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Investigations into matrix components affecting the performance of the official bioassay reference method for quantitation of paralytic shellfish poisoning toxins in oysters

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ABSTRACT

Significant differences previously observed in the determination of paralytic shellfish poisoning toxins (PSTs) in oysters using official method AOAC 2005.06 and 959.08 were investigated in detail with regard to possible matrix effects. Method AOAC 2005.06 gave results 2–3 times higher than the mouse bioassay method, 959.08, differences thought to be due to underestimation of PSTs by the mouse bioassay. In order to prove the cause of these large differences, work was conducted here to examine the presence and effects of matrix components on the performance of each of the two assays. A range of oyster, cockle and mussel samples were extracted using the AOAC 959.08 hydrochloric acid (HCl) extraction method and analysed for PSP by both MBA and LC-FLD. In addition, extracts were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP–MS) for metals as well as being subjected to a range of nutritional testing methods. Whilst there was no evidence for effect of nutritional components on either assay, ICP–MS analysis revealed a relationship between samples exhibiting the largest differences in relative method performance, specifically those with the largest LC-FLD/MBA toxicity ratio, and samples containing the highest concentrations of zinc and manganese. In order to prove the potential effect of the metals on either the LC-FLD and/or MBA assays, HCl extracts of a range of shellfish were subjected to a number of matrix modifications. Firstly, a number of PSP-positive oyster samples were processed to reduce the concentrations of metals within the extracts, without significantly reducing the concentrations of PSTs. Secondly, a range of mussel and cockle extracts, plus a standard solution of saxitoxin di-hydrochloride were spiked at variable concentrations of zinc. All treated and non-treated extracts, plus a number of controls were subjected to ICP–MS, LC-FLD and MBA testing. Results proved the absence of any effect of metals on the performance of the LC-FLD, whilst showing a large suppressive effect of the metals on the MBA. As such, the results show the performance of the official MBA is potentially unsafe for application to the routine monitoring of PSP toxicity in oysters or in any other shellfish found to contain high concentrations of metal ions.

1. Introduction

Paralytic shellfish poisoning toxins (PSTs) are a family of tetrahydropurine compounds based on saxitoxin (STX; Fig. 1). These compounds are present in certain

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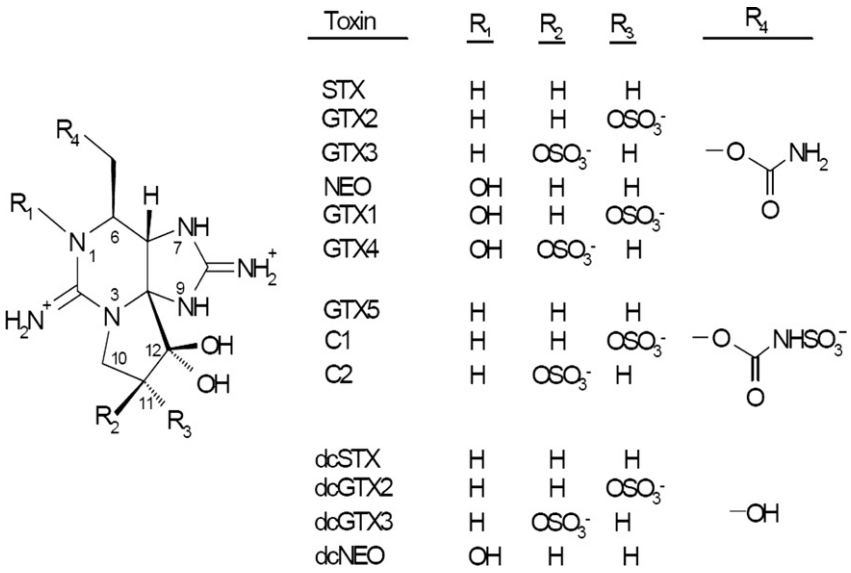

Fig. 1. Chemical structures of saxitoxin analogues currently available as certified reference standards.

species of marine algae (Llewellyn, 2006; Wright, 1995), which during algal blooms may accumulate in filter-feeding bivalve shellfish, subsequently posing serious risks to human shellfish consumers (Luckas et al., 2004; Etheridge, 2010). As a result, European legislation stipulates the requirement to monitor for the presence of PSP toxins in shellfish products before these may be placed on the market (Anon, 2004, 2006). The current official reference method for this testing in European law is the mouse bioassay (MBA), as described by AOAC 959.08 official method (OM) (Anon, 2005a). In the United Kingdom, the MBA procedure was widely used by regulatory bodies to test for PSTs until 2006 when instrumental alternatives began to be introduced. In Canada, the MBA procedure has also been widely used, however, a different chemical based method has been recently developed and implemented in these labs. The MBA procedure involves the replicate injection of mice with filtered, hydrochloric acid extracts of shellfish with subsequent death times being used to calculate sample toxicity. In more recent years, alternative non-animal methods have become increasingly available (Etheridge, 2010; Humpage et al., 2010), with one pre-column oxidation liquid chromatography with fluorescence detection (LC-FLD) method (official method AOAC 2005.06) becoming formally validated and adopted into European law as an approved alternative method for the detection of PSTs in shellfish (Anon, 2005b, 2006; Lawrence and Menard, 1991; Lawrence et al., 1995, 2004, 2005) and more recently a post-column oxidation LC-FLD being validated and approved as an official method (AOAC 2011.02; van de Riet et al., 2009, 2011). The pre-column oxidation method, involving the extraction of shellfish homogenates in acetic acid, sample clean up and oxidation prior to LC-FLD analysis, allows quantitation of individual PSTs. Total PSP toxicity is subsequently estimated by summing individual toxin concentrations and applying toxicity equivalents factors (TEF) determined for each toxin. Previous work at Cefas has refined and validated AOAC 2005.06 OM to include additional toxins and a more repeatable and automated ion-exchange clean-up step (Turner et al., 2009). The method has been validated for mussels, common cockles (*Cerastoderma edule*), Pacific oysters (*Crassostrea gigas*), native oysters (*Ostrea edulis*), hard clams (*Mercenaria mercenaria*) and razor clams (*Ensis* sp.) (Turner et al., 2009, 2010) and the method is currently implemented into the UK official control monitoring program for mussels, razors, cockles and hard clams. Comparisons conducted between the toxicity results obtained by both MBA and LC-FLD methods highlighted good correlations for all species, with the exception of native and Pacific oysters where large differences were recorded in results generated by the two methods (Turner et al., 2009, 2010, 2011). Additional collaborative work on a range of oyster samples highlighted good agreement between a number of different non-bioassay methodologies, including a post-column oxidation LC-FLD method (LC-ox-FLD; Anon, 2011), LC with tandem mass spectrometry (LC–MS/MS) and a functional Electrophysiological Assay (EA) (Turner et al., 2011). With no evidence for fluorescence enhancement in the oyster samples artificially increasing the fluorescence signal in the LC-FLD methods and further data showing no issues relating to reproducibility of the MBA or assumptions regarding toxicity equivalence factors (TEF), evidence suggested that the cause of the observed differences in method performance was likely to result from a suppression of toxicity in the MBA test, causing the toxicity results returned by the official reference method in oysters to be significantly under-estimated (Turner et al., 2011).

The aims of this work were therefore to conduct a range of tests to determine the potential causes of the differences observed between MBA and other methods. Specifically, matrix components present in the sample extracts would be analysed to assess whether any would positively correlate with the differences in method performance observed.

Previous studies have described the effects of transition metals (Aune et al., 1998; McCulloch et al., 1989; Vale and de Sampayo, 2001; Wiberg and Stephenson, 1960, 1961) and other salts (Wiberg and Stephenson, 1960, 1961) on the performance of the MBA, occurring as a consequence of the competitive displacement of saxitoxin from the sodium channels (Doyle et al., 1993; Grissmer, 1984) as well as on the performance of the LC-FLD method itself (Guevremont et al., 1991). As such, the concentrations of a broad suite of metals were quantified in each of the samples and conductivity measurements were carried out to determine any variability of extract salinity. To complete the assessment of the non-toxin components of shellfish extracts, a full suite of nutritional testing was conducted, including the analysis of lipids, proteins, sugars and carbohydrates. Results from this testing would highlight any potential correlations between matrix components and relative method performance for the determination of PSP toxins. Subsequent work then focused on conducting modifications to the sample extracts to determine whether removal of any interfering compounds would have any subsequent effect on the performance of either the MBA or the LC-FLD method.

2. Experimental

2.1. Reagents and chemicals

HPLC-grade solvents and analytical grade chemicals were used throughout for sample preparation and LC analysis. Certified reference materials (GTX1&4, NEO, dcSTX, 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, Nova Scotia, 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 Cefas LC-FLD analysis. Zinc chloride ($ZnCl_2$) was obtained from Sigma Aldrich (Poole, UK) at 99.999% purity. All references to STX equivalents in this manuscript refer to STX di-HCl equivalents.

