A. Turner. November 2010. Rev June 2011

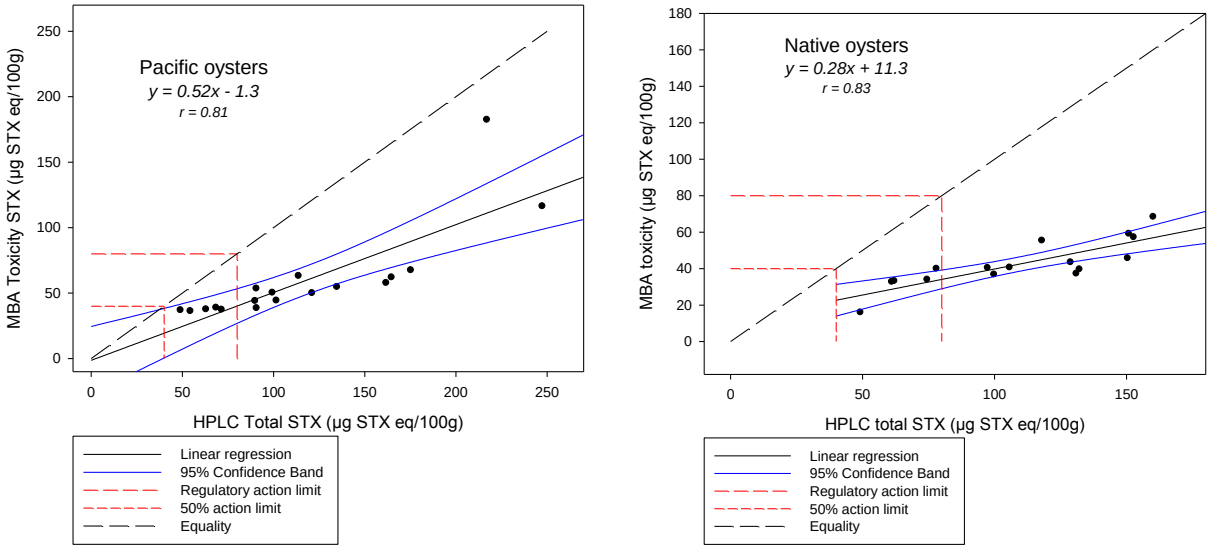
Summary of investigations conducted at Cefas into the effects of oyster matrix on HPLC and MBA PSP results

1. Background

Validation work conducted at Cefas as part of contract C2934 (FSA-ZB1807 / FS235002) previously highlighted significant differences in method performance between the High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD) and Mouse Bioassay (MBA) official control methods for the quantitation of PSP toxins (PSTs) in oysters. A proposal was submitted to the FSA in September 2009 for investigations to be conducted into the potential effects of oyster matrix components on HPLC and MBA results. This report summarises the previous findings, the work originally proposed and the work conducted at Cefas over the past year to determine the cause of the method performance issues.

2. Conclusions from C2934 validation work on oysters

Analysis of PSP-contaminated Pacific oyster and native oyster samples by HPLC-FLD (pre-column oxidation, official method AOAC 2005.06) and MBA methods was conducted as part of the FSA-funded Cefas validation program (Cefas project code C2934) into the validation and implementation of an HPLC PSP method. Results showed that the total PSP toxicities determined using HPLC are on average 200% and 280% of the toxicities determined by the reference MBA method for Pacific oysters (**Figure 1**) and native oysters (**Figure 2**) respectively. In comparison, the data generated for mussels and cockles (**Figure 3**) showed a better correlation between the two methods (mean HPLC/MBA = 101% and 133% for mussels and cockles respectively). It was therefore possible that matrix components within the oysters are causing either an over-estimation of toxicity by HPLC or an under-estimation of toxicity by MBA. Given the importance of demonstrating the equivalence of the two methodologies prior to any potential implementation of HPLC methods into routine official control programmes, there was therefore a strong need to further investigate the potential causes of these findings.



Figures 1-2: Comparison of total saxitoxin equivalents quantified by pre-column oxidation HPLC-FLD with the MBA reference method for UK Pacific and native oysters, showing 95% confidence, regulatory limits and equality.

