ATYPICAL 'DSP' POSITIVES IN COCKLES (*Cerstoderma edule*) FROM THE ROUTINE ALGAL BIOTOXIN MONITORING PROGRAMME FOR ENGLAND, WALES AND NORTHERN IRELAND

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Introduction

Figure 1: Locations of atypical DSP MB positives

During the 2001 and 2002 algal biotoxin monitoring programmes atypical DSP positives were detected by the Diarrhetic shellfish poisoning (DSP) mouse bioassay (MB) in cockles from several areas around the coast of England and Wales. The most significant areas affected were the Thames Estuary, the Burry Inlet and the Wash (Figure 1). All areas have extensive commercial cockle industries and the subsequent extensive and prolonged closure of these fisheries has had a major impact on the shellfish industry. Concurrently similar problems were being experience in Northern Ireland in cockle beds within Strangford Lough and Dundrum Bay which were closed for prolonged periods due to identical atypical DSP MB responses (Figure 1). Although the atypical DSP MB's have been predominately found in cockles other species have occasionally been affected. Identical clinical signs in the DSP MB have been observed in mussels, oysters and clams.

Currently with in the UK a modified version of Yasumoto's DSP MB is employed using acetone and diethyl-ether to extract lipophilic components for interperitoneal injection of 3 outbred mice (Yasumoto *et al.*, 1984). Mice are observed for 5-24 hours with a positive result being the death of more than one mouse during this period. The usual clinical signs observed during a typical DSP reaction in the mice is listed in Table 1. The clinical signs being observed during the atypical DSP MB were neurotoxic with rapid onset (Table 1). These were atypical and did not correspond to known clinical signs induce by any DSP toxin in mice.

Table 1: Typical and atypical DSP clinical signs observed in the DSP MB

Comparison between Typical and Atypical DSP clinical signs	
Typical Mouse Bioassay clinical signs	Atypical 'DSP' mouse clinical signs
death usually occurs between 1 and 24 hours diarrhoea unresponsive partial paralysis bluish discolouration cold to the touch erratic breathing	rapid symptom onset 1-2 minutes prostration with marked, rapid jerking of back legs and rapid breathing. In severe cases convulsions and spasm which cause jumping. 2-3 minutes rapid rear leg twitching, spasms and laboured breathing 3-15 minutes continued rear leg twitching and gasping/laboured breathing 3-15 minutes death Death usually within 10-20minutes or mice recover completely

Global occurrences of similar atypical DSP MB

The Yasumoto *et al.*, (1984) method extracts all lipophilic compounds from the shellfish matrix. Other uncharacterised lipid compounds which are toxic to mice may also be extracted by this method and may give positive results in the DSP MB. Certainly unknown 'toxins' have been found to cause atypical DSP MB responses in other countries including Norway, Canada and Scotland. In all these cases the onset of clinical signs in the mice was rapid and potentially neurotoxic (pers. com. Prof A. Tore, Norweign School of Veterinary Science; Dr. D. Richards, Canadian Food Inspection Agency; Prof. T. Yasumoto, Japan Food Research Laboratories).

Stability issues

When it was apparent that both English/Welsh and Northern Irish cockle were producing atypical DSP MB results samples were exchanged between the laboratories. The problem with the stability of the 'toxin' was discovered on these exchanges. Whole shellfish and homogenated shellfish were exchanged which had been stored at +5°C or –20°C for several days. In either form or at either temperature the clinical signs observed were less severe or absent on retesting. To ensure that this did not reflect test variables between laboratories similar storage conditions were emulated within the laboratory. Again a marked decrease was observed in severity of response in the DSP MB. A similar problem with the lability of the 'toxin' had been found in shellfish which caused atypical DSP MB results in Canada and Norway.

Investigation

Metals

The occurrence of high levels of zinc in shellfish tested using the PSP bioassay has been linked to the death of the mice through neurotoxic symptoms (McCulloch *et al.*, 1989). Although this has not been found using the DSP mouse bioassay, and there is some question over whether metals would be present at high concentrations in the final DSP extract, 6 samples (four positive and two negative by mouse bioassay) were tested for a range of metals.

The method employed was based on AEP analytical methods No 11, 1994. Results are reported in Table 2.

