	0.646
Linear regression slope equation	y=0.61x+14	y=0.61x+20	y=0.52x+0.1	y=0.37x+34
Other method>AL; MBA<AL	4 (9%)	1 (3%)	3 (27%)	1 (10%)
Other method<AL; MBA>AL	0	1 (3%)	0	0
Other method and MBA both < or > AL	42 (91%)	34 (94%)	8 (73%)	9 (90%)

AL action limit (80 µg STX di-HCl eq./100 g)

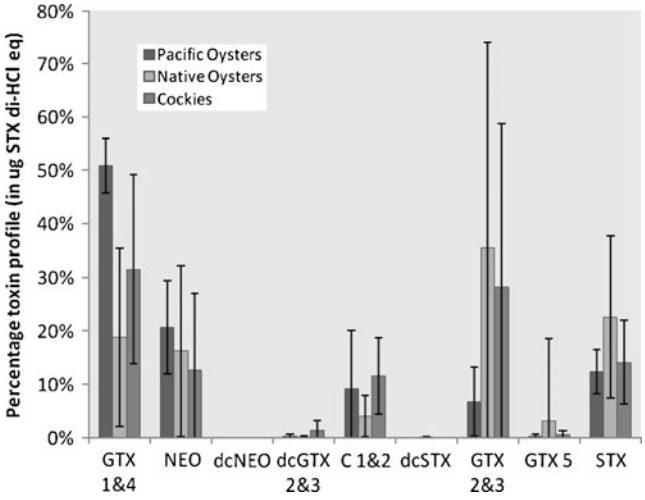

Fig. 6 Comparison of total PSP toxin percentage profile (in terms of STX di-HCl equivalence, ±1 SD) as quantified by ox-LC-FLD in UK cockles, Pacific oysters and native oysters

glucose and 10 HEPES, pH 7.4. The patch pipette (1–2 MΩ) contained (in millimolar) 140 CsF, 5 NaCl, 1 $MgCl_2$, 10 EGTA and 10 HEPES, pH 7.2.

Results and discussion

Comparison of pre-column oxidation LC-FLD with MBA

Comparisons between shellfish toxicity, as determined by MBA [7], and total saxitoxin equivalents, as quantified by pre-column LC-FLD following [12, 13], are illustrated in Figs. 1, 2 and 3 for cockles (n=22), Pacific oysters (n=18) and native oysters, respectively (n=16). Visual inspection of the results indicates that there are correlations between the data sets but with a noticeable and variable positive bias in the LC results for each species as compared to the MBA.

The mean LC/MBA ratio for the 22 cockle samples is 1.36, showing there is a degree of positive bias in the cockle LC results. Four out of the 22 cockle samples exhibit LC toxicities above the action limit (AL; 80 μg STX di-HCl eq./100 g) with the MBA below AL (Table 2), although the LC results from these return total saxitoxin equivalents close to the action level (81, 82, 83 and 91 μg STX di-HCl eq./100 g). Figure 1 shows the visual comparison of toxicity results, highlighting the linear regression between the two methods and showing its relation to equality. Confidence bands are the 95% confidence for the predicted mean at each x value and give further evidence for a slight positive bias in the LC method as compared with the MBA (Table 2). It is noted that whilst the differences observed between the two methods fall within the uncertainty of measurement determined earlier for the LC method [13, 14], any such bias can be explained primarily by the use of the highest TEF for each epimeric pair. The strains of *Alexandrium* used to contaminate cockles at Cefas and SAMS contained high proportions of GTX1&4, quantitation of which using the pre-column oxidation LC method involved the assumption that the highest toxicity epimer (GTX1) is exclusively present. Re-calculations using the lowest TEF for all epimeric pairs result in a mean LC/MBA ratio of close to 1.0 for all cockles analysed (data not shown) and a 100% agreement between LC and MBA results in relation to the action limit.

Results for Pacific oysters, illustrated in Fig. 2 and summarised in Table 2, demonstrate a clear positive bias for the LC results as compared with the MBA reference method. A mean LC/MBA ratio of 2.03 (23% RSD; n=18) results in a significant number of samples (>60%) being found lower than the action limit by MBA but higher by LC (Table 2). Given such a high relative bias in the LC results, the assumption regarding exclusive use of the highest TEF for each epimeric pair does not fully explain the observed bias, even with the relatively high proportion of GTX1&4 present in the samples. The assumption for the exclusive presence of the lowest TEFs for each epimeric pair would only reduce the mean LC/MBA value to 1.56.

