THE USE OF NON-ANIMAL ASSAYS WITHIN A BIOTOXIN MONITORING PROGRAMME

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Two alternative methods to the mouse bioassay for biotoxin detection in shellfish, the RIDASCREEN Saxitoxin ELISA for PSP detection and the KB cytotoxicity assay for DSP detection, were incorporated into a shadow monitoring programme undertaken in England and Wales. This was to establish the reliability and feasibility of carrying out these alternatives as a negative screen to the mouse bioassay within an active monitoring programme.

There was good agreement between the KB assay and DSP mouse bioassay in all samples tested with no false positives or negatives. Results from this study suggest that the KB assay would be a good potential candidate for incorporating into a routine biotoxin monitoring programme as a negative screen and could help reduce dependence on the DSP mouse bioassay.

The predominance of GTX's and lack of STX in shellfish samples resulted in the underestimation of toxin content by the ELISA kit on comparison to the PSP mouse bioassay. Although the kit was easy and quick to use, in areas in which STX is not the dominant toxin in shellfish the implementation of this test kit as a replacement to the mouse bioassay would not be recommended.

Introduction

Consumption of bivalves presents a health risk if shellfish originate from areas which have toxic algae problems. During periods of toxic algae occurrence bivalves can bioaccumulate toxins from the algae which can present serious, possibly fatal, health risks to the consumer. The UK is required to undertake a biotoxin monitoring programme under the EC Shellfish Hygiene Directive (91/492/EEC). The biotoxin monitoring programme currently in place in England/Wales involves the monitoring of water for toxic algae and shellfish flesh for biotoxins. The flesh monitoring programme relies on the EC approved AOAC mouse bioassay for detection of PSP toxins (AOAC 1990) and Yasumoto's mouse bioassay for detection of DSP in shellfish flesh (Yasumoto *et al.* 1984). Both are lethal assays and as with all mammalian bioassays are susceptible to a number of inaccuracies which can cause false reactions (Fernandez and Cembella 1995).

Historically Paralytic Shellfish Poisoning (PSP) episodes have principally been associated with the genus *Alexandrium*, however in the last 10 years, PSP outbreaks due to *Pyrodinium bahamense* var. *compressum* and *Gymnodinium catenatum* have caused considerable human mortalities. Within Europe PSP is usually associated with *Alexandrium* spp. there are presently 27 identified species of *Alexandrium* worldwide and of these 10 have been found to be toxic (Balech 1995). These species produce an array of chemically similar neurotoxins (saxitoxins) with more than 20 natural analogues of saxitoxin so far isolated (Oshima *et al.* 1995). The largest reported outbreak of PSP in Britain occurred in 1968 when 78 people were affected by paralytic illness following consumption of local gathered mussels (*Mytilus edulis*) (Ayres and Cullum, 1978). All UK-associated clinical cases of PSP which have been reported this century have involved mussels (Ayres, 1975). Prior to 1995, all positive tests for PSP in shellfish flesh in England and Wales had been from samples taken off the north-east coast of England. However, during 1995 toxic incidents occurred in the Fal and Milford Haven (Figure 1). Since this incident, PSP has since been found within the Fowey. PSP toxicity is usually an annual event at these sites and the north east coast although levels may not exceed the action limit.

Diarrhoeic Shellfish Poisoning (DSP) was first recorded in association with mussels consumed in The Netherlands during the 1960's (Kat 1979). This form of poisoning has been linked to the occurrence of several toxic species of the genus *Dinophysis* and *Prorocentrum lima*. There are several toxins within the DSP complex, the toxins associated with DSP symptoms are the okadaic acid/dinophysistoxin group. The first reported clinical cases of DSP involving UK produced shellfish occurred during 1997 (49 cases). There had previously been a small number of clinical cases associated with imported shellfish. Positive shellfish results for DSP have principally been found in mussels in the north-east of England and in the Solway Firth on the north-west coast. Sporadic positive results have occurred at a small number of other sites, but these have usually not been confirmed upon testing of repeat samples.

The use of animal bioassays presents ethical considerations, especially as within England and Wales the occurrence of biotoxins is sporadic and with the occasional exception low. Usually less than 10% of mouse bioassays performed within the biotoxin monitoring programme are found to contain biotoxins. The implementation of a negative screen to the biotoxin monitoring programme based on a non-animal assay would greatly reduce the number of mouse bioassays performed. With such an aim in mind two alternative methods for PSP and DSP detection were incorporated into a shadow monitoring programme at areas which had a previous history of biotoxins. This was to establish the reliability and feasibility of carrying out these assays within an active monitoring programme.