Table 2: Trace and Heavy metals concentrations in cockle samples from the Burry Inlet and Thames Estuary

Sample No.	Location	Atypical DSP MB results	Metals											
BTX/2001			Total Solid	CR	NI	CU	ZN	AS	CD	PB	HG	SE	AG	FE
			%	PPM	PPM	PPM	PPM	PPM	PPM	PPM	PPM	PPM	PPM	PPM
88	Thames	PS	16.6	1.8	5.8	1.8	11	1.5	0.06	0.96	0.02	0.61	0.54	643
313	Burry	NG	11.4	0.73	5.5	0.88	10	1.2	0.08	0.27	0.01	0.37	0.01	138
437	Burry	PS	11.1	0.23	4.6	0.89	8.7	1.1	0.07	0.25	0.01	0.29	0.06	23
460	Burry	PS	14.2	0.64	3.1	0.68	11	1	0.06	0.19	<0.01	0.35	<0.01	117
634	Thames	PS	16.4	1.2	4.9	0.9	9.9	1.1	0.05	0.37	0.02	0.41	0.26	304
635	Thames	NG	12.9	0.76	5	0.79	9.2	1.1	0.05	0.26	0.02	0.43	0.16	258

PPM= $\mu g\ g^{-1}$

Metal concentrations were within acceptable ranges for shellfish in all samples tested (FSA report 'Review of monitoring for chemical contaminates in shellfish in support of the shellfish hygiene directive'). On comparison between negative and positive shellfish there appears to be no significant difference observed between positive and non-positive shellfish in the Burry. McCulloch *et al.*, (1988) found that a level of more than 900 $\mu g\ g^{-1}$ zinc was required to cause mouse death. The Zinc levels detected in cockles were two orders of magnitude less than this concentration.

The results would indicate that metals are not responsible for the unusual DSP positives currently being detected in cockles.

Free Fatty Acids

The extraction procedures employed for the DSP MB have been developed to extract lipophilic toxins but also can co-extract other low polarity compounds such as free fatty acids (FFA). The lethal effects of FFA extracted from various shellfish species on mice has been documented (Mori *et al.*, 1995; Sajiki *et al* 1995; Takagi *et al.*, 1982; Suzuki *et al.*, 1996). The symptoms observed in the mice were crouching down, jerky head movements, laboured breathing and inactivity (Lawrence *et al.*, 1994). These symptoms are not consistent with those observed in the unusual cockle DSP positives (see Table 1).

Although the symptoms did not support FFA as being the cause of the cockle problem to confirm and eliminate these chemicals from the investigation a number of cockle samples were tested for a selection of FFA. As bound FFA are dominant in shellfish (Exler and Weihrauch, 1977) twenty two bound FFA were tested for in cockle samples.

Both positive and negative cockle samples were extracted using the standard DSP procedure (Yasumoto 1984). The residues were resuspended in methanol and derivatised to methyl esters using the method of Lepage and Roy (1988). These extracts were then analysed by Gas Chromatography Mass Spectrometry (GC-MS) and quantified using selected ion monitoring deployed diagnostic ions.

Table 3: Saturated FFA concentrations in cockle samples from the Burry Inlet, Camel Estuary and Thames Estuary

Sample No.	Location	Atypical DSP MB results	Saturated FFA $\mu g\ g^{-1}$ C12:0	C13:0	C14:0	C15:0	C16:0	C17:0	C18:0	C19:0	C20: 0	C21:0	C22:0	C23:0	C24:0	Tot Sat FFA
BTX/2001																
437	Burry	PS	0.06	0.02	ND	ND	0.09	3.2	0.7	2.1	0.9	0.6	1.3	0.4	ND	8.7
634	Thames	PS	ND	ND	0.3	0.07	ND	0.9	ND	1.6	1.9	ND	2.7	1.7	1.1	10.3
BTX/2002																
88	Thames	PS	0.2	0.5	ND	0.2	0.9	ND	3.5	0.2	1.0	1.1	0.5	0.3	1.1	9.5
215	Burry	NG*	ND	0.03	0.8	ND	ND	1.4	1.9	1.1	ND	ND	0.6	1.9	2.4	10.1
228	Thames	PS	ND	ND	1.5	0.04	0.02	0.8	0.05	2.6	0.8	0.7	ND	3.1	0.4	10.0
275	Thames	NG	0.02	0.1	0.1	0.9	1.2	1.7	ND	ND	ND	0.07	0.08	1.1	ND	5.3
285	Camel	NG	ND	0.7	0.3	1.2	0.4	ND	0.05	2.8	1.7	ND	0.9	2.6	0.07	10.7
298	Burry	NG	1.5	ND	ND	3.5	0.09	3.4	0.06	ND	0.08	1.3	ND	0.05	0.02	10.0