Results illustrated graphically in Fig. 3 for native oysters and summarised in Table 2 highlight the clear large positive bias in LC results for the 16 samples analysed as compared with the MBA reference method. A mean LC/MBA ratio of 2.59 for native oysters (RSD=20%) resulted in 11 samples being above AL by LC whilst below AL by MBA (Table 2). In addition, one further sample showed a toxicity of 49 μg STX di-HCl eq./100 g by LC but was negative by MBA. As with Pacific oysters, a relatively high proportion of GTX1&4 and GTX2&3 is present, resulting in the potential for over-estimation of total toxicity when using the highest TEF for each epimeric pair. Again, however, this effect does not fully explain the significant bias observed in these results.

Comparison of extraction methods

One factor potentially affecting the correlation between the two methods is the difference in concentrations of PSP toxins extracted with the two different extraction methods. There are noted examples of varying proportions of different PSP toxins between the acetic acid and hydrochloric acid extracts, with evidence of different extraction efficiencies and/or transformation of toxin analogues in the hydrochloric acid extracts [25–28], with the latter predominantly relating to the transformation of the C toxins into the more toxic carbamates counterparts [25, 28–30]. Results from the analysis of both acetic and HCl extracts of both Pacific and native oysters by pre-column LC are illustrated in Fig. 4 and show a clear positive bias in the LC results in both extracts as compared with the MBA results. The mean LC bias in acetic

Table 6 Comparison of total saxitoxin equivalence (microgramme STX dc-HCl eq./100 g) in oysters (Pacific and native) generated following MBA, ox-LC-FLD, LC-ox-FLD, LC-MS/MS and electrophysiological assay

Sample	Species	MBA Cefas	ox-LC-FLD Cefas	LC-ox-FLD CFIA	LCMS NRCC	EA UChile
RM4	NO	59[a]	151			
NO 49	NO	68	160	121		
NO 50	NO	41	106	76		
NO 51	NO	37	131	121		
NO 55	NO	46	150	106		
NO 60	NO	40	97	108		
RM9 131	NO	nd	49			
NO 169	NO	33	62	50	83	110
NO 170	NO	33	61	58	96	72
NO 171	NO	34	75	64	132	115
NO 172	NO	40	132	103	212	88
NO 173	NO	55	118	106	217	125
NO 174	NO	44	129	120	236	197
NO 175	NO	37	100	82	164	97
NO 176	NO	57	153	132	274	174
NO 177	NO	nd	2	2	1	nd
BTX2010/1605	NO	40	78			
RM1	PO	37[a]	49			
RM2	PO	182[a]	217	155		
BTX2008/1604	PO	44	90	68		
RM3	PO	58[a]	162			
PO 1	PO	53	91	85		
PO 5	PO	55	135	110		
PO 6	PO	36	54	41		
PO 7	PO	37	72	49		
PO 9	PO	68	175	120		
PO 12	PO	63	114	101		
PO 13	PO	39	68	58		
PO 15	PO	44	102	86		
PO 18	PO	116	247	175		
PO 19	PO	50	99	77		
PO 197	PO	62	165	132	154	92
PO 198	PO	50	121	101	137	129
PO 199	PO	38	63	44	71	65
PO 200	PO	39	91	74	103	110
PO 201	PO	nd	19	7	10	nd

NO native oysters, *PO* Pacific oysters, *nd* not detected

[a] Samples analysed by MBA at AFBINI

acid extracts for all oysters as compared to MBA was 2.17 (RSD=22%), whereas the bias for LC results following hydrochloric acid extraction was higher (2.40, RSD=30%). Consequently, the mean bias in results generated from LC analysis of acetic acid and HCl extracts was 0.94 (RSD=21%). Data therefore shows that the toxicity estimated by LC analysis of HCl extracts is, if anything, higher than the values determined from acetic acid extracts, inferring that the different extraction methods and any subsequent transformation of toxin analogues in either of the acidic extracts are not responsible for the significant positive bias in the LC results as compared with the reference MBA.