Several methods were examined for rapid easy inclusion into the biotoxin monitoring programme and the KB cytotoxicity assay for DSP detection and RIDASCREEN Saxitoxin ELISA for PSP were decided upon. The KB uses oral epidermoid carcinoma cells which when exposed to medium polarity protein phosphatase inhibitors (PPI) such as DSP toxins - okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2), undergo morphological changes which cause rounding of the cells. The PPI enzymes inhibit the cytoskeletal proteins which are naturally phosphorylated by protein kinases, these proteins can no longer be dephosphorylated and accumulate altering cell confirmation (Pouchus *et al.* 1997). By visually estimating the proportion of viable cells in the presence of these toxins it is possible to calculate a minimal active concentration (MAC) at which >30% of the cells are affected. This MAC can then be used to determine toxin concentration in shellfish samples. The RIDASCREEN Saxitoxin ELISA is a competitive enzyme immunoassay which is specific to saxitoxin (STX) but has cross reactivity of between 25-55% for gonyautoxin (GTX) II, III, C1, C2 and neosaxitoxin (NEOSTX).

Materials and methods

Shellfish were collected from 5 sample areas (Figure 1) involved in the 1999 England/Wales biotoxin monitoring programme which had histories of PSP and/or DSP episodes. Samples were collected throughout the year by local authority officers and sent to the CEFAS, Weymouth Laboratory where they were immediately extracted for PSP and DSP testing. Analysis by mouse bioassay and alternative assays was performed within 10 days of sample extraction.

DSP monitoring

Mussel samples (*Mytilus edulis*) were dissected and digestive glands (DG) extracted and analysed by mouse bioassay in accordance with the Yasumoto's procedure (Yasumoto *et al.*1984). A total of 35 shellfish samples were analysed by mouse bioassay and simultaneously tested by KB assay. Extraction for the KB assay was as the mouse bioassay extraction with the exception that residue was resuspended in ethanol. The KB assay was based on that developed by Amzil *et al.* (1992). Aliquots of digestive gland homogenate were frozen (-20°C) for liquid chromatography-mass spectrometry (LC-MS) testing which was performed throughout the sampling programme. The LC-MS extraction method was based on that developed by Lawrence *et al.* (1994). All techniques were calibrated with certified OA and DTX-1 solutions obtained from the National Research Council (NRC), Canada and DTX-2 from Dr K. James of the Cork Institute.

The semi-quantitative KB assay was based on Amzil *et al.* (1992) with the following amendments: 100µl of media containing 10,000 cells were dispensed into each well of a 96 well test plate and left for at least 24 hours to attach. 100µl of OA standard or test solution were added to wells and left for 48 hours with cell examination at 24 hours and 48 hours to determine minimum active concentration (MAC) when >30% cell rounding occurred. The plates consisted of 6 test solutions with dilutions ranging from 1/400 to 1/4000, 6 OA concentrations were used ranging from 25ng/ml-2.5ng/ml. A standards and test samples were run in duplicate on 2 test plates for each run. Tissue culture conditions are as Amzil but with a reduction in fetal calf serum concentration to 5%.

PSP monitoring

Shellfish samples were extracted and analysed by mouse bioassay in accordance with the AOAC procedure (AOAC 1990). A total of 33 shellfish samples were analysed by mouse bioassay and simultaneously tested by ELISA kit (Ridascreen, R-Biopharm Gmbh, Germany). Aliquots of shellfish homogenate were frozen (-20°C) for high performance liquid chromatography (HPLC) testing which was performed during November 1999. The HPLC method was based on that developed by Lawrence *et al.* (1991) using acetic acid extraction and pre-column derivatisation. All techniques were certified calibrated PSP solutions obtained from the National Research Council (NRC), Canada.

The ELISA was performed in accordance with the manufacturers instructions except that shellfish samples were diluted 1/2000 with buffer and STX was prepared from NRC standard.

