THE FIRST REPORT OF THE CO-OCCURRENCE OF PECTENOTOXINS, OKADAIC ACID AND DINOPHYSISTOXIN-2 IN SHELLFISH FROM ENGLAND

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Introduction to Toxin Monitoring in England and Wales

The monitoring programme for algal biotoxins is a requirement of the Shellfish Hygiene Directive 91/492/EEC, and is implemented in England and Wales by the Food Safety (Fishery Products and Live Shellfish Hygiene) Regulations 1998, as amended. This legislation requires that EU member states monitor for the presence of toxin producing plankton in production and relaying areas, and biotoxins in live, bivalve molluscs. The Food Standards Agency is responsible for ensuring that this programme is carried out, effectively.

A four-year, rolling programme of monthly phytoplankton monitoring is in place to ensure that all shellfish harvesting areas are monitored. This provides an early warning for the potential of shellfish contamination by harmful algae. Regional Food Authorities are responsible for collecting the water and shellfish samples from the designated sites. Phytoplankton samples are delivered to the CEFAS, Lowestoft Laboratory for algal identification and enumeration, whereas the Weymouth Laboratory receives shellfish for toxin analyses. When concentrations of potentially toxic algal species are evident at or above action levels [e.g. 100 cells L^{-1} for any diarrhetic shellfish poison (DSP) producing algae], further phytoplankton samples are acquired along with shellfish from the same location. Weekly water samples are then analysed until the bloom event has dissipated.

Shellfish samples from all commercial harvesting areas are also collected on a monthly basis. For those areas with a history of shellfish contamination or demonstrating a regular presence of toxic phytoplankton, samples are taken fortnightly (April to September). The mouse bioassay (MBA) is the regulatory tool deployed for the detection of toxicity associated with DSP, and the qualitative analysis is performed using a method based on Yasumoto *et al.* (1984). Following reports of DSP-positive samples, shellfish from the impacted sites are then tested on a weekly basis until two consecutive negative results are obtained. For the past two years, liquid chromatography coupled with electrospray ionisation mass spectrometry (LC-ESI-MS) has been applied to screen DSP-positive shellfish samples, to provide information of the specific toxin profiles and, where possible, to determine toxin quantities.

In late September 2002, positive mouse bioassay results were reported for blue mussels (*Mytilis edulis*) harvested from a non-commercial shellfish bed located in the northeast of England. To protect the health of casual shellfish gatherers, public warnings were issued. This event provided an opportunity to apply LC-ESI-MS to confirm the findings of the MBA result, and to identify the toxin(s) associated with this sample. The following describes the analytical approaches taken, and presents the qualitative, quantitative, and confirmatory LC-MS and MS/MS toxin data.

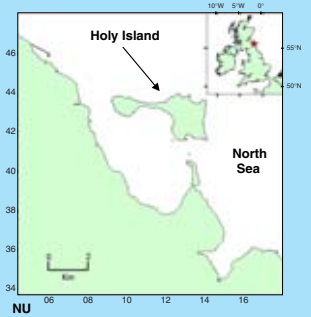

Figure 1: Sample location map of mussels (M. edulis) and phytoplankton from Holy Island, northeast England.

Materials and Methods

Phytoplankton sampling and identification

A depth-integrated water sample was collected from Holy Island, Northumberland (Figure 1) on 17 September 2002. A sub-sample was filtered (10 µm gauze), and the gauze and isolated phytoplankton were preserved in 2 % formaldehyde. The gauze plus formaldehyde solution was washed over a 10 µm sieve, and the contents of the sieve were placed into a 25 ml settling chamber. Identification and enumeration by microscopy was undertaken after settling.

Shellfish Flesh Extraction for LC-MS Analysis

A 4 g sub-sample of the whole shellfish flesh homogenate (2002/936) prepared for the MBA, was extracted with 16 ml of methanol and de-ionised water (8:2 v/v) for one min using an Ultra Turrax™. Following centrifugation (3000 g for 20 mins), 1 mL of the supernatant was filtered through a 0.25 µm filter and transferred to a 2 mL borosilicate autosampler vial. This was then stored at –20 °C prior to analysis.

