DEVELOPMENT AND EVALUATION OF PASSIVE SAMPLING AND LC-MS BASED TECHNIQUES FOR THE DETECTION AND MONITORING OF LIPOPHILIC MARINE TOXINS IN MESOCOSM AND FIELD STUDIES

ELIE FUX

Thesis submitted for the examination for the award of PhD in Chemistry

2008

DUBLIN INSTITUTE OF TECHNOLOGY
THE MARINE INSTITUTE
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Supervised by Dr Philipp Hess - Marine Institute

and Dr Barry Foley - DIT

Submitted in November 2008

DUBLIN INSTITUTE OF TECHNOLOGY

School of Chemical and Pharmaceutical Sciences

THE MARINE INSTITUTE

Shellfish Safety – Biotoxins Chemistry
Abstract

The consumption of shellfish that accumulate marine biotoxins produced by harmful algae can result in severe gastrointestinal or neurotoxic illnesses depending on the nature of the toxin. Therefore, it is essential that suitable monitoring programs are implemented in order to protect public health. This PhD study focused on aspects of the characteristics and monitoring of azaspiracids and toxins from *Dinophysis* spp.

Passive samplers were used in this study in parallel with both indigenous and transplanted mussels that did not contain toxins to investigate the accumulation of toxins on the West Coast of Ireland during 2005. This approach allowed for a number of observations including the persistence and contamination characteristics of azaspiracids, high levels of okadaic acid toxins in the water which did not induce shellfish contamination and non-suitability of passive sampling for early warning of shellfish contamination when placed in the same location as the shellfish. Furthermore, several sampling materials were compared in mesocosm experiments (culture of *Prorocentrum lima*) where the adsorption and desorption behaviours of okadaic acid and dinophysistoxin-1 were examined. A rapid and efficient extraction method was developed for a wide range of toxins.

The current EU reference method for the determination of lipophilic marine toxins is the mouse bioassay. Efforts towards the replacement of the non ethical and non specific animal test have been suggested and LC-MS is perceived as an alternative method of choice. However, LC-MS methods suffer from highly variable matrix effects affecting quantitation of the toxins. This study has evaluated three approaches for the evaluation of matrix effects in the analysis of okadaic acid, azaspiracid-1 and pectenotoxin-2. The influence of shellfish species, heat treatment and amount of dry residue in the shellfish extracts on the degree of matrix effects were evaluated. Additionally, a rapid Ultra-Performance LC-MS method for the analysis of 21 lipophilic marine toxins in 6.5 min was developed and matrix effects were evaluated.

A phytoplankton survey was carried out in the Celtic Sea in July 2007. *Dinophysis acuta* was found at high concentrations. Seawater was sampled from specific depths. The toxin profile of the phytoplankton was established in samples collected over a 14 h cycle. Pectenotoxins, okadaic acid and dinophysistoxin-2 were detected in the phytoplankton and were not found to be produced at a particular time of the day. The toxins accumulated and their concentration ratios in passive samplers were identical to those observed in *D. acuta*. Passive samplers deployed at 110 m depth and a phytoplankton sample from 80 m depth allowed for the establishment of the toxin profiles. In both cases, the toxin profiles and concentration ratios compared well with the samples obtained from other depths suggesting that *D. acuta* can occur and produce toxins in the absence of light.

Recent developments in the field of chromatography have shown that the water octanol partition coefficient (logP<sub>ow</sub>) of chemicals can be evaluated from the retention time. The method uses a calibration curve from compounds with known logP<sub>ow</sub>. The partition coefficient of toxins was investigated as an important physicochemical parameter governing the accumulation of toxins in passive samplers. Knowledge of the acidity constant (pK<sub>a</sub>) is essential as logP<sub>ow</sub> should be established in non dissociated molecules.
I certify that this thesis which I now submit for examination for the award of PhD in Chemistry, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute’s guidelines for ethics in research.

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Signature
Date
Candidate
Acknowledgments

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• Elie Fux, Daniela Rode, Ronel Bire and Philipp Hess, 2008. Approaches to the evaluation of matrix effects in the liquid chromatography- mass spectrometry analysis of three regulated lipophilic toxin groups in mussel matrix (*Mytilus edulis*). Food additives and contaminants, 25, 1024-1032

• Elie Fux, Claire Marcaillou, Florence Mondeguer, Ronel Bire and Philipp Hess, 2008. Field and mesocosm trials on passive sampling for the study of adsorption and desorption behaviour of lipophilic toxins with a focus on OA and DTX1. Harmful Algae, 7 (5), 574-583


Conference Proceedings


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Technical note

Oral Presentations

Nov 08  “D.acuta and its toxins at depths from the Celtic Sea” at the 13th International conference on Harmful Algae Bloom – Hong-Kong

Jun 08  “Advances in passive sampling for the detection of lipophilic marine toxins” 11th International conference of phycology – National University of Ireland Galway


Jun 07  “Challenges for the implementation of LC-MS based methods as an alternative to the mouse bioassay” Ifremer/ CNRS Brest – France

May 07  “Description of matrix effects observed in the LC-MS analysis of the lipophilic toxins okadaic acid, azaspiracid-1 and pectenotoxin-2 in mussel matrices” at the 12th International Union for Pure and Applied Chemistry (IUPAC) Symposium on Mycotoxins and Phycotoxins – Istanbul – Turkey

April 07  “Passive sampling for the detection of lipophilic marine toxins” - Annual Safefood conference – Cork – Ireland

Nov 06  “Update on BIOTOX research- SPATT technology and recent LC-MS developments” 7th Irish shellfish workshop, Marine Institute - Galway

Sept 06  “Comparison of the accumulation of lipophilic marine biotoxins in passive samplers, transplanted mussels and indigenous mussels on the Irish Coast” at the 12th International conference on Harmful Algae Bloom – Copenhagen – Denmark

June 06  “Comparison between passive sampler, mussel samplers and indigenous mussels for the monitoring of marine biotoxins” The 58th Irish universities chemistry research colloquium – National University of Ireland Galway
Abbreviations list

ACN: Acetonitrile
APCI: Atmospheric Pressure Chemical Ionisation
ASP: Amnesic Shellfish Poisoning
AZA: Azaspiracid
AZP: Azapiracid Shellfish Poisoning
CRM: Certified Reference Material
CV: Coefficient of variation
DAD: Diode Array Detector
DCA: Deoxycholic acid
DCM: Dichloromethane
DG: Digestive gland
DSP: Diarrhetic Shellfish Poisoning
DTX: Dionophysistoxin
DVB: Divinyl-benzene
ELISA: Enzyme-Linked ImmunoSorbent Assay
ESI: Electrospray
EU: European Union
FDA: US Food and Drug Administration
FLD: Fluorescence Detector
GYM: Gymnodimine
h: Hours
HAB: Harmful Algae Bloom
HP: Hepatopancreas
HPLC: High Performance Liquid Chromatography
i.p.: intraperitoneal injection
Hex: Hexane
HT: Heat Treatment
ILV: Inter-Laboratory Validation
IS: Internal Standard
ISp: Ion spray
ISO: International Standard Organisation
IT: Ion Trap Mass Spectrometer
LD50: Lethal dose at 50 %
LFIA: Lateral flow immunoassay
LLE: Liquid – Liquid Extraction
LOD: Limit of detection
LOQ: Limit of quantification
LRM: Laboratory reference material
MBA: Mouse Bioassay
ME: Matrix effects
MeOH: Methanol
MESCO: Membrane-enclosed sorptive coatings
MI: Marine Institute
min: Minutes
MLD: Minimum Lethal Dose
MRM: Multiple Reaction Monitoring
MS: Mass Spectrometry
<table>
<thead>
<tr>
<th>Abbreviation</th>
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</tr>
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<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>MU</td>
<td>Mouse Unit</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>NSP</td>
<td>Neurotoxic Shellfish Poisoning</td>
</tr>
<tr>
<td>OA</td>
<td>Okadaic Acid</td>
</tr>
<tr>
<td>PCI</td>
<td>Post-column infusion</td>
</tr>
<tr>
<td>PEEK</td>
<td>Polyetheretherketone</td>
</tr>
<tr>
<td>PEA</td>
<td>Post-Extraction Addition</td>
</tr>
<tr>
<td>PrOH</td>
<td>Propanol</td>
</tr>
<tr>
<td>PSD</td>
<td>Passive Sampling Device</td>
</tr>
<tr>
<td>PP</td>
<td>Protein Phosphatase</td>
</tr>
<tr>
<td>PSP</td>
<td>Paralytic Shellfish Poisoning</td>
</tr>
<tr>
<td>PTX</td>
<td>Pectenotoxin</td>
</tr>
<tr>
<td>QqQ</td>
<td>Triple Quadrupole Mass Spectrometer</td>
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<tr>
<td>QToF</td>
<td>Hybrid Quadrupole Time of Flight Mass Spectrometer</td>
</tr>
<tr>
<td>RM</td>
<td>Raw Mussels</td>
</tr>
<tr>
<td>RBA</td>
<td>Rat Bisoassay</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SA</td>
<td>Standard Addition</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SIM</td>
<td>Single Ion monitoring</td>
</tr>
<tr>
<td>SLV</td>
<td>Single Laboratory Validation</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>SPATT</td>
<td>Solid Phase Adsorption Toxin Tracking</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasma Resonance</td>
</tr>
<tr>
<td>SPX</td>
<td>Spirolide</td>
</tr>
<tr>
<td>SQ</td>
<td>Single Quadrupole</td>
</tr>
<tr>
<td>SSR</td>
<td>Solvent to Sample Ratio</td>
</tr>
<tr>
<td>SSI</td>
<td>Sonic Spray Interface</td>
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<tr>
<td>STX</td>
<td>Saxitoxin</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<tr>
<td>TIC</td>
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<tr>
<td>TIS</td>
<td>Turbo Ion Spray</td>
</tr>
<tr>
<td>TQ</td>
<td>Triple Quadrupole</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-Violet</td>
</tr>
<tr>
<td>WF</td>
<td>Whole Flesh</td>
</tr>
<tr>
<td>WP</td>
<td>Work Package</td>
</tr>
<tr>
<td>YTX</td>
<td>Yessotoxin</td>
</tr>
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1. **Background Information and Objectives**

1.1. **General introduction – Research scope**

Research in the field of marine biotoxins has become an area of interest as a consequence of several food poisonings across the world. Human intoxication with marine biotoxins can occur when shellfish that are contaminated with marine biotoxins are consumed. The symptoms can range from relatively benign gastrointestinal illnesses to the death of the shellfish consumers in extreme cases. The nature of the symptoms depends on the type of toxin that has been ingested. The toxins were classified in five distinct types of shellfish poisoning namely diarrhetic shellfish poisoning (DSP), azaspiracid shellfish poisoning (AZP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP) and paralytic shellfish poisoning (PSP). Several events of shellfish poisoning have been reported in the media, the scientific community or by governmental bodies. For instance, shellfish poisoning made the headlines when the BBC reported that in July 1998 a total of 49 people who ate at two London restaurants suffered from acute nausea, vomiting, diarrhoea, abdominal pain, and feverishness lasting eight hours (BBC News, 1998). All had been served dishes of mussels originating from the UK, including one who was made ill by mussel soup. Doctors diagnosed DSP in all patients. One of the largest food poisonings with DSP occurred in Belgium. On the 1st of February 2002, the Antwerp Department of the Health Inspection was notified that a presumptive foodborne illness had occurred in a large group of inhabitants of Brasschaat and Schoten, two towns in the vicinity of Antwerp, shortly after they had eaten boiled mussels. In total, 403 cases meeting the case definition of gastrointestinal disease were reported. Symptoms were characterised by a short onset (half an hour to three hours), vomiting, abdominal pain, nausea, and diarrhoea. One individual was admitted to hospital for extreme abdominal pain (Schrijver De et al., 2002). ASP first came to the attention of public health
authorities in 1987 when 156 cases of acute intoxication occurred as a result of ingestion of cultured blue mussels (*Mytilus edulis*) harvested off Prince Edward Island, in eastern Canada. As a consequence, 22 individuals were hospitalized and three elderly patients eventually died (U.S. Food and Drug Administration, 1992). PSP is also a very serious public health problem and led to tragic incidents in Guatemala where an outbreak of 187 cases with 26 deaths was recorded in 1987 as a result of the ingestion of a clam soup. The outbreak led to the establishment of a control program over shellfish harvested in Guatemala (U.S. Food and Drug Administration, 1992).

These examples of shellfish poisoning demonstrate the need for gathering scientific knowledge about the nature of the toxins involved in the different shellfish poisoning outbreaks as well as their origin and the dose to effect at short and long term exposures. It is extremely important to control and regulate the presence of shellfish toxins for the protection of public health as well as to maintain consumer’s confidence regarding the safe consumption of shellfish products. Indeed, regulatory authorities have the responsibility of food safety on one hand, but also must ensure that prevention measures are adequate because of the detrimental economical impact that repeated closure of shellfish production can have.

The different steps that follow human intoxication following shellfish poisoning are depicted in Figure 1.1.
In reality it is difficult to complete entirely one step before moving to the other and progress in the different fields, e.g. toxicology, chemistry, biochemistry, is usually made as a joint effort and constantly being reassessed using new information before an efficient surveillance programme is put in place. The different steps that are necessary to the implementation of a monitoring system are further discussed below.

1- **Human Intoxication**: Symptoms that people or animals exhibit provide important information to the nature of the toxin involved (Quilliam, 2003). When cases of food poisoning are reported and shellfish poisoning is associated with the symptoms, the exposure level can be evaluated from the amount and the nature of shellfish eaten.

2- **Identification and isolation of bioactive compound**: the toxicity of the shellfish responsible for poisoning is evaluated. Extractions of shellfish are performed using a range of solvent polarities in order to gain information about the physico-chemical parameters of the contaminant (lipophilic or hydrophilic, stability). Eventually, a structure of the compound responsible will tentatively be assigned using a chemical method such as LC-MS/MS. However, very good confidence in structure assignment can only be obtained by NMR spectroscopy. This technique requires mg amount of
toxin of high purity and therefore can only be used once isolation of the contaminant has taken place.

3- **Reference material:**

Monitoring laboratories must provide evidence that shellfish testing is carried out following strict guidelines that consequently ensure high level of confidence in the data generated. This is achieved by quality accreditation such as Good Laboratory Practices (GLP) or International Organisation for Standardisation (ISO) that requires the use of validated methods. Method validation implies that certified reference material (CRM) and calibration standards are used in order to demonstrate the suitability of a given method for the detection and quantification of the analytes of interest.

The National Research Council’s Certified Reference Materials Program initiated by the group of Michael Quilliam in Canada began in 1987 with the production of a CRM for ASP testing (Quilliam, 2003). By 2008, the program has generated many CRMs with 9 PSP calibration solutions, one ASP (Domoic acid) calibration solution and the second generation of shellfish tissue CRM contaminated with domoic acid. One shellfish tissue CRM is also available for OA and DTX1 with concentrations of 10.1 and 1.3 mg/kg, respectively. Calibration solution of the lipophilic toxins and their concentrations are shown in Table 1.1.
Table 1.1: Concentrations of calibration solutions of lipophilic toxins available from the NRC. Concentrations are given in µmol/l as given on the NRC website.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Concentration [µmol L⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>17</td>
</tr>
<tr>
<td>PTX2</td>
<td>10.0</td>
</tr>
<tr>
<td>PTX2sa</td>
<td>Similar to PTX2 conc.</td>
</tr>
<tr>
<td>AZA1</td>
<td>1.47 ± 0.08</td>
</tr>
<tr>
<td>YTX</td>
<td>4.6</td>
</tr>
<tr>
<td>SPX-13-DesMe-C</td>
<td>10.2</td>
</tr>
<tr>
<td>GYM</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Because of the limited availability of certified reference material, the production of in-house reference material and use in proficiency testing is strongly encouraged (Hess et al., 2007). Several studies dealt with the stabilisation of reference materials for shellfish toxins using anti-oxidants, freeze drying, gamma irradiation and heat treatment (Hess et al., 2005b; McCarron and Hess, 2006; McCarron et al., 2007a; McCarron et al., 2007b; McCarron et al., 2007c; McCarron et al., 2008).

4- Risk assessment / Regulatory limits: This is discussed in detail in the next section.

1.1.1. Regulatory limits and monitoring in the EU

The regulation (EC) No 853/2004 (Official Journal of the European Union L 139 of 30 April 2004) lays the criteria for placing live bivalve molluscs on the EU market for human consumption. Live bivalve molluscs should not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the limits given in Table 1.2. The limits are given for toxin class and each toxin class contains a number of individual toxins that contribute to the shellfish toxicity. Toxins that are regulated under the same toxin class are not necessarily structurally related to each other (e.g. PTXs and DTXs).

Official controls on the production of live bivalve molluscs include marine toxins monitoring. The EU accepted definition for monitoring is given in the Regulation (EC) No 882/2004:

“Monitoring means conducting a planned sequence of observations for measurements with a view to obtaining an overview of the state of compliance with feed or food law, animal health and animal welfare rules”.

Thus, classified relaying and production areas must be periodically monitored to check for the presence of biotoxins in live bivalve molluscs and of toxin-producing plankton in production and relaying waters. Sampling plans must take particular account of possible variations in the presence of plankton containing marine biotoxins and must comprise:

1- periodic sampling to detect changes in the composition of plankton containing toxins and their geographical distribution. Results suggesting an accumulation of toxins in mollusc flesh must be followed by intensive sampling;

2- periodic toxicity tests using those molluscs from the affected area most susceptible to contamination.

The sampling frequency for toxin analysis in the molluscs is, as a general rule, to be weekly during the periods at which harvesting is allowed. This frequency may be reduced in specific areas, or for specific types of molluscs, if a risk assessment on toxins or phytoplankton

---

### Table 1.2: Maximum levels of marine toxins for placing live bivalves of the market - EU regulation (EC) No 853/2004

<table>
<thead>
<tr>
<th>Toxin Class</th>
<th>Limit µg /kg</th>
<th>Regulated toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSP</td>
<td>800</td>
<td>Saxitoxins (STX)</td>
</tr>
<tr>
<td>ASP</td>
<td>20 000</td>
<td>Domoic acid (DA), epi domoic acid</td>
</tr>
<tr>
<td>DSP</td>
<td>160</td>
<td>OA, DTX1, DTX2, DTX3, PTX1, PTX2</td>
</tr>
<tr>
<td>YTX</td>
<td>1 000</td>
<td>YTX, 45-hydroxy-YTX, homo-YTX, 45-OH-homo-YTX</td>
</tr>
<tr>
<td>AZA</td>
<td>160</td>
<td>AZA1, AZA2, AZA3</td>
</tr>
</tbody>
</table>

1: whole body or edible part
occurrence suggests a very low risk of toxic episodes. It is to be increased where such an
assessment suggests that weekly sampling would not be sufficient. The risk assessment is to
be periodically reviewed in order to assess the risk of toxins occurring in the live bivalve
molluscs from these areas.

With regard to the monitoring of plankton, the samples are to be representative of the water
column and to provide information on the presence of toxic species as well as on population
trends. If any changes in toxic populations that may lead to toxin accumulation are detected,
the sampling frequency of molluscs is to be increased or precautionary closures of the areas
are to be established until results of toxin analysis are obtained.

1.1.2. Official methods for marine biotoxins analyses in shellfish

*Directive 91/492/EEC* specifies the reference methods for the detection of the different toxin
classes. For the detection of toxins belonging to the PSP group (STX-group) the mouse
bioassay (MBA) is the reference method for end product testing. DSP toxin can be detected
through a series of MBA procedures, differing according to the test sample (hepatopancreas
(HP) or whole body (WB)) and to the solvents used for extraction and purification steps
(selectivity can be obtained to some extent through sample preparation). No animal tests are
required for the monitoring of ASP, which are determined by using HPLC methods, as laid
down in *Council Directive 97/61/EC*. Both HPLC-UV and LC-MS were used during an inter-
laboratory trial for DA quantitation and acceptable degree of comparability was obtained
between the participants (Hess et al., 2005a).

In addition to biological testing methods (the term biological methods covers several *in vivo*
assays, including the MBA and the RBA), alternative detection methods, such as chemical
methods and *in vitro* assays, are allowed if it is demonstrated that the performance of the
chosen methods is at least as effective as the biological method and that their implementation
provides an equivalent level of public health protection. However, Article 5 of *Decision
2002/225/EEC explicitly states that, in case of discrepancies between the results obtained with different methods, the DSP MBA should be considered as the reference method for the biotoxins specified in the decision.

1.1.3. Marine toxins origin: occurrence and detection of HAB

Marine toxins are produced by toxic phytoplankton also referred to as harmful algae. The occurrence of a toxic event is a result of a Harmful Algal Bloom (HAB), where a bloom is a dense accumulation of phytoplankton (up to millions of cells/L). Among the 5 000 known species of marine phytoplankton, 300 species can occur in such high number that they discolour the surface of the water (known as “red tide”) and 80 have the capacity of producing toxins (Hallegraeff et al., 2003). The water can appear coloured when organisms that contain coloured pigments bloom, leading to the terms "red tide" or "brown tide". However, not all red tides are harmful and not all HABs are red tides. Furthermore, many of the most harmful blooms have no colour associated with them at all. Many toxins (DSP, PSP, NSP) are produced by dinoflagellates which are one group of unicellular eukaryotic organisms with the largest number of harmful species (Taylor et al., 2003). Toxins associated with ASP are produced by diatoms. These unicellular organisms with siliceous cell covering constitute the largest group of algae known (Fryxell and Hasle, 2003).

Three types of phytoplankton blooms can be distinguished:

1- Species that produce harmless water discoloration that can in the case of very dense blooms lead to fish and invertebrate deaths as a result of oxygen depletion.

2- Species that produce potent toxins (some dinoflagellates, diatoms, cyanobacteria).

3- Species that are non-toxic to humans but harmful to fish and invertebrates by damaging fish gills mechanically or through the production of haemolytic substances and neurotoxins (some diatoms and dinoflagellates).
Phytoplankton blooms are a natural phenomenon but their proliferation may be partly attributed to agricultural runoff and sewage in coastal water, redistribution of microalgae via ship ballast water and transfer of shellfish stocks (Quilliam, 2003). Environmental factors such as temperature, light, amount of nutrients are expected to have a great influence on the development of algal blooms. However, the spatio-temporal dynamics for the formation of a bloom are not well understood.

Beside the interest in marine biotoxins from the viewpoint of food safety, many marine biotoxins are potential sources of therapeutic and anti-cancer agents (Konishi et al., 2004). HAB can be monitored at different levels including analysis of phytoplankton cells, analysis of shellfish that feed on harmful phytoplankton or analysis of passive samplers that accumulate toxins that were released in the water. Figure 1.1 summarises the different detection techniques that can be used to study HAB.

Figure 1.2: Detection techniques following a harmful algae bloom of marine biotoxins
1.1.4. Economic impacts of marine biotoxins

The occurrence of marine biotoxins in shellfish production areas can cause the closure of the site. Figure 1.3 shows the number of weeks of closure due to the occurrence of ASP (yellow boxes), DSP (red boxes), AZP (blue boxes) and the co-occurrence of DSP and AZP (green boxes) in Irish production areas in 2005 (Clarke et al., 2007). In some areas, harvesting was banned for several consecutive months due to the successive presence of DSP and AZP in Bantry Bay (North Chapel, South Chapel, Whiddy point), or their co-occurrence in McSwynes Bay. Such long-lasting closures have huge economical impacts on the industry. It must be emphasised that the occurrence of toxins is highly variable and that 2005 was particularly affected by shellfish toxins.
### Table: Irish Shellfish Production Closures in 2005

<table>
<thead>
<tr>
<th>Production Area</th>
<th>Jan-Feb</th>
<th>Mar-May</th>
<th>Jun-Aug</th>
<th>Sep-Nov</th>
<th>Dec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southwest</td>
<td>1 2 3</td>
<td>4 5 6</td>
<td>7 8</td>
<td>9-10</td>
<td>11</td>
</tr>
<tr>
<td>Ardgole</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ardgroom</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bane Finich</td>
<td></td>
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<tr>
<td>Bear Island</td>
<td></td>
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<tr>
<td>Castletownbere</td>
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<tr>
<td>Cleandra</td>
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<tr>
<td>Cromane East</td>
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<tr>
<td>Dunmanns Bay</td>
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<tr>
<td>Gearhies</td>
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<tr>
<td>Glengarriff</td>
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<tr>
<td>Gouladoo</td>
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<tr>
<td>Killakillogue</td>
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<tr>
<td>Newtown</td>
<td></td>
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<td></td>
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<tr>
<td>North Chapel</td>
<td></td>
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<tr>
<td>Roaringwater</td>
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<td>Snare</td>
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<tr>
<td>South Chapel</td>
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<td>Tahilla</td>
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<td>Templeoie</td>
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<tr>
<td>Whiddy Point</td>
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<td></td>
</tr>
<tr>
<td>West</td>
<td>11 12</td>
<td>13 14</td>
<td>15 16</td>
<td>17 18</td>
<td>19</td>
</tr>
<tr>
<td>Curraun</td>
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</tr>
<tr>
<td>Poulnaclough</td>
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<td>McSwynes Bay</td>
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<td>Mountcharles</td>
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<td>Mulroy Channel</td>
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<tr>
<td>Mulroy Bay</td>
<td>38 39</td>
<td>40 41</td>
<td>42 43</td>
<td>44 45</td>
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</tbody>
</table>

Figure 1.3: Irish shellfish production closures in 2005 due to ASP, DSP, and AZP. Reproduced from Clarke et al., 2007.
1.1.5. European research projects

A call for research projects on the validation of alternative (i.e., non-animal based) methods for the toxins problematic in the EU was performed by the EU commission. In late 2004, the EU funded three projects in this area, BIOTOX (www.biotox.org), BIOTOXmarin (www.biotoxmarin.de) and DETECTOX (www.detectox.eu), all due to finish in 2007.

