Chapter 18
A Comparison of Assay Techniques for the Analysis of Diarrhetic Shellfish Poisoning Toxins in Shellfish

R. Raine, A.M. Wilson, G. Hermann, and J.P. Lacaze

Introduction

Contamination of shellfish with Diarrhetic Shellfish Poisoning (DSP) toxins principally derived from *Dinophysis* spp. is the biggest problem for shellfish producers with respect to algal biotoxins along the west of the European Atlantic seaboard (Raine et al. 2010). The onset of these harmful algal events can occur in a matter of days due to rapid transport of toxic cells into an enclosed area by oceanographic processes. Rapid analysis of biotoxins in shellfish is therefore paramount. The standard method within Europe for the analysis of DSP toxins has been the mouse bioassay (MBA) (Yasumoto et al. 1978), which is often used in tandem with chemical methods such as liquid chromatography with mass spectrometry (LC-MS) (EC reg. 2074/2005). These methods have numerous limitations including their expense and use in a restricted number of laboratories which can cause long lag times, often exceeding that of the onset of a harmful algal event. These issues are particularly prevalent in geographically remote and peripheral regions, and have prompted the requirement of new analytical technologies for the analysis of algal biotoxins in shellfish to be performed rapidly, inexpensively, and locally which has particular relevance for local end product testing. This study investigates the use of two rapid techniques; an immunoassay and a functional assay, taking advantage of a national monitoring programme where results can be compared with MBA and LC-MS analysis in order to assess the accuracy, reliability and ease of use and applicability of these methods.

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Methods and Materials

Sample Collection

Edible blue mussels (*Mytulis edulis*) were collected fortnightly during the period June–September 2009 and weekly from May–September 2010 from Killary Harbour (53° N 37′ W, 09° 48′ W) Connemara, Co. Galway, Ireland (Fig. 18.1). Approximately 40 individuals were collected as sub-samples of those collected under the Irish National Biotoxin Monitoring Programme (NMP) operated by the Marine Institute (MI), from three stations; inner: GY-KH-KI, middle: GY-KH-KM and outer: GY-KH-KO, covering the length of the fjord (16 km). Environmental parameters were recorded on each sampling occasion. Samples for phytoplankton analysis were collected using a 12 mm i.d. tube to achieve an integrated water sample over the depth range 0–10 m (Lindahl 1986). Discrete water samples at various depths (2, 5 and 10 m) dependent on the sample site were taken in addition to integrated samples in 2010. All samples were preserved with Lugol's iodine before analysis using an inverted microscope (McDermott and Raine 2010).

Mussels collected during 2009 were stored whole at −20 °C. For analysis, the mussels were thawed, cleaned and the shellfish removed by cutting the abductor muscles. At least 100 g of flesh from each sample was rinsed with deionised water and homogenised using a hand held blender for approximately 2 min. Homogenates were stored in graded polypropylene centrifuge tubes (50 ml) at −20 °C. Mussels collected during the 2010 period were prepared immediately to eliminate any suspected freeze-thaw storage effects. Samples were thawed and refrozen as required.

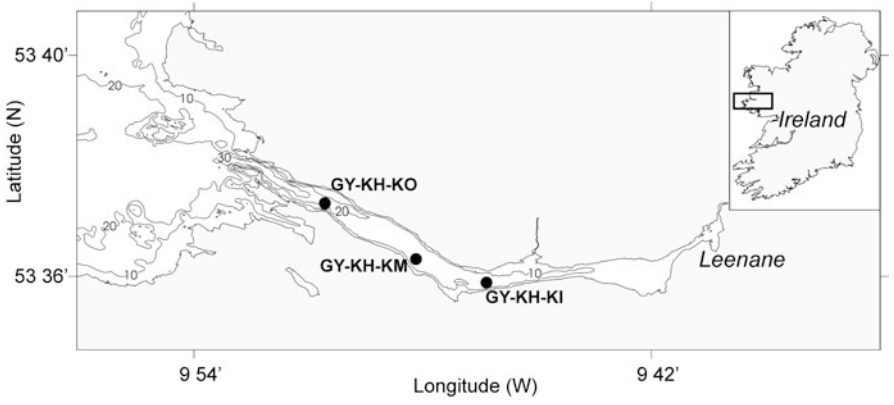

Fig. 18.1 Map of Killary Harbour, Co. Galway, Ireland showing the location of sampling sites and location of sampling stations in the outer (GY-KH-KO), middle (GY-KH-KM) and inner (GY-KH-KI) sections of the fjord