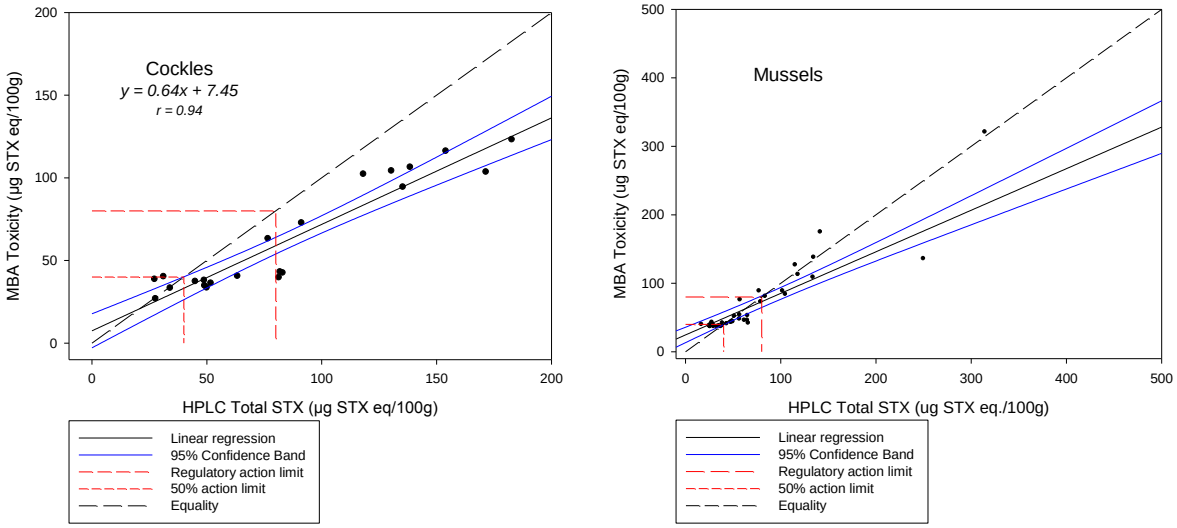


Figure 3: Comparison of total saxitoxin equivalents quantified by pre-column oxidation HPLC-FLD with the MBA reference method for UK cockles and mussels, showing 95% confidence, regulatory limits and equality.

3. Proposed work

The proposed work (detailed in quote ref: FSA-ZB183 dated 28th September 2009) comprised the following work packages.

- Nutritional analysis of range of shellfish homogenates for determination of metals concentrations and other nutrients present. Data to be analysed to determine any potential correlation between toxicity results and nutritional content.
- Following any noticeable correlation metals concentrations, the potential effects of high concentrations of metals (particularly zinc) within the oysters were to be investigated.
- Additional investigations into the effects of other matrix components such as fatty acids if the results from metals work are inconclusive
- In addition to the work proposed, further work was to be conducted involving additional methodologies conducted externally at other laboratories, specifically the use of post-column oxidation HPLC, LC-MS and a functional assay (electrophysiological assay).

4. Work conducted at Cefas

4.1 Effects of matrix components on fluorescence response

An investigation into the effects of matrix components on fluorescence response in the HPLC analysis was conducted by comparing the response of calibration standards over a range of concentrations spiked into both solvent and Pacific oyster extract. No evidence was obtained showing any indication of signal enhancement (**Table 1**).

Table 1. Linear regression gradients and coefficients calculated for PSP toxin calibration standards prepared in extracts of Pacific oysters and solvent over the working calibration range (0-100 µg STX eq/100g 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%

4.2 Effects of extraction solvent on HPLC analysis

The official MBA involves extraction of shellfish homogenates in hydrochloric acid, where as the HPLC method requires extraction in acetic acid. Given some noted differences in PSP toxin concentrations extracted and quantified in the two extraction methods, both extractions were employed on a range of MBA-positive Pacific and native oyster samples and the PSP toxin results compared. Results indicated on average slightly higher toxicities quantified from the HCl extracts, showing that the extraction procedure employed in the routine HPLC method (acetic acid) was not responsible for the differences observed with the MBA following HCl extraction (**Figure 4**).

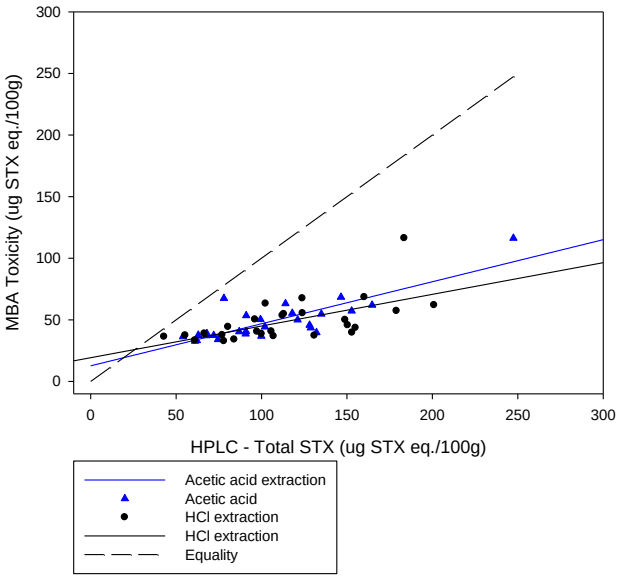


Figure 4. Comparison of total saxitoxin equivalents quantified by pre-column HPLC-FLD and the MBA reference method for UK oysters in both hydrochloric and acetic acid extracts.

4.3 Analysis of PSP toxins by other methodologies (Tables 2 and 3)

Additional oyster, cockle and mussel samples were analysed for PSTs at Cefas (pre-column oxidation HPLC and MBA), with additional sub-samples sent to the Canadian Food Inspection Agency (CFIA) for post-column oxidation HPLC (PCOX), National Research Council of Canada (NRCC) for HPLC with tandem mass spectrometry detection (LC-MS/MS) and the University of Chile for Electrophysiological assay (EA) and repeat MBA.

- Comparative results between Cefas and CFIA HPLC results were close in agreement (**Figure 5a,b)**. As the PCOX method differentiates and separately quantifies individual toxin isomers, results indicated that assumptions inherent in the pre-column oxidation HPLC method regarding the sole presence of the most toxic epimers were not responsible for the high bias in HPLC results observed in oysters (**Table 3**).
- LC-MS/MS and EA results have also returned similar or high PSP toxicity results as compared to the LC data in all species (**Figure 5a,b**) providing evidence that all four non-bioassay methods are consistently higher than results returned by MBA in both oyster species (**Table 3**).