BIOTOXmarin consist in developing fast, simple and cost-effective detection methods for marine biotoxins in seafood as well as patient. The techniques are based on the application of high-affinity capture antibodies and novel artificial receptor mimics against the toxins. The toxins include DSP, ASP, PSP and NSP toxins.

The objective of the DETECTOX project is to develop a multi-channel, high-throughput biosensor for the detection of DSP toxins, YTXs, PTXs, AZAs and GYM. The proposed biosensor will exploit the phenomenon of Surface Plasmon Resonance (SPR) to detect and measure these lipophilic phycotoxins in seafood residues. The sensor will be designed as an inhibition assay capable of rapid, reliable multi-toxin detection. This is an attractive technology as it would allow for real-time, automated, multiresidue analysis of food products, both in laboratories and at on-site locations.

In January 2005, the project BIOTOX was launched and focused on the development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood and priorities were set in ten distinct work packages (WP). The Marine Institute was involved in several work packages, such as the development (WP2) and validation (WP5) of analytical reference method, the preparation of reference material (WP6) and the development of an early warning system using passive samplers to forecast lipophilic toxins accumulation in bivalves (WP7).
1.2. Objectives

This PhD project was designed to participate in the research conducted by the Marine Institute as part of the EU project BIOTOX. Particular aspects of the research included the development of LC-MS methods as a replacement to the MBA and the assessment of passive samplers as early warning devices.

A passive sampler suitable for the detection of lipophilic marine toxins was developed in New Zealand prior to commencing this PhD and was referred to as SPATT sampler. During the trials in New Zealand it was observed that the solid phas adsorption toxin tracking (SPATT) technique can provide an early warning to forecast shellfish contamination. This led research groups in Europe to express the need for the evaluation of the SPATT technique in European waters.

The objectives of this PhD work were:

1. To test the usefulness of passive samplers to detect lipophilic toxins in seawater as an early warning. Results will be compared with routine monitoring of phytoplankton and shellfish flesh testing to analyse if the samplers are useful as an early warning system in case of harmful algae blooms.

2. To investigate the accumulation of lipophilic toxins released by algae on different sorbent materials on a laboratory scale (mesocosm experiment). This includes the comparison of adsorption rates of the different sorbent materials and the development of efficient extraction methods suitable for all sorbents materials chosen.

Several hundreds of passive samplers were obtained after the field work was completed and preliminary analyses revealed that a wide range of toxins accumulated on the samplers. This
implied the development of a high throughput detection method capable of quantifying numerous toxins in a single analytical run. The additional goals set to facilitate the above objectives were thus:

3. Development of a UPLC-MS/MS method for the detection of numerous lipophilic marine toxins
4. Assessment of matrix effects of the newly developed UPLC method
5. Comparison of matrix effects between UPLC and HPLC methods
6. Influence of heat treatment and shellfish species on matrix effects

These secondary goals also contributed significantly to the validation of a LC-MS method in BIOTOX. The findings of the relationship between the occurrence of toxin producing phytoplankton and the accumulation of toxins on the passive samplers and shellfish led us to investigate two particular aspects:

7. LC-MS analysis for the toxin profile determination in phytoplankton cells
8. Comparison of toxin profile in phytoplankton, in shellfish and in passive samplers in the field

Finally, it was felt necessary to evaluate the physio chemical parameters of some lipophilic marine toxins in order to allow for further work for the passive sampling of toxins. This included:

9. The evaluation of the acidity constant and the lipophilicity of lipophilic marine toxins by LC-MS.

The objectives of this study are summarised in Figure 1.4.
1.3. Lipophilic marine toxins

1.3.1. Okadaic acid group

The first reported OA outbreaks occurred in Japan in 1976 and 1977 (Yasumoto et al., 1978) and led to food poisoning after mussels and scallops ingestions. The disease was named diarrhetic shellfish poisoning after its predominant human symptoms. In the following 30 years, new compounds belonging to the OA group were discovered in various places around the world as a result of the occurrence of HAB.
1.3.1.1. Chemical structures and properties

The OA group includes a number of compounds that are either naturally produced by dinoflagellates or are the result of shellfish metabolisation of these naturally produced toxins. These compounds exhibit a wide range of different properties and can be sub-divided according to their solubilities.

Lipophilic compounds of the OA group include OA isolated from sponges *Halichondria okadaii* from which it gets its name and *Halichondria melanodocia* (Tachibana and Scheuer, 1981), its isomer DTX2 (Hu et al., 1992a) and its methyl derivative 35-S-methyl okadaic acid, named DTX1 (Murata et al., 1982). In addition, ester derivatives of these toxins were also identified. Two additional isomers of OA and DTX were discovered, DTX2b was obtained from Irish mussels (James et al., 1997b) and DTX2c (Draiscti et al., 1998a) in *D. acuta* samples collected from the south-west coast of Ireland. However, the structures of both isomers have not been elucidated yet.

The first derivatives discovered were the 7-0-acyl-ester derivatives of DTX1, subsequently named DTX3, as a result of DTX1 acylation with several fatty acids with carbon chains ranging from C14 to C22, with unsaturation numbers from 0 to 6 (Yasumoto et al., 1985). Later, the development of LC-MS techniques enabled direct identification of 7-0-acyl ester derivatives of OA and of DTX2 from purified mussel HP from Ireland (Marr et al., 1992).

The OA group also consists of ester derivatives such as OA diol ester. This sub-group of compounds resulting from the acylation of the OA carboxyl group with different unsaturated C4 to C10 diols to form allylic diol-esters, was discovered in *P. lima* cultures (Yasumoto et al., 1989; Hu et al., 1992b; Norte et al., 1994) and *P. maculosum* (Hu et al., 1992b). These esters appear to derive from enzymatic hydrolysis of the sulfated precursors such as DTX4 and DTX5 (Hu et al., 1995a, b) catalyzed by esteras in the *Prorocentrum* cells (Quilliam et al., 1996; Windust et al., 1997).
Recently, several OA diol-esters were also identified in *D. acuta* samples from New Zealand (Suzuki et al., 2004; Miles et al., 2004b). The comparison of hydrolysed and non-hydrolysed *D. acuta* sample has shown that the peak area of OA was approximately 20 times higher in the hydrolysed sample. Analysis by LC-MS and HPLC with diode array detector (DAD) analyses established that the predominant homologue was the *trans*-C8 OA diol-ester (Suzuki et al., 2004). In addition, two OA-D8 isomers were also detected by LC-MS/MS and had retention times slightly longer than OA-D8. Information on a *cis*-OA-D8, also produced from *D. acuta* from New Zealand became available later (Miles et al., 2006a). The latter study suggested that one of the unknown OA-D8 isomers described by Suzuki et al. (2004) is probably the *cis*-OA-D8 (Miles et al., 2006a).

A water soluble sulfated ester derivative of OA named DTX4, was isolated and structurally identified from a *P. lima* culture (Hu et al., 1995b). This was followed by the discovery of new sulfated esters, DTX5a, DTX5b (Hu et al., 1995a), and DTX5c (Cruz et al., 2006). The latter study suggested that sulphate derivatives related to each reported diol ester exists and that different phytoplankton species can be associated with the production of typical enol esters.

Additional OA related compounds were recently discovered from cultures of *Prorocentrum spp* which possess elongated side chains with unsaturation number ranging from 1 to 3. One compound was named DTX6 (Suarez-Gomez et al., 2001) while the additional four compounds were not named and are referred to as unknowns 1 to 4 in Table 1.3 (Suarez-Gomez et al., 2005).

Data on the stability of OA group toxins are scarce. Heat stability of OA and DTX2 was investigated in shellfish tissue and indicated that OA starts to degrade significantly from 120 °C and DTX2 from 100 °C (McCarron et al., 2007).
Figure 1.5: Structure of toxins belonging to the OA group. Please refer to Table 1.3 in which the identity of the R groups for each toxin in this group is shown.
Table 1.3: Structure of toxins belonging to the OA group

<table>
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<tr>
<th>Toxin name</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$R_4$</th>
<th>$R_5$</th>
<th>$R_6$</th>
<th>[M-H](^{+})</th>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>-</td>
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</tr>
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<td>DTX1</td>
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<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>-</td>
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</tr>
<tr>
<td>DTX2</td>
<td>H</td>
<td>H</td>
<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>-</td>
<td>803.5</td>
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<tr>
<td>DTX3</td>
<td>H or CH$_3$</td>
<td>H or CH$_3$</td>
<td>H or CH$_3$</td>
<td>Acyl</td>
<td>H</td>
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<td>H</td>
<td>H</td>
<td>c</td>
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<td>H</td>
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<td>d</td>
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<td>d</td>
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<td>H</td>
<td>H</td>
<td>g</td>
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<td>H</td>
<td>H</td>
<td>a</td>
<td>OH</td>
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<td>H</td>
<td>H</td>
<td>b</td>
<td>OH</td>
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</tr>
<tr>
<td>OA-D8 (cis)</td>
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<td>H</td>
<td>H</td>
<td>c</td>
<td>OH</td>
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<td>H</td>
<td>H</td>
<td>d</td>
<td>OH</td>
<td>927.6</td>
</tr>
<tr>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>e</td>
<td>OH</td>
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<td>H</td>
<td>H</td>
<td>f</td>
<td>OH</td>
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<td>H</td>
<td>H</td>
<td>h</td>
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<td>H</td>
<td>H</td>
<td>i</td>
<td>-</td>
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</tr>
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<td>H</td>
<td>H</td>
<td>j</td>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>k</td>
<td>-</td>
<td>953.1</td>
</tr>
</tbody>
</table>

1.3.1.2. Causative organisms

Biosynthesized toxins within the OA group are produced by two dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum*. Initially, *D. fortii* has been reported as the causative organism in Japanese DSP outbreak in 1976 (Yasumoto et al., 1980). In the following years, the toxin profile of several samples of *D. fortii* collected in Japan was established (Lee et al., 1989; Sato et al., 1996; Suzuki et al., 1996c; Suzuki and Mitsuya, 2001). Although, the toxin content of the cells from the same geographical origin is highly variable (Lee et al., 1989; Suzuki et al., 1996c), DTX1 and PTX2 (Section 1.3.2) are generally dominating the toxin profile in *D. fortii* from Japan. *D. fortii* was also reported to be the predominant *Dinophysis*
*spp (with *D. caudata*) in Emilia Romagna – Italy, at the beginning of autumn and was found to contain OA at 15 pg/cell (Draisci et al., 1996).

As a result of the identification of *D. fortii* as the toxin producer organism, extensive surveys of plankton and shellfish toxicity were conducted in Japan and mussel toxicity did not always coincide with high levels of *D. fortii* but with high levels of *D. acuminata*. Subsequent analysis of mussel specimens collected in early spring, when *D. acuminata* was present at high concentration in the absence of *D. fortii*, revealed that OA was the compound associated with the mussel toxicity, while DTX1 was not detected (Yasumoto et al., 1985). DSP toxins were detected in *D. acuminata* from Spain, France, USA, Japan and Scandinavia, as shown in Table 1.5. The toxins produced by *D. acuminata* can be different depending on its origin. Thus, in addition to the production of OA, low amounts of DTX1 were also detected in samples from Japan (Lee et al., 1989) and New Zealand (MacKenzie et al., 2005) but not from France (Marcaillou et al., 2001; Mondeguer et al., 2002; Marcaillou et al., 2005).

Seasonal variability of OA in *D. acuminata* from France was investigated and showed that from mid May to beginning of June the toxicity reached 76 pg/cell while in October the concentration dropped to 4 pg/cell (Mondeguer et al., 2002). Additionally, it was observed that the most toxic cells were loaded with starch and not very pigmented while the less toxic cells collected in autumn were more pigmented and irregularly loaded with starch.

*D. acuta* was found to produce OA group toxins in Spain, Scandinavia, Ireland, Portugal, New Zealand and in very low amounts in Japan (Table 1.4). *D. acuta* also showed differences in the toxin produced depending on the geographical location. The major difference is that strains of *D. acuta* from Ireland, Portugal and Spain contained DTX2 at similar or greater concentration than OA while DTX2 was below the LOD in the strains of *D. acuta* from Scandinavia and New Zealand. DTX1 was detected in *D. acuta* from Norway at 4.2 pg/cell (Lee et al., 1989) and at trace levels in a strain from New Zealand (MacKenzie et al., 2005).
Analysis of *D. norvegica* revealed the presence of OA in strains from USA, Canada and Norway where DTX1 was also detected (Table 1.6). DTX1 was found in *Dinophysis spp.* *(acuminata, norvegica and rotundata)* from the Baltic sea at concentrations up to 149 pg/cells (Kuuppo et al., 2006).

Culture of *Dinophysis* has only been successful recently where the growth of *D. acuminata* required the prey species *Myrionecta rubra* (Park et al., 2006). This will allow for the unambiguous confirmation that the above toxins are produced by *Dinophysis* as well as for the examination of the toxin profiles under different conditions and toxin production cycle.

The benthic species *Prorocentrum lima* is a producer of OA and DTX1 as well as DTX4, a sulphated water soluble toxin (Hu et al., 1995b) and several OA diol ester derivatives (Hu et al., 1995a). *P. lima* has been found to occur in Japan (Koike et al., 1998), Canada, France, UK and in Spain (Table 1.8).

Studies on the production of these toxins in *P. lima* cultures over a 90 days period showed that OA is either solely or partially due to enzymatic hydrolysis of DTX4 (Quilliam et al., 1996). This was suggested following the discovery of high intracellular DTX4 concentration with only low concentrations of OA and DTX1 in boiled extracts, where enzymatic activity was stopped. Since OA was found to be the predominant toxin in the culture medium, followed by DTX1 and DTX4 in trace amounts, it was suggested that OA could be produced in the cell in low amount similar to DTX1, and that the majority of OA found in the medium is the result of a very fast enzymatic hydrolysis into OA diol ester derivatives, which are further hydrolysed at a lower rate to yield OA. Studies on *P. lima* cell cycle and their related toxin production showed that DTX4 and OA/DTX1 are produced at different phases of the cell cycle (Pan et al., 1999). The same study reported that DTX4, OA, DTX1 and OA diol esters accounted for 54, 30, 12 and 4 % of the total intracellular toxin content respectively, while only OA and
trace amounts of DTX1 were detected in the culture medium. However, this study did not provide any evidence of OA being directly produced by the cell and as of yet, there is no study to the author’s knowledge that demonstrated the potential production of free OA from *P. lima* cells. DTX1 production is not believed to occur through the same mechanism as there is no evidence of DTX1 derivatives being produced in *P. lima* cultures (Quilliam et al., 1996; Bravo et al., 2001; Nascimento et al., 2005). Strains of *P. lima* from Spain were found to produce low amounts of DTX2 (Bravo et al., 2001), DTX6 (Suarez-Gomez et al., 2001) and three ester derivatives of OA that were not named (Suarez-Gomez et al., 2005).

*P. maculosum*, previously identified as *P. concavum* (Faust, 1996) was found to produce OA. (Dickey et al., 1990; Zhou and Fritz, 1994). In addition, the OA diol ester D7b, and the compounds DTX5a, and DTX5b were also isolated from *P. maculosum* cultures (Hu et al., 1992b; Hu et al., 1995b).

OA was reported in cells of *P. belizeanum* from tropical origins at 12.5 pg/cell (Morton et al., 1998). Two additional new compounds were also discovered in *P. belizeanum* including DTX6 (Cruz et al., 2006) as well as one unnamed compound refered to as Unknown 4 in Table 1.3 (Suarez-Gomez et al., 2005).
Table 1.4: *D. acuta* origin and published OA group toxins cell content determined by HPLC-FLD or LC-MS. Concentrations are given in pg/cell.

<table>
<thead>
<tr>
<th><em>D. acuta</em> origin (ref)</th>
<th>OA</th>
<th>DTX1</th>
<th>DTX2</th>
<th>DTX2b</th>
<th>DTX2c</th>
<th>OA esters</th>
<th>trans-OA-D8</th>
<th>cis-OA-D8</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Lee et al., 1989)</td>
<td>4.0</td>
<td>4.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>S (Lee et al., 1989)</td>
<td>9.4</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sweden (Edler and Hagelton, 1990)</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Yasumoto, 1990)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>S (Blanco et al., 1995)</td>
<td>0.6 - 94</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IE (James et al., 1997b)</td>
<td>58 ± 7</td>
<td>nd</td>
<td>78 ± 14</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IE (Draisci et al., 1998a)</td>
<td>p</td>
<td>-</td>
<td>p</td>
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<td>nd</td>
<td>80</td>
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<td>-</td>
<td>4.4 – 22</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (MacKenzie et al., 2002)</td>
<td>1.7 – 2.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.5 – 19.4</td>
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<td>-</td>
</tr>
<tr>
<td>IE (Fernandez - Puente et al., 2004)</td>
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<td>-</td>
<td>77</td>
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<td>nd</td>
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<td>P (Vale, 2004a)</td>
<td>15.6</td>
<td>nd</td>
<td>10</td>
<td>0.4(^{c})</td>
<td>0.4(^{c})</td>
<td>p</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (Suzuki et al., 2004)</td>
<td>2-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>p(^{a})</td>
<td>p(^{b})</td>
</tr>
<tr>
<td>NZ (MacKenzie et al., 2005)</td>
<td>0.8 – 2.7</td>
<td>nd-0.1</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>6.5-10.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (Miles et al., 2006a)</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>p(^{d})</td>
<td>P</td>
</tr>
<tr>
<td>S (Pizarro et al., 2008)</td>
<td>11 - 58</td>
<td>-</td>
<td>8 - 37</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 - 83</td>
<td>-</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified; \(^{a}\): 20 fold increase OA conc. after hydrolysis; \(^{b}\): compound not known at time of study tentatively assigned to trans-OA-D8 (Miles et al., 2006a) \(^{c}\): late eluting OA/DTX2 isomer; \(^{d}\): predominant. N: Norway; S: Spain; Jpn: Japan; IE: Ireland; NZ: New Zealand; P: Portugal
Table 1.5: *D. acuminata* origin and published OA group toxins cell content determined by HPLC-FFLD or LC-MS. Concentrations are given in pg/cell.

<table>
<thead>
<tr>
<th><em>D. acuminata</em> origin (ref)</th>
<th>OA</th>
<th>DTX1</th>
<th>DTX2</th>
<th>DTX2b</th>
<th>DTX2c</th>
<th>OA esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jpn (Lee et al., 1989)</td>
<td>trace</td>
<td>1.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F (Lee et al., 1989)</td>
<td>1.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>US (Cembella, 1989)</td>
<td>25.5 ± 6.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Yasumoto, 1990)</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E (Blanco et al., 1995)</td>
<td>1 – 37</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sweden (Johansson et al., 1996)</td>
<td>1.1- 23</td>
<td>nd – traces</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Denmark (Andersen et al., 1996)</td>
<td>nd -40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>S (Fernández et al., 2001)</td>
<td>0.7 – 21.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F (Marcaillou et al., 2001)</td>
<td>3-50</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F (Mondeguer et al., 2002)</td>
<td>76 (spring)</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 (autumn)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E (Fernández et al., 2003)</td>
<td>9.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>E (Morono et al., 2003))</td>
<td>nd – 11.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F (Marcaillou et al., 2005)</td>
<td>nd – 158</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd – 68</td>
</tr>
<tr>
<td>NZ (Miles et al., 2005)</td>
<td>Trace – 1.2</td>
<td>0.1 – 2.4</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>Jpn (Suzuki et al., 2008)</td>
<td>nd</td>
<td>nd - 1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd¹</td>
</tr>
<tr>
<td>Jpn (Kamiyama and Suzuki, 2008)</td>
<td>nd</td>
<td>2.5 – 4.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd¹</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified; ¹: only DTX3 were monitored. F: France
Table 1.6: *D. norvegica* origin and published OA group toxin cell content determined by HPLC-FFLD or LC-MS. Concentrations are given in pg/cell.

<table>
<thead>
<tr>
<th><em>D. norvegica</em> origin (ref)</th>
<th>OA</th>
<th>DTX1</th>
<th>DTX2</th>
<th>DTX2b</th>
<th>DTX2c</th>
<th>OA esters</th>
<th>trans-OA-D8</th>
<th>cis-OA-D8</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Lee et al., 1989)</td>
<td>nd-0.8</td>
<td>2.5 – 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>US (Cembella, 1989)</td>
<td>32.6±5.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CA (Subba Rao et al., 1993)</td>
<td>0.07 – 54.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Jpn (Suzuki et al., 2008)</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified;<sup>a</sup>: sum of OA and DTX1 as ELISA kit was not able to distinguish the two toxins; CA: Canada

Table 1.7: *D. fortii* origin and published OA group toxins cell content determined by HPLC-FFLD or LC-MS. Concentrations are given in pg/cell.

<table>
<thead>
<tr>
<th><em>D. fortii</em> origin (ref)</th>
<th>OA</th>
<th>DTX1</th>
<th>DTX2</th>
<th>DTX2b</th>
<th>DTX2c</th>
<th>OA esters</th>
<th>trans-OA-D8</th>
<th>cis-OA-D8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jpn (Lee et al., 1989)</td>
<td>-</td>
<td>13.0 - 191.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>I (Draisci et al., 1996)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Sato et al., 1996)</td>
<td>nd-57.7</td>
<td>nd - 16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Suzuki et al., 1996b)</td>
<td>-</td>
<td>5-252</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Suzuki et al., 1999)</td>
<td>nd</td>
<td>118-155</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Suzuki and Mitsuya, 2001)</td>
<td>-</td>
<td>189 - 209</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>-</td>
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</tr>
<tr>
<td>Jpn (Suzuki et al., 2008)</td>
<td>nd</td>
<td>1.1 – 10.9</td>
<td>-</td>
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</table>

- : not available; nd: not detected; p: present but not quantified
Table 1.8: *P. lima* strain origin and published OA group toxins cell content determined by HPLC-FFLD or LC-MS. Concentrations are given in pg/cell.

<table>
<thead>
<tr>
<th><em>P. lima</em> origin (ref)</th>
<th>OA</th>
<th>DTX1</th>
<th>DTX2</th>
<th>DTX4</th>
<th>DTX6</th>
<th>OA-D7a</th>
<th>OA-D7b</th>
<th>trans-OA-D8</th>
<th>cis-OA-D8</th>
<th>OA-D9a</th>
<th>OA-D9b</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>- (Yasumoto et al., 1987)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>p</td>
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<td>- (Yasumoto et al., 1989)</td>
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<td>p</td>
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<tr>
<td>CA (Jackson et al., 1993)</td>
<td>1.4 – 8</td>
<td>-</td>
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<tr>
<td>(Norte et al., 1994)</td>
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<td>-</td>
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<td>p</td>
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<tr>
<td>CA (Hu et al., 1992)</td>
<td>10-15</td>
<td>p</td>
<td>trace</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>p</td>
<td>-</td>
<td>p</td>
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<tr>
<td>CA (Hu et al., 1999)</td>
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<tr>
<td>CA (Hu et al., 1995b)</td>
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<td>p</td>
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<tr>
<td>CA (Quilliam et al., 1996)</td>
<td>p</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>p</td>
<td>p</td>
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</tr>
<tr>
<td>Jpn (Koike et al., 1998)</td>
<td>0.3 – 1.3</td>
<td>nd</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>F (Barbier et al., 1999)</td>
<td>1.9</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
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<tr>
<td>- (Pan et al., 1999)</td>
<td>0.3 – 5.3</td>
<td>0.03– 2.1</td>
<td>2.6 – 11.5</td>
<td>-</td>
<td>-</td>
<td>0.02 - 1.4</td>
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<tr>
<td>- (Suarez-Gomez et al., 2001)</td>
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<td>p</td>
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<tr>
<td>ES (Bravo et al., 2001)</td>
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<tr>
<td>UK (Foden et al., 2005)</td>
<td>0.1 -1.8</td>
<td>0.2 – 6.3</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>UK (Nascimento et al., 2005)</td>
<td>0.4 - 17.1</td>
<td>0.4 – 11.3</td>
<td>-</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>S (Suarez-Gomez et al., 2005)</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>p</td>
<td>p</td>
<td>p</td>
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</tr>
</tbody>
</table>
In addition, Lee et al., (1989) reported *D. mitra* to contain 10 pg/cell of DTX1, *D. rotundata* to contain 101 pg/cell, *D. tripos* to contain 36 pg/cell of DTX1. All species were sampled in Japan. Recently, *D tripos* from Japan was analysed for OA, DTX1 as well as DTX3 and none of the toxins were detected (Suzuki et al., 2008). Three other *Dinophysis* species, *D. caudata*, *D. hastata* and *D. sacculus*, are also suspected (Hallegraeff, 2003). In the Philippines analysis of *D. caudata* and *D. miles* revealed 7.2–53.9 and 3.1–10.7 pg/cell of DTX1, respectively (Marasigan et al., 2001). *D. caudata* from the Galicia Southern Rias contained 0.73 pg/cell (Fernández et al., 2001).

OA and DTX1 were detected in *D. sacculus* at concentrations of 110-400 and 8-65 fg/cell, respectively (Giacobbe et al., 2000). Maximum DSP toxins (OA+DTX1 455 fg/cell) were found in early spring blooms. The authors suggested that the role of *D. sacculus* in harmful events in the Mediterranean area may be far from negligible despite their low toxicity. The organism *P. redfieldi* is also believed to produce DSP toxins (Viviani, 1992). *P. Hoffmannianum* was found to contain OA in the USA at levels of 3.46 – 20 and 5.6 – 53.8 pg/cell (Aikman et al., 1993; Morton et al., 1994).