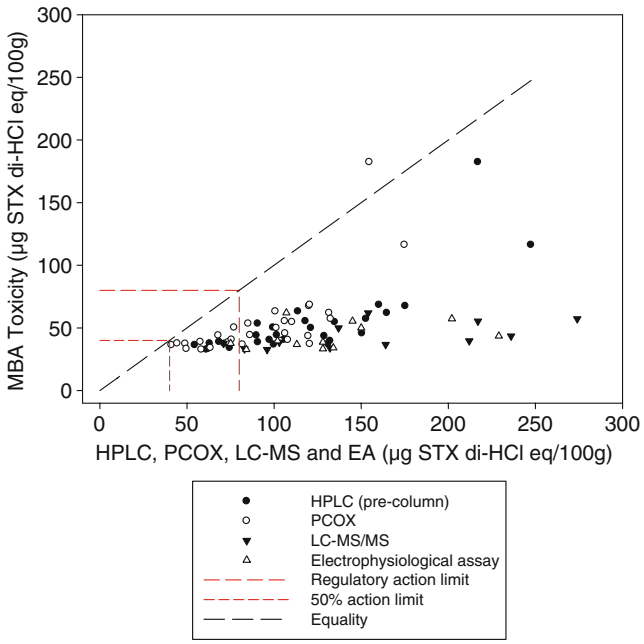

Fig. 7 Total saxitoxin equivalents in oysters (Pacific and native) quantified by ox-LC-FLD, LC-ox-FLD, HPLC-MS/MS and electrophysiological assay as compared with the MBA PSP toxicity reference method

Matrix fluorescence enhancement

The possibility of fluorescence enhancement of PSP toxins in the oyster matrix was investigated given the potential for this to occur as highlighted in Pacific oysters during previous validation studies [13, 14]. Experiments were carried out using calibration standards diluted in either solvent or extracts of PSP-free Pacific oysters from exactly the same source (temporal and spatial) as the Pacific oysters PO1-19 (Table 1). Table 3 summarises the effects of this specific Pacific oyster matrix on the fluorescence response of each of the PSP toxin analogues, with results indicating only a small amount of fluorescence suppression noted for GTX1&4 and NEO and with the absence of any noticeable enhancement in the oyster matrix. As such, there is no evidence for matrix components in these particular oysters falsely enhancing the toxin signals following LC separation. Low levels of fluorescence enhancement have been observed previously for some toxins in the native oyster matrix [14], but these enhancements were minor compared to the relative differences in final toxicity results observed here. It is possible that variations in fluorescence enhancement or suppression previously observed between different shellfish species [13, 14] may also occur between different samples of the same species with different spatial and temporal origins. The set of samples utilised in this study, however, was comprised of a variety of shellfish samples with origins from around the UK, including Scotland, South West England, Southern England and North East England. Shellfish utilised for feeding experiments were contaminated with a number of different strains of *Alexandrium* at different times of the year and were originally sourced from different growing environments over a 2-year period. As such, the samples utilised here are not constrained to one isolated source of shellfish, with variability in spatial and temporal source of shellfish still resulting in samples exhibiting the significant differences in method performance observed.

Analysis of shellfish samples by post-column oxidation LC-FLD, HILIC-MS/MS and electrophysiological assay

With data indicating significant differences in the results returned by the two current official control methodologies for determination of PSTs in bivalve shellfish, further analysis was conducted on a sub-set of the above samples using additional methodologies in order to further examine relative method performance for these groups of samples. Results obtained from LC-ox-FLD [19], LC-MS/MS [21, 29, 31] and EA [22] were compared to those results calculated from both ox-LC-FLD and MBA. Relative method performance was examined for the determination of PSTs in non-oyster samples (Table 4). Figure 5 displays

Table 7 Summary of results in Pacific and native oysters comparing ox-LC-FLD, LC-ox-FLD, LC-MS/MS and electrophysiological assay against the reference MBA

	ox-LC-FLD	LC-ox-FLD	LC-MS/MS	EA
Number of samples	35	30	12	12
Mean ratio to MBA (RSD%)	232% (55%)	187% (52%)	359% (120%)	268% (82%)
Pearson correlation coefficient (r)	0.776	0.690	0.584	0.480
Linear regression slope equation	y=0.47x−2	y=0.61x−4	y=0.10x+32	y=0.10x+32
Other method>AL; MBA<AL	23 (66%)	16 (53%)	11 (92%)	10 (83%)
Other method<AL; MBA>AL	0	0	0	0
Other method and MBA both < or > AL	12 (34%)	14 (47%)	1 (8%)	2 (17%)