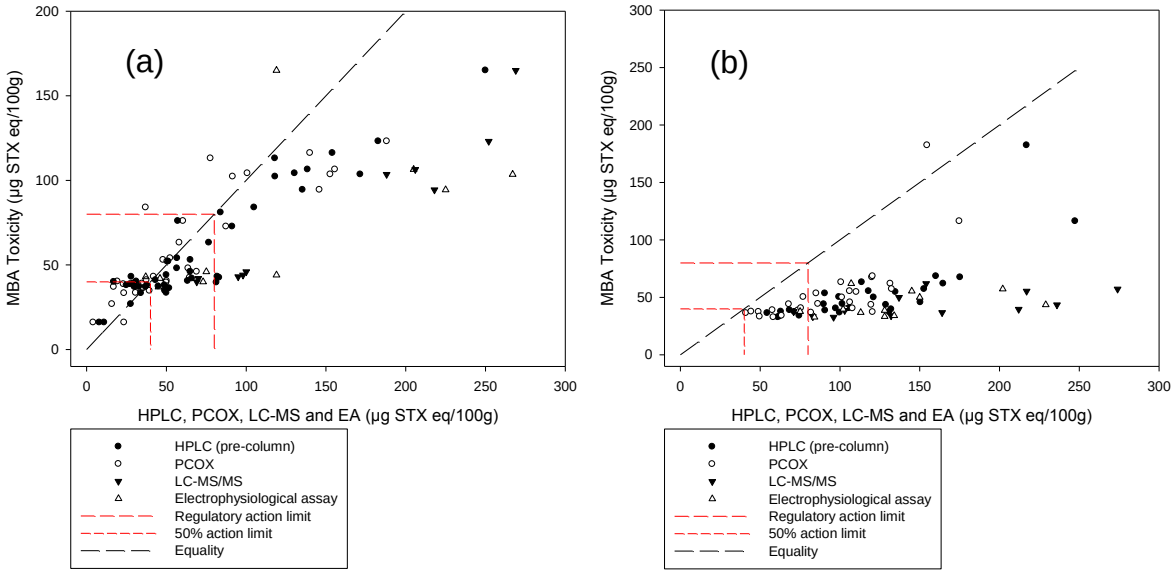


Figure 5. Total saxitoxin equivalents in a) mussels and cockles and b) oysters (Pacific and native) quantified by pre-column HPLC-FLD, post-column HPLC-FLD, HPLC-MS/MS and Electrophysiological assay as compared with the MBA PSP toxicity reference method.

Table 2. Comparison of total saxitoxin equivalence (µg STX di-HCl eq./100g) in cockles (n=22) and mussels (n=24) generated following MBA, pre-column oxidation HPLC-FLD, PCOX, LC-MS/MS and Electrophysiological assay.

Sample	Species	MBA	HPLC	PCOX	LCMS/MS	EA
		Cefas	Cefas	CFIA	NRCC	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*	82	42		
CO RM6b	Co	40*	81	50		
CO RM6c	Co	36*	52	36		
CO RM6d	Co	34*	50	31		
CO RM6e	Co	38*	49	33		
CO RM7a	Co	43*	83	65		
CO RM7b	Co	41*	63	50		
CO RM7c	Co	35*	49	39		

M = mussels, Co = cockles, nd = not detected. *Samples analysed by MBA at AFBINI

Table 3. Comparison of total saxitoxin equivalence (µg STX dc-HCl eq./100g) in oysters (Pacific and native) generated following MBA, pre-column oxidation HPLC-FLD, PCOX, LC-MS/MS and Electrophysiological assay.

Sample	Species	MBA	HPLC	PCOX	LCMS	EA
		Cefas	Cefas	CFIA	NRCC	UChile
RM4	NO	59*	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*	49			
RM2	PO	182*	217	155		
BTX2008/1604	PO	44	90	68		
RM3	PO	58*	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. *Samples analysed by MBA at AFBINI

4.4 Reproducibility of MBA

Extracts of mussels, cockles and oysters analysed at Cefas for PSP toxicity testing by MBA were sent to Chile for repeat analysis. Results compared well indicating that MBA method reproducibility was not responsible for the differences in relative method performance observed (**Figure 6**).

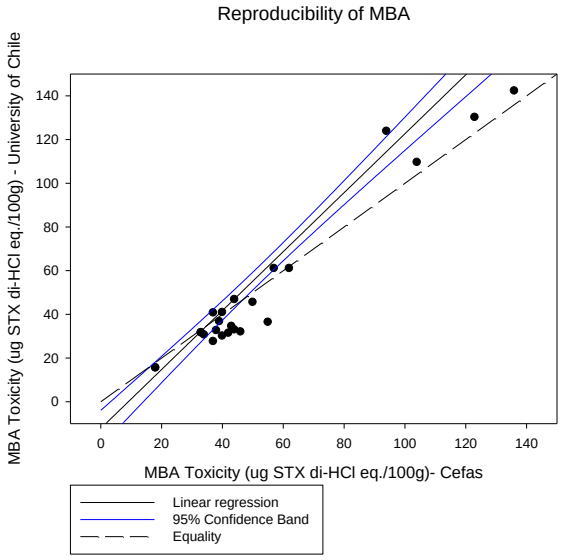


Figure 6. Comparison of MBA results generated at two laboratories (Cefas and University of Chile) for determination of reproducibility of toxicity results.

4.5 Nutritional analysis of shellfish extracts

a) Analysis was conducted at Campden laboratory, UK, for the determination of total carbohydrate, protein, fat and sugars present in the HCl extracts of a range of shellfish. Results from this analysis are illustrated in **Figure 7**, where nutritional concentrations are plotted against the observed HPLC/MBA toxicity ratio. On the whole, nutritional content was low, especially for fat. Whilst carbohydrate results indicate a slight visual relationship between total carbohydrate levels and the observed difference between HPLC and MBA results, it is not clear from these results alone whether such a correlation is significant. Fatty acid profile was also determined, but due to the low levels present in the samples, no clear results were obtained.