C12:00 lauric acid, C13:0 tridecanoic acid, C14:0 myristic acid, C15:0 pentadecanoic acid, C16:0 palmitic acid, C17:0 heptadecanoic acid, C18:0 stearic acid, C19:0 nonadecanoic acid, C20:0 arachidic acid, C21:0 heneicosanoic acid, C22:0 behenic acid, C23:0 tricosanoic acid, C24:0 lignoceric acid

* moderate symptoms ND = Not detected (value = 0 when summing totals)

Table 4: Monosaturated FFA concentrations in cockle samples from the Burry Inlet, Camel Estuary and Thames Estuary

Sample No.	Location	Atypical DSP MB results	Monosaturated FFA $\mu g\ g^{-1}$ C14:1	C16:1	C18:1	C20:1	C22:1	C24:1	Tot Sat FFA
BTX/2001									
437	Burry	Pos	ND	0.02	0.03	0.01	ND	0.04	0.10
634	Thames	Pos	0.01	0.08	0.02	ND	ND	ND	0.11
BTX/2002									
88	Thames	Pos	0.03	0.02	ND	ND	0.03	0.01	0.09
215	Burry	Neg*	ND	ND	0.06	0.01	ND	ND	0.07
228	Thames	Pos	ND	0.03	0.01	0.04	0.01	ND	0.09
275	Thames	Neg	ND	ND	0.04	ND	0.03	0.01	0.08
285	Camel	Neg	0.02	0.05	0.01	ND	ND	ND	0.08
298	Burry	Neg	ND	ND	ND	0.06	0.01	0.01	0.08

C14:1 (cis-9-tetradecanoic acid), C16:1 (cis-9-hexadecanoic acid), C18:1 (cis-9-octadecanoic acid), C20:1 (cis-11-eicosenoic acid), C22:1 (cis-13-docosenoic acid), C24:1 (cis-15-tetracosenoic acid)

* moderate symptoms ND = Not detected (value = 0 when summing totals)

Table 5: Polyunsaturated FFA concentrations in cockle samples from the Burry Inlet, Camel Estuary and Thames Estuary

Sample No.	Location	Atypical DSP MB results	Polyunsaturated FFA $\mu g\ g^{-1}$ C18:2	C20:5	C22:6	Tot Sat FFA
BTX/2001						
0437	Burry	Pos	0.02	0.01	ND	0.03
0634	Thames	Pos	ND	0.06	ND	0.06
BTX/2002						
0088	Thames	Pos	0.04	0.02	0.05	1.1
0215	Burry	Neg*	ND	ND	0.03	0.03
0228	Thames	Pos	ND	0.03	0.01	0.04
0275	Thames	Neg	0.02	0.01	ND	0.03
0285	Camel	Neg	0.07	0.01	0.04	0.12
0298	Burry	Neg	ND	0.06	ND	0.06

C18:2 (9,12-octadecadienoic acid or linoleic acid)
C20:5 (5,8,11,14,17-eicosapentaenoic acid)
C22:6 (4,7,10,13,16,19-docosahexaenoic acid)

* moderate symptoms ND = Not detected (value = 0 when summing totals)

The total concentrations of FFA (saturated, monosaturated and polyunsaturated) in cockles were low (5.4-11.9 $\mu g\ g^{-1}$) (Table 3-5) and three orders of magnitude less than shellfish which had induced 'false' DSP positives (1.2-2.9 mg g^{-1}) as found by Lawrence *et al* (1994) and Yasumoto *et al* (1984). The small dataset made comparisons between negative and positive samples statistically impossible, but with the exception of sample 275, negative and positive shellfish showed very similar concentrations of FFA. Sample 275, a negative sample, had total concentrations of FFA half of that of other samples.

Although only a selection of FFA were tested for in cockles, their overall low concentration at levels of magnitude less than those found in shellfish which have cause 'false' positives, coupled with the lack of mouse symptom agreement would initially suggest that FFA are not responsible for the atypical DSP positives detected in cockles. However, their implication in the unusual DSP positives cannot be eliminated without further investigation of an increased range of FFA, something which was not possible within the confines of this work.