Figure 1: Sample locations 1999

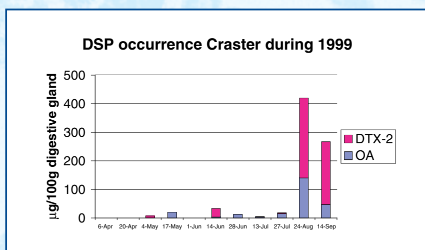

Figure 2

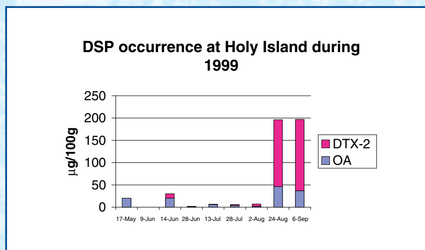

Figure 3

Results

DSP KB cell assay method comparison

The 3 sites involved in DSP testing were Craster and Holy Island in England and Milford Haven in Wales, all had previous histories of DSP. During the trial no DSP toxins were detected at Milford Haven by either LC-MS or mouse bioassay. However, at this site an isomeric form of OA was detected in one sample taken on the 7 September. DSP was detected at both Craster and Holy Island during the sampling period (Figures 2 and 3). A low concentration of DSP toxins was detected from May in both areas by LC-MS but levels did not rise sufficiently to cause positives in the mouse bioassay until late July/early August. Only 2 DSP toxins were found, OA and DTX-2, DTX-2 was the predominant toxin and comprised 71% of toxin encountered.

The MAC for OA, DTX-1 and DTX-2 are listed in Table 1. The MAC for OA was used to calculate the toxin concentration in shellfish digestive glands as OA is commercially available and has a similar activity to DTX-2. OA and DTX-2 were the dominant toxins found in England/Wales DSP incidents. By performing a range of sample dilutions and using the MAC it was possible to detect a range of toxin concentrations in test samples (Table 2). The minimum detectable level was 4-8µg/100g and maximum detectable level was 40-80µg/100g depending on the test duration time.

The KB assay showed good agreement with the mouse bioassay, in all samples where a positive mouse bioassay resulted the KB assay showed levels sufficiently high to cause mouse death (Table 3). Generally good agreement was found in samples taken from Craster and Holy Island with the LC-MS within the semi-quantitative parameters of the KB assay. At lower concentrations of OA and DTX-2 some variation occurred within the KB assay. Overestimation was found in 4 instances, however, in the 2 of these instances the mouse bioassay also showed the presence of toxins at levels sufficient to cause DSP mouse symptoms or death. The overestimation could therefore indicate the presence of compounds other than those searched for, which affected both the cells and mouse bioassay. Underestimation occurred in 3 samples, in these instances levels were insufficient to cause mouse death. There were no false negatives by KB assay on comparison to the mouse bioassay during the trial.

At Milford Haven toxin was detected at low levels on 2 occasions by the KB cells (Table 3). One of the occurrences coincided with an LC-MS peak of an unknown lipid soluble toxin which could have caused cytotoxic activity.

There was little variation between results obtained over the 24 hour and 48 hour exposure times for the KB assay, nearly all results conformed within the ranges expected within each test examination time.

Table 1

DSP toxin MAC obtained during the KB assay

Toxin	MAC 24hr ng/ml	Limit of detection ng	MAC 48hr ng/ml	Limit of detection ng
OA	5	1	2.5	0.5
DTX-1	2.5	0.5	1.25	0.25
DTX-2	3.25	0.75	≥ 2.5	≥ 0.5

Table 2

Dilution factors employed and corresponding detection level of DSP toxin in 100g of mussel DG

Dilution Factor	24hr OA equiv µg/100g	48 hr OA equiv µg/100g
1/400	8	4
1/500	10	5
1/670	13	8
1/1000	20	10
1/2000	40	20
1/4000	80	40

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Table 3

DSP content in mussel DG by KB assay, LC-MS and mouse bioassay

Sample Location	Sample Date 1999	24 hr KB (µg/100g)	48 hr KB (µg/100g)	OA	DTX-2	24hr Mouse Bioassay
Craster	06-Apr	ND	ND	0	0	NG
	20-Apr	ND	10	0	0	NG
	04-May	8	8	0	7	NG
	17-May	8	5	20	0	NG
	01-Jun	ND	ND	0	0	NG
	14-Jun	20	20	3	30	NG
	28-Jun	13	20	12	0.3	NG
	13-Jul	13	10	3	2	NG*
	27-Jul	40	40	15	3	PS
	24-Aug	>80	>40	140	280	PS
	14-Sep	>80	>40	47	220	PS
Holy island	17-May	8	8	20	0	NG*
	09-Jun	ND	ND	0	0	NG
	14-Jun	8	8	20	10	NG
	28-Jun	10	5	1	0.7	NG*
	13-Jul	13	10	6	0.2	NG
	28-Jul	13	10	4	2	NG
	02-Aug	40	40	2	5	PS
	24-Aug	>80	>40	46	150	PS
	06-Sep	>80	>40	37	160	PS
Milford Haven	12-Apr	ND	ND	ND	ND	NG
	27-Apr	ND	ND	ND	ND	NG
	11-May	ND	ND	ND	ND	NG
	26-May	ND	ND	ND	ND	NG
	11-Jun	ND	ND/10	ND	ND	NG
	23-Jun	ND	ND	ND	ND	NG
	12-Jul	ND	ND	ND	ND	NG
	28-Jul	ND	ND	ND	ND	NG
	16-Aug	ND	ND	ND	ND	NG
	25-Aug	10	5	ND	ND	NG
	07-Sep	20	7.5	ND[f]	ND	NG
	22-Sep	ND	ND	ND	ND	NG
	21-Oct	ND	ND	ND	ND	NG