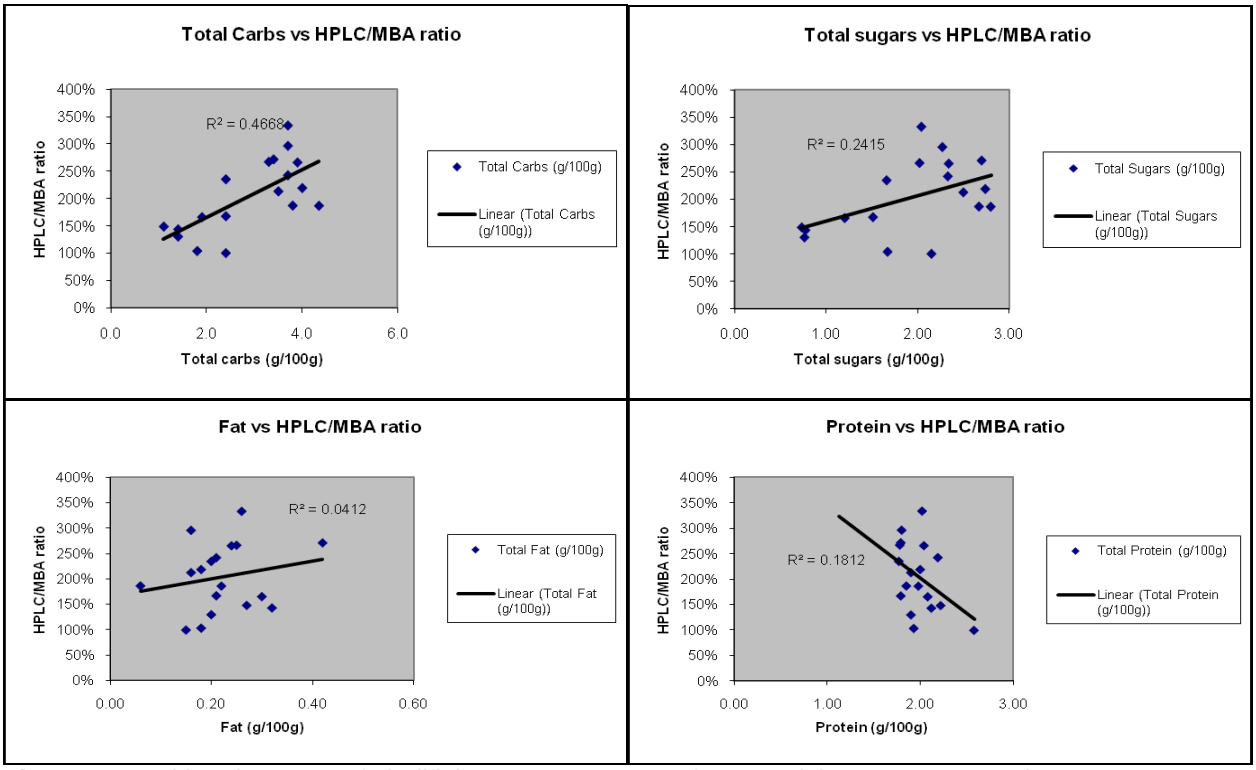


Figure 7. Nutritional content of shellfish extracts vs. HPLC/MBA toxicity percentage ratio

b) Due to previously published issues relating to the performance of MBA in the presence of high concentrations of salt, analysis was conducted at Cefas for the determination of conductivity / salinity in each of the above shellfish samples. Results indicated very little variability in salinity in all samples analysed, consequently showing no relationship between this and the observed differences in HPLC and MBA method performance.

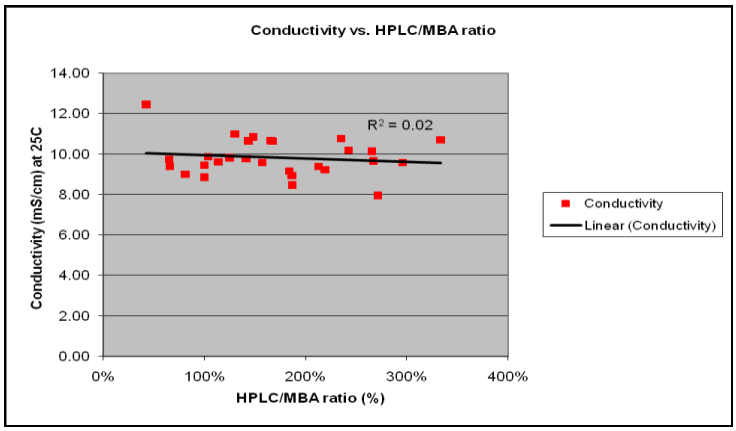


Figure 8. Comparison of observed HPLC/MBA ratio against conductivity of sample HCl extract

4.6 Analysis of shellfish extracts for metals

ICP-MS analysis was conducted at CFIA for the determination of metals concentrations in HCl extracts of mussels, cockles and oysters. The full data is displayed in **Appendix 1**. A comparison was conducted between metals concentrations against the observed bias between the HPLC and MBA PSP toxicity results. As expected, the oyster samples exhibited significantly higher levels of some metals (particularly zinc) as compared to levels in other shellfish species. Consequently, two metals (Zn and Mn) showed some degree of relationship between higher metals concentrations and a higher bias in the HPLC results as compared to MBA (**Figure 9**). None of the other metals showed any noticeable positive correlation between metal concentration and HPLC/MBA toxicity ratios.