Toxin Extraction and Analysis

Toxins were analysed using commercially available kits. Both immunoassay kit (DSP ELISA, Abraxis) and an enzymatic protein phosphatase (PP2A) kit (OKATEST, ZEU-Inmunotec, Spain) were used for the detection of DSP toxins in the mussel extracts. The toxins were extracted from the shellfish homogenates using the manufacturer's instructions supplied with each kit. Briefly, DSP ELISA extracts were prepared by vortex mixing 1 g of mussel flesh with 9 ml 80 % (v/v) methanol followed by centrifugation (3,000 g for 10 min). Cleaned methanolic shellfish extracts were used for toxin analysis after filtration through 25 mm 3 μm pore size filter (Whatman, GF/C). PP2A (OKATEST) extracts were prepared in a similar manner by vortex mixing 5 g mussel flesh with 25 ml 100 % (v/v) methanol in a 50 ml centrifuge tube followed by centrifugation (2,000 g for 10 min at 4 °C). In 2009 the performance of the DSP ELISA kit only was used on relatively fresh extracts, a decision made on logistical grounds in the initial investigative period. Both methods were applied in 2010.

Both protocols were modified using an additional hydrolysis step in order to quantify the total DSP toxin content including esters and DTX-3. Extracts were diluted using sample dilution buffer supplied. All extracts were hydrolysed as part of the procedure and diluted accordingly.

Assays were carried out in 96-well microtitre plates supplied with the kits and incubated according to the manufacturers' instructions. Both assays operate on a colour reaction, the intensity being inversely proportional to the concentration of toxin present in the sample. Absorbance readings of the test mixtures and calibration standards were performed at 450 nm for the DSP ELISA and 405 nm for the DSP OKATEST using a plate reader (Biotek) with Gen5 software. Results were expressed as the concentration of okadaic acid and its equivalents, i.e. okadaic acid (OA) and its derivative dinophysistoxins DTX-1, DTX-2 and 7-O-acyl ester derivatives (DTX-3). Toxin concentrations were determined by external calibration using OA standards of known concentrations supplied with each kit.

Results

Levels of DSP toxins in mussel flesh from three monitoring sites in Killary Harbour through the summer of 2009 are summarised in Fig. 18.2a. These results were derived from LC-MS analysis as part of the Irish National Biotoxin Monitoring Programme. Contamination of mussel flesh with DSP toxins appeared in mid June and lasted through July until early August. DSP toxins levels rose to values exceeding the EU Maximum Permitted Level (MPL) of 0.16 μg OA eq $\cdot g^{-1}$ on 22 June at the outer and middle sites and on 29 June at the inner site. DSP toxin levels subsequently rapidly increased at all three sites to ca. 1.2 μg OA eq $\cdot g^{-1}$ on 5 July, with toxicity increasing faster at the outer and middle sites than the inner site

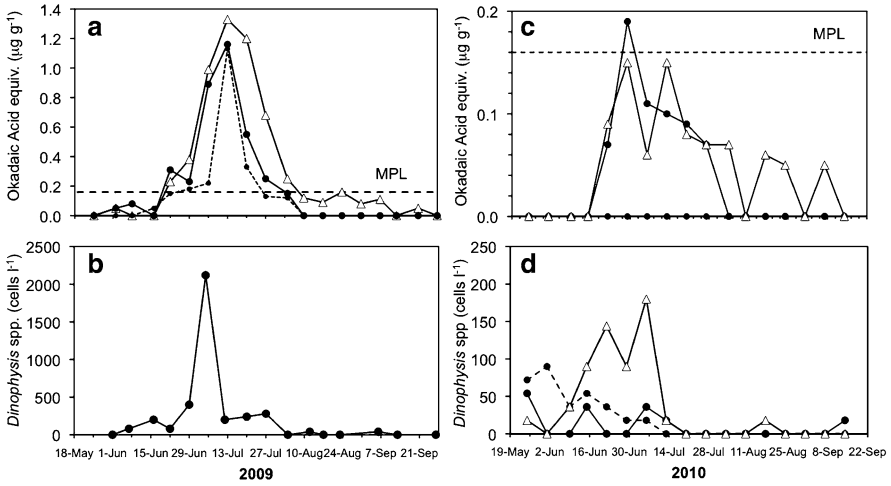

Fig. 18.2 Diarrhetic Shellfish Poisoning toxin levels in mussel flesh analysed by LC-MS and *Dinophysis* spp. levels in depth integrated water samples taken from the outer (*open triangles*) middle (*closed circles*, *solid line*) and inner (*closed circles*, *dotted line*) sampling sites in Killary Harbour, Ireland. (**a**) DSP toxin levels in 2009; (**b**) *Dinophysis* spp. levels at the middle site during 2009; (**c**) DSP toxin levels in 2010; (**d**) *Dinophysis* spp. levels during the summer of 2010. Note that no measurable levels of toxins were observed in samples taken from the inner site during 2010

suggesting that contamination was being transported into the harbour from outside. DSP toxin levels then decreased to levels below the MPL after mid-July at the inner and middle sites and from 10 August at the outer site. These dates co-incided with positive MBA results and enforced the closure of harvesting sites over a period of 7 weeks.