2.2. Sample preparation

PSP-contaminated oyster, cockle and mussel samples utilised for the first part of this study were the same as some of those generated and analysed previously (Turner et al., 2011). Bulk shellfish samples were shucked, homogenised and stored frozen (≤−20 °C) until required. Each sample (typically 100–150 g homogenate) was extracted using the AOAC 959.08 (Anon, 2005a) hydrochloric acid (HCl) extraction method. Subsamples forwarded to LC-FLD analysis were additionally cleaned using C18 solid phase extraction (SPE) and ion-exchange fractionation as detailed in Anon (2005b) and Turner et al. (2009).

2.3. Sample testing protocols

2.3.1. Cefas pre-column oxidation LC-FLD method

An Agilent 1200 LC module was used (Stockport, UK) with fluorescence detector (excitation 340 nm and emission 395 nm). Mobile phases were prepared according to Anon (2005b), and chromatography was conducted following exactly the same conditions as detailed in Turner et al. (2009). PSP toxin concentrations present in the HCl extracts of shellfish samples were quantified against six-point calibration standards, with toxicity equivalents factors (TEFs) taken from Oshima (1995). In the case of isomeric pairs (GTX1&4, GTX2&3, C1&2 and dcGTX2&3), the highest toxicity equivalents factor was used for each pair. Individual toxin concentrations were reported as μg STX di-HCl 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 μg STX di-HCl eq./100 g.

2.3.2. MBA testing

The MBA method was followed precisely as stipulated by the AOAC 959.08 official method.

Specifically, the assays utilised triplicate mice (CF-1 strain developed by the National Institute of Public Health, Chile, weight range 19–21 g, bred in a standard facility at the Faculty of Medicine with no gender separation). Testing was conducted on both modified and unmodified HCl extracts of UK oyster, cockle and mussel samples.

2.3.3. Metals testing

HCl extracts of oysters, cockles and mussels were analysed at the Canadian Food Inspection Agency (CFIA, Dartmouth, Nova Scotia, Canada) using an in-house method for metal ion concentrations. 2.0 mL aliquots of HCl extracts of shellfish samples were digested with nitric acid and hydrogen peroxide in a closed vessel microwave. The digest was quantitatively transferred to a 50 mL tube and made to volume with deionised water. A 2.5 mL aliquot of this digest was then diluted to 10 mL with deionised water resulting in a final dilution factor of 100. Diluted digests were analysed by ICP–MS (Perkin Elmer, Elan DRC II, Woodbridge, ON, CAN) using a multi-element scan. Samples were run alongside a Certified Reference Material (NIST 2976 Mussel Tissue), with data corrected for recovery only if results from a CRM fell outside 90–110% (Pb only). For metals showing very high concentrations (Zn^{2+} only), linear calibration curves were extended to encompass the appropriate range.

2.3.4. Conductivity testing

A Hach Lange, Manchester, UK, HQ40d conductivity meter was used to determine the conductivity of HCl extracts. Triplicate measurements were recorded and the results corrected for temperature to 25 °C.

2.3.5. Nutritional analysis

HCl extracts of a number of Pacific oyster, native oyster, cockle and mussel samples were processed by Campden Technology Ltd (Gloucestershire, UK) for a suite of nutritional tests. Each extract was split into appropriately sized subsamples to allow the determination of protein, sugars, fat content, fatty-acid profile, moisture and ash content. For determination of protein, samples were digested in sulphuric acid with catalyst, with the resulting organic nitrogen steam distilled into boric acid to convert nitrogen

to ammonia. Titration against standard hydrochloric acid enabled calculation of nitrogen as protein using a standard factor of $N \times 6.25$. Sugar enzymatic test kits manufactured by Rhone-Diagnostics were employed to measure the sugar profile, as glucose, fructose and sucrose. For the determination of total fat content, subsamples were predried using Microwave then placed into a low resolution NMR magnet and the hydrogen protons measured as the fat content. Fatty-acid profile was determined following sample extraction with chloroform and methanol and saponification, with production of methyl esters using boron trifluoride and subsequent analysis by GC–MS. A full fatty-acid profile (C4:0–C24:0) provided, by calculation, the saturates, mono-unsaturates, poly-unsaturates and trans-fatty acids plus omega fats if present. Moisture levels were calculated gravimetrically following drying in an analytical microwave moisture analyser. Ash content was also calculated gravimetrically after first placing samples under IR lamps to dry/char, before treatment in a cool muffle furnace heated to 525 °C using a programmer to regulate the temperature rise per minute (to avoid loss of sample). Finally, total carbohydrate was calculated from

$$\textit{Total Carbohydrate} = 100 - (\textit{moisture} + \textit{ash} + \textit{fat} + \textit{protein})$$

2.4. Matrix-modification protocols

Results comparing specific matrix components indicated whether any of these were potentially affecting the performance of either the MBA or LC-FLD PSP methods. Sample extracts were subsequently modified to allow an assessment of the potential effects of matrix components. Matrix modifications, specifically the removal and addition of metals, were conducted at Cefas along with LC-FLD analysis, ICP–MS analysis was conducted at CFIA with additional ICP–MS performed at Harwell Scientifics, UK during the demetallation refinement process. MBA was conducted at the University of Chile.

2.4.1. Demetallation of oyster extracts

McCulloch et al. (1989) described the removal of zinc and other transition metals from HCl extracts of oysters through use of base precipitation, metal sulphide precipitation with use of hydrogen sulphide and use of ion-exchange resins. However, this work was conducted on extracts which were free of any PSP toxins. The aim was to conduct clean-up procedures on PSP-positive oyster extracts to eliminate or reduce concentrations of metal ions without large reductions in PST concentrations, noting the presence of localised positive and negative charged substituents in the latter (Fig. 1), depending on both the structure of the specific saxitoxin analogue and the pH of the extract (Rogers and Rapoport, 1980). Although some toxin recovery losses are to be expected through the use of any clean-up procedure, any oyster extracts returning positive PSP toxicity results by MBA were to be cleaned up where possible without reducing the toxicity of the samples to levels below the MBA limit of detection (LOD). A major effort was dedicated at Cefas to trialling numerous clean-up methodologies for the removal of metals in PSP-positive oyster extracts, including the use of base precipitation and clean-up with a variety of solid phase extraction (SPE) phases. Owing to health and safety concerns, precipitation through use of bubbling hydrogen sulphide was not permitted within the laboratory.

2.4.1.1. Reduction in metal concentrations through base-induced precipitation. 20–25 mL of HCl extracts of oyster samples previously adjusted to pH 3.5 were adjusted to pH 12.5 (±0.5) with the addition of granular sodium hydroxide (NaOH). After thorough mixing, samples were centrifuged (4500 g, 10 min), the supernatant removed and readjusted quickly to pH 3.5 before diluting to initial volume. This process was conducted in less than 30 min in order to minimise any possible base-induced toxin degradation or transformation of PSP toxin analogues that may have affected the total toxicity of the sample. Aliquots of the extracts both before and after the precipitation step were tested by ICP–MS for metals and PSP toxins by both LC-FLD and MBA.

2.4.1.2. Metal removal through use of primary secondary amine SPE cartridges. 18 mL of HCl extracts of oyster samples previously adjusted to pH 3.5 were adjusted to pH 12.5 (±0.5) as described above. Each oyster sample was then treated as follows. 12 Primary Secondary Amine (PSA) cation-exchange sorbent cartridges (UCT Inc, Bristol, PA; 1 g/6 mL cartridge size) were pre-conditioned with 6 mL 0.1 M HCl, 6 mL deionised water and finally 6 mL deionised water adjusted to pH 12.3 with NaOH. 1.5 mL aliquots of each sample adjusted to pH 12.5 (±0.5) were added to the 12 conditioned SPE cartridges, using vacuum to elute the sample effluent at a flow rate of 1–2 mL/min. Columns were then washed with the further addition of 12 mL deionised water adjusted to pH 12.3, collecting the column effluent in the same collection tubes. Column washings were combined from all 12 SPE clean ups and adjusted to pH 3.5 prior to rotary evaporation to dryness. Dry samples were subsequently re-dissolved in 18 mL deionised water re-adjusting the final pH of the solution to 3.5. Aliquots of the extracts both before and after the demetallation step were tested for metal concentrations and PSP toxin concentrations by both LC-FLD and MBA.

2.4.2. Zinc fortification in mussel and cockle extracts and STX di-HCl solution

Variable volumes of a stock solution of 100 mg/mL $ZnCl_2$ were spiked into a range of HCl extracts of mussel and cockle samples previously found to be positive by MBA and LC-FLD. In order to produce samples of a large enough volume to enable all assays to be conducted, some samples of smaller volume were pooled. Additionally, a stock solution of 0.47 μg STX di-HCl/mL prepared from dilution of an NRCC STX di-HCl CRM was prepared and variable concentrations of Zn^{2+} added (0, 50, 100, 200 and 300 μg/mL). Aliquots of the extracts both before and after the Zn^{2+} fortification were tested for metal concentrations (to confirm the expected Zn^{2+} concentrations) prior to determination of PST content by both LC-FLD and MBA.