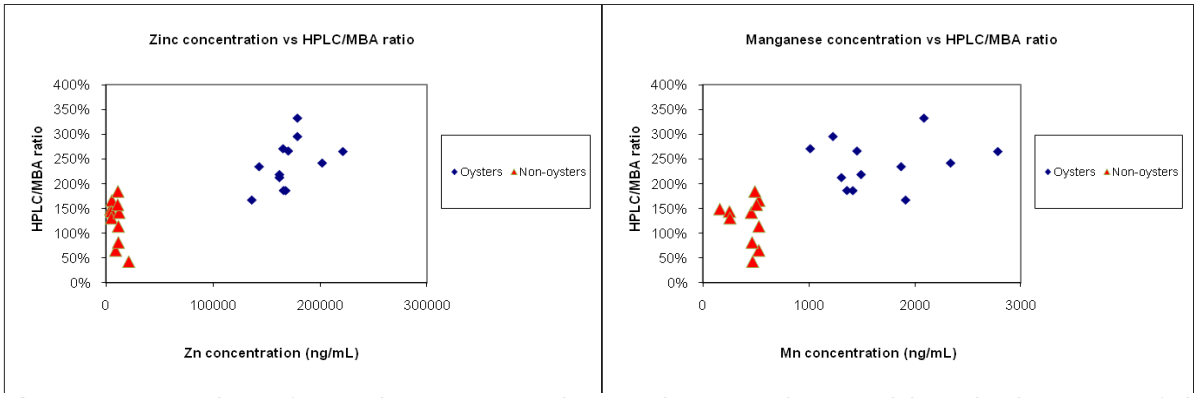


Figure 9. Comparison of Zn and Mn concentrations against HPLC/MBA toxicity ratios in a range of shellfish extracts, including mussels, cockles and oysters.

4.7 Investigations into the potential effects of zinc concentrations on the relative method performance of HPLC and MBA.

4.7.1 Demetallation of oyster extracts high in Zn

- 13 MBA-positive and 2 MBA-negative oyster bulk samples (Pacific and native) were extracted using the standard HCl extraction procedure. Each sample extract was split into 2 sub-samples. The first untreated sub-sample was tested for PSP toxins by HPLC (Cefas), MBA (University of Chile) and for metals by ICP-MS (CFIA). The second sub-sample was submitted to two clean-up methods, conducted to either reduce or eliminate the concentrations of zinc present in the extracts, the methods being chosen following a lengthy period of testing and optimisation at

Cefas. The challenge was to reduce the concentrations of the metal ions dissolved within shellfish extracts, without removal of the PSP toxins. A reduction in metal concentrations (30-60%) was achieved through use of a high pH precipitation and centrifugation step and a near complete demetallation (for zinc) was conducted through the additional clean up of extracts using Primary Secondary amine (PSA) cation exchange SPE cartridges (manufactured by UCT). These results are illustrated in **Figure 10**. Each demetallated extract, alongside a range of control samples (demetallated extracts from MBA-negative samples) was subsequently submitted for HPLC analysis (Cefas), metals analysis (CFIA) and MBA (University of Chile).

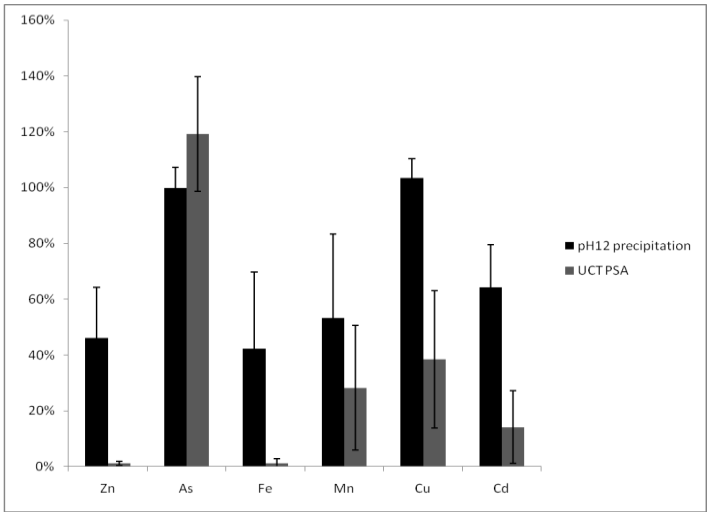


Figure 10. Mean percentages of metals concentrations remaining in HCl extracts of shellfish after a) pH12 precipitation and b) demetallation using UCT PSA SPE cartridges (±1 sd).

- **Table 4** shows the comparison of PSP toxicities determined in the oyster samples by both HPLC and MBA in untreated and demetallated HCl extracts. As expected, both demetallation procedures have resulted in some reductions in PSP toxin levels, but the majority of PSP-positive samples still exhibit total toxicities above the MBA LOD. The table shows the clear overall reduction in LC/MBA ratio with removal of metals from the extracts