* some DSP symptoms [f] unknown DSP isomer detected
ND DSP toxins not detectable NG negative mouse bioassay PS positive mouse bioassay

Results

PSP ELISA method comparison

During the study the levels of PSP found in shellfish from the 5 areas never exceeded the EC action level of 80µg STX equiv./100g shellfish flesh. The mouse bioassay only detected PSP toxins in 3 samples (Table 4). The ELISA in comparison with the mouse bioassay underestimated these values by up to 81%. The ELISA found no PSP toxins or levels well below the detectable limit of the mouse bioassay in all other tests. No false positives were obtained using the ELISA. HPLC examination of samples showed the dominance of GTX 1/4 and GTX 2/3 in samples, no STX was detected (Figure 4).

Table 4

PSP levels detected by mouse bioassay and ELISA

Sample	Mouse Bioassay µg STX equiv./100g	ELISA µg STX equiv./100g
Craster 1/6	33	8
Fal 22/7	33	6
Holy Island 9/6	36	9

The PSP profile of mussels during the study

NEOSTX
GTX 2/3
GTX 1/4

Figure 4

Discussion

DSP KB assay method

The current study was to evaluate the reliability and practicality of the KB assay as a negative screen to the mouse bioassay. The method was based on that developed by Amzil *et al.* (1992). The method employed in this study was extended 48 hours with cell examination at 24 and 48 hour intervals rather than 4 hours as used by Amzil and colleagues. This allowed a large dilution of mussel DG extract which increased ease of reading plates whilst maintaining a minimum detection limit considerable higher than the mouse bioassay. Determination of DSP in shellfish extracts by KB assay would appear to be a valid method. The KB cells shows clear morphological changes on exposure to OA, DTX-1 and DTX-2 which can easily be discerned by light microscope without staining. Reproducibility was good with 98% concurrence between duplicate wells and plates examined during the same time durations. The agreement between the 24 and 48 hour time intervals for OA concentration was also good with 96% of results falling within expected ranges. There was also good agreement between the KB assay and mouse bioassay in all samples tested with no false positives or negatives.

Incorporation of the KB assay into an active monitoring programme did not present any difficulties. The KB cell line has good temporal stability (Shoemaker *et al.* 1983) and was easy to maintain. The KB plates could be prepared and stored for up to 3 days before use, a constant supply of plates for testing could therefore be provided throughout the week with minimal time commitment. Each plate can be used for 8 samples or 4 if samples are run in duplicate. The time involved in preparing and running this assay over 24 hours is comparable to that of the mouse bioassay. In addition the KB assay format could be adjusted to be comparative in sensitivity to the mouse bioassay, which would allow a reduction in the time required for the assay run.

The KB assay has only been developed for the detection of PPI's, toxins such as yessotoxin and azaspiracid which can be detected by the DSP mouse bioassay may not be detected by this method. However, during this study only OA and DTX-2 were found to be responsible for toxicity occurrences in mussels in England and Wales, the major toxins currently associated with DSP in the UK would therefore be detected by this method. Results from this study suggest that the KB assay would be a good potential candidate for incorporating into a routine biotoxin monitoring programme as a negative screen and could help reduce dependence on the DSP mouse bioassay.

PSP ELISA method

In the 3 samples in which toxin was detected by mouse bioassay the ELISA underestimated total toxin concentration. The PSP profile of toxins in the mussels showed the predominance of GTX 1/4 and GTX 2/3 but no STX. Kasuga *et al.* (1996) found that cross-reactivity to GTX-1 and GTX-4 was less than 0.1% and the RIDASCREEN Saxitoxin ELISA instruction manual describes the relative cross-reactivity to GTX-2 and GTX-3 as 30% and 55% respectively. The lack of STX in samples taken during this study resulted in the underestimation of toxin content by the ELISA kit. Kasuga and colleagues found a similar problem with kits exhibiting unpredictable cross-reactivities to a mixture of GTX's, and underestimation of the toxicity of some naturally contaminated shellfish samples (Kasuga *et al.* 1996). The variation in cross-reactivity against PSP toxins with this kit is clearly very important when applied to real samples. Although the kit was easy and quick to use, in areas in which STX is not the dominant toxin in shellfish the implementation of this test kit as a replacement to the mouse bioassay could not be recommended.

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