AL action limit (80 µg STX di-HCl eq./100 g)

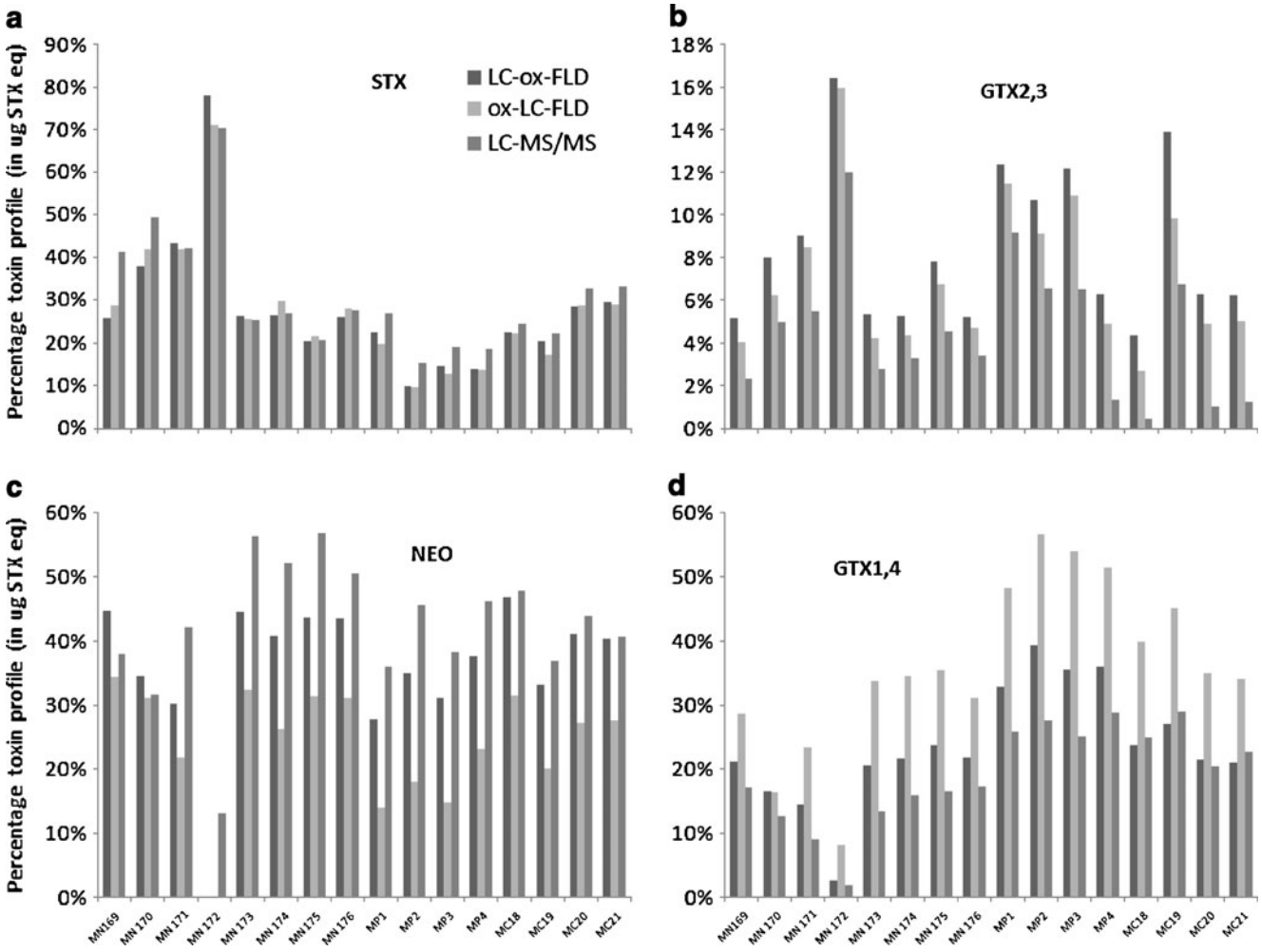

Fig. 8 Comparison of PSP toxin percentage profile for four dominant toxins (in terms of STX di-HCl equivalence) as quantified by ox-LC-FLD, LC-ox-FLD and LC-MS/MS in UK cockles and oysters