1.3.1.3. Occurrence in shellfish and geographic distribution

1.3.1.3.1. Okadaic acid and dinophysistoxins

OA group toxins have been found in most regions of the world as a consequence of the worldwide distribution of *Dinophysis spp* as described above.

Following the first documented DSP intoxication in 1976 in Japan, Yasumoto et al. (1978) carried out epidemiological studies on mussels *Mytilus edulis*, scallops *Patinopsecten yessoensis* and *Chlamys nipponensis akazara*, oysters *Crassostrea gigas* and *Ostrea edulis* as well as sea squirt *Halocynthia roretzi*. Mussels were found to be the most toxic followed by scallops and oysters while the sea squirts specimens did not exhibit any toxicity. The study of
the anatomical distribution of the toxin(s) in mussels indicated that the hepatopancreas (HP) was the most toxic tissue followed by the gills, while the scallops and oysters only contained the toxin(s) in the HP (Yasumoto et al., 1978). Later, Murata et al. (1982) identified the toxin involved in the latter DSP event from the HP of the contaminated mussel. The comparison of this toxin with OA that was previously isolated from sponges (Tachibana and Scheuer, 1981) and from *P. lima* (Murakami et al., 1982) enabled them to establish that DTX1 was the major toxin involved in the DSP event reported above. Subsequent analysis of the digestive glands of toxic Japanese mussels and scallops revealed that DTX1 may not have been the only toxin involved in DSP outbreaks and that DTX1 7-0-acyl derivative, OA and several pectenotoxins (see PTX section) could have contributed to the overall shellfish toxicities (Yasumoto et al., 1985). In 1994, OA and DTX1 were also detected in mussels (*Mytilus coruscus*) from Niigata (central coast of Honshi, Sea of Japan) as a consequence of the occurrence of *D. fortii* (Andersen et al., 1996).

In Italy, the first reported case of DSP occurred in 1989 after the consumption of mussels *Mytilus galloprovincialis* harvested in the northern Adriatic Sea and believed to be a consequence of the occurrence of *Dinophysis spp* (Boni et al., 1993). The presence of OA was then confirmed in shellfish during the summer of the following year (Zhao et al., 1993; Della Logia et al., 1993).

In Spain, DSP monitoring began following the intoxication of 5 000 people in 1981 after the consumption of mussels from the Galician Rias. The occurrence of *D. acuta* and *D. acuminata* was investigated during the summer of 1990 and coincided with the accumulation of OA in mussels (Reguera et al., 1993). Further HPLC-FLD analysis of mussels, clams and razor clams HP from Western Spain and Portugal collected between 1987-1991 demonstrated that OA was the main component associated with DSP cases (Gago-Martinez et al., 1993).
Later, it was demonstrated that in addition to OA, DTX2 also occurred in Spain (Gago-Martinez et al., 1996) and Portugal (Vale et al., 1998). Accumulation of DSP toxins in different shellfish species was investigated in Portugal. Mussels and cockles were more contaminated than carpet shells, clams or razor clams while oysters were the bivalve that accumulated the least toxins (Vale and de M. Sampayo, 2002).

The presence of DSP toxins in shellfish from 1993 to 2002 is shown in Figure 1.6 (not exhaustive) and included Ireland, England, Scotland, France, Spain, Portugal, Italy, Belgium, Germany, Scandinavia and Baltic countries.

In Greece, a DSP episode was first recorded in 2000, when many people exhibited gastrointestinal symptoms after consuming mussels (*Mytilus galloprovincialis*) originating from Thermaikos Gulf (NW Aegean Sea) where the majority of Greek shellfish production farms are located (Mouratidou et al., 2006). In 2002, HPLC analysis of mussels from the same location detected OA at concentrations up to 36 µg/g (HP) as well as traces of DTX1 as a consequence of the occurrence of *D. acuminata* (Mouratidou et al., 2006).

Another OA outbreak occurred in Greece in 2004 and resulted in the contamination of *M. galloprovincialis* at levels ranging from 0.1 to 0.2 µg/g (Ciminiello et al., 2006).

The first case of DSP in North America (Nova Scotia, Canada) proven through chemical toxin analysis occurred in 1990 with levels of DTX1 reaching 1 µg/g in mussel HP (Quilliam et al., 1993). A DSP contamination of scallops (*Placopecten magellanicus*) was also reported from Nova Scotia in 1990 where the levels of OA/DTX1 determined by ELISA reached 469 ng/g in the digestive glands (DG) and coincided with the occurrence of *D. norvegica* (Subba Rao et al., 1993).

In New Zealand the impact of DSP toxins has been limited (MacKenzie et al., 2005). OA was detected by ELISA during spring 1996 in blue mussels (*M. edulis*) with maximum
concentrations of 750 µg/kg (WF) while only very low levels were detected in Greenshell mussels (*Perna canaliculus*) that were feeding on the same phytoplankton (MacKenzie et al., 1998). Hydrolysis of the greenshell mussel tissue did not explain for the difference in concentration and therefore the authors suggested that toxin absorption can be dependent on the shellfish species. In 2001 the LC-MS analysis of Greenshell mussels (*P. canaliculus*) collected in the North-West of New Zealand revealed OA concentrations ranging from 17 – 41 µg/kg (WF) and OA ester derivatives from 65 – 228 µg/kg (MacKenzie et al., 2002). In 2002, the occurrence of a low toxicity event of *D. acuminata* resulted in the contamination of blue (*M. edulis*) and greenshell mussels (*P. canaliculus*) with OA and DTX1 at very similar levels in most cases and concentrations up to 50 µg/kg (MacKenzie et al., 2004).

DSP was first reported in Chile in 1970 when 100 people exhibited DSP symptoms after the consumption of ribbed mussels (*Aulacomya ater*). A monitoring program was implemented in 1991 and during the same year the MBA developed by Yasumoto et al. (1978) revealed high toxicities in several mussel species (*Mytilus chilensis* and *Aulacomya ater*) and in clams (*Ameghinomya antiqua*) (Lembeye et al., 1993). Subsequent mussel LC-FLD analysis of the mussels revealed that OA and DTX1 were responsible for the toxicity (Zhao et al., 1993).

Recently, smoked shellfish from Patagonia fjords that included blue mussels (*Mytilus chilensis*), ribbed mussels (*Aulacomya ater*) and razor clams (*Tangelus dombeii*) were reported to contain essentially ester derivatives of DTX1 (Garcia et al., 2004).

The occurrence of OA in China (Hong-Kong) was investigated in 2005 in green lipped mussels (*Perna viridis*) in seven production areas over four seasons and concentrations ranged from 70 to 131 ng/g (WF) (Mak et al., 2005).
1.3.1.3.2. Ester derivatives of OA group toxins

The first ester derivative discovered was the $7\text{-}O\text{-}\text{acyl}$ derivative of DTX1 and was detected in Japanese scallops (P. yessoensis) (Yasumoto et al., 1985). When the scallops (P. yessoensis) were fed twice a day with D. fortii that produced exclusively DTX1, it was demonstrated that after 5 days the esters of DTX1 accounted for at least 75 to 90 % of the total DTX1 (Suzuki et al., 1999). The kinetics of the esterification of DTX1 (produced by D. fortii in Japan as above) in scallops (P. yessoensis) and mussels (Mytilus galloprovincialis) were compared over 12 days (Suzuki and Mitsuya, 2001). On day 1 of the experiment DTX1 and DTX1 esters were not detected in any of the shellfish species while esterified DTX1 accounted for more than 91 % and 94 % of the total DTX1 in scallops on day 8 and 12 respectively. The esterification rate was slower in mussels as esters of DTX1 accounted for 17 % and 34 % of the total DTX1 on day 8 and 12 respectively.

$7\text{-}O\text{-}\text{acyl}$ ester derivatives of OA and of DTX2 were detected in purified mussel extracts from Ireland (Marr et al., 1992). OA and DTX2 were found to be acylated to the same proportions (in percentage of free toxins).

Ester derivatives of OA were also found in Spanish mussels (M. galloprovincialis) at concentrations ranging from 0.01 to 2.5 µg/g (HP) accounting for 3 to 39 % of the total OA (Fernandez et al., 1996). Mixtures of OA and DTX2 esters were also found in Spanish mussels and accounted for 7.4 % of OA (12.2 µg/g HP) and 1.9 % of DTX2 (4.3 µg/g HP), respectively (Fernandez et al., 1998).

In Portugal, the occurrence of D. acuta led to the contamination of mussels (M. edulis) with OA and DTX2, and consecutively to their ester derivatives which accounted for 80 -90 % and 50 % of the total OA and DTX2, respectively (Vale et al., 1999). Clams (Donax trunculus) collected from the South Coast of Portugal exhibited a similar trend with OA esters ranging from 70 to 90 % of the total OA while DTX2 esters that did not exceed 50 % of the total
DTX2, suggesting that OA is preferentially esterified than DTX2 (Vale et al., 1999). Further studies on the esterification of OA and DTX2 was carried out in other shellfish species as well as in edibles parts and HP of mussels (*M. edulis*) (Vale and Sampayo, 2002a). In mussels, OA esters accounted for less than 50% of the total OA and were lower for DTX2, confirming the above findings. Furthermore, the level of esters in mussels DG was lower than in the edible parts while esters in the HP of cockles (*Cerastoderma edule*) accounted for 100% of the toxins present. The percentages of OA esters in peppery furrow shell (*Scrobicularia plana*), carpet shell (*Venerupis pullastra*), oyster (*Crassostrea japonica*), razor clam (*Ensis spp.*), clam (*Ruditapes decussata*) and cockles (*Cerastoderma edule*) were more than 95% of the total OA detected while free DTX2 was rarely observed.

The fatty acids (FA) C14:0, C16:0, C20:5 and C22:6 were identified as the major FA involved in the production of DTX1 esters in DG of scallops (*P. yessoensis*) (Yasumoto et al., 1985) and of OA and DTX2 esters in DG of mussels (*M. edulis*), cockles (*Cerastoderma edule*), razor clams (*Solen marginatus*), clams (*Ruditapes decussates* and *Venerupis*) and green crabs (*Carcinus maeanas*) (Vale, 2006a). The FAs C18 with unsaturation numbers from 0 to 4 were also found to contribute to the production of esters as well as iso-C16:0, C16:1, C15:0, C17:0 and C17:1.
1.3.1.4. Toxicity

OA group toxins induce DSP which cause diarrhea, nausea, vomiting and abdominal pain to consumers of contaminated shellfish.

The opinion of the scientific panel on contaminants in the food chain regarding the toxicity of the OA group and analogues has been published by the European food safety authority (Anonymous, 2008). Details regarding exposure assessment, toxicokinetics and toxicity assessment are outlined in the report. The panel established toxic equivalence factors based on LD50 experiments by intraperitoneal (i.p.) injection in mice. The same toxicity factors were attributed to OA and DTX1 while DTX2 only accounted for 60% of the toxicity relative to OA (Aune et al., 2007).
1.3.1.5. Methods of detection

1.3.1.5.1. Animal tests

The MBA is currently the EU reference method despite the fact that it has not been validated or even harmonised through the EU countries.

Yasumoto et al. (1978) developed an extraction method prior to the MBA consisting in a triplicate extraction of the shellfish digestive glands with acetone. The solvent was subsequently evaporated and the residue resuspended in 1 % Tween 60 for intraperitoneal injection into three female mice of approximately 20 g of body weight.

Survival time is then checked for 24 hours and the minimum dose to kill a mouse over that period was defined as one mouse unit (MU) (Yasumoto et al., 1980). Shellfish toxicity is expressed as mouse unit per gram of HP (MU/g). The assay is considered positive when at least two mice die within 24 hours. In order to limit false positives, an additional step consisting of partitioning shellfish extract between water and diethyl ether was then introduced in the Japanese MBA protocol in order to remove the water-soluble saxitoxin prior to mice injection as the latter can lead to a false positive (Yasumoto et al., 1984).

The introduction of a partition step allows for more specificity. For example, acetone extraction prior to MBA enables the detection of OA/DTXs, PTXs, AZAs and YTXs while the introduction of a partitioning step against diethyl ether prevents the detection of YTXs as the latter remain in the aqueous phase.

A modification to the method was introduced by reducing the survival time down to 5 hours since no significant difference between the amount of OA corresponding to mouse dead time of 5 and 24 hours were observed (Marcaillou and Masselin, 1990).
The MBA is a simple procedure and has the advantage of providing a total toxicity response based on the animal biological response and therefore to detect unknown toxic components. Nevertheless, besides being ethically not acceptable to use animals in the laboratory, the MBA also suffers from many disadvantages. As mentioned above, the choice of the solvents clearly affects the result which led the CRL to produce a harmonised SOP protocol in 2007 in order to harmonise the assays within the member states (Community Reference Laboratory for Marine Biotoxins, 2007). However, despite the harmonised protocol, high variability between laboratories is frequently encountered as a consequence of specific animal characteristics (strain, sex, age, weight, diet, stress) which can lead to false negatives and false positives. The presence of polyunsaturated free fatty acids in shellfish have been found responsible for false positives in the MBA (Lawrence et al., 1994; Suzuki et al., 1996a). Furthermore, the MBA suffers from interferences from fast active cyclic imine toxins such as spirolides and gymnodimines (Gill et al., 2003; Munday et al., 2004) and is therefore not specific to the OA group. Finally, i.p. injection of 1 ml (as stated in the SOP) exceeds the good practice guidelines (< 0.5 ml) and the i.p. route is not appropriate for the detection of the ester derivatives.

The issues associated with the use of MBA for shellfish testing in the UK were addressed (Combes, 2003) and it was concluded that the overall method was not adequate for regulatory testing because of the absence of control (positive and negative) as well as discrepancies. A semi-quantitative assay was developed using rats in the Netherlands (Kat, 1983). Although variations between animals also exist and specificity is not optimal as other diarrhetic agents would be detected, the rat bioassay has the advantage of avoiding the extraction of shellfish.

1.3.1.5.2. Immunoenzymatic assays

Immunoaffinity methods are based on the specificity of compounds to bind to antibodies which are raised in animals (rabbits, sheep and mice) or in cell cultures. Isolation of the
produced antibodies can be used in enzyme-linked immunosorbent assays (ELISA), lateral flow immunoassay (LFIA) and biosensors based on surface plasma resonance (SPR). The detection of immunochemical techniques is based on the antigen-antibody interaction. It must be emphasised that antibody based methods rely on molecular recognition which is sensitive to specific sites of a molecule.

Two ELISA test kits sensitive to OA were developed and one of them, the “DSP-check kit” is commercially available from R-Biopharm (Germany). The latter was developed by Usagawa (Usagawa et al., 1989) and uses monoclonal antibodies sensitive to OA with a LOD of 20 ng/g. The assay demonstrated cross reactivity to DTX1 (70 %) (Usagawa et al., 1989) and DTX2 (40 %) (Aune et al., 2007) but not to DTX3, PTXs or YTXs. HPLC-FLD (ADAM derivatisation) and the “DSP-Check” kit were compared (Vale et al., 1999). The kit was capable of quantitatively detecting DSP toxins in all the tested contaminated samples containing only okadaic acid and high correlation was observed between the two methods. The immunoassay kit tested also appeared to be more sensitive and faster than HPLC.

The second assay “Okadaic acid ELISA Kit” commercialised by Rougier Bio-Tech (Canada) is a competitive indirect ELISA (Shewstowsky et al., 1992). In addition to OA, this kit has been shown to detect with equal sensitivity other kinds of OA derivatives such as: the methyl ester, diol ester (Chin et al., 1995), DTX4 and DTX5 (Lawrence et al., 1998) but not DTX3. The affinity of the OA antibody was found to be lower for DTX2 and DTX1 than for OA by 10- and 20-fold respectively (Chin et al., 1995).

The differences in the cross-reactivities of individual toxins with the antibodies restrict the use of ELISA assays to shellfish contaminated predominantly with OA as false negatives will occur if the sample is predominantly contaminated with DTX1 and DTX2.

1.3.1.5.3. Protein phosphatase assays
Protein phosphatase (PP) assays are based on the potency of DSP toxins to inhibit serine/threonine protein phosphatases. Due to the inhibition of PP1 and PP2A, a LC-linked phosphatase radioassay was developed (Holmes, 1991). However because of handling of radioisotopes and enzymes the method is not well suited to be implemented in routine laboratories.

A colorimetric phosphatase assay has also been developed and proved to be sensitive with a limit of detection of 2 ng/g of mussel HP (Tubaro et al., 1996). The PP2A-based functional methods for the detection of OA and its analogues are being commercialised, and are likely to be validated in the near future (Hess et al., 2006).

The main advantages of the PP2A assays include high sensitivity and specificity. The major drawbacks are that reliable good quality enzymes need to be available and no information on toxin profile is obtained.

1.3.1.5.4. HPLC-FLD

The absence of chromophores in OA and DTXs does not allow for their analysis by HPLC-UV. Therefore, fluorimetric HPLC of the 9-anthrylmethyl derivatives of OA and DTX1 has been developed by Lee et al. (1987) and was one of the established chemical methods for shellfish before the development of LC-MS technique. The method consists of consecutive extractions of shellfish tissue using methanol, di-ethyl ether and chloroform, followed by a derivatising step of OA and DTX1 using 9-anthryldiazomethane (ADAM). The extract is then subjected to a clean-up step using silica before injection on HPLC. Both OA and DTX1 were quantified at an excitation of 365 nm and an emission at 412 nm. The method was found to be linear from 1 to 80 ng of OA and DTX1 injected (equivalent to 0.1 - 8 mg/ml) and a LOD of 400 ng/g was obtained for both toxins in shellfish extracts. Recoveries were assessed from spiking experiments with OA and DTX1 in both mussels and scallops. Three independent levels were assessed for each combination where 1, 2 and 4 µg of toxin was spiked in 1 g of
digestive gland. Excellent recoveries ranging from 95.4 to 98.1 % were obtained as well as good reproducibility of 2.8 and 2.1 % for OA and DTX1 respectively (n=5). Although the first level of spiking exceeds the present EU limit for OA (0.8 µg/g OA equivalent in digestive glands) quantification in shellfish extracts does not seem to suffer from any interferences due to the good selectivity of the fluorescent detector and offers good reproducibility.

![Chemical reaction](image)

**R** = OA or DTX1

**Figure 1.7**: Fluorescence labelling of OA or DTX1 using ADAM derivative.

Alternative derivatising reagents were assessed in order to improve the stability of the derivatised product and the limit of detection because of the unstable nature of ADAM above -70 °C and its limited availability. Examples of other derivatising agents include 1-bromoactylpyren (BAP) (Dickey et al., 1993; James et al., 1997b), 9-chloromethylantracene (Zonta et al., 1992; Lawrence et al., 1996), 4-bromoethyl-7-methoxycoumarin (Shen et al., 1997), and 2,3-(anthracenedicarboximido) ethyl trifluoromethansulfonate that was used for the determination of synthesised DTX1 ester derivatives (Akasaka et al., 1996) and N-(9-acridinyl)-bromoacetamide (Pleasance et al., 1992).

Derivatisation using BAP resulted in less peak artefacts compared to ADAM (Carmody et al., 1995; Kelly et al., 1996).

Modified procedures of Lee et al. (1987) were also developed as a consequence of losses of DTX1 in the washing step of the SPE clean-up. Modification of the washing step procedure by replacing chloroform and methanol by dichloromethane, acetone and acetonitrile was
proposed (Stabell et al., 1991). Alternatively, a two steps SPE washing protocol was applied with 1- DCM-Hexane (1:1) and 2- DCM, and eluted with chloroform-methanol (Aase and Rogstad, 1997).

1.3.1.5.5. LC-MS analysis

1.3.1.5.5.1. Toxin extraction prior to LC-MS and LC-MS/MS analysis

a. Toxin extraction and clean-up from shellfish

*Dispersive single step extraction*

A single step extraction consisting of the extraction of 1 g of mussel or scallop HP with 4 ml of 80 % methanol followed by liquid-liquid extractions (LLEs) with diethyl ether to remove non polar lipids and with chloroform to extract the OA and DTX1 was developed by Lee et al. (1987). Quilliam (1995) investigated the recoveries obtained using this procedure and reported that 99 % of OA present in the aqueous methanol extract transferred into the chloroform layer and 1 % into the hexane layer during the clean-up process. Acetonitrile and methanol were compared for their efficiencies in single-step extraction. The results obtained were in agreement with Lee et al. (1987) as 75 % aqueous methanol or higher gave the best recoveries. Quilliam (1995) emphasized that a dispersive extraction is not as accurate as an exhaustive extraction because of the bias introduced by the non dissolved solids, estimated to 5 % when a (4:1) extraction solvent to tissue ratio was used. However, it was observed that the positive bias is systematically cancelled by a small amount of OA that is not recovered in the aqueous methanol.

McNabb et al. (2005) also used a dispersive extraction consisting of 2 g of mussel, oyster, scallop or cockle tissue homogeneised with 18 ml of 90 % methanol. An aliquot (2 ml) of the extract obtained was then partitioned against hexane for sample clean-up. Recoveries were assessed for OA using spiked sample and fortified mussels (using contaminated mussel HP) at concentrations of 0.024, 0.1 to 1.0 mg/kg. Recoveries ranged from 72 to 104 % in mussel and
scallops and from 95 to 133 % in oysters and cockles. Recoveries greater than 100 % were observed for several samples suggesting that signal enhancement may have taken place and hence may compensate for low extraction efficiencies in other samples. Goto et al. (2001) used the same extraction method (with modification to clean-up procedure) and showed that consecutive extraction with 90 % methanol did not improve the recovery. After extensive clean up, recoveries ranging from 88 to 104 % for OA and from 82 to 101 % for DTX1 were obtained in scallop muscle and digestive glands. Goto et al. (2001) successfully used silica column combined to LLE clean-ups and did not experience irreversible losses of the DSP toxins on the column as reported in other studies (Quilliam et al., 1995). The washing step of the silica clean-up was carried out with acetone and the DSP toxins eluted with acetone/MeOH (3:7).

In the course of the study of matrix effects Ito et al. (2001) evaluated the hexane LLE clean-up and no effect on signal suppression was observed. Matrix effects were corrected using single level standard addition (see matrix effects section 1.4.3).

Stobo et al. (2005) achieved OA recoveries ranging from 91 to 94 % in mussel tissue (WF) spiked at 40, 80 and 160 µg/kg using a dispersive extraction with 80 % methanol at a solvent to sample ratio (SSR) of 4. In addition, the recoveries in WF of cockle, oyster and scallop tissues were also determined and ranged from 72 to 105 %. The deviations of the recoveries for the different spiked levels obtained from other species were higher than for mussels.

Quilliam et al. (1995) evaluated C18 and aminopropylsilica SPE cartridges, as well as alumina and silica columns as a complementary clean-up to LLE. Only aminopropylsilica SPE was identified as a suitable clean-up as 95 % recovery was obtained for the DSP toxins. The author outlined that the recovery dropped to 75% when the methanolic extract is not acidified prior to the LLE step with chloroform. Acidification step prior to LLE has also been carried out in other studies (Lee et al., 1987; Suzuki et al., 2000; Goto et al., 2001).
Suzuki et al. (2005) used a single step 90 % methanolic extraction at a SSR of 9 and obtained recoveries ranging from 84 to 102 % for OA and from 78 to 100 % for DTX1 in mussels. In scallops, OA recoveries ranged from 82 to 98 % and DTX1 from 87 to 103 %. The different extraction methods and the recoveries that were achieved are summarised in Figure 1.8. Clean-up steps can also be achieved using imunoaffinity extraction that consists of raising analyte antibodies and in binding them covalently to silica to form the immunosorbent. Since antibodies show a strong affinity and are highly specific towards the analyte used to initiate the immune response, the corresponding immunosorbent may extract and isolate this analyte from complex matrices in a single step. The subsequent elution of the analyte can afford a matrix-free extract to be analysed. This approach was assessed for OA analysis by HPLC-FLD and LC-MS and compared with conventional LLE clean-up (Delaunay et al., 2000). The results presented were encouraging as the concentration obtained for the OA CRM tissue from the NRC was within the certified concentration. Good clean-up efficiency was reported when immunosorbent clean-up was used for HPLC-FLD analysis based on the observation of the reduction of background interferences (Delaunay et al., 2000).
SSR 9
McNabb et al., 2005
OA (WF): 85 – 100 %
DTX2 (WF): 95 – 100 %
OA esters (WF): 96 – 102 %
DTX2 esters (WF): 92 – 103 %

SSR 4
McNabb et al., 2005
OA: 70 – 95 %
DTX2: 89 – 99 %
OA esters: 33 – 77 %
DTX2 esters: 65 – 95 %

Extraction MeOH
(8:2) SSR 4

Extraction MeOH
(9:1) SSR 9

LLE Hex. (x 2)
LLE HCCl3 (x 2)

Aminopropyl silica column

Resusp. MeOH + DCA

SSR 4
Stobo et al., 2005
OA (WF):
91 – 94 %
72 – 93 %
78 – 105 %
85 – 97 %

Figure 1.8: Published extraction methods for OA and analogues from mussels, scallops, cockles and oysters

a Results reported before correction by standard addition
b Results reported after standard addition

DCA: deoxycholic acid

OA (WF): octadecenoic acid
OA (HP): octadecenoic acid (high-performance)
DTX2 (WF): dTX2 (octadecenoic acid)
DTX2 (HP): dTX2 (octadecenoic acid) (high-performance)

SSR 9
Suzuki et al., 2005

OA (WF): 84 – 102 %
DTX1 (WF): 75 – 107 %
DTX2 (WF): 98 – 120 %

SSR 2
Draisci et al., 1998
OA (WF): 97 – 104 %
DTX1 (WF): 93 – 102 %
DTX2 (WF): 96 – 102 %

SSR 0.5
Quilliam, 1995
OA (WF): > 98 %

SSR 2
Quilliam, 1995
OA (WF): > 98 %

SSR 9
Suzuki and Yasumoto, 2000
OA (WF): > 95 %
DTX1 (WF): > 95 %

Silica column

Resusp. MeOH + DCA

SSR 1
Ito and Tsukada, 2001
OA (WF):
81 – 89 %
a
94 – 103 %
b
DTX1 (WF):
66 – 72 %
a
102 – 112 %
b

LLE Hex. (x 2)

LLE HCCl3 (x 2)

Resusp. MeOH

SSR 1
Quilliam, 1995
OA (WF): > 95 %
DTX1 (WF): > 95 %

LLE HCCl3 (x 2)

Resusp. MeOH + DCA

Resusp. MeOH

OA (WF): 70 – 95 %
DTX2 (WF): 89 – 99 %
OA esters (WF): 33 – 77 %
DTX2 esters (WF): 65 – 95 %

SSR 2
Goto et al., 2001
OA (WF): 88 – 102 %
DTX1 (WF): 73 – 105 %

OA esters (WF): 96 – 102 %
DTX2 esters (WF): > 98 %

OA esters (WF): 96 – 102 %
DTX2 esters (WF): > 98 %

OA (WF): 85 – 100 %
DTX2 (WF): 95 – 100 %
OA esters (WF): 96 – 102 %
DTX2 esters (WF): 92 – 103 %
**Exhaustive extraction procedure**

Quilliam (1995) reported an exhaustive extraction consisting of the homogenisation of 2 g of mussel HP with ca. 8 ml of 100 % methanol followed by centrifugation. The pellet was consecutively re-extracted twice with 8 ml of 80 % methanol and with 6 ml of 80 % methanol using a vortex mixer. After centrifugation, the supernatants were combined in a 25 ml volumetric flask and volume made up to the mark with 80 % methanol. OA quantification was performed on extracts obtained from four consecutive tissue extractions using 80 % methanol (4:1). A triplicate extraction was found to be sufficient as recoveries were 87.0, 11.6, 1.4, and less than 0.2 % in fractions 1 to 4, respectively.