3. Results

3.1. Analysis of HCl extracts of oysters, cockles and mussels

Table 1 summarises the toxicity results obtained from the LC-FLD and MBA PSP of the HCl extracts of a range of 20 oysters, cockles and mussels. The results show the large differences in PSP toxicity estimated in oysters by the two techniques, as described in earlier work for a larger group of samples (Turner et al., 2011). Table 1 also summarises the individual metals concentrations determined following ICP–MS analysis of the acidic shellfish extracts. Concentrations of 12 metals were determined and results in Table 1 indicate some degree of correlation between high metals concentrations for some of the analytes and samples which are found to exhibit the largest differences in PSP toxicity returned by the two PSP assays. This correlation is shown visually in Fig. 2 for selected metals, with results indicating a noticeable positive correlation between the concentrations of zinc and manganese and the ratio between the differences in toxicity determined ($r = 0.81$ and 0.77 respectively, with the former exhibiting a clear bimodal distribution). Copper and cadmium showed a lower degree of correlation ($r = 0.52$ and 0.49 respectively) with all other metals showing no evidence of any positive correlation between concentration and differences in PSP toxicity results. Consequently there is some evidence for the potential effect of high concentrations of zinc and manganese resulting in either an enhanced response in the LC-FLD analysis or in a suppression of the MBA response.

Table 1 also summarises the results returned from the nutritional testing of the same samples. In general the levels determined are low, as expected given the nature of the acidic sample extraction. In particular levels of lipids are consistently very low (<0.5% w/w) eliminating the possibility that components of the total fat, including free fatty acids, may be affecting the performance of the bioassay. As a consequence of the low levels of total lipids present, no meaningful data were obtained from the GC–MS analysis of fatty-acid profile within the samples (data not shown). Levels of protein were also low and remained consistent between samples, indicating no relationship between protein in the sample extracts and performance of either assay. Levels of total carbohydrates and total sugars were also relatively low in the sample extracts, although the amounts determined were found to vary between samples (Table 1).

Fig. 3 illustrates some degree of correlation between the samples exhibiting higher levels of carbohydrates and sugars and those showing evidence for differences in PSP toxicity results between the LC-FLD and MBA assays ($r = 0.68$ and 0.46), but the wide scatter of points evident in the graphs does not point to any conclusive relationship. Conductivity measurements taken from the same samples indicate no meaningful variability in the salinity of the extracts and as summarised in Table 1, there is no apparent relationship in these samples between conductivity and the differences in toxicity results obtained. In addition, no relationships were found between any combination of the matrix components quantified and their synergistic effect on assay performance (stepwise linear regression (forward selection). As such, there is no evidence for multiple matrix components to be simultaneously affecting either the MBA or LC-FLD method, confirming that the effect of the metals is the most significant.

3.2. Analysis of demetallated HCl extracts of oysters

Following metals reduction and demetallation, sample extracts were analysed by ICP–MS to confirm the level of metals reduction in each of the treated samples. Fig. 4 illustrates these results for the most prevalent metal ions, including the two metals (Zn^{2+} and Mn^{2+}) showing the clearest indication of a correlation between metals concentration and differences in toxicities returned by the LC-FLD and MBA assays. For both these metals, a percentage reduction in concentration of around 60% was achieved through use of the pH 12 precipitation process. Some degree of success was also achieved for the reduction in concentration of the other metals, with the exception of As and Cu. Demetallation through use of the UCT PSA SPE cartridges resulted in a near total removal of Zn^{2+} and Fe^{2+}, with an average 70% reduction in Mn^{2+} observed (Fig. 4). As such, both treatments were successful in significantly reducing the levels of both Zn^{2+} and Mn^{2+} in the oyster extracts.

Nutritional analysis was also conducted on the oyster extracts to determine whether any modifications to the nutritional content of the samples would occur through either of the treatment processes. Table 2 summarises the results from this analysis and indicates, as expected, that levels of protein and total carbohydrate remain unaffected after treatment. Levels of sugars appear to drop slightly after demetallation and amounts of total lipids appear to rise, although reasons for this increase are unknown. Levels of moisture and ash indicate the slightly higher levels of solid material in the treated extracts, although the differences observed are relatively low.

Results from the analysis of the untreated and demetallated oyster extracts by LC-FLD are summarised in Table 3. As expected, given the level of treatment required to remove the metals from the extract solutions, the data shows some differences in total STX equivalents between the treated and untreated extracts. However, the majority of samples still exhibit toxicities higher than the MBA LOD after both metal reduction treatment processes.

Table 3 also summarises the PSP toxicities obtained by MBA on the untreated and demetallated extracts of the oyster samples. The mean LC-FLD/MBA percentage ratio in the untreated oyster extracts was found to be 277%. Following metals reduction by both pH 12 precipitation and SPE clean-up, this ratio dropped to a mean of 178% and 124% respectively. Fig. 5 illustrates the comparison of the LC-FLD/MBA toxicity percentage ratios in the untreated oyster sample extracts with the percentage ratios in the treated samples following both metals reduction procedures. The graph shows that on average there is a correlation between decreasing concentrations of zinc and a reduction in the LC-FLD/MBA toxicity percentage ratio. As such, this data provides further evidence for the effect of high concentrations of metals such as zinc on the suppression of the MBA.

Table 1
Summary of PSP toxicity results (LC-FLD and MBA), metals concentrations (ICP–MS), conductivity and nutritional testing determined in a range of UK Pacific oysters, native oysters, cockles and mussels (na = not analysed).

Samples	PSP toxicity (μg STX di-HCl eq./100 g)		Metals ICP–MS analysis (ng/mL)												Nutritional testing (g/100 g)						Conductivity (mS/cm)
	LC-FLD	MBA	Zn	As	Al	Cr	Fe	Mn	Ni	Cu	Se	Cd	Sb	Pb	Carbohydrate	Total sugars	Protein	Fat	Moisture	Ash	
PO 197	201	61	221,551	1329	79	94	6877	2786	58	1993	199	147	16.07	35.5	3.9	2.34	2.04	0.24	92.6	1.23	10.13
PO 198	149	46	202,229	1276	104	89	6828	2339	43	1642	186	160	5.83	35.2	3.7	2.33	2.19	0.21	92.6	1.30	10.18
PO 199	77	33	136,515	1119	221	120	5357	1914	30	885	178	163	2.41	29.2	2.4	1.51	1.79	0.21	94.4	1.19	10.63
PO 200	100	37	143,322	1058	84	133	4099	1872	73	781	191	160	36.68	31.3	2.4	1.66	1.77	0.20	94.5	1.17	10.76
PO 201	16	nd	143,496	869	394	495	4303	1734	334	1256	172	192	5.66	29.7	2.3	1.98	2.14	0.21	94.2	1.18	10.28
NO 169	61	32	166,164	601	127	82	8888	1359	0.00	1503	141	254	1.10	14.3	3.8	2.67	1.85	0.06	93.4	0.93	8.46
NO 170	78	32	168,115	600	256	367	9791	1415	252	1546	167	255	5.43	16.6	4.4	2.80	1.98	0.22	92.4	1.05	8.93
NO 171	84	31	162,333	585	54	154	10,732	1493	60	1597	200	248	36.77	19.0	4.0	2.74	2.00	0.18	92.8	1.06	9.21
NO 172	153	41	179,104	660	143	181	12,634	2088	221	1686	232	275	37.02	22.7	3.7	2.04	2.02	0.26	92.9	1.11	10.69
NO 173	124	36	162,307	629	117	81	8202	1307	0.51	1399	134	244	0.98	17.0	3.5	2.50	1.90	0.16	93.3	1.10	9.38
NO 174	155	47	179,096	585	149	86	18,292	1227	12	1439	133	219	1.02	16.4	3.7	2.27	1.80	0.16	93.3	1.09	9.57
NO 175	107	41	165,675	505	138	65	8302	1012	0.00	2203	116	167	0.67	14.0	3.4	2.70	1.79	0.42	93.5	0.91	7.95
NO 176	179	61	170,532	581	151	85	8708	1455	0.00	1377	121	219	0.69	16.9	3.3	2.02	1.78	0.25	93.5	1.20	9.66
NO 177	3	nd	266,060	401	188	83	7526	362	0.00	1361	104	343	0.32	10.6	2.1	1.80	1.13	0.12	95.6	1.03	8.62
Co 202	187	130	4177	841	109	170	10,290	159	1250	80	244	62	36.35	8.4	1.1	0.73	2.22	0.27	95.2	1.20	10.83
Co 203	247	110	5281	791	432	188	19,187	527	327	4260	304	35	36.40	31.9	1.9	1.20	2.08	0.30	94.6	1.12	10.66
Co 204	196	124	4796	958	507	388	10,382	249	1347	280	257	69	27.54	9.4	1.4	0.77	2.12	0.32	95.0	1.21	10.65
Co 205	202	na	4776	901	358	488	9684	254	1469	312	227	62	8.81	7.0	1.4	0.76	1.90	0.20	95.3	1.21	10.99
M 1535	28	34	8832	899	272	122	5383	525	18	148	348	71	1.71	53.9	2.4	2.15	2.58	0.15	93.8	1.05	9.73
M 1780	65	32	12,178	556	155	121	4912	455	48	281	274	130	1.44	77.8	1.8	1.67	1.93	0.18	95.1	0.96	9.76
M 2319.	17	30	21,369	942	269	119	7528	467	47	579	289	204	1.22	146.9	na	na	na	na	na	na	12.45
M 2360	30	28	11,474	625	198	112	7873	462	7.78	451	235	175	0.85	55.2	na	na	na	na	na	na	8.99
M 2432	66	31	10,827	814	226	90	7112	503	17	432	253	170	0.68	52.0	na	na	na	na	na	na	9.57
M 2445.	250	142	11,062	604	304	135	4693	489	38	428	244	105	0.68	59.1	na	na	na	na	na	na	9.16
M 2451	50	33	11,480	1354	275	111	5404	527	81	291	220	111	0.50	56.1	na	na	na	na	na	na	9.60