	a) Untreated				b) pH12 ppt				c) UCT PSA SPE			
Samples	**LC-FLD**	**Zn**	**MBA**	**LC/MBA %**	**LC-FLD**	**Zn**	**MBA**	**LC/MBA %**	**LC-FLD**	**Zn**	**MBA**	**LC/MBA %**
PO 197	201	221551	61	324%	100	93300	42	238%	73	865	46	159%
PO 198	149	202229	46	298%	108	82290	38	284%	121	2618	142	85%
PO 199	77	136515	33	205%	19	41500	31	61%	18	1832	Nd	Nd
PO 200	100	143322	37	259%	63	25020	35	180%	81	1243	46	176%
PO 201	16	143496	nd	nd	28	41620	nd	nd	35	1952	Nd	Nd
NO 169	61	166164	32	184%	55	43740	33	167%	57	1275	42	136%
NO 170	78	168115	32	238%	31	96120	nd	nd	35	6270	72	49%
NO 171	84	162333	31	247%	60	108100	30	200%	48	1376	37	130%
NO 172	153	179104	41	386%	94	73530	76	124%	130	1042	106	123%
NO 173	124	162307	36	224%	81	117100	30	270%	72	1412	63	114%
NO 174	155	179096	47	356%	90	121800	80	113%	83	2566	59	141%
NO 175	107	165675	41	291%	50	113700	33	152%	42	2030	Nd	Nd
NO 176	179	170532	61	313%	106	53190	43	247%	128	1340	Na	Na
NO 177	3	266060	nd	nd	2	143400	Nd	nd	2	4110	Nd	Nd
NO 178	na	na	na	nd	84	71750	80	105%	72	1522	nd	nd

Table 4. Summary of PSP toxicity results obtained by LC-FLD and MBA (µg STX di-HCl eq./100g) and concentrations of Zn (ng/mL) in a) untreated and treated extracts of Pacific oysters and native oysters following b) metals reduction by pH12 precipitation c) demetallation by UCT PSA SPE. Nd = not detected, na = not analysed

- The mean HPLC/MBA ratio in the untreated oyster extracts was found to be 277%. Following demetallation by pH12 precipitation and SPE clean-up, this ratio dropped to a mean of 178% and 124% respectively.
- **Figure 11** illustrates the comparison of the HPLC/MBA toxicity ratios in the untreated oyster sample extracts with the toxicity ratios in the treated samples following both demetallation procedures. The graph shows that on average there is a clear relationship between decreasing concentrations of zinc and a reduction in the HPLC/MBA toxicity ratio. As such, this data provides good further evidence for the effect of high concentrations of metals such as zinc on the suppression of the MBA.

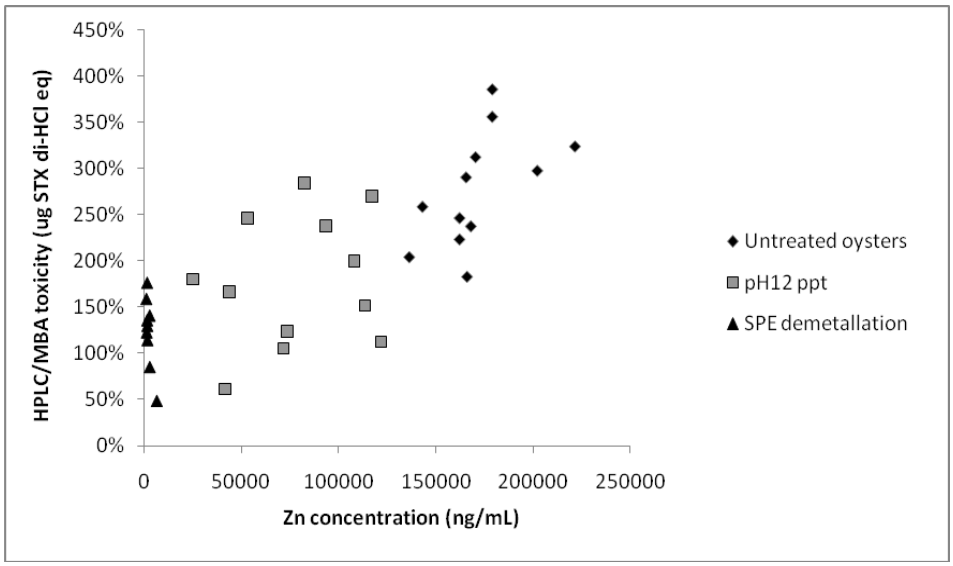


Figure 11. Comparison of HPLC/MBA toxicity ratio (µg STX di-HCl eq) against zinc concentration in a) untreated oysters b) oysters demetallated following pH12 precipitation c) oysters demetallated following SPE clean up

4.7.2 Fortification of mussel and cockle extracts with zinc

- A range of mussel and cockle samples showing a good average agreement between HPLC and MBA results and shown to be low in zinc concentrations as compared to oysters were spiked with varying concentrations of zinc. Samples were subsequently analysed by HPLC, ICP-MS and MBA.
- Results obtained from metals testing by ICP-MS indicated that the Zn spiking was successful in terms of increasing the concentrations of Zn within each sample to levels similar to those detected in oysters.
- HPLC results obtained from spiked and non-spiked extracts confirmed that the addition of Zn to mussel and cockle extracts had no effect on the results returned by the HPLC method (**Figure 12**).

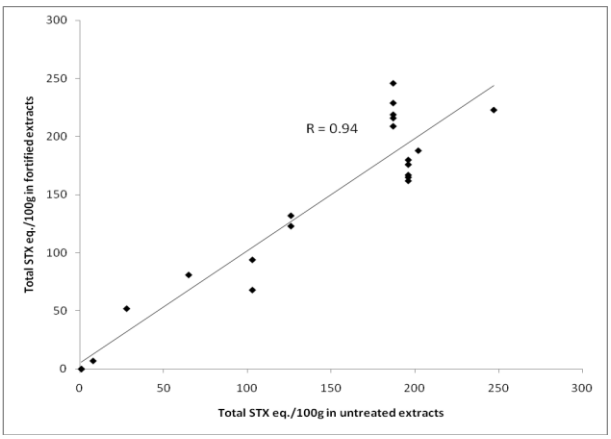


Figure 12. Comparison of total saxitoxin equivalents (µg STX di-HCl eq./100g) determined by LC-FLD in both untreated mussel and cockle extracts and extracts fortified with elevated concentrations of zinc.