A cockler in the Burry Inlet

Algal Biotoxins

A selection of positive, negative with symptoms and negative MB cockle samples from England and Wales were extracted and screened using LC-MS for known algal biotoxins. These included all known lipophilic toxins and PSP toxins. Although it was unlikely that PSP could be involved in the unusual DSP positives, due to its hydrophilic nature, the neurotoxic symptoms in mice necessitated its elimination.

Lipophilic compounds (OA, DTX's, AZA's and YTX's) were extracted with methanol:deionised water (8:2) (screen extraction) or acetone and di-ethyl ether (MB extraction). Extracts were then centrifuged before screening. The LC-MS method was that of Quilliam *et al.*, (2001). Certified reference material was available for OA and DTX-1 comparison and contaminated shellfish material for DTX-2, AZA's, YTX and PTX-2. The presence or absence of spirolides was based on abstracted mass to charge ratios taken from Quilliam *et al.*, (2002).

Hydrophilic compounds (PSP toxins) were extracted and tested as per Franco (1993). Samples were analysed by reverse phase HPLC with fluorescence detection. Certified calibration solutions were available for STX, NEO and GTX 1-4 confirmation.

The results did not indicate the presence at a detectable level of any toxin for which standards or contaminated material was available in any sample. However, peaks within the spirolide mass range were detected in one sample. To investigate the possible presence of spirolides samples were sent to Dr Allan Cembella and Dr Mike Quilliam at the NRC, Canada. On investigation of samples with standards available at the NRC for a range of spirolide toxins the shellfish were found not to contain spirolides at detectable concentrations.

Additional investigation of atypical DSP 'Toxin' by Professor Yasumoto

Prof. Yasumoto performed MB's on this material and detected 30 Mouse Units (MU) with the mice exhibiting atypical DSP clinical signs. The results indicated high polarity of the 'toxin'. Further investigation by Professor Yasumoto indicated that the 'toxin' differed from any known polyether toxin of dinoflagellate origin. Also the high polarity of the toxin suggests that the acetone extraction method is inappropriate for its extraction and this may explain the stability issues.

Causative organism

Although it has not yet be ascertained that the causative chemical component is algae derived, water samples have been collected on a weekly basis from two points in the Burry Inlet. In total 16 samples have been examined.

During the period of investigation no known toxic algal species was found at high concentration within the phyto or zooplankton assemblages. However, several potentially toxic algae were identified: *Prorocentrum minimum*, *Pfiesteria shumwayae*, *Protoperidinium* spp., *Pseudonitzscia* spp., *Amphidinium* spp., and cyanobacteria. *P. lima* has also been identified in areas affected by atypical DSP MB in Northern Ireland. The findings of Prof. Yasumoto suggest a non-dinoflagellate origin for the 'toxin' this coupled with the low concentration of all the potentially toxic dinoflagellates would make it unlikely that these species are the source of the 'toxin'. However, this does not exclude cyanobacteria and further investigation into the presence of cyanobacteria toxins needs to be undertaken.

The Burry Inlet

Discussion

The short time scale and limited financial resources meant that all research had to be undertaken in association with the routine algal biotoxin monitoring programme. The additional problems encountered with sporadic nature of the positives and the poor stability and/or repeat extractability of the causative 'toxin' on storage considerably slowed the investigation.

However, progress was made in eliminating potential anthropogenic contaminates and some algal toxins as the source of the atypical DSP MB. Investigation of trace and heavy metals in the shellfish showed normal concentrations when compared to previously analysed cockle and mussel samples. Levels of Zinc previously associated with false PSP positives were at concentrations two magnitudes below toxic levels. It is therefore unlikely that metals are the cause of the atypical DSP positives. Analysis of a selection of FFA's showed levels of three magnitudes below concentrations associated with 'false' (FFA induced) DSP MB positives. Although the limited number of FFA's tested makes it impossible to completely eliminate these chemicals as a possible cause of the atypical DSP positives, the relatively low concentrations detected would make it unlikely that FFA's were responsible.

Analytical investigation of samples has eliminated many algal biotoxins as being the cause of the atypical DSP MB: OA, DTX's, YTX, AZA's, PTX's, STX, NEO, GTX's, C-toxins and spirolides. This coupled with the findings of Prof. Yasumoto which suggest a non-dinoflagellate origin of the 'toxin' recommends the investigation of cockle metabolites as a potential source of the 'toxin'.

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