McNabb et al. (2005) investigated two exhaustive extractions consisting of three successive extractions using 90 % methanol at a SSR of 9 and 80 % at a SSR of 4. The recoveries at each stage of the three successive extractions were obtained for 10 lipophilic toxins e.g. AZA1, GYM, PTX2sa, YTX, 45-OH-YTX, carboxy-YTX as well as the DSP toxins OA, OA esters, DTX2 and DTX2 esters. At SSR of 9, OA and DTX2 were recovered in the two first fractions while a small amount of their ester derivatives were still present in the third extract (1.8 – 3.6 %). At SSR of 4 a small amount of DTX2 was found in the third extract of one sample (3 %) whereas their ester derivatives still accounted for 6.5 to 12.4 %. Therefore, a double methanolic (90 %) extraction seems to be adequate for all tested DSP toxins except for the ester derivatives at a SSR of 4.

Exhaustive extraction using acetone was also reported by Draisci et al. (1998). Recoveries of OA, DTX1 and DTX2 were assessed at two concentrations (0.2 and 1 µg/g HP) and were found acceptable as recoveries ranging from 97 to 104 %; 93 to 102 and 96 to 102 % were obtained for OA, DTX1 and DTX2, respectively.
b. Toxin extraction from phytoplankton

**Extraction from Dinophysis spp.**

Extractions of OA, OA diol esters and PTXs have been carried out using both SPE and LLE. Miles et al. (2004b) applied 50 or 240 ml aliquot of net hauls concentrates on C-18 SPE cartridges (500 mg; Varian). Small wads of cotton wool were packed on the top of these columns to reduce clogging by algal cells. The washing step was performed with freshwater (25 ml) and the column was eluted with methanol (5 ml). The sample was evaporated and resuspended for LC-MS analysis. Another SPE method was also used for extraction from *D. acuta* samples from New Zealand (Suzuki et al., 2003; Suzuki et al., 2004; MacKenzie et al., 2005). The latter consisted of a filtration of condensed phytoplankton sample (150 ml) through GF/C filter on which the cells were subsequently rinsed with methanol (5 ml). The aqueous filtrate and methanol were combined and transferred to a 500 mg C18-silica SPE cartridge (Strata, Phenomenex, Torrance, CA, USA). Conditioning of the column consisted of 10 ml of methanol and 10 ml methanol:water (1:9). Elution was performed with equal fractions (15 ml) of a mixture methanol:water at 3:7, 6:4 and 8:2 ratios. The eluates were combined and washed once with hexane (1:1, v/v). After adding water to reduce the methanol concentration to 60%, the toxins were extracted twice with chloroform (1:1, v/v). The combined chloroform extracts were evaporated and resuspended in methanol. LLE were also reported and the procedure consisted of an aqueous phytoplankton concentrate (200 ml) that was repeatedly frozen and thawed and sonicated with 8:2 methanol water (200 ml) for 15 min (Draisci et al., 1998a; James et al., 1999a). After centrifugation the supernatant was partitioned against chloroform (2 x 200 ml) and evaporated to dryness before resuspension. A slightly modified method where after repeated freeze thawing the sample was sonicated using acetonitrile:methanol:water (80:5:15) was also reported (James et al., 1999b). Miles and coworkers (Miles et al., 2004a; Miles et al., 2006a) used a filtration step under vacuum
through a glass fibre filter after sample freeze thawing and sonication. The filtrates were each extracted with diethyl ether (2 x 400 ml) and then with dichloromethane (2 x 400 ml). The extracts were dried (Na$_2$SO$_4$), evaporated in vacuo and resuspended. Vale P. (2004) used a method that included a boiling step to stop enzymatic activity that was previously evaluated for analysis in *P. lima* cells (Quilliam, et al., 1996). Toxin extraction was performed from plankton sampled from sub-superficial layers that were obtained with a Hansen bottle. Two sub-samples (7 l) were immediately vacuum filtered onto 10 µm nylon membranes (F 47-mm, three membranes were used per sub-sample). The filters of each sub-sample were placed in two test tubes and 2-ml diluted seawater was added to each (one part of seawater was diluted with three parts of distilled water). One of the tubes was immediately placed in boiling water for 5 min and the other stayed at room temperature. The samples were frozen and thawed before 4 ml MeOH was added to each tube and the toxins were extracted by sonication at 100 W for 2.5 min. Extracts were left for 2 h at room temperature. After centrifugation, 5.0 ml of each supernatant was split into duplicate tubes (2.5 ml each). After addition of 0.5 ml water, all four tubes were extracted twice with 2 ml dichloromethane. The combined dichloromethane layers were evaporated. From each pair of duplicate samples, one was directly resuspended with 500 ml 80% MeOH and the other was hydrolysed by alkaline hydolysis. For the remaining duplicate aqueous methanol layers, one was directly filtered into a vial while the other was hydrolysed.

*Extraction from Prorocentrum species*

Hu et al. (1992b) described an extraction method where the culture was centrifuged and methanol was added to the pellet in the centrifuge tube. After two hours the sample was sonicated (3 x 15 s) and centrifuged. The pellet was extracted two more times as described above. The three supernatants were combined, evaporated and resuspended before isolation. Quilliam et al. (1996) compared several extraction methods for the analysis of toxins.
produced by *P. lima* cultures. Aliquots (50 ml) of culture were transferred to 50 ml centrifuge tubes and centrifuged for 10 min at 6600 g. The supernatants were decanted without disturbing the cell pellets. Several different extraction method were investigated, the principal ones being as follows: Method 1 (80% methanol): Each cell pellet was resuspended in 2 ml of 8:2 methanol/water and sonicated for 1 min in pulse mode (50% duty cycle, 375 watts) while cooling in an ice bath. After centrifugation for 10 min at 6600 g., the supernatant was decanted. The pellet was rinsed twice (vortex mixing, centrifugation) with 1 ml methanol/water (8:2). Supernatants were combined and made to 5.0 ml. Extracts were passed through a 0.45 µm filter prior to analysis. Method 2 (French Press): Four cell pellets were each resuspended in 0.2 ml 50 mM TrisHCl pH 7.4, combined and passed through a chilled French press at pressure > 10 Kpsi. A 1 ml aliquot of buffer was used to wash remaining residues through the press and the sample was then brought to 2.0 ml with buffer. Aliquots (0.5 ml) were mixed with 2.0 ml methanol and processed as in method 1. Method 3 (freeze/thaw): Each cell pellet was resuspended in 0.5 ml of TrisHCl buffer and immersed in liquid nitrogen. The sample was then allowed to thaw at room temperature and left in the dark for 24 hours. Then 2 ml of methanol were added and the sample was sonicated and extracted as in method 1. Method 4 (boiling): Each cell pellet was resuspended in 0.5 ml of TrisHCl buffer and immersed in boiling water for 3 min. Following this, 2 ml of methanol was added, followed by sonication and extraction as in method 1. A modified boiling method where no buffer was used and the pellet extracted with methanol:water (8:2) was also reported (Pan et al., 1999).
1.3.1.5.5.2. LC-MS method for the analysis of DSP toxins

The development of LC-MS techniques in the late 1980’s led researchers to investigate the instrument performances for the analysis of lipophilic marine toxins. LC-MS is a powerful technique as it allows highly sensitive and selective measurement without the need of a derivatising step thereby reducing sample preparation.

The first reported method using LC-MS for the analysis of DSP toxins used fast-atom-bombardment (FAB) (Lee et al., 1988). Electrospray source (ESI) was then assessed as interface for LC and MS coupling for neurotoxins analysis (Quilliam et al., 1989). The main advantage of the ESI source is that the protonated molecule \([M+H]^+\) with little or no fragmentation can be obtained as a result of the mild ionisation/desolvation process (Bruins et al., 1987).

Pleasance et al., (1990) used a reversed phase isocratic elution consisting of 60 % ACN in water containing 0.1 % TFA and a 2.1 x 250 mm microbore column packed with 5 µm Vydac 201TP (C18) stationary phase to separate OA and DTX1.

The subsequent modification of the method by combining a gradient elution consisting of a linear increase of 40 to 100 % ACN (containing 0.1 % TFA) in 20 min and the reduction of the internal diameter of the column (2.5 to 1 mm) improved LOD by a factor 10 (Pleasance et al., 1992). The use of smaller ID column, also referred to as micro LC, allowed for operation at a lower flow rate and for the introduction of the totality of the eluent from the LC system column to the MS without flow splitting as typical flow rates vary from 20 to 50 µl/min. Column eluent splitting was also carried out in order to ensure that the best signal/noise is achieved. Because the ESI source is concentration dependent, flow splitting has little impact on the analyte signal and strongly reduces background noise (Quilliam, 1995).

Draisci et al. (1995) investigated the influence of TFA concentration on LC-MS sensitivity towards OA and DTX1. The assessed concentrations ranged from 0.02 to 0.12 % and 0.1 %
TFA was found to give the best sensitivity. Draisci et al. (1995) also evaluated the differences in analysis performance when 0.1 % acetic acid (Quilliam et al., 1989) and 0.1 % TFA (Pleasance et al., 1990) were used as mobile phase additives for the separation of OA and DTX1. TFA was preferred as acetic acid led to the formation of sodium adduct ions as abundant as the protonated molecular ion (Draisci et al., 1995). The formation of additional ammonium and potassium adducts of OA and DTX1 when acetic acid was used were also reported elsewhere (Luckas et al., 1994). The use of TFA reduced the number and the relative abundance of adducts as only \([\text{M+Na}]^+\) ions of OA and DTX1 were observed at a 1:4 ratio compared to \([\text{M + H}]^+\) allowing for a three fold sensitivity gain (Draisci et al., 1995).

Although, triple quadrupole mass analysers were used in the previously described LC-MS methods, quantitation was performed by single ion monitoring (SIM) on the molecular ion \([\text{M+H}]^+\). The use of tandem (MS/MS) analysers offers the advantage of highly selective measurements. Quilliam (1995) investigated the fragmentation pattern of OA in positive ionisation mode. Low collision energies resulted in successive loss of water \((m/z\ 787, 769, 751\) and 733) and a further increase to 40 eV resulted in the complete destruction of \([\text{M+H}]^+\).

Further experiments on fragmentation pathway elucidation were carried out by in-source fragmentation to overcome the poor transmission yield of ions through the collision chamber thereby reducing sensitivity in the old generation instruments. Cone voltage of 10 V resulted in the exclusive production of \([\text{M+H}]^+\) ion while an increase to 20-40 V resulted in successive losses of water \([\text{M+H-H}_2\text{O}]^+,\ [\text{M+H-2 H}_2\text{O}]^+,\ [\text{M+H-3 H}_2\text{O}]^+\) and \([\text{M+H-4 H}_2\text{O}]^+.\) High cone voltage of 70 V resulted in the fragmentation of the molecule, yielding more specific ions such as \(m/z:\ 169, 223, 287\) for OA and DTX2 while the same fragments ions with a 14 amu increase were observed for DTX1, indicating that these fragments involve the rings with carbon 31 and 35. The proposed fragmentation pattern of OA is shown in Figure 1.9.
James et al. (1999) introduced a micro LC-MS/MS method for OA and DTX2 analysis in plankton and shellfish with multiple reaction monitoring (MRM) acquisition where the precursor ion [M+H]$^+$ was fragmented in the collision cell to yield the fragment ions m/z: 733, 751, 769 corresponding to successive water losses.

One of the main advantages of LC-MS technique is its ability to implement a multi-analyte method enabling the quantitation of numerous compounds in one single analysis run. Due to the growing concern regarding shellfish contamination with YTX in Italy, LC-MS methods were developed for its quantitative determination in shellfish (Draisci et al., 1998a). Because YTX is not amendable to positive ionisation, it was chosen to investigate the possibility of the direct determination of toxin belonging to the OA, PTX and YTX groups in negative ionisation mode (Draisci et al., 1999a). It was shown previously that similar detection limits can be obtained in negative ionisation when ammonium acetate is used as buffer (1 mM) and where the molecular anion of OA [M+H]$^-$ corresponding to m/z 803.5 is obtained, than in
positive ionisation mode (Quilliam and Ross, 1996). The method reported by Draisci et al. (1999) did not achieve complete chromatographic separation between YTX and DSP toxins while maintaining satisfactory resolution within OA isomers (DTX2, -2b and -2c), PTX2 analogues (PTX2 sa and 7-epi-PTX2 sa) and DTX1 and PTX2. The authors suggested that this approach could be used as a screening technique. Samples that would be found to contain OA analogues could then be re-analysed using a modified method from Draisci et al. (1995) consisting of an isocratic separation using ACN/water doped with TFA followed by positive ionisation for SIM quantitation on [M+H]+. Trials were carried out on the improvement of the method sensitivity in both positive and negative ionisation method (Quilliam et al., 2001). A gradient elution consisting of water/aqueous acetonitrile both containing 2 mM ammonium formate and 50 mM formic acid was employed on a Hypersil BDS C8 (3 µm, 250 x 2 mm) followed by positive and negative detection. This provided a 20 to 50-fold sensitivity increase compared to when TFA was used in positive mode demonstrating the applicability of LC-MS technique for the detection of OA group toxins in negative ionisation mode. Furthermore, a buffer different than TFA was desirable because of the formation of strong ion pairs with amines. This resulted in signal suppression and sensitivity loss as well as its persistence in the MS system (due to the strong anion CF3COO−) when negative ionisation mode was used (Quilliam et al., 2001 and 2003).

The recent methods that were reported for the analysis of OA and analogues are performed in the negative mode and use either acetic acid, formic acid, ammonium acetate or the conjugated couple formic acid / ammonium formate (Suzuki and Yasumoto, 2000; Goto et al., 2001; Ito and Tsukada, 2002; Stobo et al., 2005; McNabb et al., 2005). The LOD obtained ranged from 5 to 51 pg on-column. The details of the methods are given in Table 1.9. The proposed fragmentation pattern of OA in the negative mode is shown in Figure 1.10 (Gerssen
et al., 2008). When MS/MS was performed in the negative mode out the fragment 255 was chosen for OA quantitation (McNabb et al., 2005).

Figure 1.10: Proposed OA fragmentation pathway in negative mode (Gerssen et al., 2008)
Table 1.9: Examples of LC-MS methods developed for the analysis of DSP toxins. Linearities and LOD are given for OA unless otherwise stated. LOD are given from methanolic solution unless otherwise stated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Detector</th>
<th>Source</th>
<th>Column</th>
<th>Elution</th>
<th>Flow Rate µL/min</th>
<th>V Inj / µL</th>
<th>Linearity range / ng injected</th>
<th>LOD / pg injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Pleasance et al., 1992)</td>
<td>TQ/SIM (+)</td>
<td>ISp</td>
<td>Microbore C18 (5 µm, 250 x 1 mm)</td>
<td>Gradient ACN/water + TFA</td>
<td>50</td>
<td>1</td>
<td>1 – 75</td>
<td>400¹</td>
</tr>
<tr>
<td>(Draisci et al., 1995)</td>
<td>SQ/SIM (+)</td>
<td>ISp</td>
<td>Supelcosil LC18-DB (5 µm, 250 x 4.6 mm)</td>
<td>Isocratic ACN/water (90:10) + TFA</td>
<td>800 (20:1 split to MS)</td>
<td>20</td>
<td>2 – 20</td>
<td>400</td>
</tr>
<tr>
<td>(Quilliam, 1995)</td>
<td>TQ/FIS (+)</td>
<td>ISp</td>
<td>Vydac 201TP C8 (5 µm, 250 x 2.1 mm)</td>
<td>Isocratic MeOH/water (7:3) + 0.1 % TFA</td>
<td>300 (10:1 split)</td>
<td>5</td>
<td>na</td>
<td>400¹              (DTX1)</td>
</tr>
<tr>
<td>(Quilliam and Ross, 1996)</td>
<td>TQ/SIM (+)</td>
<td>ISp</td>
<td>Vydac 201TP C8 (5 µm, 250 x 1 mm)</td>
<td>Isocratic MeOH/water (8:7:3-2) + TFA</td>
<td>50</td>
<td>1</td>
<td>na</td>
<td>40</td>
</tr>
<tr>
<td>(James et al., 1997b)</td>
<td>TQ / MRM (+)</td>
<td>ISp</td>
<td>Supelcosil LC18-DB (5 µm, 300 x 1 mm)</td>
<td>Isocratic ACN/water (85:15) + TFA</td>
<td>30</td>
<td>0.2</td>
<td>na</td>
<td>25</td>
</tr>
<tr>
<td>(Draisci et al., 1998a)</td>
<td>TQ / MRM (+)</td>
<td>ISp</td>
<td>Supelcosil LC18-DB (5 µm, 300 x 1.0 mm)</td>
<td>Isocratic ACN/water (80:20) + TFA</td>
<td>10</td>
<td>1</td>
<td>0.05 - 1</td>
<td>15</td>
</tr>
<tr>
<td>(Goto et al., 2001)</td>
<td>TQ/SIM (-)</td>
<td>ESI</td>
<td>Symmetry C18 (3.5 µm; 150 x 2.1 mm)</td>
<td>Isocratic ACN/water (7:3) + acetic acid</td>
<td>100</td>
<td>1</td>
<td>0.05-0.2 OA</td>
<td>28 OA²</td>
</tr>
<tr>
<td>(Suzuki and Yasumoto, 2000)</td>
<td>SQ/SIM (-)</td>
<td>ESI</td>
<td>Mightysil RP-18 C8 (5 µm; 150 x 2 mm)</td>
<td>Isocratic ACN/Water (7:3) + acetic acid</td>
<td>200</td>
<td>2</td>
<td>0.01- 30</td>
<td>5</td>
</tr>
<tr>
<td>(Ito and Tsukada, 2002)</td>
<td>SQ/SIM (-)</td>
<td>SSI¹</td>
<td>Inertil ODS-2 (5 µm; 150 x 2.1 mm)</td>
<td>Gradient MeOH/water + ammonium acetate</td>
<td>200</td>
<td>10</td>
<td>0.5 -5 OA &amp; DTX1</td>
<td>30 OA &amp; DTX1²</td>
</tr>
<tr>
<td>(Stobo et al., 2005)</td>
<td>SQ/SIM (-)</td>
<td>TIS⁶</td>
<td>BDS Hypersil C8 (3 µm; 50 x 2.1 mm)</td>
<td>Gradient ACN/water + ammonium acetate</td>
<td>250</td>
<td>5</td>
<td>0.013 – 0.25</td>
<td>33²</td>
</tr>
<tr>
<td>(McNabb et al., 2005)</td>
<td>TQ/MRM (-)</td>
<td>ESI</td>
<td>Luna C18 (5 µm, 50 x 2 mm)</td>
<td>Gradient ACN/water + ammonium formate + formic acid</td>
<td>200</td>
<td>10</td>
<td>0.05 – 1</td>
<td>11¹</td>
</tr>
</tbody>
</table>

¹ determined in mussel tissue, ² determined in scallop adductor muscle; ³ determined in cockles ⁴ determined in oysters ⁵ SSI: Sonic spray interface (Hitachi);⁶ TIS: Turboionspray® (Applied Biosystem)
1.3.1.5.5.3. Internal standard

One internal standard (IS) was prepared by acetylation of OA followed by purification by preparative chromatography. The compound obtained, 7-O-acetyl derivative of OA, was evaluated and quantitated using the IS and was compared against external calibration. The latter led to a 7.1 % uncertainty against 1.7 % when the IS was used (Quilliam, 1995). No improvement in the precision was observed for DTX1 in this study, although the author mentioned that the use of the IS did improve it on another set of samples. The article suggested that a 7-O-acetyl derivative of DTX1 could also be prepared and would elute closer to DTX1 and hence may be more suitable to correct for DTX1 losses.
1.3.2. Pectenotoxins group

PTXs were discovered following the Japanese incident in 1976 and 1977, where more than 160 people were poisoned as a result of the consumption of contaminated scallops *Patinopecten yessoensis* (Yasumoto, 1978). Beside OA and DTX1, two PTXs were isolated from the shellfish and named PTX1 and PTX2 (Yasumoto et al., 1985). Since, more than a dozen compounds have been discovered in phytoplankton and in bivalves.

1.3.2.1. Chemical structures and properties

PTXs are macrocyclic compounds that possess a large lactone ring as shown in Figure 1.11 a-d. The most frequently encountered compound is PTX2 and has been detected in shellfish, phytoplankton cells and concentrates as well as in sea water.

![Figure 1.11: Identified structures of PTXs. Structure a- corresponds to PTX 1-4, 6, 7, 11, 13](image-url)
Table 1.10: Structures of PTX 1-4, 6, 7, 11, 13

<table>
<thead>
<tr>
<th></th>
<th>C_7</th>
<th>R_1</th>
<th>R_2</th>
<th>R_3</th>
<th>[M+NH_4]^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX1</td>
<td>R</td>
<td>H</td>
<td>H</td>
<td>CH_2OH</td>
<td>892.5</td>
</tr>
<tr>
<td>PTX2</td>
<td>R</td>
<td>H</td>
<td>H</td>
<td>CH_3</td>
<td>876.5</td>
</tr>
<tr>
<td>PTX3</td>
<td>R</td>
<td>H</td>
<td>H</td>
<td>CHO</td>
<td>890.5</td>
</tr>
<tr>
<td>PTX4</td>
<td>S</td>
<td>H</td>
<td>H</td>
<td>CHO</td>
<td>890.5</td>
</tr>
<tr>
<td>PTX6</td>
<td>R</td>
<td>H</td>
<td>H</td>
<td>CO_2H</td>
<td>906.5</td>
</tr>
<tr>
<td>PTX7</td>
<td>S</td>
<td>H</td>
<td>H</td>
<td>CO_2H</td>
<td>906.5</td>
</tr>
<tr>
<td>PTX11</td>
<td>R</td>
<td>OH</td>
<td>H</td>
<td>CH_3</td>
<td>892.5</td>
</tr>
<tr>
<td>PTX13</td>
<td>R</td>
<td>H</td>
<td>OH</td>
<td>CH_3</td>
<td>892.5</td>
</tr>
</tbody>
</table>

Table 1.11: Structures of PTX8, 9, 12, 1, PTX2 sa and 7-epi-PTX2 sa

<table>
<thead>
<tr>
<th></th>
<th>C_7</th>
<th>R_1</th>
<th>R_2</th>
<th>R_3</th>
<th>[M+NH_4]^+</th>
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</thead>
<tbody>
<tr>
<td>PTX8</td>
<td>b</td>
<td>CH_2OH</td>
<td>CH_2OH</td>
<td>892.5</td>
<td></td>
</tr>
<tr>
<td>PTX9</td>
<td>b</td>
<td>CO_2H</td>
<td>CH_3</td>
<td>906.5</td>
<td></td>
</tr>
<tr>
<td>PTX12</td>
<td>c</td>
<td>-</td>
<td>CHO</td>
<td>874.5</td>
<td></td>
</tr>
<tr>
<td>PTX14</td>
<td>d</td>
<td>-</td>
<td>CHO</td>
<td>874.5</td>
<td></td>
</tr>
<tr>
<td>PTX2 sa</td>
<td>R</td>
<td>e</td>
<td>-</td>
<td>CO_2H</td>
<td>894.5</td>
</tr>
<tr>
<td>7-epi-PTX2 sa</td>
<td>S</td>
<td>e</td>
<td>-</td>
<td>CO_2H</td>
<td>894.5</td>
</tr>
</tbody>
</table>

PTX2 can undergo hydrolysis or oxidation depending on the source organism. Several studies demonstrated that PTX2 yields PTX2sa (Figure 1.11 -e) as a result of enzymatic hydrolysis in mid-guts extracts of greenshell mussel from New Zealand (Suzuki et al., 2001b); in digestive gland extracts of scallops from New Zealand (Suzuki et al., 2001a) as well as digestive glands from Norwegian blue mussels (Miles et al., 2004a). The latter finding was used to produce PTX2sa from isolated PTX2 for toxicological studies as attempts of hydrolysing PTX2 using commercial porcine esterase was not successful (Miles et al., 2004a). The authors also investigated the kinetics of this enzymatic hydrolysis using HP extracts of greenshell mussels from New Zealand as well as of blue mussel from Norway and reported hydrolysis of PTX2 to PTX2sa with half lives of 7 and 15 min, respectively (Miles et al., 2004a). The authors observed that PTX2sa subsequently epimerised to its thermodynamically more stable
stereoisomer 7-epi-PTX2sa within hours but does not result from by PTX2 hydrolysis as suspected by other studies (Suzuki et al., 2001b).