the comparative results obtained from the four different non-animal methods against the reference MBA, with comparisons summarised in Table 5. Results in Table 5 show the MBA and two LC methods agreeing closer on average with the LC-MS/MS and EA methods over-estimating to an extent. Comparison of LC-ox-FLD and ox-LC-FLD results for 46 samples in Table 4 shows similar results for the two methods in these species, with a small degree of positive bias in the Cefas ox-LC-FLD method evident for some of the cockle samples. This bias is fully attributable to the assumptions regarding the sole presence of the highest toxicity epimer, whereas the LC-ox-FLD method quantifies each isomer individually, given that high proportions of GTX1&4 and GTX2&3 were found to be present in this sample group. Figure 6 illustrates the average complete toxicity profile of the cockle samples, showing the high relative proportions of these toxins along with STX and C1&2, albeit with a high variability of some toxins due to the different sources of algae strains used for some of the feeding experiments (Table 1). Additional data from LC-MS/MS and EA shows total saxitoxin equivalents values either similar to or higher than those returned by the two LC methods. Table 5 also demonstrates the effect of application of each method result in relation to the regulatory action limit (80 μg STX di-HCl eq./100 g). As shown previously for the pre-column oxidation LC method in mussels [13], the effects of implementation of both the LC methods for mussels and cockles in comparison with the MBA would be similar, with only a very low percentage of samples showing total saxitoxin equivalents greater than the regulatory limit by LC and less than the regulatory limit by MBA. For the LC-MS and EA analysis, such an analysis of comparative results is more difficult with the lower number of samples analysed, but importantly, none of the methods are shown to under-estimate the toxicity of the mussel and cockles samples as compared with the reference method. It is also noted that previous work has demonstrated a significant correlation between the results returned

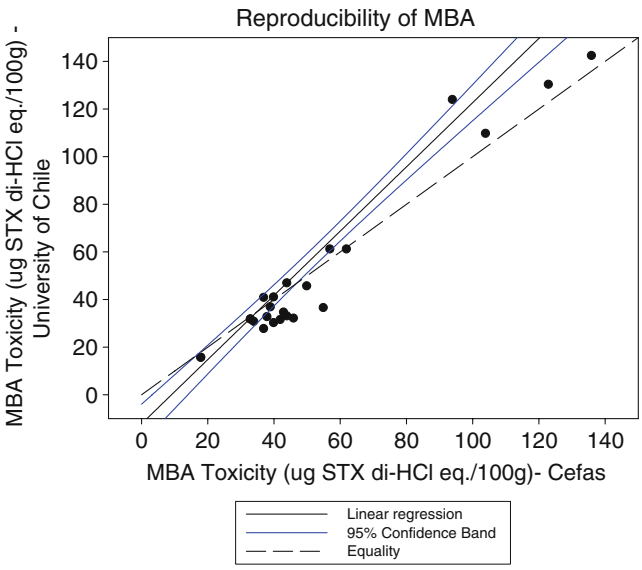

Fig. 9 Comparison of MBA toxicity results (microgramme STX di-HCl eq./100 g) generated at two laboratories (Cefas and University of Chile) for determination of reproducibility of toxicity results

by the EA as compared with the MBA in a large sample data set [22, 32]

Table 6 summarises the total saxitoxin equivalents results obtained from the five methodologies in both Pacific and native oysters. Results highlight a good agreement between the pre-column and post-column LC results for both oyster species, with a slight positive bias in the pre-column LC results as compared with the LC-ox-FLD results being again fully attributable to the assumption in presence of higher TEFs for epimeric pairs in the former method. Figure 6 again illustrates the high average relative proportions of GTX1&4 and GTX2&3 in these two sample matrices. In addition, comparison of results from the four non-animal methodologies shows a significant positive bias in comparison with the MBA reference method, illustrated by the correlation graph in Fig. 7. Results from the five methods therefore strongly indicate that the total toxicity of the oyster samples is significantly higher than the values returned by the MBA. Table 7 summarises the overall bias of each method and the potential effects of application of each method in relation to the regulatory action limit. For both LC methods, as well as LC-MS/MS and EA, results indicate a high proportion of samples with total saxitoxin equivalents greater than 80 μg STX di-HCl eq./100 g but with the MBA showing values significantly less than the regulatory limit. The close agreement between pre-column and post-column oxidation LC methods gives further evidence that the effects of TEF assumption in the former method are not solely responsible for the large bias seen in the results from these species.