3.3. Analysis of zinc fortified samples

3.3.1. Mussel and cockle samples

Mussel and cockle samples found previously to contain low levels of zinc in the HCl extracts (between 4 and 21 μg/mL; Table 1), were analysed by ICP–MS and concentrations of zinc determined in both untreated samples and samples subsequently fortified with zinc. Spiking concentrations were calculated so as to achieve final zinc concentrations similar to those occurring naturally in extracts of oyster samples. Specifically, a number of samples were spiked to final Zn concentrations at close to 150 and 250 μg/mL respectively. The results from the ICP–MS analysis were used to confirm the success of the fortification processes, so that MBA analysis was not conducted on any samples containing unnaturally high levels of zinc which may cause a toxic response from the zinc alone (Aune et al., 1998; McCulloch et al., 1989). Subsequently all fortified and non-fortified mussel and cockle extracts were subjected to analysis by both LC-FLD and MBA. Results from these analyses are summarised in Table 4. The LC-FLD data confirms that samples containing elevated concentrations of zinc in HCl extracts do not result in any enhancement of the fluorescence signal. Fig. 6 illustrates a clear correlation between the two datasets, with a coefficient of 0.94 indicating a good agreement between the toxicities determined in both untreated and fortified non-oyster samples. It is noted that whilst high concentrations of zinc do not affect the pre-column oxidation LC-FLD method, there is the potential for metal ions to interfere with the ion-pairing based post-column oxidation method (LC-ox-FLD; Anon, 2011). Indeed, LC-ox-FLD analysis conducted at the Cefas has shown strong evidence for differences in chromatographic retention times for early eluting PSP toxins in oyster samples as compared to the retention characteristics in non-oyster samples such as mussels (data not shown). However, it is also evident from previous comparative analysis (Turner et al., 2011) that the toxicities estimated using the two LC methods are in close agreement. Given also that both methods employ the same detection (FLD) method, there is good evidence that the high concentration of zinc and other metal ions will not affect the accuracy of the LC-ox-FLD method. Table 4 also summarises the toxicity results obtained following MBA on both the untreated and fortified samples, confirming that the addition of zinc to mussel and cockle extracts can affect the performance of the bioassay. Control samples (PSP-negative extracts of mussels and cockles) indicated no effect of enhanced levels of zinc on the performance of the MBA. However, with zinc fortification concentrations targeted at both 150 and 250 ng/mL, results indicate on average some degree of MBA suppression at both these concentrations (Fig. 7). Notably, the largest suppressive effect is observed in samples containing the highest concentrations of zinc (>180 μg/mL) where the MBA results are found to drop to 30% of their original levels. Overall, the mean LC-FLD/MBA ratio in untreated mussel and cockle samples was 125%, whereas the mean LC-FLD/MBA ratio in the same samples spiked with zinc was found to rise to 161%, with the samples fortified to a total concentration of >180 μg/mL zinc exhibiting a mean LC-FLD/MBA ratio of 306%, similar to the ratio determined in the natural native

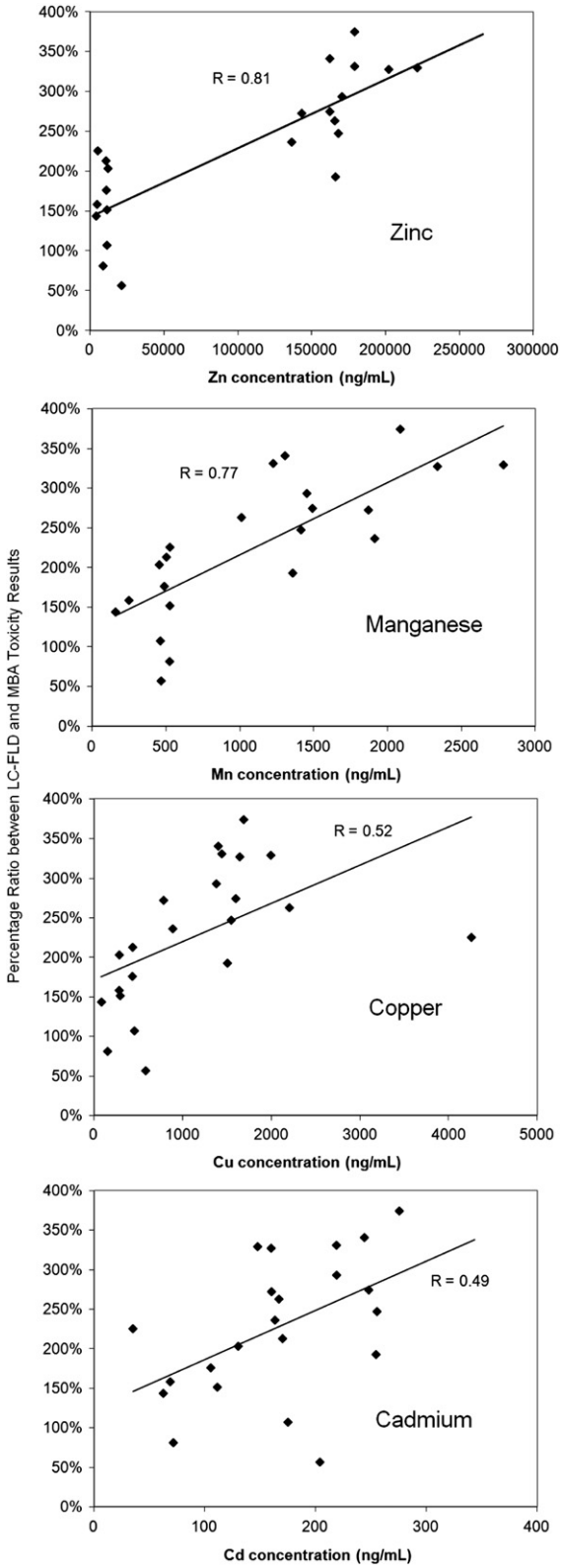

Fig. 2. Percentage toxicity ratios between LC-FLD and MBA PSP (μg STX di-HCl eq./100 g) in comparison with selected metals concentrations (ng/mL) in UK oysters, cockles and mussels.

oyster samples (Turner et al., 2011). It is stressed that these concentrations of zinc are similar to those present naturally in the oyster samples (Table 1).

3.3.2. Saxitoxin di-hydrochloride standard

Solutions of pure saxitoxin di-hydrochloride fortified with a range of zinc concentrations and submitted for MBA testing showed an interesting correlation (Fig. 8). A clear suppression in the MBA was again evident in the fortified standard solutions, but with suppression evident at lower concentrations of zinc, than in some of the samples (Fig. 7). In addition, the results also seem to show that the degree of suppression is reduced when zinc is present at 300 ng/mL. However, these results do confirm that suppression is evident when metals are present in conjunction with zinc concentrations typically present naturally in oysters.