The contamination of mussel tissue with DSP biotoxins coincided with an increase in *Dinophysis acuminata* and *D. acuta* numbers in the water column (Fig. 18.2b). *Dinophysis* spp. cell densities in integrated samples increased to 2,100 cells $\cdot l^{-1}$ on 5 July corresponding to the initial sharp increase in DSP toxin levels in mussel flesh at this time. This clearly indicated that the DSP event in the fjord resulted from the influx of cells of *Dinophysis* spp., which is a known DSP producer.

Figure 18.2c shows DSP toxin levels in mussel flesh detected by LC-MS during the summer of 2010, during which a DSP contamination event also occurred. The event began in late June with a low, steady increase in toxin levels in mussel flesh. Relative to 2009, this DSP event was much smaller but still resulted in the closure of harvesting sites. DSP toxin concentrations in mussels detected by LC-MS reached the EU MPL on 28 June with levels of 0.19 µg OA eq $\cdot g^{-1}$ at the middle site and 0.15 µg OA eq $\cdot g^{-1}$ at the outer site. Co-incident positive MBA results on this date resulted in harvest closures. After 28 June at the middle site, DSP levels fell and remained below the MPL. However, levels reached 0.16 µg OA eq $\cdot g^{-1}$ on 12 July at the outer site, and a positive MBA resulted in further closure. Subsequently, toxin levels fell and remained within the range of 0.05–0.06 µg OA eq $\cdot g^{-1}$ in August

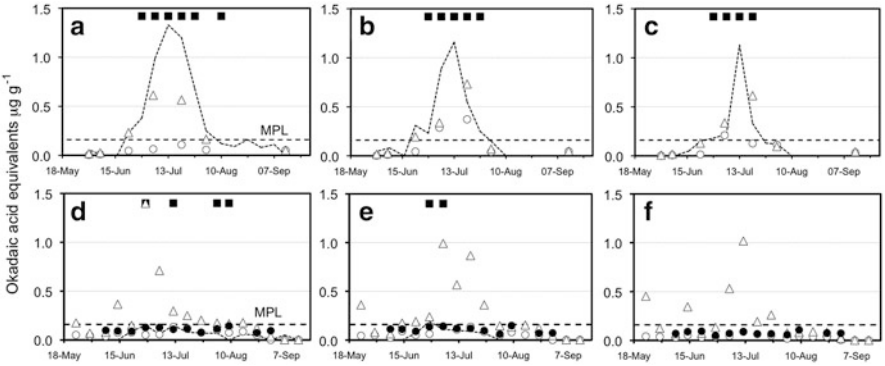

Fig. 18.3 Comparison of DSP toxin levels results using rapid assay techniques on mussel samples taken from Killary Harbour, Ireland in 2009 and 2010. Samples were taken from (**a**) outer site, (**b**) middle site and (**c**) inner site during 2009 and (**d**) outer site, (**e**) middle site and (**f**) inner site during 2010. *Open symbols* are results obtained before (*circles*) and after (*triangles*) hydrolysis using the DSP ELISA kit; *closed circles* for 2010 are results obtained using the protein phosphatase assay. Data from LC-MS are shown by comparison (*dashed line*) and can be referenced to Fig. 18.2a, c. The maximum permissible level (*MPL*) of DSP toxin in shellfish flesh is indicated by the *horizontal dashed line*, and the dates when positive mouse bioassay results were obtained are shown with *filled squares*. Sampling site locations are shown in Fig. 18.1

and September. Positive MBA on 3 and 9 August at the outer site resulted in a short closure. DSP levels remained below the limit of detection (LOD) at the inner site throughout the summer.