- MBA results obtained from spiked and non-spiked extracts confirmed that the addition of Zn to mussel and cockle extracts had some effect on the results returned by the MBA method (**Table 5**). Control samples (PSP-negative extracts of mussels and cockles) indicated no effect of enhanced levels of zinc on the performance of the MBA. With zinc fortification concentrations targeted at both 150 and 250 ng/L, results indicate some degree of MBA suppression at both these concentrations on average. However, the largest suppressive effect is noted in samples with the higher concentrations of Zn (samples shaded in **Table 5**) where the MBA results are found to drop to less than a third of their original levels. Overall, the mean HPLC/MBA ratio in untreated mussel and cockle samples was 125%, whereas the mean HPLC/MBA ratio in the same samples spiked with zinc was found to rise to 161%, with the samples fortified to a total concentration of >180,000 ng/mL zinc exhibiting a mean HPLC/MBA ratio of 306%, similar to the ratio determined in the natural native oyster samples (**Figure 2**). It is stressed that these concentrations of zinc are similar to those present naturally in the oyster samples (**Table 4**).

	Initial samples (no spiked zinc)				Samples spiked with zinc				Change in HPLC/MBA ratio with Zn spike
Species	Zinc	HPLC	MBA	HPLC/MBA	Zinc	HPLC	MBA	HPLC/MBA	
M	6483	126	135	93%	130900	123	64	192%	206%
M	6483	126	135	93%	231500	132	37	357%	382%
M	10540	103	100	103%	136800	68	61	111%	108%
M	10540	103	100	103%	214900	94	37	254%	247%
M	8832	28	40	70%	157100	52	53	98%	140%
M	12178	65	37	176%	163500	81	45	180%	102%
M	6235	1	nd		165600	nd	nd		
M	6825	8	nd		163500	7	na		
Co	5281	247	127	194%	153800	223	130	172%	88%
Co	4776	202	na		143200	188	110	171%	
Co	4177	187	151	124%	47070	229	na		
Co	4177	187	151	124%	95040	209	116	180%	145%
Co	4177	187	151	124%	127300	219	138	159%	128%
Co	4177	187	151	124%	183100	216	72	300%	242%
Co	4177	187	151	124%	266200	246	na		
Co	4796	196	144	136%	45190	176	140	126%	92%
Co	4796	196	144	136%	77870	180	130	138%	102%
Co	4796	196	144	136%	124200	162	122	133%	98%
Co	4796	196	144	136%	176100	167	151	111%	81%
Co	4796	196	144	136%	245300	165	53	311%	229%
Co	4383	1	nd		140800	0	nd		
Co	3954	0	nd		147500	0	nd		

Table 5. Summary of PSP toxicity results obtained by LC-FLD and MBA (µg STX di-HCl eq./100g) and concentrations of Zn (ng/mL) in a) untreated mussels and cockles and b) mussels and cockles fortified with zinc. Nd = not detected, na = not analysed

5. Potential effects of HPLC method implementation

During the program of HPLC method validation for oysters, samples received at Cefas as part of the routine official control monitoring program were analysed for PSP toxins using the current (HCl extraction) HPLC screening method, with only HPLC-positive samples being forwarded for quantitation by MBA. However, as a result of the comparative issues encountered, oyster samples received between July 2009 and June 2011 found to be positive by the HPLC screen were additionally extracted by acetic acid and submitted to quantitation by HPLC. Results from all these samples are summarised in **Table 6**. During this period, only one routine oyster sample was found to be positive by MBA, with an additional MBA-positive native oyster sample being quantified during Spring 2010 (BTX/10/1605) although a larger number were found to be HPLC screening positive and MBA negative due to the lower sensitivity of the MBA method. Given the evidence for under-estimation of the total PSP toxicity in both Pacific and native oysters using the current MBA reference method, there is the potential for an increased number of PSP-positive samples being returned following any implementation of the HPLC method in these species. Hypothetically, with an under-estimation in PSP toxicity of <50% in the MBA and in relation to the MBA limit of detection of 33 µg STX eq./100g, an oyster sample showing a negative MBA test could still be actually exhibiting PSP toxicity as determined by HPLC close to the action limit of 80 µg STX eq./100g, which with application of measurement uncertainty could result in the sample results falling higher than this limit. However, the results summarised in **Table 6**, show that from the samples analysed during this period (28 samples in total), no additional samples found positive by the HPLC screen and negative by MBA would have resulted in a PSP toxicity value by HPLC above the action limit, even with application of the higher value calculated from measurement uncertainty.

Sample	Species	HPLC 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
BTX/11/933	PO	<RL	na	Nd
BTX/11/1107	PO	<RL	na	Nd
BTX/11/1214	PO	<RL	na	Nd
BTX/11/1273	PO	<RL	na	Nd
BTX/11/1462	PO	<RL	na	Nd
BTX/11/1494	PO	<RL	na	Nd
BTX/11/1495	PO	<RL	na	Nd
BTX/11/1651	NO	<RL	na	Nd
BTX/11/1756	PO	<RL	na	Nd
BTX/11/1757	NO	<RL	na	Nd

Table 6. Summary of PSP toxicities (MBA and HPLC, both in µg STX eq./100g) in Pacific and native oyster samples showing positive HPLC screening results (RL = reporting limit; 16 µg STX eq./100g).