4. Discussion

4.1. Interpretation of results

Work previously conducted highlighted the evidence for significant differences in method performance between the official MBA and other non-bioassay methods for the determination of PSP toxins in oysters (Turner et al., 2011). This study showed that such differences observed were not resulting from matrix-related fluorescence enhancement in the LC-FLD, assumptions taken regarding toxicity equivalence factors (TEFs) and did not relate to reproducibility issues with the MBA. However, these results alone did not prove conclusively that the cause of the performance differences was resulting from the effects of oyster matrix on the MBA. The present study has taken the same samples of oysters analysed previously and has described a range of tests carried out to determine the specific cause of the observed differences in method performance. Nutritional testing conducted on a range of mussel, cockle and oyster samples showed little evidence of any relationship between levels of carbohydrates, fats, protein or sugars on the relative performance of the two assays. Similarly, no differences were observed in the conductivity of any of the extracts. However, analysis of a broad suite of metals identified two metals, zinc and manganese, that were present at higher concentrations in the oyster samples as compared with the cockles and mussels. Whilst this resulted in a correlation between Zn and Mn extract concentration and the reported difference in method performance (expressed as the percentage ratio between the LC-FLD and MBA toxicity results), this still did not provide the proof that these metals were the actual cause of either an LC-FLD enhancement or MBA suppression. Consequently, a number of oyster samples high in zinc and manganese, were subjected to processes designed to reduce or eliminate the level of metals within the extracts. Two different demetallation procedures were utilised which significantly reduced the metals concentrations but without eliminating the PSP toxins. This then enabled further LC-FLD and MBA analysis to be conducted on oyster extracts containing significantly lower concentrations of metals. Results have shown that in demetallated oyster extracts, the agreement between the LC-FLD and MBA

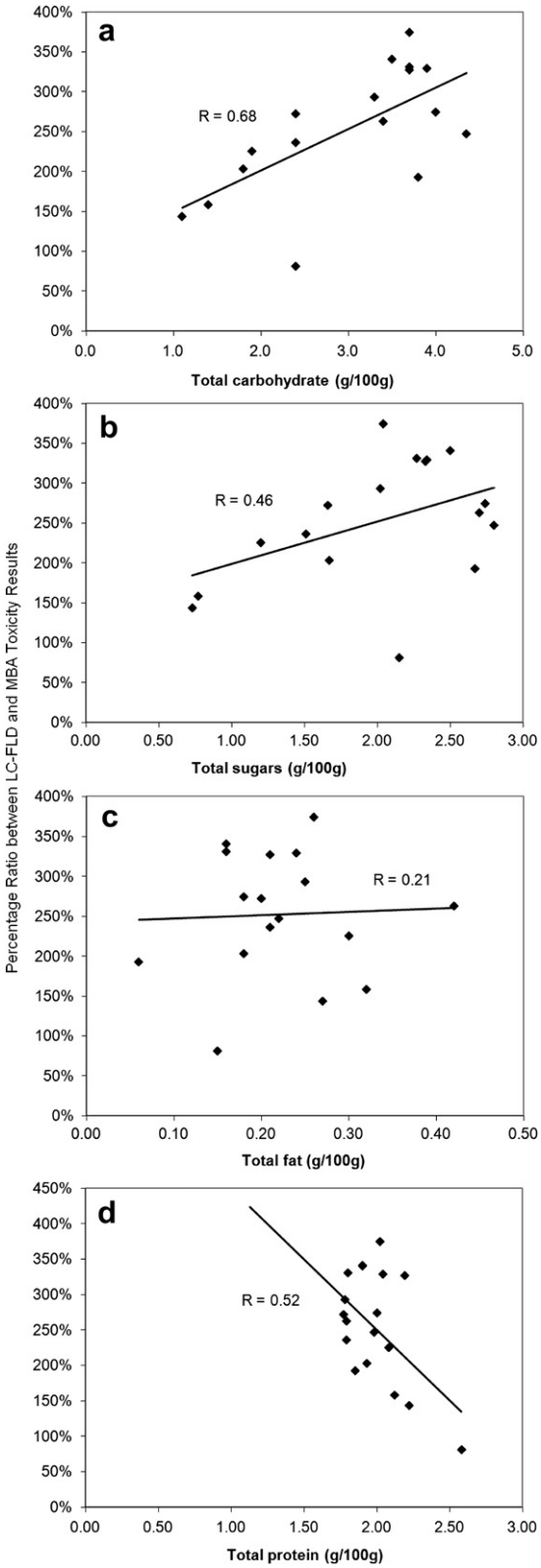

Fig. 3. Percentage toxicity ratios between LC-FLD and MBA PSP (μg STX di-HCl eq./100 g) in comparison with selected nutritional tests (all results in g/100 g) in UK oysters, cockles and mussels.

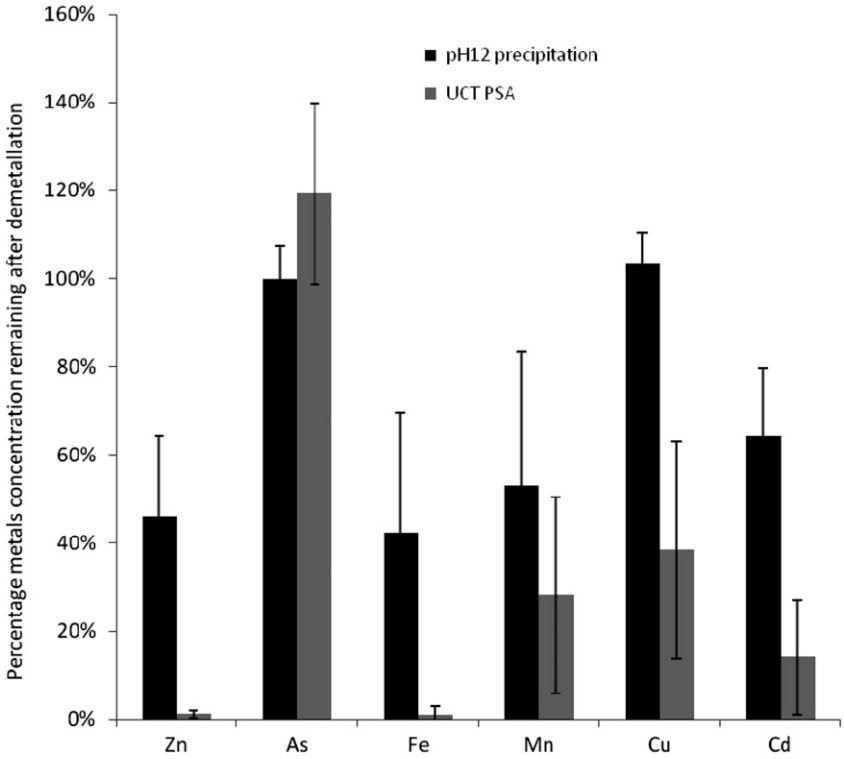

Fig. 4. Mean percentages of metals concentrations remaining in HCl extracts of shellfish after a) pH 12 precipitation and b) demetallation using UCT PSA SPE cartridges (±1sd).

analyses is closer, showing that the differences in method performance are reducing greatly when the metals are removed. Negative controls were analysed (demetallated PSP-negative oyster samples) and confirmed that no unusual responses were observed as a result of the treatment process. In order to demonstrate conclusively that the presence of high concentrations of zinc in the oyster extracts was affecting the MBA and not the LC-FLD, a number of mussel and cockle samples were spiked with a range of zinc concentrations, at levels found in the oyster samples. Results demonstrated that with the addition of zinc at these levels, no changes were observed in the LC-FLD results, but a significant level of suppression in the MBA was recorded. In particular, the suppression was very high (around 300%) when zinc was spiked at concentrations higher than 180 μg/mL, a concentration typically observed in many of the oyster samples.

4.2. Toxicity of PSP-positive oysters

The main target of saxitoxin and its analogues is a voltage gated sodium channel (Na_V), although other means of their pharmacological activity have been reported (Llewellyn, 2006). Saxitoxin is not the only binding agent of Na_V channels. Transitional metal ions were also reported to interact with Na_V channels and their mode of action has been widely studied. The order of binding potency for divalent cations is $Zn^{2+} > Cd^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$, with IC_{50} for Zn^{2+} and Cd^{2+} in much lower concentrations than for other divalent cations (Doyle et al., 1993). It has been suggested that metal ions and STX share their binding site (Backx et al., 1992; Favre et al., 1995; Heinemann et al., 1992; Henderson et al., 1974; Satin et al., 1992), so it may not be surprising that some transitional metal cations, including zinc, compete reversibly with STX (Schild and Moczydlowski, 1991; Weigele and Barchi, 1978). Interestingly, the Na_V channel isoforms affinity towards STX varies inversely with their sensitivity to metal ions.

PSTs binding to Na_V channels inhibit Na^+ currents which consequently promotes inability of excitable cells to function properly. The most obvious manifestation of this event is paralysis and respiratory failure was seen as a primary cause of death (Evans, 1965). If oysters contain high

Table 2
Summary of mean (±1sd) nutritional results (g/100 g) determined in a) untreated b) pH 12 precipitated c) UCT PSA SPE demetallated oyster extracts.