Dinophysis cell densities recorded in discrete and integrated water samples during this 2010 event again confirmed that it was caused by an influx of *Dinophysis* spp. (Fig. 18.2d). Higher cell densities were recorded in discrete samples compared with the integrated tube water samples. This is not unusual, as the organism can exist in sub-surface thin layers at high density (Farrell et al. 2012). Relatively low cell densities were recorded during the event compared to 2009. However, *Dinophysis* spp. are known to cause toxicity problems in shellfish at cell densities as low as 100–200 cells $\cdot l^{-1}$ (Botana et al. 1996). At the outer site, cell densities between 90 and 180 cells $\cdot l^{-1}$ were recorded in integrated water samples through June and the start of July. Lower densities (0–90 cells $\cdot l^{-1}$) were recorded at the middle and inner sites (Fig. 18.2d). Peak cell densities observed in water bottle samples were 300 cells $\cdot l^{-1}$ on 21 June at the outer site (10 m depth), 125 cells $\cdot l^{-1}$ on 28 July at the middle site (5 m depth), and 70 cells $\cdot l^{-1}$ on 2 August (2 m depth) at the inner site.

Comparative results of DSP toxin analysis using rapid techniques during 2009 and 2010 are shown in Fig. 18.3, where data are compared with those derived from LC_MS. In 2009, hydrolysed and non-hydrolysed ELISA samples and LC-MS data showed generally good agreement (Fig. 18.3a–c). Both data sets showed the same general trend; an initial non-toxic phase followed by a steady increase exceeding the MPL, followed by a steady decline. All three sites gave similar results using the immunoassay. Hydrolysed samples analysed by ELISA mimicked the LC-MS

results. However, the non-hydrolysed samples appeared to underestimate levels. All hydrolysed samples analysed by ELISA during the closure period produced positive results; no 'false positives' were found in hydrolysed samples determined by the ELISA. However, most non-hydrolysed samples gave results below the MPL during the closure period. Nevertheless, all positive results (i.e. >EU MPL of 0.16 μg OA eq $\cdot g^{-1}$) determined by LC-MS (and the MBA) were also positive using the ELISA method when the hydrolysis step was employed.

Figure 18.3d–f shows a comparison of DSP toxin levels in mussels flesh collected during summer 2010 when analysed by DSP ELISA (ABRAXIS), OKATEST and LC-MS methods. All the data sets show a similar pattern, with the notable exception of samples analysed by the DSP ELISA kit after the hydrolysis step. During the sampling period, only one sample from the middle sample site (28 June) gave a positive result by LC-MS (i.e. >MPL) whereas six positive results by MBA were recorded. During the 2010 sampling period, 35 samples analysed by LC-MS were below the limit of detection and/or quantification. However, both the immunoassay and enzymatic assay were able to detect DSP toxins at levels below the LC-MS LOD. Non-hydrolysed samples analysed by the ELISA method did not detect any positive DSP (>MPL) samples. DSP levels recorded by the enzymatic assay were more similar to the LC-MS data. Although no positive samples were detected during the closure period, high levels of DSP were detected by the OKATEST kit which were higher than those detected by the ELISA non-hydrolysed, and on two occasions slightly higher than the samples analysed by LC-MS. Hydrolysed samples analysed by ELISA gave significant overestimations of DSP levels in all samples. This was most likely caused by matrix effects resulting from the hydrolysis. These matrix effects were evident in samples with high and low concentrations of toxins, with 23 false positives found.

Table 18.1 shows a comparison of DSP toxin data from samples taken in 2009, stored and re-analysed using both the Protein Phosphatase (PP2A, Okatest) enzyme assay and LC-MS methods on non-hydrolysed and hydrolysed extracts from mussel flesh. Data from 2010 is also included. Good agreement is seen between the two methods in the 19 samples that were re-analysed. All but two extracts were in agreement and on both occasions the two errant results were borderline. On a sample originally taken on 20 July at the middle site, non-hydrolysed extract analysed by LC-MS gave a negative toxicity result, but when the hydrolysed sample was analysed a positive result was obtained, agreeing with the original MBA analysis, and also with the PP2A re-analysis on both hydrolysed and non-hydrolysed extracts.

Discussion

The DSP toxin group consists of the lipophilic toxin okadaic acid and its analogues dinophysistoxin-1 and -2 (DTX-1, DTX-2) and dinophysistoxin-3, a complex mixture of 7-O-acyl ester derivatives of OA, DTX-1,-2 (Suzuki and Quilliam 2011). Until 2011, detection of DSP toxins in shellfish was carried out by the MBA, as the

Table 18.1 A comparison of data on DSP toxins in mussel flesh derived from analysis using a protein phosphatase assay (PP2A) and LC-MS

