Detection and quantitation of lipophilic phycotoxins using LC-MS/MS in Pacific oyster samples to investigate the causes of the prolonged closure of a Scottish harvesting site

C. Brunet, R. Kelly, B. Stubbs and S. Morris

Cefas Weymouth Laboratory, The Nothe, Barrack Road, Weymouth, DT4 8UB, United Kingdom.
www.cefas.co.uk

1. Introduction

The Centre for Environment, Fisheries and Aquaculture Science (Cefas) is the official control (OC) laboratory contracted by the Food Standard Agency Scotland (FSAS) to carry out the testing of shellfish samples for presence of marine biotoxins, including diarrhetic shellfish poisoning (DSP) and other lipophilic toxins (LTs). The DSP method prescribed in EC regulation 2074/2005[1] as the official method is a biological assay that Cefas follows within the constraints of its home office licence and UK national reference laboratory standard operating procedure. The process entails an acetone extraction followed by liquid/liquid partition with diethyl ether and is carried out on the whole shellfish.

For the purpose of the Scottish OC monitoring programme, Pacific oysters *(Crassostrea gigas)* were collected weekly between 31st March and 13th October 2008 from one of the Scottish representative monitoring points (RMP) situated on the west coast. Of the 29 samples collected, eight recorded positive assay results with occurrences spread throughout the sampling period and led to temporary harvesting restrictions.

Whilst DSP events had taken place at this site previously, the prevalence of DSP in 2008 seemed unexpectedly high. The results recorded at this site also appeared to be an isolated occurrence as none of the other Pacific oyster sites monitored across Scotland (n=6) and none of the other sites in the area (n=26) recorded DSP positive results at a similar frequency. Indeed, DSP positive events at the site accounted for 21% of all Scottish DSP positive results between April 2008 and March 2009.

Data from the phytoplankton monitoring programme had indicated that, the potential for harmful phytoplankton to be the cause of the observed toxicity event was low for *Dinophysis* sp., medium/high for *Prorocentrum lima*, low/medium for 3D9 (the organism now thought to be responsible for azaspiracids) and medium/high for a combination of the three (data not shown; Cefas & SAMS, 2009)

In order to investigate further, FSAS agreed that archived samples should be analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) to establish toxin profiles and quantities.

2. Methods

From the 29 samples collected and assayed under the Scottish OC DSP monitoring programme, archived (frozen) tissue homogenate was available for 18 of these. Another eight samples previously extracted for LC-MS/MS analysis had existing archived extracts (stored below -15°C). Details of the OC DSP results and archived material availability are given in Table 2.

The LTs were extracted from a 2.0 g sample homogenate using a double extraction with 100% methanol as currently described by the European Union Community Reference Laboratory (EU-CRL) for marine biotoxins. The resulting solvent-to-sample ratio (SSR) was 10:1. Following a filtration step, an aliquot of the extract was analysed by LC-MS/MS without any further clean up. Another aliquot of the filtered extract was submitted to an alkaline hydrolysis[2] to chemically convert any potential acyl-esters of okadaic acid (OA) and dinophysistoxins (DTX1 and DTX2), often referred to as DTX3, to their parent compounds. The hydrolysed aliquots were subsequently also analysed by LC-MS/MS.

The instrumentation deployed to analysed hydrolysed and unhydrolysed extracts comprised of an 1100 series Agilent LC system coupled with a Waters Quattro Micro mass spectrometer detector with electrospray ionisation. The LTs were separated by applying an alkaline gradient (pH11) to a Waters XBridge analytical column. The mass spectral data was acquired using multiple reaction monitoring (MRM) and selecting two fragmentation transitions for each of the following compound of interest:

- Okadaic acid (OA)
- Dinophysistoxins (DTX1 and DTX2)
- Acyl-esters of OA, DTX1 and DTX2 (DTX3)
- Pectenotoxin 2 (PTX2) and its metabolite Pectenotoxin 2 seco-acid (PTX2sa)
- Azaspiracids (AZA1, AZA2 and AZA3)
- Yessotoxin (YTX), homoYTX (*homo* YTX), 45 hydroxyYTX (45 OHYTX), 45 hydroxy *homo* YTX (45 OH *homo* YTX)
- Gymnodimine (GYM)
- 13-desmethyl spirolide C (SPX1)