Figure 8 plots the toxin profiles as percentages of total saxitoxin equivalents for the four major toxins present in 16 MBA-positive cockle and oyster samples after analysis by ox-LC-FLD, LC-ox-FLD and LC-MS/MS. Results indicate clear similarities in profile between the three methods, particularly for STX and GTX2&3, providing further evidence for the validity of the methods. The relative proportions of GTX1&4 showed higher levels of this toxin following the ox-LC-FLD method as compared with LC-ox-FLD and LC-MS/MS which show similar ratios. This difference is again attributable to the use of the highest TEF for the GTX1&4 epimeric pair, consequently explaining the lower relative proportions of NEO quantified by the pre-column LC-FLD method as compared with LC-ox-FLD. LC-MS/MS results are consistently higher for NEO concentrations, which is partially responsible for the positive bias of the LC-MS/MS analyses compared with ox-LC-FLD, LC-ox-FLD and MBA analyses. It is believed that the problem is due to matrix enhancement of ionization in the electrospray source, which is a common problem in LC-MS/MS. This is complicated by it being difficult to have blank tissue samples available for every matrix

Table 8 Comparison of PSP toxicity results (microgramme STX di-HCl eq./100 g) obtained by MBA at two separate laboratories (Cefas, UK and University of Chile)

Species	MBA Cefas	MBA UChile
PO	62	61
PO	50	46
PO	38	33
PO	39	37
PO	nd	nd
NO	33	32
NO	33	32
NO	34	31
NO	40	41
NO	55	36
NO	44	47
NO	37	41
NO	57	61
NO	nd	nd
Co	123	130
Co	104	110
Co	94	124
M	43	34
M	46	32
M	40	30
M	37	28
M	42	31
M	136	142
M	44	33

PO Pacific oysters, *NO* native oysters, *Co* cockles, *M* mussel, *nd* not detected, *na* not applicable

investigated in order to prepare proper matrix matched calibration solutions. The implementation of a standard addition quantitation for all samples could correct this problem, and such a technique has been investigated by NRCC for PSP toxin analyses by LC-MS/MS with great success (results not yet published). However, it does require lengthy sample preparation and was not feasible for this experiment.

Reproducibility of MBA

With comparative results from the five methods for all four species indicating a potential MBA under-estimation effect in the oyster samples rather than an over-estimation in the LC-FLD results, it was important to determine how repeatable the MBA results would be for these samples. HCl extracts of mussels, cockles and oysters previously analysed by MBA in the UK and frozen (<−20 °C) post-analysis were shipped to the University of Chile for repeat MBA. The correlation between the two sets of analyses is illustrated in Fig. 9 with actual toxicity results tabulated in Table 8. A level of agreement is demonstrated between the two sets of data, indicating that the reproducibility of the MBA for these samples is acceptable and within the levels described for the method previously [33–35].

Conclusions and future work

Results illustrated in Figs. 1, 2, 3 and 4 and summarised in Table 2 show clear differences in the method performance and results obtained from the two official methods of analysis for determination of PSP toxicity in oysters. From these results alone, it is impossible to confirm whether the cause of the observed differences relates to an over-estimation of the LC-FLD method or an under-estimation of the MBA. However, work conducted on PSP-free Pacific oyster material showed the absence of any matrix-related fluorescence enhancement, which may have artificially increased toxin concentrations. Similarly, work conducted on both HCl and acetic acid extracts confirmed that the differences in extraction methods were not responsible for the differences in method performance. Further analysis by LC-ox-FLD, LC-MS/MS and EA conducted on a range of oyster samples gave estimations of total PSP toxicity significantly higher than the values returned by MBA. Analysis of non-oyster samples showed a good level of agreement between the two LC-FLD methods with LC-MS/MS and EA results either similar, or higher still. Repeat analysis by MBA showed the bias in oysters was not related to any reproducibility issues with the MBA. Together, this data gives strong evidence for the under-estimation in total PSP toxicity in Pacific oysters and native oysters by MBA as compared with the total saxitoxin equivalents values quantified by LC-FLD. Future work will continue with investigations into the presence of matrix components in the extracts of oyster samples which may affect the relative performance of the different methodologies utilised in this comparative study. In the interim, our results suggest that a precautionary approach to public health protection would imply the use of analytical methods in addition to or in replacement of the MBA for official control monitoring of PSP in oysters.