	Method:	PP2A	PP2A	LC-MS	LC-MS
	Treatment:	Non-hydrolysed	Hydrolysed	Non-hydrolysed	Hydrolysed
Site	Date	μg OA eq g^{-1}	μg OA eq g^{-1}	μg OA eq g^{-1}	μg OA eq g^{-1}
Inner	07 June 2009	<0.06	0.08	0.02	0.03
Middle	07 June 2009	<0.06	0.12	0.04	0.07
Outer	07 June 2009	<0.06	0.10	0.03	0.04
Inner	05 July 2009	0.19	>0.38	0.18	0.67
Middle	05 July 2009	0.27	>0.38	0.23	0.68
Outer	05 July 2009	0.08	0.26	0.05	0.16
Inner	20 July 2009	0.13	0.30	0.08	0.17
Middle	20 July 2009	0.29	0.37	0.15	0.35
Outer	20 July 2009	0.12	0.28	0.07	0.19
Middle	02 Aug 2009	0.10	0.23	0.05	0.13
Inner	13 Sept 2009	<0.06	0.20	0.01	0.02
Middle	13 Sept 2009	<0.06	0.16	0.00	0.03
Outer	13 Sept 2009	0.09	0.18	0.03	0.05
Inner	08 June 2010	<0.06	0.07	0.02	0.01
Middle	08 June 2010	0.08	0.11	0.01	0.02
Outer	08 June 2010	0.08	0.10	0.02	0.02
Inner	14 June 2010	0.07	0.09	0.00	0.01
Middle	14 June 2010	0.08	0.11	0.01	0.03
Outer	14 June 2010	0.08	0.09	0.03	0.02
Inner	21 June 2010	0.07	0.09	0.00	0.01
Middle	21 June 2010	<0.06	0.09	0.03	0.05
Outer	21 June 2010	<0.06	0.09	0.03	0.05
Inner	28 June 2010	<0.06	<0.06	0.02	0.02
Middle	28 June 2010	0.07	0.14	0.07	0.11
Outer	28 June 2010	0.08	0.13	0.06	0.09
Middle	05 July 2010	0.10	0.14	0.09	0.11
Inner	05 July 2010	<0.06	0.07	0.04	0.04
Outer	05 July 2010	0.09	0.13	0.06	0.09
Inner	09 Aug 2010	<0.06	0.11	0.00	0.01
Middle	09 Aug 2010	0.08	0.15	0.02	0.03
Outer	09 Aug 2010	0.09	0.14	0.05	0.07

Mussel samples were taken from the outer, middle, and inner sites of Killary Harbour indicated in Fig. 18.1 on various dates in 2009 and 2010. Samples taken in 2009 had been stored frozen for 18 months prior to analysis using both methods. Analysis was carried out on both hydrolysed and non-hydrolysed extracts

EU official testing method. Commission Regulation (EU) No 15/2011 amending Regulation (EC) No 2074/2005, established the EU RL LC-MS/MS method as the reference method for the detection of lipophilic toxins in shellfish for the purposes of official controls. However, this analytical technique requires expensive equipment and maintenance as well as highly trained staff to perform routine

shellfish monitoring analyses. Alternative methods, cheaper to run and easier to use, are required by food business operators who are expected to perform end-product testing. Commercially available to research laboratories and the industry, the DSP ELISA (Abraxis) immunoassay and the OKATEST PP2A assay are designed for the detection in shellfish of OA, DTX-1,-2 and DTX-3, with the application of the important hydrolysis step. Although the immunoassay performed initially well in 2009, serious matrix effects can be seen when the kit was used to analyse hydrolysed samples. These matrix effects were apparent when mussel flesh samples containing both high and low levels of DSP toxins were analysed.

The DSP OKATEST performed well in detecting both high and low concentrations of DSP toxins in mussel samples. There were no effects similar to the matrix effects seen with the immunoassay data, and the data sets agreed with LC-MS on both fresh and stored samples. The PP2A assay is a functional assay based on the inhibition of the phosphatase enzyme by the OA-toxin group, which has the ability to hydrolyse a specific substrate, yielding a product that can be detected colorimetrically. Samples containing toxins from the okadaic acid group inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. Based on the data achieved in this study, the enzymatic based assay (PP2A) would be recommended in preference to the Abraxis immunoassay for rapid analysis, screening and end product testing of DSP toxins in shellfish. It is however important to bear in mind that the DSP OKATEST is a specific assay and therefore will not detect other regulated lipophilic toxins such as pectenotoxins, azaspiracids and yessotoxins. This limitation implies that the OKATEST cannot replace the multi-toxin LC-MS/MS method, but could confidently be used as an end-product testing technique by the industry in the case of shellfish solely contaminated with DSP toxins.

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