Oyster extract treatment	Protein	Total carbohydrate	Total sugars	Fat	Moisture	Ash
Untreated	1.87 ± 0.25	3.3 ± 0.7	2.24 ± 0.42	0.21 ± 0.08	93.5 ± 0.9	1.11 ± 0.11
pH 12 precipitated	1.72 ± 0.06	2.4 ± 0.7	1.31 ± 0.16	0.58 ± 0.10	92.8 ± 0.6	2.55 ± 0.27
UCT PSA SPE demetallated	2.09 ± 0.24	2.3 ± 0.7	1.08 ± 0.35	0.85 ± 0.06	91.1 ± 1.3	3.64 ± 0.66

Table 3
Summary of PSP toxicity results obtained by LC-FLD and MBA (μg STX di-HCl eq./100 g) and concentrations of Zn and Mn (g/100 g) in a) untreated and treated extracts of Pacific oysters and native oysters following b) metals reduction by pH 12 precipitation c) demetallation by UCT PSA SPE. Nd = not detected, na = not analysed.

Samples	a) Untreated				b) pH 12 ppt				c) UCT PSA SPE			
	LC-FLD	Zn	Mn	MBA	LC-FLD	Zn	Mn	MBA	LC-FLD	Zn	Mn	MBA
PO 197	201	221,551	2786	61	100	93,300	934	42	73	865	269	46
PO 198	149	202,229	2339	46	108	82,290	734	38	121	2618	272	142
PO 199	77	136,515	1914	33	19	41,500	409	31	18	1832	95	nd
PO 200	100	143,322	1872	37	63	25,020	327	35	81	1243	168	46
PO 201	16	143,496	1734	nd	28	41,620	553	nd	35	1952	345	nd
NO 169	61	166,164	1359	32	55	43,740	381	33	57	1275	137	42
NO 170	78	168,115	1415	32	31	96,120	1206	nd	35	6270	910	72
NO 171	84	162,333	1493	31	60	108,100	1317	30	48	1376	812	37
NO 172	153	179,104	2088	41	94	73,530	879	76	130	1042	722	106
NO 173	124	162,307	1307	36	81	117,100	1089	30	72	1412	533	63
NO 174	155	179,096	1227	47	90	121,800	1091	80	83	2566	703	59
NO 175	107	165,675	1012	41	50	113,700	1050	33	42	2030	586	nd
NO 176	179	170,532	1455	61	106	53,190	497	43	128	1340	150	na
NO 177	3	266,060	362	nd	2	143,400	202	nd	2	4110	39	nd
NO 178	na	na	na	na	84	71,750	568	80	72	1522	311	nd

concentrations of zinc that could compete with STX for binding to Saxitoxin resistant (STX-R) and Saxitoxin sensitive (STX-S) Na_V channels, there may be potential for zinc to initiate the same effect as STX. An earlier study on STS-S channels concluded that zinc slows Na_V channel opening without effect on channel closing rates hence its mechanism of action is via modulation of gating rather than via open-channel block (Gilly and Armstrong, 1982). On the contrary, later analysis on cardiac cells (STX-R) suggests the effect of divalent ions represent voltage-dependent block of the open channel (Sheets and Hanck, 1992). However, zinc and cadmium were able to block Na^+ currents in a voltage-independent manner. Application of external zinc enhanced conversion of the open Na_V channel to a low conductance conformation which indicated the inhibitory effect of external zinc could be understood as brief interruptions in the open-channel Na^+ currents rather than as closures of channel gating resulting in zero Na^+ current (Ravindran et al., 1991; Schild et al., 1991). This may suggest that in the presence of STX, zinc would compete in binding to Na_V channel without completely occluding Na^+ influx, thus explaining different effect of zinc as compared to STX. Studies by Ravindran et al. (1991) and Schild and Moczydlowski (1991) on STX-S muscle and STX-R cardiac channels confirmed that zinc relieves blockade by STX in a competitive fashion. What is more, they showed that the frequency of STX-blocking events decreases and the duration of STX-unblocked events dramatically lengthen as the zinc concentration is raised. The average dwell time in STX-blocked state remains essentially constant.

So far, only potential involvement of zinc in saxitoxin toxicity suppression in MBA has been discussed. There is a lack of studies on interactions of external zinc and other STX analogues. We do not know how toxin profiles differences between samples would influence the outcome of the results in the presence of low or high concentrations of zinc. However, previous work (Turner et al., 2011) showed similar profiles of toxins in the samples studied here, thereby reducing the impact of any potential differences in STX/Zn interaction due as a result of this factor in this particular study. Additionally, other transitional metal ions or other compounds may be considered, although their relevance would be probably different to zinc (Fig. 2). The mean LC/MBA ratio of naturally contaminated oysters was 250%. When mussel and cockle extracts were fortified with >180 μg/mL of zinc, the mean LC/MBA ratio increased from 125% to 306%. However, zinc chloride spiked (50–200 μg/mL) to STX standards (0.47 μg/mL STX di-HCl) suppressed PSP toxicity only up to 50%. These results may indicate alone that zinc itself may not responsible for full suppression of PST induced toxicity in MBA and other matrix components may play a synergetic role with zinc. However, further study on a larger data set would be required to determine these effects.

The effect of zinc on PSP MBA has already been reported (Aune et al., 1998; McCulloch et al., 1989; Vale and de Sampayo, 2001), whereby some oyster samples containing zinc >400–450 μg/ml induced unusual symptoms or even death in mice subjected to combined Paralytic and Amnesic shellfish poisoning (PSP + ASP) MBA testing (Aune et al., 1998; McCulloch et al., 1989). The observed signs included extreme weakness, body temperature drop, cyanosis, displayed paralysis and slow deaths without cramps. As in both of the studies it was stated that these signs deviated from PSP or ASP related symptoms, we may assume that these may have been related to the zinc toxicity only. None of the symptoms indicated presence of PSTs (Aune et al., 1998; McCulloch et al., 1989; Vale and de Sampayo, 2001). The extracts used in this study contained zinc with a range of concentrations up to 250 μg/mL, ~50% lower than reported limit to induce zinc toxicity in mice after *i.p.* injection. None of the MBA tests performed on extracts in our study, whether they were PST contaminated or the PST free control samples, recorded signs atypical to PST toxicity, which would potentially indicate zinc toxicity. On the contrary, oyster samples containing zinc levels 130–250 μg/mL alongside saxitoxin analogues recorded reduced PST toxicity in the MBAs as compared to analytical assays, which was not observed in mussel and cockle samples with

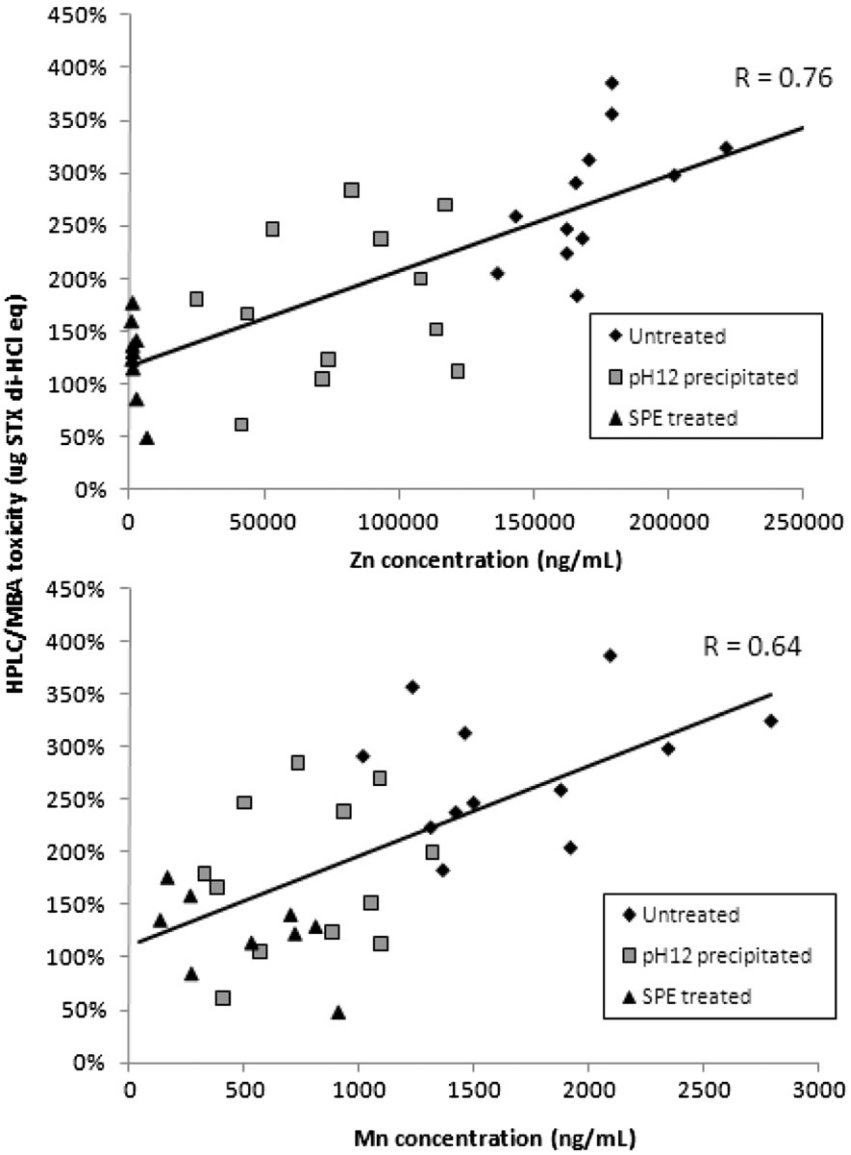

Fig. 5. Comparison of the percentage ratio between LC-FLD and MBA toxicity results (μg STX di-HCl eq./100 g) against zinc and manganese concentrations (ng/mL) in a) untreated oyster extracts b) extracts following pH12 precipitation c) extracts demetallated by SPE.

zinc levels 4–21 μg/mL. Based on these results it can be suggested that the concentration of zinc in shellfish extracts plays a decisive role how the zinc effect in MBA is manifested.