Acknowledgements The authors would like to gratefully thank and acknowledge the help of K. Hargin and C. Martins (Food Standards Agency, UK) for funding the LC analysis conducted at Cefas and shellfish feeding conducted at SAMS and VeroMara, C. Campbell (SAMS), C. Shellcock (VeroMara) for provision of some of the contaminated shellfish materials, AFBINI for conducting additional MBA, Mr. L. Coates and K. Dhanji (Cefas) and Y. Gao (NRCC) for technical help and laboratory support. Partial support from IAEA Technical Cooperation Grant CHI 07/011 to University of Chile is gratefully acknowledged.

References

1. Llewellyn LE (2006) Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat Prod Rep 23:200–233
2. Wright JLC (1995) Dealing with seafood toxins: present approaches and future options. Food Res Int 28:347–358
3. Luckas B, Hummert C, Oshima Y (2004) Analytical methods for PSP. In: Hallegraeff GM, Anderson DM, Cembella AD (eds) Manual on harmful marine microalgae. Unesco Publishing, France, p 191
4. Etheridge SM (2010) Paralytic shellfish poisoning: seafood safety and human health perspectives. Toxicon 56:108–122
5. Anonymous (2004) Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29th April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Off J Eur Union L226:83–127
6. Anonymous (2006) Commission Regulation (EC) No 1664/2006 of 6th Nov. 2006 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. Off J Eur Union L320:13–45
7. Anonymous (2005) AOAC Official Method 959.08. Paralytic shellfish poison. Biological method. Final action. In: Truckses MW (ed) AOAC official methods for analysis, 18th Edition, Chapter 49: Natural toxins. AOAC International, Gaithersburg, pp 79–80
8. Lawrence JF, Menard C (1991) Liquid chromatographic determination of paralytic shellfish poisons in shellfish after prechromatographic oxidation. J AOAC Internat 74(6):1006–1012
9. Cleroux C, Menard C, Lawrence JF (1995) Evaluation of prechromatographic oxidation for liquid chromatographic determination of paralytic shellfish poisons in shellfish. J AOAC Internat 78(2):514–520
10. Lawrence JF, Niedzwiadek B (2001) Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection. J AOAC Internat 84(4):1099–1108
11. Lawrence JF, Niedzwiadek B, Menard C (2005) Quantitative determination of paralytic shellfish poisoning toxins in shellfish