Determination of DSP Toxins by LC-ESI-MS

For the purpose of screening DSP toxins in the shellfish extract, a Hewlett Packard 1050 Series LC (Agilent Technologies Ltd., Cheshire, UK) coupled to a Platform II single quadrupole MS (Micromass, Cheshire, UK) was used. Reverse phase separation was achieved using a Phenosphere NEXT C8 column (150 x 2.0 mm, 5 µm; Phenomenex, Cheshire, UK) plus a guard column. A gradient mobile phase programme was applied following a similar method reported by Quilliam *et al.* (2001) and was composed of 100 % water and 95 % acetonitrile-water both containing 2 mM ammonium formate and 50 mM formic acid. The flow rate was 200 µL min^{-1}, and for sample extracts, and calibration solutions, the injection volume was10 µL.

The ESI interface was operated in both positive and negative modes with a capillary voltage of 3.5 kV, cone voltage of 45 V and a source temperature of 160 °C. Selected ion monitoring (SIM) was performed using the following mass-to-charge (m/z) ratios:

Okadaic acid (**OA**) and Dinophysistoxin-2 (DTX2)	*m/z* **803.5** $[M\text{-}H]^-$
Pectenotoxin-2 (**PTX2**)	*m/z* **876.5** $[M+NH_4]^+$
Pectenotoxin-2 seco acid (**PTX2sa**)	*m/z* **894.5** $[M+NH_4]^+$
7-epi-pectenotoxin-2 seco acid (**7-epi-PTX2sa**)	*m/z* **894.5** $[M+NH_4]^+$

Within the same analytical run, screening of additional toxins [DTX1, yessotoxin (YTX) and azaspiracid-1 to –3 (AZA1 to 3)] was also undertaken using either protonated or deprotonated ions. Six levels of concentration of OA and PTX2, diluted from certified reference solutions (NRC, Halifax, Canada) were used for quantification. The range of the amount of toxins on column was 0.36 to 36 ng for OA, and for PTX2, 0.26 to 26 ng. Linear calibration curves were established, with r^2 values of >0.999. Assuming a similar molar response, DTX2 was quantified using the OA calibration curve.

Confirmatory analyses by LC-MS/MS

LC-MS/MS analyses were conducted using a PE-SCIEX (Thornhill, Ontario, Canada) API-4000 triple quadruple MS with a nebuliser-assisted electrospray (TurboSpray) source. Separation was achieved using a 50 x 2.1 mm column packed with 3 µm Hypersil BDS-C8 (Keystone Scientific, Bellefonte, PA), an isocratic mobile phase composed of 2 mM ammonium formate with 50 mM formic acid in 50 % (v/v) acetonitrile, and a flow rate of 200 µL min^{-1}. Initial confirmation was accomplished by selected reaction monitoring (SRM) of ammonia adduct ions and specific product ions of DTX2, PTX2 and its analogues. After preparation of a concentrated extract (preparative scale solid phase extraction of a 20 g sample using a 6 g OASIS-HLB column), product ion spectra of *m/z* 822.5 (DTX2), 876.5 (PTX2), and 894.5 (PTX2sa) were acquired using nitrogen collision gas and a collision energy of 45 eV.

Results and Discussion

Observations of the phytoplanton sample acquired from Holy Island revealed the predominance of the dinoflagellate *Dinophysis acuta*. This was found to be at a concentration of 2 874 cells L^{-1}. *Prorocentrum lima* and *D. acuminata* were not evident although the latter was present and at <5 cells L^{-1}, in a water sample taken three weeks earlier. A sample of *M. edulis* acquired after this observation and, from the same location, produced a positive MBA result.

The application of LC-ESI-SIM-MS revealed the presence of OA, DTX2, PTX2 and its seco acid analogues (PTX2sa and 7-*epi*-PTX2sa) in this sample (Figure 2). This DTX1, YTX and AZAs were not detected. Evidence of AZAs in mussels from this location has been reported previously by James *et al.*, (2002). However, this is the first report of the co-occurrence of OA, DTX2 and PTX2s in mussels from English waters. These findings contribute to current understanding of the spatial distribution of these toxins, and in particular PTXs throughout European waters. Published observations of the presence of PTXs in shellfish and phytoplankton found in Europe extends to Italy (Draisci *et al.* 1996), Ireland (James *et al.* 1999; Draisci *et al.* 1999), and Portugal (Vale *et al.* 2002). Since PTXs and DTXs (including OA) have been found to co-occur in *Dinophysis* spp (e.g. Draisci *et al.* 1996), the presence of *D. acuta* most likely contributed to the natural contamination of shellfish detected at the Holy Island site.