The toxins concentrations are reported on a wet weight basis in µg/kg and were quantified using external calibration curves constructed using several calibration standards prepared by dilution of certified reference material for OA, PTX2, AZA1, YTX, SPX1 and GYM. Pre-certified reference materials were used for DTX1 and DTX2. The solutions were diluted with methanol. The other toxins have been quantified using the calibration curve for the toxins of the same group and assuming equi-molar responses.

Table 1: Method limits of quantitation (LOQ) concentrations (µg/kg) for selected lipophilic toxins in Pacific oysters.

Toxins	Free OA	Free DTX1	Free DTX2	PTX2	AZA1	YTX	SPX1	GYM
LOQ	48.5 ± 2.69	41.9 ± 1.85	43.5 ± 7.36	6.13 ± 0.10	3.78 ± 0.28	28.2 ± 3.24	2.53 ± 0.13	12.0 ± 0.94

3. Results

Free DTX1, DTX2 acyl-esters, AZA2, AZA3, YTX and its analogues as well as GYM were not detected in any of the samples. The concentrations for the toxins detected were low compared to the regulatory limits as stated in EC Regulation 2074/2005[1] and were all below the method LOQ so these estimated values are reported on a qualitative and indicative basis only.

The concentrations for the compounds of interest are detailed in Table 2.

Table 2: Concentrations (µg/kg) of regulated and non-regulated lipophilic toxins detected in Pacific oysters.

Cefas sample reference BTX/2008/	Date collected	DSP MBA result1	Archive material availability	Free OA	Free DTX1	Free DTX2	OA esters	DTX1 esters	DTX2 esters	Total OA / DTXs	PTX2	PTX2sa	AZA1	AZA2	AZA3	YTX	homo YTX	45 OH YTX	45 OH homo YTX	SPX1	GYM
935	31/03/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.00	ND	ND	ND	ND	ND	ND	2.43	ND
1014	07/04/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.12	ND	ND	ND	ND	ND	ND	2.50	ND
1089	14/04/2008	PS	Extract	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.04	ND	ND	ND	ND	ND	ND	2.24	ND
1175	21/04/2008	NG	Extract	ND	ND	ND	ND	ND	ND	ND	5.00	3.22	3.25	ND	ND	ND	ND	ND	ND	2.05	ND
1266	28/04/2008	NG	None																		
1404	07/05/2008	PS	Extract	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.46	ND	ND	ND	ND	ND	ND	1.80	ND
1437	12/05/2008	NGs	Extract	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.20	ND	ND	ND	ND	ND	ND	2.45	ND
1527	19/05/2008	PS	Extract	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.55	ND	ND	ND	ND	ND	ND	2.08	ND
1625	26/05/2008	NGs	None																		
1697	02/06/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	3.25	ND	ND	ND	ND	ND	ND	ND	ND	2.00	ND
1801	09/06/2008	NGs	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.53	ND
1862	16/06/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.96	ND
1969	23/06/2008	NG	None																		
2054	30/06/2008	PS	Extract	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.43	ND
2136	07/07/2008	NG	Homogenate	ND	ND	ND	6.92	ND	ND	6.92	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.39	ND
2230	14/07/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	3.15	ND	ND	ND	ND	ND	ND	ND	ND	2.58	ND
2294	21/07/2008	NG	Homogenate	ND	ND	ND	18.0	8.60	ND	26.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.57	ND
2385	28/07/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.70	ND
2462	04/08/2008	PS	Extract	7.91	ND	ND	ND	ND	ND	7.91	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.32	ND
2570	11/08/2008	NGs	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.09	ND
2662	18/08/2008	NGs	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.39	ND
2792	27/08/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.79	ND
2832	01/09/2008	PS	Extract	ND	ND	19.2	18.1	9.03	ND	46	4.59	ND	ND	ND	ND	ND	ND	ND	ND	2.06	ND
2916	08/09/2008	NGs	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.62	ND
3003	15/09/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.22	ND
3090	22/09/2008	PS	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.31	ND
3188	01/10/2008	NGs	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.43	ND
3233	06/10/2008	PS	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.88	ND
3302	13/10/2008	NG	Homogenate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.02	ND