Overall, therefore, from these 28 results alone, just two samples returned toxicities by MBA which were lower than the action limit but would have resulted in sample bed closures with application of the HPLC result with the addition of measurement uncertainty. From all other results obtained, even with the application of measurement uncertainty, none of the HPLC screen-positive / MBA-negative samples would have resulted in HPLC quantitation values higher than the action limit. As a result, the data obtained to date does not indicate any strong likelihood of any large number of bed closures resulting from the future implementation of the HPLC quantitation method in either native or Pacific oysters.

6. Conclusions

Work conducted at Cefas to date and summarised above has shown:

- A high HPLC to MBA bias reported in oyster samples was not found to relate to matrix-related fluoresence enhancement, reproducibility issues with the MBA, the use of inappropriate toxicity equivalence factors, or the use of different extraction protocols.
- Potential supression in the MBA as opposed to enhanced HPLC results was evidenced through quantitative data generated from additional non-bioassay methods (PCOX, LC-MS/MS and EA) which all returned toxicity results either similar to or higher than those generated by the Cefas HPLC method.
- Supression in the MBA was not found to relate to levels of salinity within the extracts and limited or no relationship was observed between nutritional content (fat, protein and carbohydrates) and the method performance.
- High HPLC/MBA bias is shown to occur in oyster samples found to be high in concentrations of zinc and manganese.
- Higher concentrations of zinc were not found to have any effect on the performance of the HPLC method
- MBA of demetallated oyster extracts has shown an overall relative increase in the MBA results returned, resulting in the reduction of the HPLC/MBA toxicity ratios observed in these species, providing further strong evidence for the suppresive effects of metals on the MBA.
- Analysis of mussel and cockle extracts spiked with concentrations of zinc similar to those found in oysters has shown a reduction in toxicity levels determined by MBA. Consequently, mussel and cockle samples found previously to give a good correlation in toxicities determined by the HPLC and MBA methods, were found on average to show higher HPLC/MBA ratios in the fortified samples, thus providing strong further evidence for the effects of zinc of the suppression of 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 now clear evidence from all the analysis conducted 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 HPLC method should be implemented for oysters, discontinuing the MBA for these species.
- Should a severe algal bloom occur in oyster beds, the MBA could potentially fail to trigger closures. However, from data gathered to date, the effects on industry should be minimal in terms of numbers of additional bed closures. With the implementation of the HPLC method in oysters, only two oyster samples received over the past three years would have been found to result in additional closures with use of HPLC quantitation results.

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Weymouth
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Appendix 1. Results from analysis of shellfish extracts for metals concentrations (ng/mL) and PSP toxins (µg STX eq./100g)

Sp.	HPLC	MBA	HPLC/MBA	Zn	As	Al	Cr	Fe	Mn	Ni	Cu	Se	Cd	Sb	Pb
PO	165	62	266%	221551	1329	79	94	6877	2786	58	1993	199	147	16.07	35.5
PO	121	50	242%	202229	1276	104	89	6828	2339	43	1642	186	160	5.83	35.2
PO	63	38	167%	136515	1119	221	120	5357	1914	30	885	178	163	2.41	29.2
PO	91	39	235%	143322	1058	84	133	4099	1872	73	781	191	160	36.68	31.3
PO	19	nd	na	143496	869	394	495	4303	1734	334	1256	172	192	5.66	29.7
NO	62	33	187%	166164	601	127	82	8888	1359	0.00	1503	141	254	1.10	14.3
NO	61	33	186%	168115	600	256	367	9791	1415	252	1546	167	255	5.43	16.6
NO	75	34	219%	162333	585	54	154	10732	1493	60	1597	200	248	36.77	19.0
NO	132	40	333%	179104	660	143	181	12634	2088	221	1686	232	275	37.02	22.7
NO	118	55	213%	162307	629	117	81	8202	1307	0.51	1399	134	244	0.98	17.0
NO	129	44	296%	179096	585	149	86	18292	1227	12	1439	133	219	1.02	16.4
NO	100	37	271%	165675	505	138	65	8302	1012	0.00	2203	116	167	0.67	14.0
NO	153	57	267%	170532	581	151	85	8708	1455	0.00	1377	121	219	0.69	16.9
NO	2	nd	na	266060	401	188	83	7526	362	0.00	1361	104	343	0.32	10.6
Co	183	123	148%	4177	841	109	170	10290	159	1250	80	244	62	36.35	8.4
Co	172	104	166%	5281	791	432	188	19187	527	327	4260	304	35	36.40	31.9
Co	135	94	143%	4796	958	507	388	10382	249	1347	280	257	69	27.54	9.4
Co	139	106	130%	4776	901	358	488	9684	254	1469	312	227	62	8.81	7.0
M	28	43	65%	8832	899	272	122	5383	525	18	148	348	71	1.71	53.9
M	65	46	141%	12178	556	155	121	4912	455	48	281	274	130	1.44	77.8
M	17	40	43%	21369	942	269	119	7528	467	47	579	289	204	1.22	146.9
M	30	37	81%	11474	625	198	112	7873	462	7.78	451	235	175	0.85	55.2
M	66	42	157%	10827	814	226	90	7112	503	17	432	253	170	0.68	52.0
M	250	136	184%	11062	604	304	135	4693	489	38	428	244	105	0.68	59.1
M	50	44	114%	11480	1354	275	111	5404	527	81	291	220	111	0.50	56.1