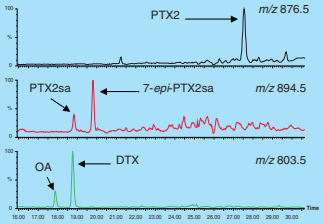

Figure 2: LC-ESI-SIM-MS chromatograms of the mussel extract (2002/936) showing the presence of OA, DTX2, PTX2, PTX2sa and 7-epi-PTX2sa.

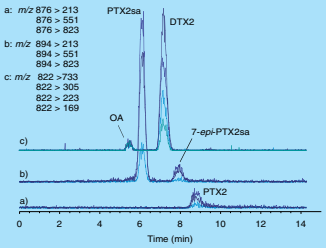

Figure 3: Confirmation of the presence of PTX2, PTX2 seco acids, OA and DTX2 in a crude extract of mussel homogenate (2002/936) using positive ESI with selected reaction monitoring (3 or 4 transitions per group as marked). The retention times and relative peak areas for each transition match those of standard toxins.

Mass spectrometric analyses performed on an API-4000 triple quadrupole MS/MS system confirmed the toxin profile obtained from initial LC-ESI-SIM-MS analyses. Figure 3 shows the confirmatory (SRM) chromatograms of the concentrated extract, and the presence of all five toxins. Mass spectra for DTX2, PTX2 and PTX2sa, produced from MS/MS analyses of the concentrated toxin isolates, are presented in Figure 4, and these correlate with those spectra obtained from authentic standards. They also show the characteristic fragment ions due to the sequential loss of up to six water molecules.

Quantification of the toxins revealed that the profile was dominated by DTX2, and the concentrations of each toxin of whole shellfish flesh were

Toxin	Conc. (ng g^{-1} whole flesh)	% contribution to total DSP toxicity
OA	120	15
DTX2	610	76
PTX2	70	9
Total DSP toxicity	800	100

The persistence of the contamination was short-lived, as these toxins were not detected after seven days. Although the concentration of PTX2 was less than the guideline level of 160 ng g^{-1} issued by the European Community Reference Laboratory (SANCO/2227/2001), the summation of DSP toxins exceeded the maximum limit by a factor of five. Currently, it is uncertain as to the contribution that the PTX2 metabolites have to the total toxicity of this sample.

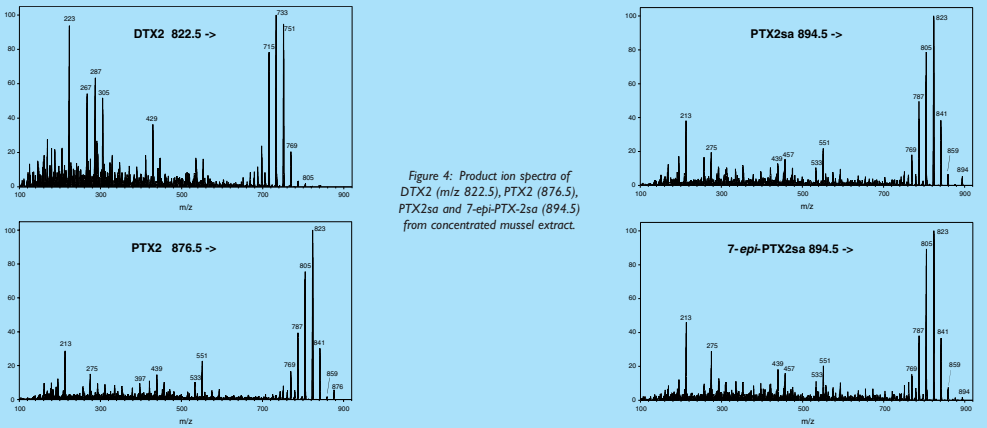

Figure 4: Product ion spectra of DTX2 (m/z 822.5), PTX2 (876.5), PTX2sa and 7-epi-PTX-2sa (894.5) from concentrated mussel extract.

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Acknowledgements

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