1: PS: positive – NG: Negative – NGs: Negative showing clinical signs – ND: Not detected

In all samples collected between 31/03/2008 and 19/05/2008, AZA1 was detected at concentration levels varying between 2.5 - 4.0 µg/kg. It was not detected in any of the subsequent samples. In contrast, evidence of OA and DTXs were detected later in the year and in four samples collected between 07/07/2008 and 01/09/2008. The toxins from this group were mainly observed in their esterified form with free OA and free DTX2 detected in only two separate samples (BTX/2008/2462 collected 04/08/2008; BTX/2008/2832 collected 01/09/2008, respectively). The maximum concentration value for the sum of OA+DTXs was calculated at 46 µg/kg in BTX/2008/2832. Throughout the sampling period, SPX1 was present at low concentration levels (1.8 – 3.6 µg/kg). Only four samples contained PTX2 and one of these (BTX/2008/1175) also contained its metabolite - PTX2sa. The occurrence of PTX2 and PTX2sa was sporadic and evident between 21/04/2008 and 01/09/2008 but since only a few samples contained these toxins, it would not necessarily reflect any trend.

On analysis of the samples that recorded positive assay results, the samples collected between 14/04/2008 and 19/05/2008 all contained only AZA1 and SPX1 below 10 µg/kg. The samples collected 04/08/2008 and 01/09/2008 both contained toxins from the OA and DTXs group as well as SPX1. The latter sample also contained PTX2. The remaining three samples (BTX/2008/2054, 3090, 3233) only contained the non-regulated SPX1 toxin.

For samples collected between 31/03/2008 and 19/05/2008, the amounts of AZA1 and SPX1 detected were similar whether the samples recorded a negative or positive assay result for DSP. It is therefore difficult to attribute the positive results solely to the presence of such low quantities. This is illustrated in Figure 1. For ease, the corresponding assay result has been included.

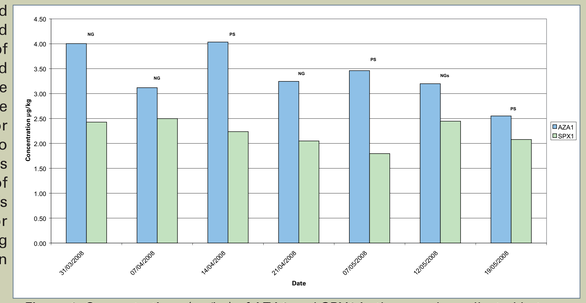

Figure 1: Concentrations (µg/kg) of AZA1 and SPX1 in the samples collected between 31/03/2008 and 19/05/2008

4. Further investigations

Since SPX1 was detected in all samples, further experiments were carried out to:

- confirm presence of SPX1
- investigate possible presence of other spirolides

Initially, eight selected extracts were submitted to a solid phase extraction step[3] and were then concentrated (SSR 17:1).

In order to confirm the presence of SPX1, a product ion scan was carried out on the SPX1 precursor ion (*m/z* 692.5). Three potential fragment ions were identified (*m/z* 164, 444 and 177) and the related MRM transitions used to re-analysed the extracts by LC-MS/MS. Figure 2 shows the chromatograms for each transition in sample BTX/2008/2294 and for transition 692>164 in the SPX1 standard.

To investigate the potential for presence of other spirolide compounds, identify potential precursor ions and determine the possible molecular weights of the compounds detected, a parent ion scan was carried out for the fragment ion *m/z* 164; characteristic of spirolides[4]. Alongside the SPX1 peak confirmed previously, most chromatograms exhibited additional peaks. In particular a peak was observed at a retention time of 7.8 min. with a precursor ion *m/z* 712.5 that may represent SPXF. Another peak eluted at a retention time of 13.2 min. with a precursor ion *m/z* 706.5 that may correspond to either SPXC or 20-methyl SPXG. A minor peak at a retention time of 13.5 min. with a possible precursor ion *m/z* 710.5 which may relate to SPXE. With the exception of the latter toxin, product ion scans were carried out and the presence of the *m/z* 164 fragment ion confirmed the selectivity of the transitions and the spirolide origin.