using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J AOAC Internat 88(6):1714–1732
12. Anonymous (2005) AOAC Official method 2005.06 Quantitative determination of paralytic shellfish poisoning toxins in shellfish using pre-chromatographic oxidation and liquid chromatography with fluorescence detection. AOAC International, Gaithersburg
13. Turner AD, Norton DM, Hatfield RG, Morris S, Reese AR, Algoet M, Lees DN (2009) Single laboratory validation of the AOAC LC method (2005.06) for mussels: refinement and extension of the method to additional toxins. J AOAC Internat 92(1):190–207
14. Turner AD, Norton DM, Hatfield RG, Rapkova M, Algoet M, Lees DN (2010) Single laboratory validation of a refined AOAC HPLC method (2005.06) for oysters, cockles and clams in UK shellfish. J AOAC Int 93(5):1482–1493
15. Quilliam MA (2007) Supplemental information for PSP toxin CRMs: structures, molecular weights, concentrations and toxicities. CRMP Technical Report CRM-PSP-20070411. National Research Council, Halifax
16. Quilliam MA, Cembella AD, Windust A, Richard DJA (1999) Comparison of saxitoxin calibration standards by mouse bioassay and chemical analysis methods. In: Martin JL, Haya K (eds) Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae. St. Andrew, NB, 27–29 May 1998. Can Tech Rep Fish Aquat Sci 2261:52–57
17. Higman WA, Turner AD (2010) A feasibility study into the provision of paralytic shellfish toxins laboratory reference materials by mass culture of *Alexandrium* and shellfish feeding experiments. Toxicon 56:497–501
18. Collins C, Graham J, Brown L, Bresnan E, Lacaze J-P, Turrell EA (2009) Identification and toxicity of *Alexandrium tamarense* (Dinophyceae) in Scottish waters. J Phycology 45(3):692–703
19. Van de Riet JM, Gibbs RS, Chou F, Muggah PM, Rourke WA, Burns G, Thomas K, Quilliam MA (2010) Liquid chromatographic post-column oxidation method for analysis of paralytic shellfish toxins in mussels, clams, scallops and oysters: single-laboratory validation. J AOAC Internat 92(6):1690–1704
20. Rourke WA, Murphy CJ, Pitcher G, van de Riet JM, Burns BG, Thomas KM, Quilliam MA (2008) Rapid post-column methodology for determination of paralytic shellfish toxins in shellfish tissue. J AOAC Internat 91:589–597
21. Dell'Aversano C, Hess P, Quilliam MA (2005) Hydrophilic interaction liquid chromatography–mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. J Chrom A 1081:190–201
22. Velez P, Sierralta J, Alcayaga C, Fonseca M, Loyola H, Johns DC, Tomaselli GF, Marban E, Suarez-Isla BA (2001) A functional assay for paralytic shellfish toxins that uses recombinant sodium channels. Toxicon 39:929–935
23. Oshima Y (1995) Post-column derivatisation liquid chromatography method for paralytic shellfish toxins. J AOAC Internat 78:528–532
24. Goldin AL, Barchi RL, Caldwell JH, Hofmann F, Howe JR, Hunter JC, Kallen RG, Mandel G, Meisler MH, Berwald Netter Y et al (2000) Nomenclature of the voltage-gated sodium channels. Neuron 28:365–368
25. Anderson DM, Kulis DM, Qi YZ, Zheng L, Lu SH, Lin YT (1996) Paralytic shellfish poisoning in Southern China. Toxicon 34(5):579–590
26. Jiang T, Jiang T-J (2008) Investigation of extraction method for paralytic shellfish poisoning toxins in shellfish. Chin J Anal Chemistry 36(11):1460–1464
27. Ravn H, Anthoni U, Christophersen C, Nielsen PH, Oshima Y (1995) Standardised extraction method for paralytic shellfish poisoning toxins in phytoplankton. J Appl Phycol 7:589–594
28. Indrasena WM, Gill TA (2000) Storage stability of paralytic shellfish poisoning toxins. Food Chem 71:71–77
29. Reeves K, Thomas K, Quilliam MA (2004) A mussel tissue certified reference material for paralytic shellfish poisoning toxins. In: Henshilwood K, Deegan B, McMahon T, Cusack C, Keaveney S, Silke J, O'Cinneide M, Lyons D, Hess P (eds) Proceedings from 5th international conference on Molluscan shellfish safety. The Marine Institute, Galway, Ireland, pp 116–122
30. Rossini GP, Hess P (2010) Phycotoxins: chemistry, mechanisms of action and shellfish poisoning. Mol Clin Environ Toxicology 2:65–121
31. Quilliam MA, Hess P, Dell'Aversano C (2001) Recent development in the analysis of phycotoxins by liquid chromatography-mass spectrometry. In: de Koe WJ, Samson RA, van Egmond HP, Gilbert J, Sabino M (eds) Mycotoxins and phycotoxins in perspective at the turn of the century. Ponsen & Looijen, Wageningen, pp 383–391
32. Suarez-Isla BA, Cordova M (2009) The need for biosensors and new field methods electrophysiological assay and MBA results from a recent outbreak of PSP in Chile. 123rd AOAC Annual Meeting & Exposition, Philadelphia
33. Ledox M, Hall S (2000) Proficiency testing of eight French laboratories in using the AOAC mouse bioassay for paralytic shellfish poisoning: interlaboratory collaborative study. J AOAC Internat 83(2):305–310
34. McFarren EF (1959) Report on collaborative studies of the bioassay for paralytic shellfish poison. J AOAC Internat 42:263–271
35. CRLMB (2009) Report on the CRLMB 2009 proficiency testing on saxitoxin group (PSP) toxins determination. Community Reference Laboratory for Marine Biotoxins, Vigo