Results presented here suggest that continued use of the MBA for routine monitoring of PSP in oysters is unsafe, given the large degree of suppression shown to occur in the MBA as a result of high concentrations of zinc and potentially other metals. It is noted however that the MBA toxicity test involves intraperoteneal (IP) injection of shellfish extracts into the mice and it is the interference of the zinc ions on this specific test that we are reporting here. Given the large differences in the acute toxicity of saxitoxins following oral and intraperitoneal routes (Mons et al., 1998), it is recognised that the effects of PSP toxins in combination with high concentrations of zinc may potentially differ in the gut of mammals as compared with the effects described following IP. Currently, there is limited data available on the toxicological effects of PSP toxins in humans (review in COT, 2008) and to our knowledge there is no clinical data currently available on the toxicological effects of PSP toxins in combination with high concentrations of zinc. Without this information it is impossible for the results described here to be extrapolated to the clinical level. However, it is important to note that until this information is available, it is must be assumed that the toxicity described by the official reference IP test is a true reflection of the toxicity likely to be experienced following consumption of the poisoned shellfish and any noted suppressive effects on toxicity in the presence of suppressive agents must be assumed to occur within mammalian systems until any contradictory evidence is presented.

Table 4
Summary of PSP toxicity results obtained by LC-FLD and MBA (μg STX di-HCl eq./100 g) and concentrations of Zn (ng/mL) in a) untreated HCl extracts of mussels and cockles and b) mussels and cockles fortified with zinc. Nd = not detected, na = not analysed.

Species	Initial samples (unfortified)				Samples fortified with zinc				Change in LC/MBA ratio (%) with Zn spike
	Zinc	LC-FLD	MBA	LC/MBA (%)	Zinc	LC-FLD	MBA	LC/MBA (%)	
M	6483	126	135	93%	130,900	123	64	192%	206%
M	6483	126	135	93%	231,500	132	37	357%	382%
M	10,540	103	100	103%	136,800	68	61	111%	108%
M	10,540	103	100	103%	214,900	94	37	254%	247%
M	8832	28	40	70%	157,100	52	53	98%	140%
M	12,178	65	37	176%	163,500	81	45	180%	102%
M	6235	1	nd		165,600	nd	nd		
M	6825	8	nd		163,500	7	na		
Co	5281	247	127	194%	153,800	223	130	172%	88%
Co	4776	202	na		143,200	188	110	171%	
Co	4177	187	151	124%	47,070	229	na		
Co	4177	187	151	124%	95,040	209	116	180%	145%
Co	4177	187	151	124%	127,300	219	138	159%	128%
Co	4177	187	151	124%	183,100	216	72	300%	242%
Co	4177	187	151	124%	266,200	246	na		
Co	4796	196	144	136%	45,190	176	140	126%	92%
Co	4796	196	144	136%	77,870	180	130	138%	102%
Co	4796	196	144	136%	124,200	162	122	133%	98%
Co	4796	196	144	136%	176,100	167	151	111%	81%
Co	4796	196	144	136%	245,300	165	53	311%	229%
Co	4383	1	nd		140,800	0	nd		
Co	3954	0	nd		147,500	0	nd		

4.3. Potential effects on industry

4.3.1. Potential effects of LC method implementation for UK oysters

Since November 2006, the protocol approved by the competent authority for testing of official control samples under the scope of the national statutory monitoring programme, involves in the first instance a qualitative LC-FLD screen of samples. Specifically, HCl extracts of oyster samples received have been screened by LC-FLD prior to any positive samples being quantified by MBA. As a result of the method performance issues described here, oyster samples received between July 2009 and December 2009 found to be positive by the LC-FLD screen were additionally

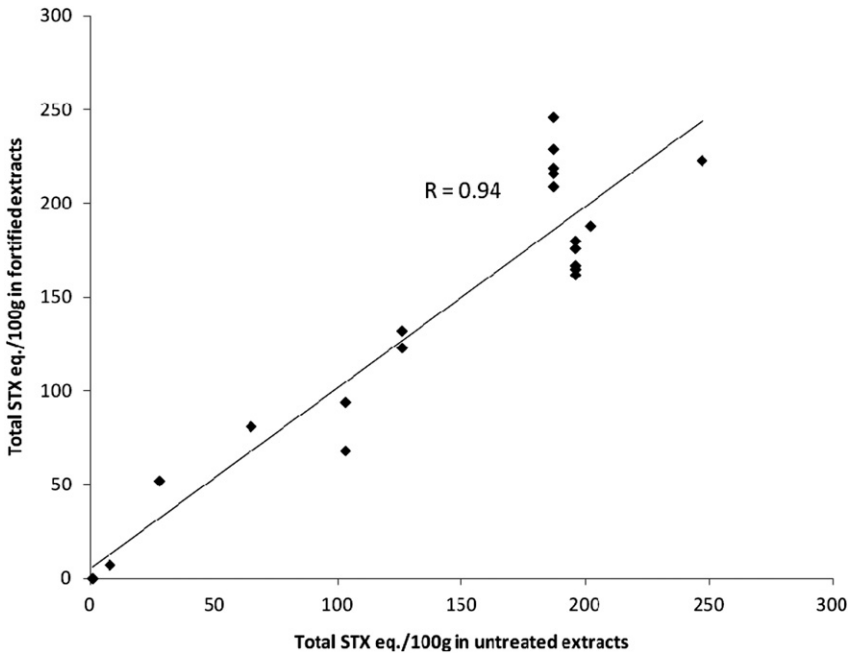

Fig. 6. Comparison of total saxitoxin equivalents (μg STX di-HCl eq./100 g) in oysters determined by LC-FLD in both untreated extracts and extracts fortified with elevated concentrations of zinc.

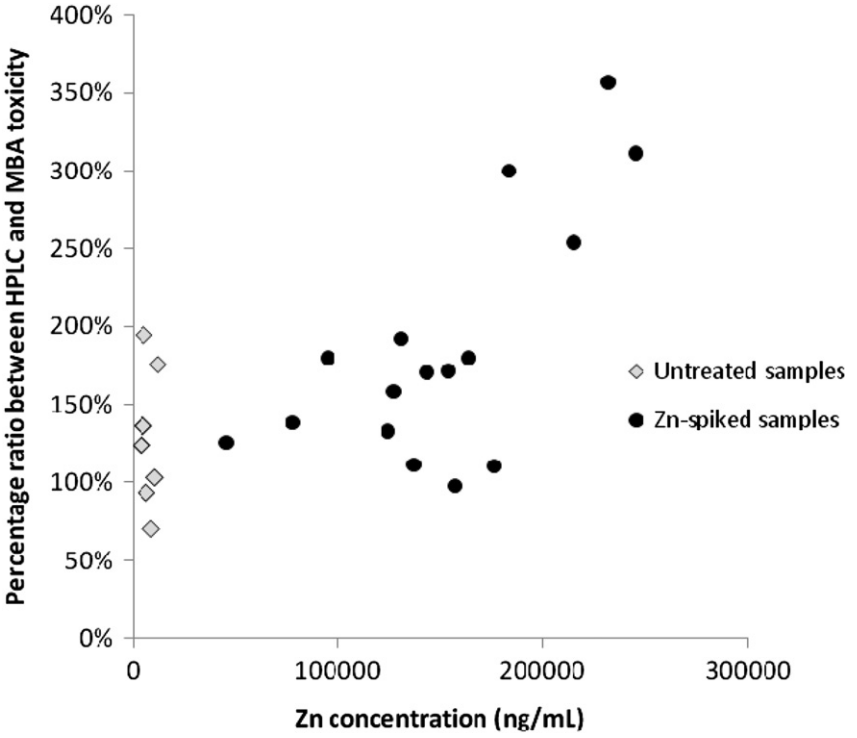

Fig. 7. Comparison of the percentage ratios between LC-FLD and MBA toxicity results (μg STX di-HCl eq./100 g) against zinc concentrations (ng/mL) in a) untreated mussel and cockle extracts b) mussel and cockle extracts fortified with zinc.

extracted by acetic acid and submitted to quantitation by LC-FLD. Results from these samples are summarised in Table 5. During this period, only one oyster official control sample was found to be positive by MBA, with an additional MBA-positive native oyster sample being quantified during Spring 2010 (BTX/10/1605). A number of samples ($n = 16$) analysed between 2008 and 2009 were also found to be LC-FLD screen positive and MBA negative, such results being explained by the lower sensitivity of the MBA method.