Several studies also observed oxidation of the C18-methyl group of PTX2 in Japanese scallops *Patinopecten yessoensis*, yielding consecutively PTX1, PTX3 and PTX6 equivalent to the different degrees of oxidation alcohol, aldehyde and carboxylic acid, respectively (Murata et al., 1986; Lee et al., 1989; Yasumoto et al., 1989; Suzuki et al., 1998). However, these compounds do not seem to occur in mussels.

PTX11 is another oxidised PTX2 that possesses an additional hydroxyl group at C34 as well as a methyl side chain at C18 and has recently been found in *D. acuta* from New Zealand (Suzuki et al., 2003). As of yet, products resulting from consecutive oxidation of PTX11 at C34 i.e. aldehyde and carboxylic acid have not been identified but can theoretically exist.

PTX2 is destroyed under strong acidic solutions but was found to be stable for 24 hours in dilute acidic and dilute alkaline solutions with pH ranging from 4.5 to 9.1 obtained by addition of phosphate buffers (Suzuki et al., 2001). The ketal centre at C7 is sensitive to epimerisation especially under acidic conditions. Hence, acidic treatment partially converts PTX1 and PTX6 to their spiroketal isomers PTX4, -8 and PTX 7, -9; respectively (Sasaki et al., 1998) while PTX2 and PTX11 are converted to PTX2b, -c and PTX11b, -c; respectively (Suzuki et al., 2003). The interconversions of PTXs are shown in Figure 1.12.
1.3.2.2. Causative organisms

PTXs have been associated with *Dinophysis spp* in many parts of the world including Europe, Japan, US and Australasia and have been detected in *D. acuta* (Table 1.12), *D. fortii* (Table 1.14) and *D. acuminata* (Table 1.13). PTX11 has been identified in several *D. acuta* strains from New Zealand (Suzuki et al., 2003; Miles et al., 2004a; Suzuki et al., 2006) but was not detected in Norwegian strains (Miles et al., 2004b). To our knowledge, there is no report of the presence of PTX11 in European strains of *Dinophysis*.

High PTX2 and PTX11 concentrations were found in *D. acuta* strains from New Zealand in 2001 with 81-82 pg/cell and 22-47 pg/cell, respectively (MacKenzie et al., 2002) and in 2002 with 33-108 pg/cell and 5-65 pg/cell, respectively (MacKenzie et al., 2002; MacKenzie et al., 2005). *D. acuta* from Portugal also contained relatively high amounts of PTX2 (48 pg/cell) (Vale, 2004a). PTX12 was detected in Norwegian strain of *D. acuta* (Miles et al., 2004b) and PTX13 in *D. acuta* from New Zealand (Suzuki et al., 2003; Miles et al., 2006b; Suzuki et al., 2006).
As outlined for the DSP toxins, the toxin production from an organism from the same geographical area can be highly variable. The PTX2 content of *D. fortii* from Japan was determined in four studies and the concentrations ranged from 10 to 182 pg/cell (Suzuki et al., 1998; Burgess and Shaw, 2001). The concentrations are summarised in Table 1.14.

PTX2 was detected in hand picked cells of *D. norvegica* at concentrations ranging from 50.8 to 67.4 pg/cell (Suzuki et al., 2008). In addition to the above Dinophysis species, it was recently demonstrated that PTX2 can be produced by *D. infundibulus* (Suzuki et al., 2008). PTX2sa has been occasionally reported in phytoplankton and is not believed to be produced by the cells but as a result of PTX2 hydrolysis from enzymes released by stressed cells (Mackenzie et al., 2002).
Table 1.12 PTXs detected and quantified in *D. acuta* strains worldwide. Detection was performed by LC-MS and concentrations are given in pg/cell

<table>
<thead>
<tr>
<th></th>
<th>PTX2</th>
<th>PTX2-sa</th>
<th>PTX11</th>
<th>PTX11sa</th>
<th>PTX12</th>
<th>PTX12sa</th>
<th>PTX13</th>
<th>PTX14</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Draisci et al., 1996)</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IE (James et al., 1999b)</td>
<td>-</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (MacKenzie et al., 2002)</td>
<td>81-82</td>
<td>2-3</td>
<td>22-47</td>
<td>0.5-1</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>P (Vale and Sampayo, 2002b)</td>
<td>p</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (Suzuki et al., 2003)</td>
<td>-</td>
<td>-</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>p</td>
<td>-</td>
</tr>
<tr>
<td>P (Vale, 2004a)</td>
<td>48</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>NZ (Miles et al., 2004a)</td>
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<td>p</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N (Miles et al., 2004b)</td>
<td>0.7-4</td>
<td>nd</td>
<td>nd-</td>
<td>nd</td>
<td>9-26</td>
<td>nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IE (Fernandez - Puente et al., 2004)</td>
<td>7-14</td>
<td>nd-13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (MacKenzie et al., 2005)</td>
<td>33-108</td>
<td>-</td>
<td>5-65</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (Suzuki et al., 2006)</td>
<td>-</td>
<td>-</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>p</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (Miles et al., 2006b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>p</td>
<td>P</td>
<td>-</td>
</tr>
<tr>
<td>S (Pizzaro et al., 2008)</td>
<td>10 - 38</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified
### Table 1.13 PTXs detected and quantified in *D. acuminata* strains worldwide. Detection was performed by LC-MS and concentrations are given in pg/cell

<table>
<thead>
<tr>
<th></th>
<th>PTX2</th>
<th>PTX2-sa</th>
<th>PTX11</th>
<th>PTX11sa</th>
<th>PTX12</th>
<th>PTX12sa</th>
<th>PTX1</th>
<th>PTX6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO (Miles et al., 2004b)</td>
<td>1 – 12</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.4 – 5-</td>
<td>nd</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (MacKenzie et al., 2005)</td>
<td>2-26</td>
<td>-</td>
<td>0.4-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Suzuki et al., 2008)</td>
<td>10.7-50.2</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Jpn (Kamiyama and Suzuki, 2008)</td>
<td>14.7-14.9</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified

### Table 1.14 PTXs detected and quantified in *D. fortii* strains from Japan. Detection was performed by LC-MS and concentrations are given in pg/cell

<table>
<thead>
<tr>
<th></th>
<th>PTX2</th>
<th>PTX2-sa</th>
<th>PTX11</th>
<th>PTX6</th>
<th>PTX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jpn (Lee et al., 1989)</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Suzuki et al., 1998)</td>
<td>182</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Burgess et al., 2003)</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Suzuki et al., 2008)</td>
<td>51.4-63.8</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified
1.3.2.3. Occurrence in shellfish and geographical distribution

PTXs were discovered in Japan from scallops (*P. yessoensis*) and PTX1 to PTX7 were isolated from the DG (Yasumoto et al., 1985; Yasumoto et al., 1989; Sasaki et al., 1998). PTX6 was detected at levels up to 13 µg/g in DG of Japanese scallops collected in Mutsu Bay in 1996 (Andersen et al., 1996) and up to 5.71 µg/g in 1995 (Suzuki and Yasumoto, 2000). Mussels (*M. galloprovincialis*) collected from the latter site were also analysed for PTX6 but was not detected (Suzuki and Yasumoto, 2000). PTX2sa were detected in mussels (*M. galloprovincialis*) from Japan (Suzuki et al., 2005) but has not been reported in scallops.

In Europe, PTX2sa were detected in mussels from Italy (*M. galloprovincialis*) and Ireland (*M. edulis*) (Draisci et al., 1999a; James et al., 1999b; Puente et al., 2004). Recently, fatty acid esters of PTX2 were discovered in mussels from Ireland (Wilkins et al., 2006). Low levels of PTX2sa were also reported in clams (*Donax trunculus*), cockles (*C. edule*) and mussels (*M. galloprovincialis*) from Portugal and Spain (Fernandez et al., 2002; Vale and de M. Sampayo, 2002; Vale, 2004a; Vale, 2006b). In Norway, several PTXs were identified in mussels (*M. edulis*) and cockles (*C. edule*) including PTX2, PTX12, PTX2sa and PTX12 seco acids (Miles et al., 2004b). PTX2sa and 7-epi-PTX2sa were also detected in mussels (*M. galloprovincialis*) from the Adriatic Coast of Croatia (Pavela-Vrancic et al., 2001). Although the detection of PTX2 in shellfish is not common due to the different metabolisation processes low levels were found in mussels from Spain (Fernandez - Puente et al., 2004) and in mussels (*M. edulis*) and oysters (*C. gigas*) from France at levels up to 36 µg/kg (Amzil et al., 2007). Recently mussels from the White Sea in Russia were also found to contain PTX2sa and PTX2 at low levels (2 ng/g) (Vershinin et al., 2006).
1.3.2.4. Toxicity

PTXs have only been administered to mice and PTX 1-4, PTX6, PTX7 and PTX11 were found to be the most toxic analogues with minimum lethal doses (MLD) ranging from 192 to 770 µg/kg by intraperitoneal injection (Yasumoto et al., 1985; Yasumoto et al., 1989; Miles et al., 2004a; Suzuki et al., 2006). The remaining analogues PTX8, PTX9, PTX2sa and 7-epi-PTX2sa did not exhibit any toxicity at levels up to 5 mg/kg (Sasaki et al., 1998; Miles et al., 2004a; Miles et al., 2006c).

Differences in toxicity can be attributed to the structure activity relationships of the different PTXs, illustrated by the decline in toxicity from the 7-S epimers PTXs (PTX1 and PTX6) to the 7-R epimers (PTX4 and PTX7) and consecutively to the the 6-membered ring B (PTX8 and PTX9) (Miles, 2007).

1.3.2.5. Methods of detection

1.3.2.5.1. Non – Chromatographic methods

PTXs can be detected using the MBA where shellfish are extracted using acetone or methanol and the extract is injected to mice with survival times of 5 or 24 hours as described in the MBA for the DSP toxin detection.

ELISA assays for the detection of PTXs are under development and preliminary work has been reported (Suzuki et al., 2008).

TLC can be used to detect PTXs in semi-purified samples and therefore can be useful as a screening technique (Yasumoto et al., 1985).

1.3.2.5.2. HPLC-UV

All PTXs absorb UV light at 235 nm (Yasumoto 1985) as a consequence of the presence of the 1, 3-dienyl moiety at C28-C31 present in all analogues except for PTX2sa and its epimer
that absorb at 237 nm (Miles et al., 2004). Quantification of PTX2 by isocratic LC-UV detection at 235 nm has been reported (Lee et al., 1989; Draisci et al., 1996; Suzuki et al., 1998). The separation of PTXs from OA and YTX group toxins was achieved on basic alumina columns (Yasumoto et al., 1984; Kumagai et al., 1986; Draisci et al., 1996). After loading the methanolic extract containing the toxins, a neutral fraction was obtained by elution with a mixture of dichloromethane and methanol and an acidic fraction was obtained by a second elution step with a basic mixture of ammonium hydroxide and methanol. PTXs were found in the neutral fraction while acidic compounds (OA and YTX group toxins) were obtained in the acidic fraction. The detection limit of PTX2 by LC-UV detection is approximately 2.0 µg/g hepatopancreas (Suzuki et al., 1998).

1.3.2.5.3. LC-FLD

Derivatisation of PTXs is applicable to the analogues that possess a carboxylic group. ADAM derivatisation has been used for the detection of PTX2sa (James et al., 1999b; Pavela-Vrancic et al., 2001; Vale and Sampayo, 2002b) and for the detection of PTX6 (Suzuki et al., 1998). BAP derivatisation has also been used for the detection of PTX2sa (James et al., 1999b).

1.3.2.5.4. LC-MS analysis

1.3.2.5.4.1. Extraction method prior to LC-MS analysis

A summary of the published extraction methods suitable for the extraction of PTX1, -2, -6 and PTX2sa and their respective recoveries are shown in Figure 1.13. All extraction methods except the method for PTX1 and PTX6 reported by Goto et al. (2001), were the same as those reported by the same authors for the DSP toxins.
PTXs are neutral compounds and can be analysed in positive and in negative modes. Some studies reported that the signal was “markedly” increased when PTXs were monitored in the negative mode using [M-H]- (Goto et al. 2000). Typically, the analysis of PTXs in positive mode yield the ions [M + H]+, [M + Na]+ and [M + NH₄]+ (Suzuki et al., 2000; Goto et al., 2001.), although this may be dependent on the source as other studies observed [M + H]+ as the predominant ion (Draisci et al., 1996).

The influence of pH modifier was investigated in the process of improving the detection of PTX2. The mobile phase consisting of ACN/water (7:3) was assessed with formic acid at 0.1%, acetic acid at 0.1 %, ammonium formate at 5 mM and without modifier. Acetic and
formic acid were found to provide equal sensitivity while no modifier and ammonium acetate resulted in lower sensitivity (Suzuki et al., 2000). This observation suggests that optimum sensitivity of PTXs is achieved at acidic pH. Suzuki et al. (2000) also observed that increasing the capillary temperature resulted in an increased ionisation of PTX2 and that the sodium adduct was predominantly observed at higher temperatures. LC-MS methods that were reported for the detection of PTXs are summarised in Table 1.15.

In MS/MS analyses the fragmentation of PTX2 leads to specific ions such as m/z 551 as well as m/z 213. The proposed fragmentation pattern of PTX is shown in Figure 1.14.

Some LC-MS methods that have been reported for the analysis of PTXs are summarised in Table 1.15.

Figure 1.14: Fragmentation pattern of PTX2 - reproduced from Miles et al., 2004
Table 1.15: Examples of LC-MS methods developed for the analysis of PTX toxins. LODs are obtained for PTX2 and from methanolic solution unless otherwise stated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Analyte</th>
<th>Detector</th>
<th>Source</th>
<th>Column</th>
<th>Elution</th>
<th>Flow Rate / µL/min</th>
<th>V Inj./µL</th>
<th>Linearity range / ng injected</th>
<th>LOD / pg injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Goto et al., 2001)</td>
<td>PTX1, -2, -6 and PTX2sa</td>
<td>TQ/SIM (+) PTX1, -2 (-) PTX6, PTX2sa</td>
<td>ESI</td>
<td>PTX6: Symmetry C18 (3.5 µm; 150 x 2.1 mm) PTX1, -2: Develosil ODS-MG5 (5 µm; 150 x 2.1 mm)</td>
<td>Isocratic ACN/water (7:3) + acetic acid</td>
<td>100</td>
<td>1</td>
<td>0.04 – 1.6³</td>
<td>224 1.2³</td>
</tr>
<tr>
<td>(Suzuki and Yasumoto, 2000)(Ito and Tsukada, 2002)</td>
<td>PTX6</td>
<td>SQ/SIM (-)</td>
<td>ESI</td>
<td>Mightysil RP-18 C8 (5 µm; 150 x 2 mm) Inertil ODS-2 (5 µm; 150 x 2.1 mm)</td>
<td>Isocratic ACN/Water (7:3) + acetic acid Gradient MeOH/water + ammonium acetate</td>
<td>200</td>
<td>2</td>
<td>0.01 – 30</td>
<td>5</td>
</tr>
<tr>
<td>(Stobo et al., 2005)</td>
<td>PTX2</td>
<td>SQ/SIM (+)</td>
<td>TIS⁷</td>
<td>BDS Hypersil C8 (3 µm; 50 x 2.1 mm)</td>
<td>Gradient ACN/water + ammonium acetate</td>
<td>250</td>
<td>5</td>
<td>0.013 – 0.25</td>
<td>11 ³ 11 ⁴ 11 ⁵ 14 ²</td>
</tr>
<tr>
<td>(McNabb et al., 2005)</td>
<td>PTX2</td>
<td>TQ/MRM (+)</td>
<td>ESI</td>
<td>Luna C18 (5 µm, 50 x 2 mm)</td>
<td>Gradient ACN/water + ammonium formate + formic acid</td>
<td>200</td>
<td>10</td>
<td>0.05 – 2</td>
<td>6</td>
</tr>
</tbody>
</table>

¹ Obtained for PTX1, -2 and -6 ² Determined in scallop adductor muscle extract ³ Determined in mussel extract ⁴ Determined in cockle extract ⁵ Determined in oyster extract ⁶ SSI: Sonic spray interface (Hitachi);⁷ TIS: Turboionspray® (Applied Biosystem)
1.3.3. Azaspiracid group

Azaspiracids have been identified after a poisoning incident in the Netherlands in 1995 where the consumption of Irish mussels harvested in Killa ry Harbour (McMahon and Silke, 1996) led to DSP – like symptoms in 8 people. Satake et al. (1998) isolated and elucidated the structure of the bioactive compound which was named AZA1. Shortly after, a second AZA outbreak occurred in Arranmore Island where AZA1 was found to be responsible for human intoxication after mussel consumption (McMahon and Silke, 1998). Further isolation of mussel samples led to the discovery of AZA2 and AZA3 (Ofuji et al., 1999b) and of hydroxyl derivatives AZA4 -5 (Ofuji et al., 2001), AZA7-11 and of AZA6 (James et al., 2003), an isomer of AZA1.

1.3.3.1. Chemical structures and properties

Azaspiracids possess two spiro ring assemblies, a cyclic amine and a carboxylic acid. Based on chromatographic retention times AZAs are the most lipophilic toxins of the regulated toxins.

Attempts in preparing reference material using gamma irradiation as stabilisation technique led to the observation that when irradiated, AZAs were stable in mussel tissues and not in methanolic solution (McCarron et al., 2007).

The AZA structures were reported with an incorrect stereochemistry. The full synthesis of AZA1 allowed for a modified stereochemistry to be assigned (Nicolaou et al., 2004) (Figure 1.15).
Figure 1.15: Structures of Azaspiracids 1-11. Structures revised by Nicolaou (2004). Please refer to Table 1.16 in which the identity of the R groups for each toxin in this group is shown.
Table 1.16: AZA structures and mass of molecular ion [M+H]*

<table>
<thead>
<tr>
<th>AZAs</th>
<th>$R_1$ (C8)</th>
<th>$R_2$ (C22)</th>
<th>$R_3$ (C3)</th>
<th>$R_4$ (C23)</th>
<th>[M+H]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZA1</td>
<td>H</td>
<td>H</td>
<td>CH$_3$</td>
<td>H</td>
<td>842.5</td>
</tr>
<tr>
<td>AZA2</td>
<td>H</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>H</td>
<td>856.5</td>
</tr>
<tr>
<td>AZA3</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>828.5</td>
</tr>
<tr>
<td>AZA4</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>844.5</td>
</tr>
<tr>
<td>AZA5</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>844.5</td>
</tr>
<tr>
<td>AZA6</td>
<td>H</td>
<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>841.5</td>
</tr>
<tr>
<td>AZA7</td>
<td>H</td>
<td>H</td>
<td>CH$_3$</td>
<td>OH</td>
<td>857.5</td>
</tr>
<tr>
<td>AZA8</td>
<td>H</td>
<td>H</td>
<td>CH$_3$</td>
<td>OH</td>
<td>857.5</td>
</tr>
<tr>
<td>AZA9</td>
<td>OH</td>
<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>857.5</td>
</tr>
<tr>
<td>AZA10</td>
<td>H</td>
<td>CH$_3$</td>
<td>H</td>
<td>OH</td>
<td>857.5</td>
</tr>
<tr>
<td>AZA11</td>
<td>OH</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>H</td>
<td>871.5</td>
</tr>
<tr>
<td>AZA12</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>H</td>
<td>OH</td>
<td>871.5</td>
</tr>
<tr>
<td>AZA13</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>859.5</td>
</tr>
<tr>
<td>AZA14</td>
<td>CH$_3$</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>873.5</td>
</tr>
<tr>
<td>AZA15</td>
<td>H</td>
<td>CH$_3$</td>
<td>OH</td>
<td>OH</td>
<td>873.5</td>
</tr>
<tr>
<td>AZA16</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>OH</td>
<td>OH</td>
<td>887.5</td>
</tr>
<tr>
<td>AZA17</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>871.5</td>
</tr>
<tr>
<td>AZA18*</td>
<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>885.5</td>
</tr>
<tr>
<td>AZA19*</td>
<td>H</td>
<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>885.5</td>
</tr>
<tr>
<td>AZA20*</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>899.5</td>
</tr>
<tr>
<td>AZA21*</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>887.5</td>
</tr>
<tr>
<td>AZA22*</td>
<td>CH$_3$</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>901.5</td>
</tr>
<tr>
<td>AZA23*</td>
<td>H</td>
<td>CH$_3$</td>
<td>OH</td>
<td>H</td>
<td>901.5</td>
</tr>
<tr>
<td>AZA24*</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>OH</td>
<td>H</td>
<td>915.5</td>
</tr>
</tbody>
</table>

Compounds marked with * indicate that a carboxylic function is also present. The positions of these groups have not been elucidated yet. It is however hypothesised that they occur at the C22 or C23 position (Rehmann et al., 2008)

1.3.3.2. Causative organisms

The first report on the detection of azaspiracids in phytoplankton was in *Protoperidinium Crassipes* (James et al., 2003). This led the authors to conclude that *P. crassipes* was the producer of AZAs. Subsequently, the eventual correlation between the occurrence of *P. crassipes* in the water and AZAs in shellfish was examined as part of the Irish national monitoring programme and did not result in the observation of any relationship (Cusack
personal comm.). *P. crassipes* is a heterotrophic dinoflagellate and was therefore suspected to have accumulated AZAs from prey species (Hess et al., 2005b). The ability of toxin accumulation in *P. crassipes* was demonstrated by Miles et al. (2004) by LC/MS analysis of hand-picked cells that were observed to feed on *Dinophysis spp*. The *P. crassipes* cells were found to contain DSP toxins suggesting that similarly, the eventual feeding of *P. crassipes* on the AZA producer would have resulted in AZA accumulation in the cells.

Recently, an extensive survey in the North Sea revealed that the organism that produces AZA was typically found in phytoplankton ranging from 3-20 µm (Krock et al., 2008 – In press). The producer is believed to be a new species and accurate taxonomic description will be determined in the future. The small size of this organism explains the association of AZA with larger heterotrophic organisms such as *Protoperidinium*. This dinoflagellate strain was found to produce AZA1, AZA2 and an isomer of AZA2.

1.3.3.3. Occurrence in Shellfish

AZAs have originally been discovered in mussels (*M. edulis*) from Ireland (Satake et al., 1998) and were then detected in mussels (*M. edulis*) from Norway and England (James et al., 2002; Aasen et al., 2006). AZAs have been detected in many bivalve species in Ireland including oysters (*Crassostrea gigas, Ostrea edulis*), scallops (*Pecten maximus*), clams (*Tapes philippinarium*), cockles (*Cardium edule*) and razor fish (*Ensis siliqua*) at levels up to 8 mg/kg (Furey et al., 2003; Hess et al., 2003). The occurrence of AZAs was then extended to Spain, France, Denmark and Portugal following their detection in scallops (*P. maximus*) and mussels (*M. galloprovincialis*) (Schrijver De et al., 2002; Magdalena et al., 2003; Vale, 2004b; Vale et al., 2008). AZAs were also detected outside Europe in mussels from Marocco (Blaghen et al., 1997) and from Nova Scotia (Twiner et al., 2008).
1.3.3.4. Toxicity

Following the first AZA outbreak in Killary Harbour, mussel samples from the production area were tested for the presence of DSP toxins, using both RBA and MBA, and gave strongly positive results. However, mouse symptoms induced by intraperitoneal injection of acetone extracts of mussel HP were distinctly different from those normally associated with DSP toxins, showing prominent neurological symptoms such as respiratory difficulties, spasms, paralysis of the limbs and death within 20 min at higher doses (Ito et al., 2000). During the course of toxin purification, the major toxin was concentrated in a lipid fraction coded KT3
(Satake et al., 1998). Injection (i.p) of the semi-purified toxin fraction into mice led to fatty change in the liver, degeneration of the pancreas and necrosis of the lymphoid tissues in the thymus and spleen. Minimum lethal doses of AZAs range from 110 µg/kg for AZA2 (Ofuji et al., 1999b) up to more than 1mg/kg for AZA5 (Ofuji et al., 2001).

Oral administration of KT3 caused edema in the small intestine and significant accumulation of mucous fluid in the whole of the small intestine (Ito et al., 1998). Oral administration of AZA1 isolated from mussels has however shown considerable variations in mice lethality depending on the mice age with minimum lethal dose of more than 450 µg/kg in 4 – 5 weeks old mice and more than 250 µg/kg in 5 month old mice (Ito et al., 2002). Recent efforts towards the full synthesis of AZA1 enabled the construction of a synthetic AZA1 (Nicolaou 2003). The oral toxicity of the synthetic compound was in good agreement with the toxicity of the natural AZA1. Several articles, reviews and book chapters are dealing with AZAs toxicities in more details (Anonymous, 2005b; Toyofuku H., 2006; Suzuki, 2008; Twiner et al., 2008).