Subsequently, the 26 sample extracts were re-analysed by LC-MS/MS using the MRM transitions identified above. The SPX1 calibration curve was used to estimate the concentrations for all SPXs assuming similar response factors. The data along with the total SPX concentrations are presented in Table 3.

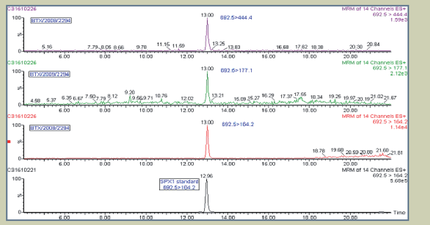

Figure 2: Confirmation of presence of SPX1 by LC-MS/MS MRM using 3 transitions (692>164, 692>444 and 692>177)

Table 3: Estimated SPXs concentrations (µg/kg)

Cefas sample reference BTX/2008/	Date collected	DSP MBA result1	Spirolide toxin concentration (µg/kg)				
			SPX1	SPXC or 20-MeSPXG	SPXE	SPXF	Estimated ΣSPXs
935	31/03/2008	NG	2.43	9.19			12
1014	07/04/2008	NG	2.50	8.82			11
1089	14/04/2008	PS	2.24	7.66			9.9
1175	21/04/2008	NG	2.05	7.40			9.4
1404	07/05/2008	PS	1.80	7.98	7.94	5.10	23
1437	12/05/2008	NGs	2.45	7.89	10.1	5.76	26
1527	19/05/2008	PS	2.08	7.90	8.61	5.94	25
1697	02/06/2008	NG	2.00	6.91	6.13	5.34	20
1801	09/06/2008	NGs	2.53	6.95	5.61	5.40	21
1862	16/06/2008	NG	1.96	7.31	5.31	5.09	20
2054	30/06/2008	PS	2.43	7.69	5.18		15
2136	07/07/2008	NG	2.39	7.76			10
2230	14/07/2008	NG	2.58	8.62		4.68	16
2294	21/07/2008	NG	3.57	9.19			13
2385	28/07/2008	NG	2.70	7.35			10
2462	04/08/2008	PS	2.32	7.20			9.5
2570	11/08/2008	NGs	2.09	8.01			10
2662	18/08/2008	NGs	2.39	7.63			10
2792	27/08/2008	NG	2.79	9.07		4.80	17
2832	01/09/2008	PS	2.06	8.11		4.75	15
2916	08/09/2008	NGs	2.62	8.01			11
3003	15/09/2008	NG	2.22	7.12			9.3
3090	22/09/2008	PS	2.31	7.40			9.7
3188	01/10/2008	NGs	2.43	7.61			10
3233	06/10/2008	PS	1.88	6.91		4.76	14
3302	13/10/2008	NG	2.02	7.19			9.2

1: PS: positive – NG: Negative – NGs: Negative showing clinical signs

5. Conclusions

The DSP positive results obtained at the site, during the period considered, were unusual for the area and isolated within Scotland. They were however unequivocal and testing was conducted in compliance with NRL procedures.

The LC-MS/MS investigations conducted on archived material indicated that the levels of individual LTs detected in the samples would not be expected to induce a positive DSP assay result. However, the effect of toxin mixtures is not well understood and the toxicological evidence related to the potential synergistic, additive or antagonistic effects of mixtures of LTs is sparse. A possible explanation for positive assay results is the presence of 'fast-acting' cyclic imines, albeit at low concentrations.

Alternatively, the LC-MS/MS method deployed in this study focuses on the detection of the regulated toxins as well as some non-regulated ones. It does not function for the detection of other compounds so the detection of other bioactive and/or natural products accumulated in the shellfish was excluded.

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