Given the evidence for underestimation of the total PSP toxicity in both Pacific and native oysters using the current MBA reference method, there is the potential for an

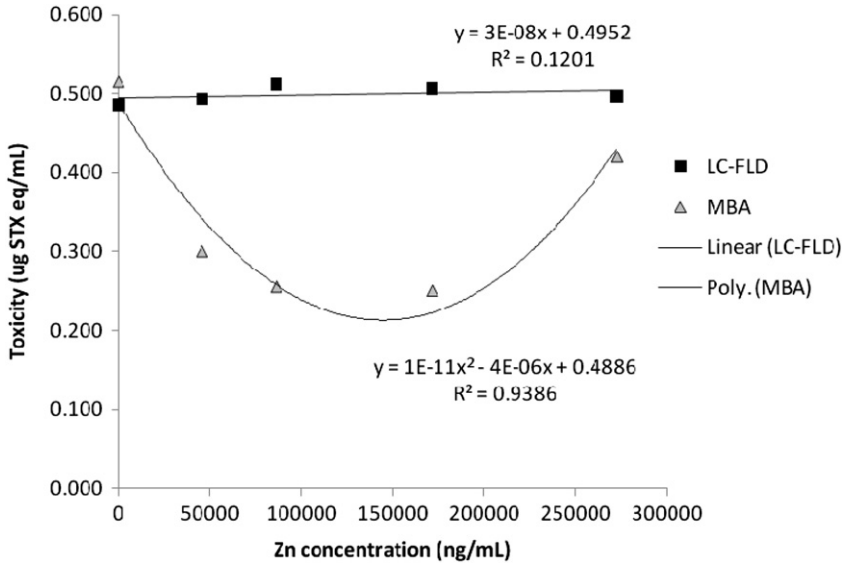

Fig. 8. MBA toxicity results (μg STX di-HCl eq./mL) against zinc concentration (ng/mL) in diluted NRCC STX di-HCl standards fortified with zinc.

Table 5
Summary of PSP toxicities (MBA and HPLC, both in μg STX eq./100 g) in Pacific and native oyster samples showing positive LC screening results (RL = reporting limit; 16 μg STX eq./100 g; na = not applicable).

Sample	Species	LC-FLD total STX equivalents	Higher HPLC value with measurement uncertainty applied (total STX equivalents)	MBA toxicity
BTX/08/1604	PO	90	125	44
BTX/08/1461	PO	<RL	na	Nd
BTX/08/1464	PO	<RL	na	Nd
BTX/08/2504	NO	<RL	na	Nd
BTX/08/2588	NO	<RL	na	Nd
BTX/08/2783	NO	<RL	na	Nd
BTX/09/2918	NO	<RL	na	Nd
BTX/09/2919	NO	<RL	na	Nd
BTX/09/2959	NO	30	44	Nd
BTX/09/2961	NO	25	34	Nd
BTX/09/3063	NO	34	49	Nd
BTX/09/3075	NO	<RL	Na	Nd
BTX/09/3160	NO	24	34	Nd
BTX/09/3206	NO	<RL	Na	Nd
BTX/09/3269	NO	<RL	Na	Nd
BTX/09/3270	NO	<RL	Na	Nd
BTX/09/3454	NO	<RL	Na	Nd
BTX/10/1605	NO	78	102	40

increased number of PSP-positive samples being returned following any implementation of the LC-FLD method in these species. Hypothetically, with an underestimation in PSP toxicity of <50% in the MBA and in relation to the MBA limit of detection of 33 μg STX eq./100 g, an oyster sample showing a negative MBA test could still be actually exhibiting PSP toxicity as determined by LC-FLD close to the action limit of 80 μg STX eq./100 g, which with application of measurement uncertainty could result in the sample results falling higher than this limit. However, the results show that from the samples analysed during this period, no additional samples found positive by the LC-FLD screen and negative by MBA would have resulted in a PSP toxicity value by LC-FLD above the action limit.

4.3.2. Global occurrences of PSP in oysters and potential effects of continued use of MBA

Given the large number of PSP-contaminated bivalve shellfish found globally in recent years, the number of oysters found to contain high concentrations of PSTs appears to be relatively small (Bricelj and Shumway, 1998). The low relative occurrence of oyster PSP toxicity is certainly likely to relate not only to the underestimation in toxicity using the reference MBA method reported here, but also the well noted lower levels of PSTs accumulated in oysters in comparison to other bivalves such as mussels due to their higher sensitivity to PSTs and subsequent higher selectivity of feeding exhibited by these species (Bricelj and Shumway, 1998; Moore et al., 2009). Outside of the UK, reports do exist for the accumulation of PSTs in oysters, such as in the US (Conte, 1984), but again the numbers of incidents of PSP due to oyster consumption is low in comparison to incidents involving consumption of mussels and clams. Specifically, out of a total of 509 PSP intoxications in California between July 1927 and June 1980, only 4 of these related to oysters, with no deaths (Sharp, 1981). However, this was followed by a single event in July 1980 involving the intoxication of 61 individuals following consumption of PST-contaminated oysters. During the same event, intoxication also occurred through consumption of mussels, with the latter affecting 36 individuals with 1 death. It was noted that the toxic poisoning following oyster consumption was less severe than the poisoning following consumption of mussels (Bricelj and Shumway, 1998). After the initial outbreak, a subsequent monitoring program revealed the highest PSP toxicity in oysters to reach 2–5000 μg STX eq./100 g flesh, where as the levels in mussels reached up to 14,000 μg STX eq./100 g (Moore et al., 2009). Between 1990 and 2000 in the Puget Sound region, out of the total number of locations giving rise to PSP toxicity in mussels, only 22% of these returned positive results by MBA following analysis of Pacific oysters from the same location (Moore et al., 2009). The number of samples returning toxicities higher than the regulatory action limit in oysters was only 1% of the total analysed, as compared with 12% of the mussels. In Canada, between 2006 and 2007, approximately 1600 samples were analysed for PSP using both MBA and HPLC methods. However, only 17 of these were oysters and none showed any levels of PSP toxicity by either methodology. In France, PSP toxicity has been detected using the MBA in both mussels and oysters to variable extents. Data obtained between 1995 and 2009 (Belin et al., 2011) allows a comparison between the numbers of toxic episodes occurring in both mussels and oysters in French waters. Examining the data from sites where both mussels and Pacific oysters were monitored simultaneously, toxicities higher than 80 μg STX eq./100 g were observed in mussels during 21 different months, but only on 7 separate occasions in the oysters submitted for monitoring. Whilst the evidence obtained from monitoring programs may highlight the difference in toxin update between species, it is still likely that the use of LC-FLD in place of MBA will ultimately result over time in an overall increase in the numbers of oyster samples returning PSP-positive results during periods of bioaccumulation of toxic phytoplankton. It is also important to

note that the suppressive effects on the MBA described here in oysters will also extend to other shellfish species, if these shellfish are grown in waters subjected to a high influx of metals.

5. Conclusions

Results showed a positive correlation between samples containing higher concentrations of zinc and manganese and those showing a poor agreement between the MBA and LC-FLD test results. No evidence was observed for the effect of nutritional components or conductivity on comparative method performance. Evidence was therefore uncovered for the effects of zinc and manganese on either or both the PSP assays. HCl extracts from a range of PSP-positive oyster samples containing naturally high concentrations of metals including zinc, were subjected to matrix modifications in order to reduce the concentrations of metals present in the extracts. Analysis of the demetallated extracts showed an improvement in the agreement between the results reported by both the LC-FLD and MBA methods. In addition, MBA and LC-FLD analysis of cockle and mussel extracts fortified with zinc found confirmed that the samples containing the higher concentrations of zinc had no effect on the LC results but resulted in a significant suppression in the MBA. In particular, samples spiked with zinc at concentrations >180,000 ng/mL, showed a high level of MBA suppression, with MBA toxicities being reduced to around 30% of their original levels. Overall, there is clear evidence that the MBA of both Pacific and native oysters, along with any other shellfish containing high concentrations of zinc, will return toxicity results significantly lower than the actual toxicity values. The evidence therefore suggests that from both a public health and animal welfare perspective, the LC-FLD method should be implemented for oysters, discontinuing the MBA for these species.

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Conflict of interest statement

Authors declare no financial or commercial conflicts of interest.

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