1.3.3.5. Methods of detection

1.3.3.5.1. Animal tests

The MBA currently in place for the detection of AZAs is the same as the one described for the DSP toxins where acetone is used as the extraction solvent. The presence of AZAs in the extract induces mice symptoms that are different to the DSP toxins with hopping, scratching and progressing paralysis (cited from the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs (Anonymous, 2005b). The shortest time for mouse death was 35 min (at 6 times of the lethal dose) and the longest was 30 h and 46 min. The test should be considered semi-quantitative at best, with a detection limit of ca. 4 µg in a 20 g mouse (Flanagan et al., 2001).
LC-MS analysis

1.3.3.5.1.1. Extraction method prior to LC-MS analysis

The first reports of the occurrence of AZAs usually used acetone as an extraction solvent because historically the MBA was performed with extracts obtained from acetone extraction. Hess et al. (2005) reported that the affinity of azaspiracids to methanol is greater for some matrices compared to acetone, and therefore that future method development should focus on using extraction protocols with methanol as solvent. Furthermore, the same authors also investigated the influence of the solvent to sample ratio (SSR) and found that the higher the SSR the better the recovery. This was also observed by other authors when a methanolic extraction with SSR 9 was compared against an extraction with SSR 4 (McNabb et al., 2005). The recoveries and extraction methods that were reported for the analysis of AZAs are summarised in Figure 1.17.
Figure 1.17: Published extraction methods for AZA1 and analogues from mussels, scallops, cockles and oysters

1.3.3.5.1.2. LC-MS methods for the detection of AZAs

The first LC-MS method developed for the detection of AZAs achieved LODs of 50 and 75 ng/ml injected, for AZA1 and AZA2, -3, respectively (Ofuji et al., 1999a). The MS was operated in the SIM mode on the molecular ions. A more selective MS/MS was developed by Draisci et al. (2000) where fragments resulting from the subsequent losses of water of the molecular ion of AZA1 were monitored. A LC-MS$^3$ method was also developed where two consecutive fragments were obtained for AZA1-5 which eluted in the order AZA4, -5, -3, -1 and -2 (Lehane et al., 2002). The MS$^3$ transitions where the first fragment obtained from the loss of one molecule water was followed by a more specific fragment resulting from the A-ring fragmentation were also assessed for quantitation and led to higher LOD (40 pg) than when two losses of water were monitored (Furey et al., 2002; Lehane et al., 2002).
The MS fragmentation pattern of AZAs can exhibit up to six losses of water molecules. (Brombacher et al., 2002). The proposed fragmentation pattern of AZA1 is shown in Figure 1.18 and the reported LC-MS methods are summarised in Table 1.17.

Figure 1.18: Proposed fragmentation pattern of AZA1 in positive ESI
Table 1.17: Examples of LC-MS methods developed for the analysis of AZA toxins. Linearities and LOD are given for OA unless otherwise stated. LOD are given from methanolic solution unless otherwise stated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Analytes</th>
<th>Detector</th>
<th>Source</th>
<th>Column</th>
<th>Elution</th>
<th>Flow mL/min</th>
<th>V Inj / µL</th>
<th>Linearity range / ng</th>
<th>LOD / pg injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ofuji et al., 1999a)</td>
<td>AZA2, -3</td>
<td>IT SIM</td>
<td>ESI+</td>
<td>Capcell Pak C18 UG-120 (150 x 2 mm)</td>
<td>Isocratic MeOH/H2O (7:3) + AcOH</td>
<td>0.2</td>
<td>1</td>
<td>0.05 – 100</td>
<td>50</td>
</tr>
<tr>
<td>(Draisci et al., 2000)</td>
<td>AZA1</td>
<td>TQ MS/MS</td>
<td>ESI+</td>
<td>Vydc 218TP51 (250 x 1 mm, 5 µm)</td>
<td>Isocratic ACN /H2O (85/15) 0+ TFA</td>
<td>0.03</td>
<td>1</td>
<td>0.1 – 1</td>
<td>20</td>
</tr>
<tr>
<td>(Lehane et al., 2002)</td>
<td>AZA1-5</td>
<td>IT MS</td>
<td>ESI+</td>
<td>Luna-2 C1, (3 µm, 150 x 2.0 mm,)</td>
<td>Isocratic acetonitrile–water (65:35 or 46:54) +0.05% TFA + 5 mM ammonium acetate</td>
<td>0.2</td>
<td>5</td>
<td>0.25 – 5</td>
<td>5</td>
</tr>
<tr>
<td>(Furey et al., 2002)</td>
<td>AZA1-3</td>
<td>IT</td>
<td>ESI+</td>
<td>Luna-2 C18, (3 µm, 150 x 2.0 mm,)</td>
<td>Isocratic acetonitrile–water (65:35 or 70:30) +0.05% TFA + 5 mM ammonium acetate</td>
<td>0.2</td>
<td>5</td>
<td>0.25 – 5</td>
<td>4</td>
</tr>
<tr>
<td>(James et al., 2003)</td>
<td>AZA1-10</td>
<td>IT</td>
<td>ESI+</td>
<td>Luna-2 C18, (3 µm, 150 x 2.0 mm,)</td>
<td>Isocratic acetonitrile–water (65:35 or 46:54) +0.05% TFA + 5 mM ammonium acetate</td>
<td>0.2</td>
<td>5</td>
<td>0.25 – 5</td>
<td>5</td>
</tr>
<tr>
<td>(Volmer et al., 2002)</td>
<td>AZA1-5</td>
<td>TQ</td>
<td>ESI+</td>
<td>Monolithic column Performance ROD (100 x 4.6 mm)</td>
<td>Isocratic acetonitrile-water (66:34) + 50 mM formic acid, 2 mM ammonium formate</td>
<td>1</td>
<td>1</td>
<td>na</td>
<td>0.4</td>
</tr>
<tr>
<td>(Volmer et al., 2002)</td>
<td>AZA1-5</td>
<td>IT</td>
<td>ESI+</td>
<td>Monolithic column Performance ROD/Speed ROD (400 x 4.6 mm)</td>
<td>Gradient acetonitrile-water + 50 mM formic acid, 2 mM ammonium formate</td>
<td>1</td>
<td>(split 1:3)</td>
<td>10</td>
<td>na</td>
</tr>
<tr>
<td>(Lehane et al., 2004)</td>
<td>AZA1-10</td>
<td>IT</td>
<td>ESI+</td>
<td>Luna-2, 3 µm (150 x 2 mm)</td>
<td>Isocratic acetonitrile/water (46:54, v/v) + 0.05% trifluoroacetic acid (TFA) and 0.004% ammonium acetate</td>
<td>0.2</td>
<td>5</td>
<td>0.013 – 0.025</td>
<td>5</td>
</tr>
<tr>
<td>(Stobo et al., 2005)</td>
<td>AZA1</td>
<td>SQ/SIM</td>
<td>TIS</td>
<td>BDS Hypersil C8 (3 µm; 50 x 2.1 mm)</td>
<td>Gradient ACN/water + ammonium acetate</td>
<td>0.25</td>
<td>5</td>
<td>0.013 – 0.025</td>
<td>19</td>
</tr>
<tr>
<td>(McNabb et al., 2005)</td>
<td>AZA1</td>
<td>TQ/ MRM</td>
<td>ESI</td>
<td>Luna C18 (5 µm, 50 x 2 mm)</td>
<td>Gradient ACN/water + ammonium formate + acetic acid</td>
<td>0.2</td>
<td>10</td>
<td>0.05 – 0.2</td>
<td>6</td>
</tr>
</tbody>
</table>

1 Determined in mussel extract 2 Determined in cockle extract 3 Determined in oyster extract 4 Determined in scallop extract
1.3.4. Yessotoxin group

YTX was named after its discovery in Japanese *Patinopecten yessoensis* (Murata et al., 1987). Initially, YTX was associated with DSP because of its co-occurrence with toxins from the OA group.

1.3.4.1. Chemical structures and properties

The yessotoxin group is composed of more than 90 analogues (Miles et al., 2005). Their structures have in common ladder shape polyether skeletons that possess two sulphated side chains. As a result of the sulphated groups, YTXs are more hydrophilic than toxins from the OA and PTX groups. In shellfish, several oxidation products of YTX were identified at C45 such as the hydroxyl derivative 45-OH-YTX and the two carboxylic acid derivatives carboxy-YTX and 45-hydroxycarboxy YTX (Satake et al., 1997; Yasumoto and Takizawa, 1997). Other analogues that possess an additional methylene in the alkyl side chain at C3 such as 1a-homoYTX, hydroxy-1a-homoYTX and carboxy-1a-homoYTX were discovered in digestive glands of mussels (Satake et al., 1997). Mono, di and tri-arabinoside analogues were also identified in *P.reticulatum* extracts and can have alkyl side chain of two or three methylene groups (Konishi et al., 2004; Miles et al., 2006d).
Table 1.18: Structures and molecular masses of YTX analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Fragment to right of part of molecule shown in Figure 1.19</th>
<th>R1</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>YTX</td>
<td>1</td>
<td><img src="image1" alt="YTX structure" /></td>
<td>H</td>
<td>1141</td>
</tr>
<tr>
<td>45-hydroxyYTX</td>
<td>1</td>
<td><img src="image2" alt="45-hydroxyYTX structure" /></td>
<td>H</td>
<td>1157</td>
</tr>
<tr>
<td>carboxyYTX</td>
<td>1</td>
<td><img src="image3" alt="carboxyYTX structure" /></td>
<td>H</td>
<td>1173</td>
</tr>
<tr>
<td>1a-homoYTX</td>
<td>2</td>
<td><img src="image4" alt="1a-homoYTX structure" /></td>
<td>H</td>
<td>1155</td>
</tr>
<tr>
<td>45, 46, 47-trinorYTX</td>
<td>1</td>
<td><img src="image5" alt="45, 46, 47-trinorYTX structure" /></td>
<td>H</td>
<td>1101</td>
</tr>
<tr>
<td>ketoYTX</td>
<td>1</td>
<td><img src="image6" alt="ketoYTX structure" /></td>
<td>H</td>
<td>1047</td>
</tr>
</tbody>
</table>

Figure 1.19: Structure of YTX and analogues Murata et al., 1987.
Table 1.18 (continue): Structures and molecular masses of YTX analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>R1</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-epi-ketoYTX</td>
<td>1</td>
<td>H</td>
<td>1047</td>
</tr>
<tr>
<td>41a-homoYTX</td>
<td>1</td>
<td>H</td>
<td>1155</td>
</tr>
<tr>
<td>9-Me-41a-homoYTX</td>
<td>1</td>
<td>CH₃</td>
<td>1169</td>
</tr>
<tr>
<td>44, 45-dihydroxyYTX</td>
<td>1</td>
<td>H</td>
<td>1175</td>
</tr>
<tr>
<td>45-hydroxy-1a-homoYTX</td>
<td>2</td>
<td>H</td>
<td>1171</td>
</tr>
<tr>
<td>carboxy-1a-homoYTX</td>
<td>2</td>
<td>H</td>
<td>1187</td>
</tr>
</tbody>
</table>

1.3.4.2. Causative organisms

Ten years after the initial discovery of YTX (Murata et al., 1987), a mixed bloom of *D. acuta* and *Protoceratium reticulatum* in New Zealand led to high toxicity levels of greenshell mussels (*Perna canaliculus*). Their subsequent HPLC and ELISA analysis for DSP toxins have shown that the shellfish were only contaminated with OA and DTX1 at trace levels, while high levels of YTX were observed using HPLC-FD detection (Yasumoto and Takizawa, 1997). YTX has been reported to be produced by cultures of *Protoceratium reticulatum* originating from many parts of the world including Japan, New Zealand, Italy, Spain and Norway (Table 1.19). The levels of YTX ranged from 0.01 up to 42.6 pg/cell (Mitrovic et al., 2005; Suzuki et al., 2007). YTX is the major toxin produced by *P. reticulatum* and sometimes
other analogues can be detected at lower levels (Ciminiello et al., 2003). Suzuki et al. (2007) analysed for 11 strains of *P. reticulatum* from Japan. YTX was in most cases the predominant toxin except in one strain where homo-YTX was the major toxin and none of the other analogues were detected. Two strains were found to contain trinorYTX, 1-homo YTX, trinor-1-homo YTX, and noroxoYTX enone at levels that accounted up to 50% of the total YTX. LC-MS analysis indicated that more than 90 analogues can exist in cultures of *P. reticulatum* (Miles et al., 2005).

YTX and homo-YTX have also been detected in *Lingulodinium polyedrum* (Tubaro et al., 1998; Draisci et al., 1999b). YTX was also detected in a culture of a Spanish strain of *L. polyedrum* and the levels were 10 times lower than in *P. reticulatum*. In 2006, ELISA analysis of cultured cells of *Gonyaulax spinifera* from New Zealand also detected YTXs (Rhodes et al., 2006).
Table 1.19: Examples of levels of YTX and analogues produced by cultures *P. reticulatum* determined by LC-MS. Concentrations are given in pg/cell.

<table>
<thead>
<tr>
<th><em>P. reticulatum</em> origin (ref)</th>
<th>YTX</th>
<th>Homo-YTX</th>
<th>45-OH-YTX</th>
<th>Carboxy-YTX</th>
<th>Noroxy-YTX</th>
<th>45, 46, 47, trinor YTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ (Satake et al., 1997)</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jpn (Satake et al., 1999)</td>
<td>nd and 14*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>P*</td>
</tr>
<tr>
<td>NZ (Satake et al., 1999)</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (MacKenzie et al., 2002)</td>
<td>5.5 (natural)</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I (Ciminiello et al., 2003; Ciminiello et al., 2003)</td>
<td>11.4</td>
<td>0.08</td>
<td>0.19</td>
<td>0.37</td>
<td>0.15</td>
<td>-</td>
</tr>
<tr>
<td>N (Samdal et al., 2004)</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (Canas et al., 2004)</td>
<td>0.011-0.020</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NZ (Mitrovic et al., 2005)</td>
<td>10-15 (stationary phase)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>J (Suzuki et al., 2007)</td>
<td>nd – 42.6</td>
<td>nd – 71.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd – 14.2</td>
</tr>
<tr>
<td>S (Paz et al., 2007)</td>
<td>1.8-18.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified
* sample from Yamada Bay that contained YTX also contained 45, 46, 47, trinor YTX
Table 1.20: Examples of levels of YTX and analogues produced by L. polyedrum determined by LC-MS. Concentrations are given in pg/cell.

<table>
<thead>
<tr>
<th>L. polyedrum origin (ref)</th>
<th>YTX</th>
<th>Homo-YTX</th>
<th>45-OH-YTX</th>
<th>Carboxy-YTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Tubaro et al., 1998)</td>
<td>1.1 – 1.53</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I (Draisci et al., 1999b)</td>
<td>p</td>
<td>P</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Culture Spain (Paz et al., 2004)</td>
<td>p</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- : not available; nd: not detected; p: present but not quantified

1.3.4.3. Occurrence in shellfish and geographic distribution

YTX was discovered in DG of scallops (P. yessoensis) harvested in Mutsu Bay – Japan in 1986 (Murata et al., 1987). The same species collected in 1993 from the same location allowed for the identification of the first two YTX analogues 45-hydroxy-YTX and 45, 46, 47-trinor YTX (Satake et al., 1996).

YTX was subsequently detected in mussels from Norway (Lee et al., 1988; Ramstad et al., 2001b), Chile (Yasumoto and Takizawa, 1997), New Zealand (Yasumoto and Takizawa, 1997) and in M. galloprovincialis harvested during the summer 1995 from the Adriatic sea in Italy (Ciminiello et al., 1997). Investigation of the toxin profile is the same mussel species also harvested in the Adriatic coast in the summer 1996 led to the discovery of two YTX analogues homoyessotoxin and 45-hydroxyhomoyessotoxin (Satake et al., 1997) and to the discovery of carboxyyessotoxin and of carboxyhomoyessotoxin in mussels harvested in 1997 and 1998 (Ciminiello et al., 2000a; Ciminiello et al., 2000b). The additional YTX analogues noroxohomo-YTX, noroxo-YTX, 1-desulfocarboxyhomo-YTX and 4-desulfocarboxyhomo-YTX were also identified in mussel from the Adriatic sea collected in 1997, 1998 and 2004 (Ciminiello et al., 2001; Ciminiello et al., 2002; Ciminiello et al., 2007) (structures not shown). In Norway, the analogues 1-desulpho YTX, 44,45-dihydroxy-YTX and 45-hydroxycarboxy-YTX were identified in mussels (M. edulis) (Daiguji et al., 1998; Aasen et al., 2005; Finch et al., 2005). The analogue 44, 45-dihydroxy-YTX was also reported in
mussels from New Zealand (*Perna canaliculus*) and from Canada (*M. edulis*) (Finch et al., 2005).

1.3.4.4. Toxicity

There are now strong evidences that YTX, as well as its analogue homo-YTX, are not a diarrhetic toxin when administered orally to mice (Tubaro et al., 1998; Aune et al., 2002; Tubaro et al., 2003; Tubaro et al., 2004). No human intoxication caused by YTX has been reported. Risk assessment of YTX was recently published by the FAO/IOC/WHO committee. 45-HydroxyYTX, desulphoYTX, homoYTX, carboxyYTX, carboxyhomoYTX and 45,46,47-trinorYTX are of similar acute toxicity to YTX (by ip injection). The *in-vivo* and *in-vitro* toxicity of YTX were also reviewed elsewhere (Munday et al., 2008).

1.3.4.5. Methods of detection

1.3.4.5.1. Animal tests

Similarly to the DSP toxins, AZAs and PTXs, YTX can be detected by the MBA. The method was already described in 1.3.1.5.1.

The symptoms of mice that have been administered with YTX differ from the DSP symptoms as the mice were restless after the injection and exhibited jumping prior to death (Aune et al., 2002; Tubaro et al., 2003).

Selectivity between the DSP and YTXs can be achieved through the extraction method followed by two separate MBA (Yasumoto, 2001). The two MBA protocols are shown in Figure 1.20. Protocol 1 uses diethyl ether partitioning of the extract and allows for quantitative recovery of the lipophilic toxins (OA, DTXs, AZAs and PTXs) but only partial recovery of YTX (about 50% in each fraction). After evaporation the residue is resuspended in Tween and injected in three mice. The test is considered as positive if 2 of the 3 mice die.
within 24 hours. The partitioning with 60 % methanol as proposed in protocol 2 achieved almost complete separation of the yessotoxins from the other lipophilic toxins. The major drawback of protocol 2 is that six mice (three mice for the DCM fraction and three mice for the methanol fraction) have to be used instead of three in protocol 1.

Figure 1.20: MBA protocols published by Yasumoto, 2001. After evaporation the dry residues are resuspended in Tween and injected in three mice.

The mouse protocol 2 has been proposed as a reference method in an Annex to the EC decision on DSP toxins (Anonymous, 2002).
1.3.4.5.2. Immunoenzymatic assays

ELISA methods have been developed for the detection of YTX (Briggs et al., 2004). The assay is very sensitive and allowed the detection of YTX in single cells of *P. reticulatum* (Samdal et al., 2004) as well as in shellfish (Aasen et al., 2005; Samdal et al., 2005). The quantification of YTX from shellfish using LC-MS and the ELISA assay were compared and correlated well (Samdal et al., 2005).

1.3.4.5.3. Functional assay

A cytotoxicity test for quantifying YTX was successfully applied to shellfish with a limit of detection of about 100 ng equivalent YTX/g of digestive gland (Pierroti, 2003). The test is based on the observation that the toxin induces a specific pattern of fragmentation of the cell-adhesion molecule E-cadherin in cultured cells. A second method is based on its ability to enhance phosphodiestersases acting on cyclic adenosine monophosphatase (Alfonso et al., 2004). The detection step in the procedures involves the immunoblotting of cell extracts (Pierroti 2003) or fluorimetric analysis (Alfonso et al., 2004). The expert group of the joint ECVAM/DG Sanco workshop recommended that the toxic equivalence factors for major analogues in each toxin class, and the characterisation of their performance in individual procedures should be estimated in order to facilitate the validation of cytotoxicity methods (Hess et al., 2006). In addition, the preparation of reference material for inter-laboratory studies as well as an increase in dissemination of methods and concepts will also contribute to the implementation of such methods.

1.3.4.5.4. HPLC- FLD

YTX does not possess any chromophore and similarly to OA, YTX can be derivatised in order to become amendable to HPLC fluorescence detection. Derivatisation of YTX can be achieved with 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-
triazoline-3,5-dione (DMEQ-TAD) (Yasumoto and Takizawa, 1997). This method was compared with the MBA where acetone was used as extraction solvent and partitions were performed with either diethyl ether or chloroform (Ramstad et al., 2001a). The HPLC method was found reliable, repeatable as well as comparable to the MBA when the extract was partitioned with diethyl ether to remove fatty acids and partitioned again with chloroform with the latter phase being evaporated, re-suspended and injected intraperitoneally.

1.3.4.5.5. LC-MS

1.3.4.5.5.1. Extraction method prior to LC-MS analysis

Comparison of chloroform and ethyl acetate for their efficiency to separate the toxins from polar contaminants by solvent partition indicated poor recovery of YTX in ethyl acetate. Chloroform was therefore preferred for the partition step (Goto et al., 2001). Poor extractability in chloroform and low recoveries from a silica cartridge column were reported in other studies, and clean-up of YTX was carried out on a reversed-phase cartridge column (Yanagi et al., 1989). Goto et al. (2001) reported that about 6% of YTXOH was lost in methanol–water (3:7 v/v) washing, but the remaining was recovered with propanol–water (2:8, v/v). The different extraction methods reported in the literature for the analysis of YTX are summarised in Figure 1.21.
1.3.4.5.5.2. LC-MS methods for the detection of YTXs

The first LC-MS method for the analysis of YTX was developed in 1998 (Draisci et al., 1998b). The toxin was monitored using the molecular ion [M – H]⁻ at m/z 1141 and the fragment ion [M-SO₃]⁻ at m/z 1061. The monitoring of the doubly charged anions [M-H]²⁻ at m/z 570.2 was also used as precursor ion (Canas et al., 2004). Examples of LC-MS methods reported for the analysis of YTX are summarised in Table 1.21.

While homoYTX coeluted with YTX using a mobile phase of acetonitrile modified with ammonium acetate at 4 mM (80:20), the two analytes were chromatographically separated by reducing the proportion of organic mobile phase to (60:40) (Draisci et al., 1999a). The authors reported that the sensitivity of the latter method was considerably lower than the former.
Numerous LC-MS methods have been developed using as a transition the loss of the sulphate group of YTX (Aasen et al., 2005; McNabb et al., 2005) and its analogues (Ciminiello et al., 2002; Miles et al., 2005). However, because many analogues of YTX exist, the transition corresponding to the neutral loss of the sulphate species does not provide enough selectivity for the unambiguous assignment of YTX compounds. Therefore, the monitoring of an additional transition is desirable. Other fragments that can be suitable for the selection of a second transition are shown in Figure 1.22.
Table 1.21: Examples of LC-MS methods developed for the analysis of YTXs. Linearities and LODs are given for YTX unless otherwise stated. LODs were obtained from methanolic solution unless otherwise stated.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Analytes</th>
<th>Detector</th>
<th>Source</th>
<th>Column</th>
<th>Elution</th>
<th>Flow Rate µl/min</th>
<th>V Inj. /µl</th>
<th>Linearity range / ng</th>
<th>LOD pg injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Draisci et al., 1998b)</td>
<td>YTX</td>
<td>TQ/MS² (-)</td>
<td>API</td>
<td>Supelcosil LC18-DB (5 µm; 300 x 1 mm)</td>
<td>Isocratic acetonitrile/water (80:20) + ammonium acetate (4 mM)</td>
<td>30</td>
<td>1</td>
<td>0.2 – 1.5</td>
<td>50</td>
</tr>
<tr>
<td>(Goto et al., 2001)</td>
<td>YTX, 45-OH-YTX</td>
<td>TQ/SIM (-)</td>
<td>ESI</td>
<td>Inertsil ODS-3 (5 µm; 150 x 2.1 mm)</td>
<td>Gradient MeOH/water + ammonium acetate</td>
<td>100</td>
<td>1</td>
<td>0.04 – 1.6</td>
<td>22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Ito and Tsukada, 2002)</td>
<td>YTX</td>
<td>SQ/SIM (-)</td>
<td>SSI&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Inertsil ODS-2 (5 µm; 150 x 2.1 mm)</td>
<td>Gradient MeOH/water + ammonium acetate</td>
<td>200</td>
<td>10</td>
<td>0.5 - 5</td>
<td>60</td>
</tr>
<tr>
<td>(Ciminiello et al., 2002)</td>
<td>YTX, homoYTX, 45-OH-YTX, 45-OH-homo-YTX, carboxy-YTX, YTX 45-OH-YTX</td>
<td>IT/SIM (-)</td>
<td>ESI</td>
<td>BDS Hypersil C8 (3 µm; 50 x 2.1 mm)</td>
<td>Isocratic ACN/water + ammonium formate + formic acid</td>
<td>200</td>
<td>5</td>
<td>0.3 – 7</td>
<td>70</td>
</tr>
<tr>
<td>(Fernandez et al., 2002)</td>
<td>YTX</td>
<td>IT/MS² (-)</td>
<td>ESI</td>
<td>Supelco RP Amide C16, (5 µm; 150x4.6 mm) PepMap C18 nanoLC /switching valve (3 µm; 150 mm x 75 µm)</td>
<td>Isocratic ACN/water (60:40) + ammonium acetate (0.5 mM)</td>
<td>200</td>
<td>3-5</td>
<td>0.6 – 25</td>
<td>30</td>
</tr>
<tr>
<td>(Canas et al., 2004)</td>
<td>YTX</td>
<td>QtoF/MS² – Accurate Mass (-)</td>
<td>ESI</td>
<td>X-terra MS C18 column (50x2 mm;3.5 µm)</td>
<td>Isocratic CAN/water (90:10) + ammonium acetate 1 mM</td>
<td>400</td>
<td>na</td>
<td>0.75 – 275</td>
<td>0.5</td>
</tr>
<tr>
<td>(Aasen et al., 2005)</td>
<td>YTX, 45-OH-YTX carboxy-YTX OH-carboxy-YTX</td>
<td>SQ/SIM(-)</td>
<td>ESI</td>
<td>X-terra MS C18 column (50x2 mm;3.5 µm)</td>
<td>Gradient H2O/ACN</td>
<td>300</td>
<td>5</td>
<td>na</td>
<td>10-45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Stobo et al., 2005)</td>
<td>YTX, 45-OH-YTX Homo-YTX 45-OH-homo-YTX</td>
<td>SQ/SIM(-)</td>
<td>TIS&lt;sup&gt;4&lt;/sup&gt;</td>
<td>BDS Hypersil C8 (3 µm; 50 x 2.1 mm)</td>
<td>Gradient ACN/water + ammonium acetate</td>
<td>250</td>
<td>5</td>
<td>0.03 – 0.63</td>
<td>40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(McNabb et al., 2005)</td>
<td>YTX</td>
<td>TQ/MS² (-)</td>
<td>ESI</td>
<td>Luna C18 (5 µm, 50 x 2 mm)</td>
<td>Gradient ACN/water + ammonium formate + formic acid</td>
<td>200</td>
<td>10</td>
<td>0.05 – 0.2</td>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Suzuki et al., 2007)</td>
<td>20 YTX analogues</td>
<td>QTrap/MS²</td>
<td>ESI</td>
<td>BDS Hypersil C8 (3 µm; 50 x 2.1 mm)</td>
<td>Gradient ACN/water + ammonium formate / formic acid</td>
<td>200</td>
<td>10</td>
<td>0.5 - 10</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined in scallop adductor muscle extract  
<sup>b</sup>Determined in mussel extract  
<sup>c</sup>Determined in cockle extract  
<sup>d</sup>Determined in oyster extract na: not available
1.3.5. Other lipophilic marine toxins

Other lipophilic toxins that do not fall into the group of regulated toxins are of particular interest because they interfere with the MBA by inducing rapid death of mice when injected i.p. Since efforts are made towards the replacement of the MBA it is important that the causes of eventual discrepancies between the MBA and other techniques are understood. These fast acting toxins are cyclic imine toxins and include gymnodimines, pinnatoxins, prorocentrolides, pteratoxins, spirolides and spiro-prorocentrime. No human toxicity has been associated with these compounds.
Spirolides are produced by *Alexandrium ostenfeldii* (Cembella et al., 2000) and occur worldwide including around the North Atlantic coast of Canada and USA, Scandinavia, France, Scotland and Adriatic coast (Cembella and Krock, 2007).

Gymnodimines are produced by *Karenia selliformis* (Miles 2003) and occurred in New Zealand (MacKenzie et al., 1995).

Prorocentrolides and spiro-prorocentrime were reported to be produced by *P. lima* and *P. concavum* (Faust, 1993).

Pinnatoxins and pteriatoxins have only been found in shellfish in Japan and their biological origin is unknown.

All detection methods of cyclic imines are based on mass spectrometry because of their lack of chromophores that prevented UV detection. The *in-vivo* and *in-vitro* toxicities of the cyclic imines have been reviewed (Munday, 2008). The author recommended that regulation of
Gymnodimine is not required however given the high toxicity of some spirolides (e.g. SPX-C, SPX-desMe-C and 20-methyl SPX-C) regulation is recommended. Since there is no oral toxicity available for pinnatoxins, prorocentrolides, pteriatoxins, and spiro-prorocentrimine, more information is required before a valid risk assessment can be made.

1.4. Liquid chromatography coupled to mass spectrometry

1.4.1. Conventional and ultra-fast liquid chromatography

Recent development in chemical synthesis has allowed for the production of column packing material with particle size smaller than 2 µm. This led to a new technology, termed ultraperformance-liquid-chromatography™ (UPLC), developed by Waters. The plate theory of chromatography states that chromatographic efficiency is assumed to remain constant under all conditions. However, experimental measurements show that efficiency varies with the flow rate of the mobile phase. The relationship between the height equivalent to a theoretical plate and the mobile phase velocity is given by the simplified Van Deemter equation (see Equation 1.1).

\[
\text{HEPT} = A + \frac{B}{u} + C \cdot u
\]

*Equation 1.1: Simplified Van Deemter equation*

- **HEPT** … Height Equivalent to a Theoretical Plate
- **A** … Eddy diffusion
- **B** … Longitudinal diffusion
- **C** … Non equilibrium mass transfer
- **u** … linear mobile phase velocity (cm·s\(^{-1}\))

The Eddy diffusion describes the effect of column packing on the flow path of solute molecules. According to the C term, higher efficiencies are obtained with thin stationary phase films and small diffusions distances (i.e. narrow column or small particle size). The magnitude of the C term increases in proportion to the mobile phase velocity.
According to the simplified Van Deemter equation and plot (see Equation 1, Figure 1.24), with the decrease in particle size there is a gain in efficiency, which is not limited by increased flow rates. As the pressure, which a pump needs to apply to push mobile phase through a column depends on both the particle size and the flow rate, a system with small particles and high flow rates requires very high pressure. Conventional HPLC is limited to ca. 400 bar (40 MPa) pressure. UPLC could operate up to 1000 bar (100 MPa), thus improving resolution, speed, and sensitivity (Schwartz, 2005). The UPLC system also allows operations in conventional HPLC conditions simply by using a HPLC column.

Multiple reaction monitoring (MRM) experiments using a tandem quadrupole mass spectrometer are generally considered as the best method for optimal quantitative and confirmatory analytical performance. However, the fast switching required between transitions in a multi-analyte method with the fast run times afforded by UPLC exceeds the capability of traditional instruments. The latest generation of mass spectrometers utilise a travelling wave device which clears the collision cell very rapidly between transitions, thus allowing fast switching and shorter dwell times, meaning more data points can be acquired.
across the narrow peaks. Improved electronics also allow fast polarity switching, allowing for analysis of positive and negative ions at the same retention time (Giles et al., 2004).

1.4.2. Electrospray ionisation

Ionisation from a liquid source can be produced using electrospray (ESI), thermospray or atmospheric chemical ionisation (APCI). An electrospray is produced by applying a strong electric field to the compounds dissolved in the mobile phase flowing through a capillary tube. An electric field is obtained by applying a potential difference of 3 to 6 kV between the capillary and a counter electrode (Hoffmann and Stroobant, 1999). The applied field will induce an accumulation of charges at the surface of the liquid which will break into highly charged droplets. These droplets will undergo desolvation from a curtain of heated inert gas or through a heated capillary. This desolvation will cause the droplets to shrink to smaller droplets thereby increasing the charge density, up to the point where the coulombic repulsions of the charges become so high that desorption of ions from the surface occurs. The ions are then transported in the MS where they can be detected.

The geometry of an electrospray ion source plays important roles in the processes of analyte desolvation, ionisation, transportation, and detection in a mass spectrometer. The different geometries of the MS manufacturers were presented and discussed in a recent article (Manisali et al., 2006). The ESI geometry is also believed to play an important role towards matrix effects (see next section.)

Electrospray ionisation and interfacing has been extensively reviewed in books and in peer reviewed articles (Cole, 1997; Niessen, 1999; Hoffmann and Stroobant, 1999; Volmer et al., 2002). Electrochemical and theoretical aspects of ESI with particular emphasis on the ion evaporation model can be found elsewhere (Rohner et al., 2004). Analytes characteristics amenable to ESI as well as dynamic range and stability of ESI were also reviewed (Cech and
Enke, 2001) as well as the internal energy and the fragmentation of ions produced in ESI (Gabelica and De Pauw, 2005).

1.4.3. Matrix effects

Different types of compounds can interfere with the LC-MS detection of the analytes of interest. These interferences can either be endogenous when they are part of the sample matrix (e.g., salts, lipids, proteins etc.) or exogenous when they are brought along during sample preparation and analysis; this is the case for instance of the compounds present in the mobile phase (Antignac et al., 2005). The existence of matrix effects in LC-MS analysis is a problem that must be addressed during method development and validation (Careri and Mangia, 2006) as the repercussions on the results could be dramatic. Matrix effects can potentially affect the sensitivity, specificity and accuracy of the analytical method (Bonfiglio et al., 1999; King et al., 2000; Antignac et al., 2005; Careri and Mangia, 2006) and that is why they must be dealt with. The situation with regards to matrix effects is different in MS compared to LC-UV for instance, where interferences are apparent as a peak (Bonfiglio et al., 1999; Annesley, 2003). Because in MS a specific ion/transition is monitored interferences may not be detected as such but would still affect the analysis. Matrix effects can result in either a loss or a gain of signal in MS; they are then respectively called ion suppression or ion enhancement. The former type of matrix effects is more common in MS detection and several papers dealt with this subject, trying to understand the mechanisms behind ion suppression (King et al., 2000; Taylor, 2005; Antignac et al., 2005), to assess the importance of these effects (Kloepfer et al., 2005; Taylor, 2005; Taylor, 2005; Kloepfer et al., 2005; Van De Steene et al., 2006; Matuszewski, 2006; Matuszewski, 2006; Van De Steene et al., 2006) or to propose solutions aiming at reducing/removing them (Yamaguchi et al., 1999; Bonfiglio et al., 1999; Annesley,
King et al. carried out a series of experiments aiming at understanding the mechanisms of ion suppression in ESI (King et al., 2000). The principles of ESI described in the previous section distinguish two consecutive processes: the solution-phase processes and the gas-phase processes. King et al. ruled out the gas-phase processes as being responsible for the ion suppression in biological samples (dog plasma) and reported that ion suppression is more likely to be related to non-volatile compounds (King et al., 2000). The latter act by changing the properties of the droplets and more precisely their surface tension and boiling point (King et al., 2000; Taylor, 2005; Antignac et al., 2005); this would explain why less analytes are transferred to the gas phase and remain in solution. Because of a slightly different ionisation process atmospheric pressure chemical ionisation (APCI) has been reported as generally being less prone to ion suppression than ESI (King et al., 2000; Van De Steene et al., 2006). The source geometry may also play a role in the matrix effects; orthogonal design were found to be better than an axial one that would direct the spray towards the orifice (Antignac et al., 2005). Aside from axial designs where contaminants/interferents may enter the ion paths through their mechanical flight path, the influence of other source design is likely to be minor as the ion suppression occurs at the early stage of ionisation. The separation parameters of the LC are also at stake as they will dictate the elution of the different compounds from the chromatographic system and therefore influence the co-elution of the endogenous or exogenous interferences with the analytes of interest (Antignac et al., 2005). In the case of exogenous compounds brought by the mobile phase the situation is somewhat different, as tuning the LC parameters will not remove/reduce the matrix effects unless the interference is removed from the mobile phase. Yamaguchi et al. reported that the presence of ammonium
acetate and acetic acid in the mobile phase was directly responsible for ion suppression observed in the detection of ibuprofen (Yamaguchi et al., 1999).

Different methods have been developed for the assessment of the nature and importance of matrix effect: post-column infusion (Bonfiglio et al., 1999; King et al., 2000) and post-extraction addition (King et al., 2000; Matuszewski, 2006). The post-column infusion consists of infusing the analyte of interest post-column while a matrix sample that does not contain the analyte is injected through the LC system. This dynamic method enables the identification of disturbances of the analyte base line on the MS analyser as a result of the presence of matrix interferences eluted from the column. The post extraction addition consists of fortifying a “blank” extract with a known amount of analyte. There are two variants of this technique; in the first one, sometimes referred to as “standard addition” different concentration levels of analyte are prepared at a given matrix strength. In the other variant the analyte level is kept constant across the different solutions but it is the matrix strength that is increased. In the first case one can assess the linearity of the analyser at a given matrix strength (generally the one corresponding to the extraction protocol), whereas the second case helps to predict the importance of the matrix effects at different matrix strengths.

Once the nature and the importance of the matrix effects have been assessed it is possible to proceed to the next level, that is putting in place procedures to reduce, remove or correct for these adverse effects. The solutions proposed can be categorised as (i) sample treatment, (ii) LC separation and (iii) MS detection. Different strategies have been reported to clean up biological extracts with the aim of reducing matrix effects and more specifically ion suppression. These include solid-phase extraction, liquid-liquid extraction (LLE) and protein precipitation (Bonfiglio et al., 1999; Antignac et al., 2005; Kloepfer et al., 2005; Van De Steene et al., 2006). The cleanup efficiency of the different treatments varies depending on the nature of the matrix, the analyte and the protocol developed. For instance Bonfiglio et al.
reported that LLE worked best for the detection of caffeine, phenacetin and a “Merck compound” in plasma samples, whereas acetonitrile precipitation gave the worst results (Bonfiglio et al., 1999). Another alternative would be to dilute the extract prior to LC-MS analysis in order to reduce matrix effects (Antignac et al., 2005) but this is subject to the sensitivity of the detector. On the LC side, the method can be tuned to enable a better separation of the analyte from the interferences as outlined before. The mobile phase composition should also be optimised to make sure that the buffers used will not compromise the detection through ion suppression mechanisms. Other strategies can be put in place in LC; flow splitting has been reported as an efficient way of dealing with matrix effects (Kloepfer et al., 2005; Van De Steene et al., 2006; Van De Steene et al., 2006) as it can improve the sensitivity while reducing matrix effects. However this approach has limitations; it can only be used with ESI which is concentration-dependent and not with APCI as this ionisation mechanism is quantity-dependent. Furthermore the spray can become unstable. On the detection side, based on the fact that problems arise during the ionisation this should be the maximum focus of attention. Although APCI may be less prone to matrix effects than ESI it may not be practical or possible to change from one type of source to the other. Similarly, the fact that negative mode is more specific than positive does not exclude the possibility of encountering matrix effects in both modes; furthermore by changing ionisation mode it may be possible to minimise/reduce matrix effect but at an unacceptable cost which is the loss of sensitivity.

Although matrix effects have been extensively studied in biological matrices and aqueous environment for pharmaceuticals, little is known about marine biotoxins. In order to protect human consumers the presence of these bioactive compounds in shellfish is regulated in the European Union (EU) (Anonymous, 2004a). Despite the fact that the mouse bioassay is still
the reference method for the analysis of lipophilic marine toxins, the EU Regulation 2074/2005 (Anonymous, 2005a) allows the use of alternative methods such as LC-MS. However no mention has been made of matrix effects. There is no legal requirement for the assessment of matrix effects or for their correction. Matrix effects were reported to have occurred in Greenshell mussel (*Perna canaliculus*), Pacific oyster (*Crassostrea gigas*), NZ cockle (*Austrovenus stutchburyi*) and scallop roe (*Pecten novaezelandiae*) (McNabb et al., 2005). Less than 10% enhancement were observed for OA and GYM in all tissues; 10-12% enhancement for PTX2; and 21-23% suppression for YTX. The authors also mentioned that the effects could be reduced by dilution in 80% MeOH. Using the same type of source but different LC-MS conditions, serious matrix effects observed in positive mode especially for DTX3 (Suzuki et al., 2005). Using a Turbo-ion spray Fernandez-Puente et al. (2004) mentioned that the post-column infusion experiment carried out with OA and a blank mussel extract did not show any matrix effect in tandem MS, whereas Stobo et al. (2005) reported significant matrix effects with the same interface but in single MS. There was 80-130% ion enhancement for OA in the assessed matrices (mussels, cockles, oysters, scallops); 27-48% ion suppression for AZA1 and 0-25% ion suppression for YTX. The differences observed in matrix effects between the above mentioned papers can be related to the shellfish and the experimental conditions used. Despite the fact that these studies are not directly comparable it can be noticed that the nature of the matrix effects was consistent with ion enhancement for OA and PTX2, and with ion suppression for AZA1 and YTX. Ito et al. (2002) also observed ion suppression for YTX using a sonic-spray interface but reported ion suppression for OA, DTX1 and PTX6 in scallops.
1.5. Passive sampling for environmental monitoring

Monitoring of pollutants in different environments (soil, outdoor and indoor air, sediments marine and fresh water) led to the development of devices that can provide a spatially and temporally integrated response as a result of passive adsorption. The passive sampling approach consists of the in-situ deployment of relatively small and simple devices that can have long exposure periods in a given medium without need of energy. Analytes are accumulated in a selected medium known as receiving phase, that can be a solvent, reagent or sorbent. Passive sampling devices (PSD) can be mailed to the laboratory where after-sampling treatment and analysis is performed, the result can be either a time averaged concentration or the concentration at which the device is at equilibrium depending on the device design (Vrana et al., 2001). Passive sampling was first introduced in the 1970’s to monitor air quality and the first passive sampler method for the aquatic environment was developed by Sodergren in 1987 at the University of Lund, Sweden. It is a simple device consisting of a tube of dialysis membrane (regenerated cellulose) that contains three milliters of organic solvent, typically hexane. The recent development of passive sampling in environmental studies including soil, air and water media have been reviewed (Namiesnik et al., 2005; Kot-Wasik et al., 2007). PSD for monitoring organic micropollutants specifically in the aquatic environment have also been reviewed (Stuer-Lauridsen, 2005).

1.5.1. Principles of passive samplers

The kinetic considerations of passive sampling described in this section are based on reviews on passive sampling techniques for monitoring pollutants in water (Vrana et al., 2005; Kot-Wasik et al., 2007; Madrid and Zayas, 2007).

The kinetic exchange between the passive sampler and the water phase can be described as a first order reaction shown in Equation 1.2.
\[
Cs(t) = C_w \frac{k_1}{k_2} (1 - e^{-k_2 t})
\]

Equation 1.2: Model of kinetic exchange between passive sampler and water phase

Where \( Cs(t) \) is the concentration of analyte in the passive sampler at time \( t \), \( C_w \) is the analyte concentration in the aqueous medium and \( k_1 \) and \( k_2 \) are the uptake and offload rate constants, respectively. Two accumulation regimes must be considered when passive sampling is used, the kinetic and the equilibrium regime, as shown in Figure 1.25. Most PSD possess a barrier between the sampling environment and the receiving phase which determines the rates at which the analytes are accumulated at given concentrations as well as, in some cases, providing enhanced selectivity towards a given class of compounds. Two types of samplers are distinguished according to the barrier type i) diffusion barrier where accumulation of analyte is obtained through diffusion through a static water layer contained in well-defined openings in the sampler and ii) permeation barrier where accumulation takes place through a porous or non-porous membrane.

Figure 1.25: Accumulation regimes in passive sampling
1.5.1.1. Kinetic regime

In the kinetic regime it is assumed that the desorption of analyte from the receiving phase is negligible and therefore Equation 1.2 reduces to the linear equation, as shown in Equation 1.3.

\[ C_s(t) = C_w k_1 t \]

**Equation 1.3: Kinetic model of passive sampler accumulation in kinetic regime**

Equation 1.3 can be rearranged to express the amount of analyte accumulated after a given exposure time.

\[ Ms(t) = CwR_s t \]

**Equation 1.4: Mass of analyte accumulated at exposure time t in kinetic regime**

Where \( Ms(t) \) is the mass of analyte accumulated in the passive sampler after exposure time \( t \) and \( R_s \) is the sampling rate. The sampling rate is the product of the first-order rate constant \( (k_1) \) and the volume of water that has been in contact with the passive sampler. For most PSD that operate in kinetic mode, the sampling rate is independent of the concentration of the media (Kot-Wasik et al., 2007). However, the sampling rate is affected by water flow, turbulences, temperature and biofouling (Vrana et al., 2005). The sampling rate is characteristic of the individual analyte of interest under given environmental conditions and can be estimated from Equation 1.5.

\[ R_s = k_0 A = k_e k_{DW} V_D \]

**Equation 1.5 : Sampling rate for membrane passive sampler**

Where \( k_0 \) is the overall mass transfer coefficient; \( A \) the surface area of a membrane, \( k_e \) the overall exchange rate constant, \( k_{DW} \) the receiving phase/water partition coefficient and \( V_D \) the volume of receiving phase. Since the majority of PSD are developed for the monitoring of
trace compounds, high sampling rate is required, key parameters are large surface area and the limitation of layers between the receiving phase and the medium. In environmental studies, any layer that contributes for more than 50% of the total resistance is considered uptake rate limiting (Kot-Wasik et al., 2007). One requirement for PSD that operate in kinetic regime is that the sampling time should be less than $t_{50}$ (Figure 1.25).

1.5.1.1.1. Calibration in kinetic regime

A review on the configurations and calibration method for passive sampling technique has been reviewed recently (Ouyang and Pawliszyn, 2007). In the theory, calibration in the kinetic regime requires the reproduction of environmental conditions. Therefore, water flow and temperature of the exposure media should be reproduced. Some studies reported the use of a constant flow rate by a circulation system through a peristaltic pump (Vrana and Schuurmann, 2002; Vrana et al., 2006a) at constant temperature for the calibration of solid phase micro-extraction (SPME) and membrane enclosed sorptive coating (MESCO) PSD (Vrana et al., 2001). The serial batch extraction is another approach to calibration and consists in reproducing a flow rate through a rotary agitation (Paschke et al., 2006). The medium is replaced every 48 h to ensure that the concentration of analyte in the media remains constant. Although these systems provide a good model on adsorption and flow rate dependencies, the influence of biofouling is not considered in these calibrations.

Similarly to quantitation in chromatographic sciences, an internal standard approach was developed, where a performance reference compound (PRC) was spiked to the passive sampler prior to its deployment (Huckins et al., 2002). PRC are analytically non-interfering organic compounds with moderate to low affinity to the passive sampler that are added to the receiving phase of the sampler prior to membrane enclosure. This approach is based on theory
and experimental evidence that PRC dissipation rate constants are related to the uptake rates of target compounds (Vrana et al., 2006b).

This approach has successfully been used for the monitoring of polyaromatic hydrocarbons, polychlorinated biphenyls and organochloride pesticides using MESCO (Vrana et al., 2006b) and for the monitoring of polyaromatic hydrocarbons and organochlorine pesticides using C18 Empore® disk (Vrana et al., 2006a).

1.5.1.2. Equilibrium regime

When the exposure time is sufficiently long, the passive sampler is operating in the equilibrium regime and Equation 1.2 reduces to Equation 1.6.

\[
C_s = C_w \frac{k_1}{k_2} = C_w K
\]

*Equation 1.6: Kinetic model of passive sampler accumulation in equilibrium regime*

The dissolved analyte concentration can be estimated from the receiving phase/water partition coefficient (\(K\)). Passive samplers operating in the equilibrium regime require that response time must be shorter than the analyte concentration fluctuations and that a stable concentration (below the maximum receiving phase capacity) is reached after a known exposure time (Mayer, 2003). Example of PSD that operates in the equilibrium regime are passive diffusion bag samplers (PDBSs) that have been used extensively for the monitoring volatile organic compounds (ITRC Diffusion Samplers Team, 2004; Vrana et al., 2005). The PDB sampler is a low-density polyethylene bag filled with deionized water, which acts as a semipermeable membrane and is suspended in a well to passively collect groundwater samples.
2. Development of LC-MS based methods for the detection of lipophilic marine toxins

This chapter deals with the development of various LC-MS based methods for the detection of lipophilic marine toxins. The methods presented have been developed for two different purposes,

1) the development of a new method suitable for shellfish monitoring that include all regulated lipophilic marine toxins

2) Methods that do not include all regulated toxins and that were developed in order to assess and minimise matrix effects

The methods that are included in this chapter were developed on three different mass spectrometers. A Hybrid quadrupole Time of Flight MS and a triple quadrupole Quattro Ultima MS that were available at the Marine Institute as well as a latest generation triple quadrupole Premier XE. The work on the latter instrument was carried out in Waters Corp. premises in Manchester – UK.

2.1. Development of LC-MS method with Time of Flight Mass spectrometer detection

The aim of this section was to develop isocratic methods for the separation of lipophilic marine toxins. Attention was brought to isocratic methods because a more stable spray can be obtained compared to a gradient elution method since the mobile phase composition remains unchanged during the time of analysis. It is expected that a more stable spray can enhance precision and reduce the bias introduced when toxins that are not commercially available as reference material are quantified against their analogue in gradient elution methods. Furthermore, the commonly used post-column infusion for the study of matrix effects also
requires acquisition with a stable ESI spray and will be considered in Chapter 3 for the
description of matrix effects.

2.1.1. Material and methods

2.1.1.1. Solvents and reagents

Acetonitrile and methanol were purchased as Pestiscan-grade solvents from Labscan (Dublin,
Ireland). Formic acid and ammonium formate were obtained from Sigma-Aldrich (Steinheim,
Germany). Water was obtained from a reversed-osmosis purification system (Barnstead,
Dublin, Ireland). OA and PTX2 certified reference materials (CRM) were purchased from the
National Research Council (Halifax, Canada). The AZA1 standard solution was purified at
the Marine Institute from naturally contaminated mussels.

2.1.1.2. LC-MS Instrumentation Acquity HPLC – QTof Ultima

HPLC analyses were performed using a Waters Acquity system. Binary mobile phase was
used, with phase A (100 % aqueous) and phase B (95 % aqueous acetonitrile), both
containing 2 mM ammonium formate and 50 mM formic acid.

The HPLC separation was achieved using a BDS Hypersil C8 column (50 × 2.1 mm, 3 µm
particle size; guard column, 10 x 2.1 mm, 3 µm particle size). The flow rate was set at
0.25 ml/min and a volume of 5 µL was injected. The column temperature was 25°C and the
sample temperature was 5°C. Three different elution conditions were used:

A1. A gradient elution was employed, starting with 30 % B, rising to 90 % B over
8 min, held for 0.5 min, then decreased to 30 % B in 0.5 min and held for 3 min to
equilibrate at initial conditions before the next run started
A2. A step gradient elution was carried out starting with 57 % B until 4.5 min, rising to 75 % B over 0.5 min, held for 10 min, decreased to 57 % in 0.5 min and held for 3 min before the next injection. All other conditions remained as in A1.

A3. A step gradient using mobile phases where no formic acid was used was carried out starting with 45 % B until 7.5 min, rising to 70 % in 0.5 min, held for 10 min, decreased to 45 % in 0.5 min and held for 3 min before the next injection. All other conditions remained as in A1.

The MS used was a Micromass Q-TOF Ultima (quadrupole-time-of-flight hybrid), equipped with a z-spray ESI source. The acquisition mode was a fragment ion scan, where the precursor ion is isolated by the first quadrupole, fragmented in the collision cell, and the final fragmentation spectrum obtained by the TOF. The transitions are shown in Table 2.1. The QTof Ultima does not have the capability of switching mode during a chromatographic run and therefore acquisitions in positive and negative ionisation were obtained in two separate analyses.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Transition for precursor ion</th>
<th>Detected ion form of precursor ion</th>
<th>Scan window for fragment ions (m/z)</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>803.5</td>
<td>[M-H]−</td>
<td>200 - 850</td>
<td>negative</td>
</tr>
<tr>
<td>DTX1</td>
<td>817.5</td>
<td>[M-H]−</td>
<td>200 - 850</td>
<td>negative</td>
</tr>
<tr>
<td>DTX2</td>
<td>803.5</td>
<td>[M-H]−</td>
<td>200 - 850</td>
<td>negative</td>
</tr>
<tr>
<td>PTX2</td>
<td>876.5</td>
<td>[M+NH₄]⁺</td>
<td>200 - 900</td>
<td>positive</td>
</tr>
<tr>
<td>AZA1</td>
<td>842.5</td>
<td>[M+H]⁺</td>
<td>300 - 900</td>
<td>positive</td>
</tr>
<tr>
<td>AZA2</td>
<td>856.5</td>
<td>[M+H]⁺</td>
<td>300 - 900</td>
<td>positive</td>
</tr>
<tr>
<td>AZA3</td>
<td>828.5</td>
<td>[M+H]⁺</td>
<td>300 - 900</td>
<td>positive</td>
</tr>
</tbody>
</table>
2.1.2. Results

2.1.2.1. HPLC Gradient method (conditions A1)

The chromatographic separation of OA, DTX2, DTX1, PTX2 and AZA1 using conditions A1 is shown in Figure 2.1. OA and DTX2 are isomers and therefore require complete chromatographic separation. The conditions used allowed for complete resolution of OA and DTX2 with a resolution factor of 1.87. It must be emphasised that analysis of the OA group toxins was performed in negative ionisation mode and PTX2 and AZA1 analyses were performed in the positive ionisation mode. PTX2 eluted between DTX2 (4.86 min) and DTX1 (5.76 min). AZA1 eluted later at 9.42 min and AZA2 and AZA3 were not monitored in this study. The peak shapes obtained for all toxins were satisfactory.

Figure 2.1: Separation of OA, DTX2, DTX1, PTX2 and AZA1 using the gradient method
2.1.2.2. HPLC Step gradient (Condition A2)

Several conditions were attempted in the course of the development of the isocratic method and the resolution obtained between OA and DTX2 was systematically calculated. The elution consisting of 60 % B did not allow for a complete separation of OA and DTX2 ($R = 1.25$) (data not shown). Acceptable resolution ($R \geq 1.5$) was obtained with a maximum of 57 % B (Figure 2.2). In these conditions OA eluted at 1.94 min, DTX2 at 2.29 min and DTX1 at 3.58 min. Similarly as the previous condition, PTX2 eluted between DTX2 and DTX1 with a retention time of 2.53 minutes. The AZAs analogues eluted much later with AZA1, AZA2 and AZA3 eluting at 28.15, 18.37 and 36.40 min, respectively (Figure 2.3). Although the peak shapes were acceptable the peak width of AZA2 was exceeding 3 min.

The introduction of a step gradient method consisting of an initial elution at 57 % B and a rapid raise to 75 % B allowed for the separation of OA and DTX2 as well as an acceptable retention time for AZA1 (Figure 2.4). The peak width of AZA1 was reduced from 2.5 min at 57 % B to 30 sec at 75 % B.

![Figure 2.2: Chromatogram at 57% B of compounds ionisable in negative mode with acidic mobile phase](image-url)
2.1.2.3. HPLC step gradient without formic acid (Conditions A3)

The selectivity of the step gradient method was modified by changing the pH of the mobile phases. Formic acid was not added to the mobile phase, which resulted in a pH of 5.83 and
7.45 for phase A and B, respectively. The higher pH of the mobile phase B was a consequence of the pH calibration in aqueous media and measurement in non-aqueous solution. This is further discussed in chapter 7. The change in pH allowed for resolution of PTX2 and DTX1 which was not achieved in the two previous methods. Thus, the resolution factor between PTX2 and DTX1 at 45 % B was 4.51 demonstrating complete separation. At 45 % the AZA analogues eluted after 20 min (data not shown). The introduction of a step gradient where the concentration in mobile phase B was rapidly rose from 45 to 70 % allowed for complete separation of OA, DTX2, -1, PTX2, AZA1, -2 and -3 in a 21.5 min run, as shown in Figure 2.5. The retention times ranged from 1.69 min for OA up to 16.5 min for AZA2.
Figure 2.5: Separation of OA, DTX1, DTX2, PTX2 and AZA1-3 from in-house LRM on the BDS Hypersil C8 with step gradient elution using neutral mobile phase. Step gradient consisted of 45% B and was raised to 70% B at 7.5 min. The total run time was 21 min.

2.1.3. Discussion

The gradient method described in this section (condition A1) is identical to the reference method used for the national shellfish monitoring in Ireland. Several research groups reported the use of the Hypersil C8 column in conjunction with the 2 mM ammonium formate / 50 mM formic acid buffer (Table 1.9). A 20 to 50-fold sensitivity enhanced sensitivity was obtained for OA analysed in the negative ionisation mode than when TFA was used and OA analysed in the positive mode (Quilliam et al., 2001). However, complete separation of DTX1 and
PTX2 cannot be achieved at this pH. Although this may be acceptable for the new generation MS QqQ instruments with capabilities of rapid switching (20 ms), older instruments can not afford to monitor compounds in positive and negative mode at the same time. Therefore, the use of mobile phase at higher pH, as in conditions A3, allow for the analysis of the OA and AZA group toxins as well as for PTX2.

Many LC-MS applications employ gradient elution for the separation of compounds as it allows for rapid elution of analytes with different polarities. The use of a gradient elution method for quantitation purposes is well suited in many cases where the sample is quantified against the analyte standard solution or its isotopically labelled analogue. However, the quantification of a compound against a structurally related compound with a different retention time introduced a bias due to the difference in ionisation efficiencies at different mobile phase composition. As mentioned in the introduction, certified reference solutions are not available for some of the regulated toxins (e.g. DTX1, DTX2, AZA2, AZA3). Therefore, quantitation is performed against their structurally related analogues that are commercially available (e.g. OA, AZA1). The use of a step gradient method can overcome the issue of ionisation efficiency differences while maintaining acceptable analysis time of less than 20 min per sample. The influence of such methods will be further investigated in the next chapter with regards to the extent of matrix effects in shellfish samples.
2.2. Development of a UPLC-MS method using Quattro Ultima

2.2.1. Materials and methods

2.2.1.1. Solvents and reagents

The solvents and reagents used were identical to those described in section 1.1.

2.2.1.2. UPLC conditions Acquity HPLC/UPLC – TQ (Quattro Ultima)

HPLC/UPLC analyses were performed using a Waters Acquity system. Binary mobile phase was used, with phase A (100 % aqueous) and phase B (95 % aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid.

B1. The HPLC separation was achieved using a BDS Hypersil C₈ column (50 x 2.1 mm, 3 µm particle size; guard column, 10 x 2.1 mm, 3 µm particle size). The flow rate was set at 0.25 ml/min and a volume of 5 µL was injected using a partially filled 10 µL loop. The column temperature was 25°C and the sample temperature was 5°C. A gradient elution was employed, starting with 30 % B, rising to 90 % B over 8 min, held for 0.5 min, then decreased to 30 % B in 0.5 min and held for 3 min to equilibrate at initial conditions before the next run started.

B2. The UPLC separation was achieved on an Acquity UPLC BEH C₈ column (100 x 2.1 mm, 1.7 µm particle size, in-line filter 0.2 µm). The flow rate was set at 0.4 ml/min and a volume of 5 µL was injected in a partially filled 10 µL loop. The column temperature was 30°C and the sample temperature was 5°C. The gradient started at 30 % B, raised to 90 % B in 3 min, and was held for 1.5 min. After
decreasing to 30 % B in 0.1 min the system was equilibrated for 2 min before the next run started. A total run time of 6.6 min was achieved.

B3. The UPLC separation was achieved on an UPLC BEH C\textsubscript{18} column (100 × 2.1 mm, 1.7 µm particle size, in-line filter 0.2 µm) using the same chromatographic conditions as described in B2.

2.2.1.3. MS detection

A triple quadrupole Quattro Ultima and a hybrid quadrupole-time of flight (Q-Tof) Ultima (Micromass Ltd., Manchester, UK) both equipped with a z-spray ESI source were used. Multiple-reaction-monitoring (MRM) acquisition mode was used for quantification using the triple quadrupole, analysing two fragment ions for OA and AZA1 and one fragment ion for PTX2. Monitored transitions were as follows (precursor > fragment): OA 803.5>255.5 and 803.5>803.5 in negative ionisation mode; AZA1 842.5>654.4 and 842.5>672.4 and PTX2 876.5>823.5 in positive ionisation mode.

2.2.2. Results

2.2.2.1. HPLC gradient (Conditions B1)

The conditions B1 are identical to the conditions A1 described above, the only difference being the type of mass spectrometer used for the detection. Slightly different retention times were obtained due to the difference in the void volumes and tubing of each system. OA, PTX2 and AZA1 eluted at 4.49, 5.12 and 9.67 min, respectively (Figure 2.6). The best peak symmetry using HPLC was observed for OA, followed by AZA1 and PTX2, respectively (Table 2.2). Complete separation of OA, PTX2 and AZA1 was achieved.
Table 2.2: Retention times, tailing factors and resolution of OA (10.7 ng/mL), PTX-2 (3.60 ng/mL) and AZA-1 (5.91 ng/mL) obtained from the HPLC method, n=7.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>RT (min) ± SD</th>
<th>Tailing factor ± SD</th>
<th>Resolution ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>4.47 ± 0.01</td>
<td>1.08 ± 0.11</td>
<td>R_{OA/PTX2} = 2.62 ± 0.24</td>
</tr>
<tr>
<td>PTX2</td>
<td>5.12 ± 0.02</td>
<td>1.21 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>AZA1</td>
<td>9.52 ± 0.13</td>
<td>1.12 ± 0.13</td>
<td>R_{PTX2/AZA1} = 17.9 ± 1.8</td>
</tr>
</tbody>
</table>

Figure 2.6: Example of chromatogram obtained using the HPLC-QqQ method, run time = 12 min

The method was assessed for its quantification performances using a set of 7 standards of OA, PTX2 and AZA1. The calibration range, slopes, intercepts, correlation coefficients (R^2), LOD (S/N = 3) and LOQ (S/N = 10) are given in Table 2.3. Excellent correlation coefficients were obtained with R^2 > 0.99 for the three toxins. The coefficient of variations obtained for the calibration curves (calculated from triplicate injection) were 13, 11 and 9 % for OA, PTX2 and AZA1, respectively. The LOD and LOQ obtained for OA (0.44 and 1.46 ng/ml,
respectively) were 10 fold higher than those obtained for PTX2 (0.04 and 0.14 ng/ml) and AZA1 (0.02 and 0.08 ng/ml).

Table 2.3: Slopes, intercepts, correlation coefficients, LODs and LOQs for the HPLC calibration, values calculated using a set of 7 standards (n=3) obtained during standard addition sequence

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Slope ± SD</th>
<th>Intercept ± SD</th>
<th>Correlation coefficient ± SD</th>
<th>LOD* (pg/mL)</th>
<th>LOQ* (pg/mL)</th>
<th>Range of standards (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>77 ± 10</td>
<td>-14 ± 152</td>
<td>0.9987 ± 0.0016</td>
<td>438</td>
<td>1460</td>
<td>2.7 - 272</td>
</tr>
<tr>
<td>PTX2</td>
<td>771 ± 81</td>
<td>119 ± 405</td>
<td>0.9994 ± 0.0007</td>
<td>43.2</td>
<td>140</td>
<td>0.9 - 91</td>
</tr>
<tr>
<td>AZA1</td>
<td>1458± 132</td>
<td>-1947± 530</td>
<td>0.9995 ± 0.0001</td>
<td>23.2</td>
<td>80</td>
<td>1.5 - 50</td>
</tr>
</tbody>
</table>

* LOD and LOQ were calculated from the lowest standard at S/N=3 and S/N=10, respectively.

2.2.2.2. UPLC gradient with C8 column (Conditions B2)

Figure 2.7: Example of chromatogram obtained using the UPLC-QqQ method, C₈ BEH column, run time = 6.6 min
Figure 2.8: Separation of LRM containing OA, DTX1, DTX2, DTX2, PTX2 and AZA1-3 on the BEH C8 column

Satisfactory separation between OA, PTX2 and AZA1 was obtained by UPLC using the C8 column (Figure 2.7, Table 2.4). Best peak symmetry using UPLC-C8 was observed for AZA1, followed by OA and PTX2, respectively. For all three toxins good separations were achieved, where the resolution factor was 3.08 between PTX2 and AZA1 and 2.38 between OA and PTX2.

However, when DTX2, DTX1 and AZA3 were added to the method, co-elution occurred. The limitation in the speed of positive/negative ionisation switching of the MS resulted in too few data points being acquired (< 15) and poor peak shapes (Figure 2.8). The Dwell time was set to the minimum of 15 ms.
Table 2.4: Retention times, tailing factors and resolution of OA (10.7 ng/mL), PTX2 (3.60 ng/mL) and AZA1 (5.91 ng/mL) obtained from the UPLC C₈ method, n=8

<table>
<thead>
<tr>
<th>Toxin</th>
<th>RT (min) ± SD</th>
<th>Tailing factor ± SD</th>
<th>Resolution ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>2.72 ± 0.01</td>
<td>1.30 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>PTX2</td>
<td>2.99 ± 0.01</td>
<td>1.31 ± 0.21</td>
<td>$R_{OA/PTX-2} = 2.38 ± 0.14$</td>
</tr>
<tr>
<td>AZA1</td>
<td>3.35 ± 0.03</td>
<td>1.23 ± 0.11</td>
<td>$R_{PTX-2/AZA-1} = 3.08 ± 0.27$</td>
</tr>
</tbody>
</table>

The method was assessed for its quantification performances using a set of 7 standards of OA, PTX2 and AZA1. The calibration range, slopes, intercepts, correlation coefficients ($R^2$), LOD ($S/N = 3$) and LOQ ($S/N = 10$) are given in Table 2.5. Similarly to conditions B1, excellent correlation coefficients were obtained ($R^2 > 0.999$). The coefficients of variations obtained from triplicate injection of the calibration solutions were 9, 6 and 6 % for OA, PTX2 and AZA1. The LOD and LOQ obtained for OA using the UPLC-C8 method were lower than when using the HPLC method (conditions B1) by a factor 2.5. However, the LOD and LOQs for PTX2 and AZA1 were higher in conditions B1.
Table 2.5: Slopes, intercepts, correlation coefficients, LODs and LOQs for the UPLC-C₈ calibration, values calculated using a set of 7 standards (n=3) obtained during standard addition sequence

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Slope ± SD</th>
<th>Intercept ± SD</th>
<th>Correlation coefficient ± SD</th>
<th>LOD* (pg/mL)</th>
<th>LOQ* (pg/mL)</th>
<th>Range of standards (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>64 ± 6</td>
<td>14 ± 121</td>
<td>0.9994 ± 0.0002</td>
<td>175</td>
<td>580</td>
<td>2.7 - 272</td>
</tr>
<tr>
<td>PTX2</td>
<td>370 ± 24</td>
<td>126 ± 131</td>
<td>0.9996 ± 0.0002</td>
<td>99.6</td>
<td>330</td>
<td>0.9 - 91</td>
</tr>
<tr>
<td>AZA1</td>
<td>712 ± 46</td>
<td>-498 ± 384</td>
<td>0.9995 ± 0.0004</td>
<td>50.8</td>
<td>170</td>
<td>1.5 - 50</td>
</tr>
</tbody>
</table>

* LOD and LOQ were calculated from the lowest standard at S/N=3 and S/N=10, respectively

2.2.2.3. UPLC gradient with C18 column (Conditions B3)

![Figure 2.9: Example of chromatogram obtained using the UPLC-QqQ method, C₁₈ column, run time = 6.6 min](image)

The retention times obtained for OA, PTX2 and AZA1 with the UPLC-C18 column were 0.3 to 0.4 min longer than those obtained with the UPLC-C8 column. OA, PTX2 and AZA1 eluted at 3.00, 3.26 and 3.72 min (Figure 2.9). Best peak symmetry using UPLC-C₁₈ was observed for OA, followed by AZA1 and PTX2, respectively. Good resolution was achieved
between PTX2 and AZA1 (4.22), while the resolution between OA and PTX2 was 2-times lower (2.17) (Table 2.6).

Table 2.6: Retention times, tailing factors and resolution of OA (10.7 ng/mL), PTX2 (3.60 ng/mL) and AZA1 (5.91 ng/mL) obtained from the UPLC-C₁₈ method, n=8

<table>
<thead>
<tr>
<th>Toxin</th>
<th>RT (min) ± SD</th>
<th>Tailing factor ± SD</th>
<th>Resolution ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>2.98 ± 0.02</td>
<td>1.25 ± 0.13</td>
<td>R_{OA/PTX-2} = 2.17 ± 0.15</td>
</tr>
<tr>
<td>PTX2</td>
<td>3.24 ± 0.02</td>
<td>1.57 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>AZA1</td>
<td>3.76 ± 0.04</td>
<td>1.31 ± 0.12</td>
<td>R_{PTX-2/AZA-1} = 4.22 ± 0.30</td>
</tr>
</tbody>
</table>

The method was assessed for its quantification performances using a set of 7 standards of OA, PTX2 and AZA1. The calibration range, slopes, intercepts, correlation coefficients (R^2), LOD (S/N =3) and LOQ (S/N = 10) are given in Table 2.7. Excellent correlation coefficients were obtained (R^2 > 0.99). The coefficients of variations obtained from triplicate injection of the calibration solutions were 5, 13 and 8 % for OA, PTX2 and AZA1. Quantification of OA using the UPLC-C18 method was significantly more repeatable than when the HPLC and the UPLC-C8 methods were used. Similarly as for the conditions B2, analysis of OA by UPLC enabled lower LOD and LOQs to be reached for OA compared to HPLC. The LOQ of AZA1 remained unchanged compared to HPLC (0.08 ng/ml) and was higher for PTX2 (0.34 ng/ml) (Table 2.7).
Table 2.7: Slopes, intercepts, correlation coefficients, LODs and LOQs for the UPLC-C₁₈ calibration, values calculated using a set of 7 standards (n=3) obtained during standard addition sequence

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Slope ± SD</th>
<th>Intercept ± SD</th>
<th>Correlation coefficient ± SD</th>
<th>LOD* (pg/mL)</th>
<th>LOQ* (pg/mL)</th>
<th>Range of standards (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>77±4</td>
<td>58±140</td>
<td>0.9946±0.0071</td>
<td>298</td>
<td>990</td>
<td>2.7 - 272</td>
</tr>
<tr>
<td>PTX2</td>
<td>225±30</td>
<td>168±292</td>
<td>0.9923±0.0106</td>
<td>103</td>
<td>340</td>
<td>0.9 - 91</td>
</tr>
<tr>
<td>AZA1</td>
<td>1017±77</td>
<td>-1095±1109</td>
<td>0.9967±0.0052</td>
<td>24.2</td>
<td>80</td>
<td>1.5 - 50</td>
</tr>
</tbody>
</table>

* LOD and LOQ were calculated from the lowest standard at S/N=3 and S/N=10, respectively

2.2.3. Discussion

The results presented above show the capability of the UPLC to reduce analysis time. However, the fast switching required between transitions in a multi-analyte method with the fast run times afforded by UPLC exceeds the capability of traditional instruments. Thus, good peak shapes were obtained using the UPLC when OA, PTX2 and AZA1 were included in the method since fast switching was not required. When additional analytes were incorporated into the method, the resulting co-elution of, for example, DTX1 and PTX2 required fast switching and resulted in bad peak shapes.

Quantitation using UPLC (C8 or C18 column) demonstrated a better repeatability as well as lower LODs for OA. For AZA1 and PTX2 the LODs obtained were higher when the UPLC C8 method was used compared to the HPLC method. The LOD of PTX2 using the UPLC C18 method was almost identical to the one using the UPLC C8 column while for AZA1 it remained the same as for the HPLC method.

When using the HPLC method the LOQ obtained for OA, PTX2 and AZA1 were equivalent to 14.6, 1.4 and 0.8 µg/kg which are 11, 114 and 200 times below the regulatory limit when a
SSR 10 is employed for shellfish extraction (WF). Therefore, only an increased limit of quantification would be desirable for OA. PTX2 is a regulated toxin and only low levels are found when detected in shellfish and the LOQ of AZA1 was quite satisfactory. Therefore the use of UPLC with the Quattro Ultima could provide a solution to improve the LOQ for OA while maintaining LOQ well below the regulatory limit for PTX2 and AZA1.

The slow positive negative ionisation mode switching can however limit the use of such method for monitoring purposes with traditional instruments. The next section demonstrates the applicability of UPLC with a latest generation mass spectrometer.

2.3. Development of an Ultra Performance Liquid Chromatography coupled to mass spectrometry – Premier XE

2.3.1. Material and methods

2.3.1.1. Reagents

HPLC grade methanol and water were purchased from Fischer Scientific, Loughborough, UK. Chromasolv HPLC Acetonitrile (ACN) and ammonium formate (97 %) were purchased from Sigma-Aldrich, Steinheim, Germany and formic acid (98 %) from BDH laboratory, Poole, UK. OA, PTX2, YTX, gymnodimine (GYM), 13-desMe-C spirolide (SPX 13-desMe-C) certified calibration solutions and mussel tissue reference material (CRM) CRM-DSP-Mus-b were obtained from the National Research Council (NRC), Halifax, Canada. AZA1 was isolated in the Marine Institute from naturally contaminated mussels from the South West of Ireland.
2.3.1.2. UPLC-MS/MS analysis

An Acquity UPLC system coupled to a Quattro premier XE mass spectrometer (Waters-Micromass, Manchester, UK) equipped with a Z-Spray ESI source was used. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm) equipped with an in-line 0.2 µm Acquity filter. Mobile phase A was 100 % aqueous and mobile phase B 95 % aqueous ACN, both containing 2 mM ammonium formate and 50 mM formic acid (Quilliam et al., 2001). A gradient from 30 % B rising to 90 % B was run over 3 min and then held for 1.5 min. At 4.5 min the gradient was set back to the initial composition and equilibrated for 2 min. The flow rate was set at 0.4 ml/min and 10 µl of each sample (maintained at 5ºC) were injected onto the column at 30 °C. The electrospray source was operated simultaneously in both positive and negative mode by rapid switching. The capillary potential was set at 2.5 kV, desolvation temperature 350 °C, source temperature 120 °C, desolvation gas flow 850 l N$_2$/h, cone gas flow 50 l N$_2$/h. All analyses were performed in MRM mode with a collision gas flow at 0.32 l/h.

When toxins were available in sufficient amount, cone voltages and collision energies were optimised on two fragment ions by infusion (quantification and confirmatory transitions). When toxins were not available in sufficient amount for infusion, one MRM transition was set based on theoretical calculation or on those reported in the literature (MacKenzie et al., 2005; Hess et al., 2005b). Cone voltages and collisions energies were set to those used for compounds with similar structures. Transitions, cone voltages and collision energies used in the method are reported in Table 2.8.
<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM</th>
<th>Mode</th>
<th>Cone voltage / V</th>
<th>Collision Energy / eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>GYM</td>
<td>508.3 &gt; 392.4, 508.3 &gt; 490.4</td>
<td>positive</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td>SPX-13-desMeC</td>
<td>692.5 &gt; 164.2, 692.5 &gt; 444.4</td>
<td>positive</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>YTX</td>
<td>1141.5 &gt; 1061.5, 1141.5 &gt; 925</td>
<td>negative</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>45-OH-YTX</td>
<td>1157.5 &gt; 1077.5</td>
<td>negative</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Carboxy-YTX</td>
<td>1173.5 &gt; 1094.5</td>
<td>negative</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Homo-YTX</td>
<td>1155.5 &gt; 1075.5</td>
<td>negative</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Carboxyhydor-YTX</td>
<td>1189.5 &gt; 1109.5</td>
<td>negative</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>OA &amp; DTX1</td>
<td>803.5 &gt; 255.2, 803.5 &gt; 113</td>
<td>negative</td>
<td>70</td>
<td>50</td>
</tr>
<tr>
<td>DTX1</td>
<td>817.5 &gt; 255.5, 817.5 &gt; 113</td>
<td>negative</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>AZA1 &amp; AZA1b</td>
<td>842.5 &gt; 654.5, 842.5 &gt; 362</td>
<td>positive</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>AZA2</td>
<td>856.5 &gt; 672.5, 856.5 &gt; 654.5</td>
<td>positive</td>
<td>30</td>
<td>55</td>
</tr>
<tr>
<td>AZA3</td>
<td>828.5 &gt; 362, 828.5 &gt; 640.5</td>
<td>positive</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>PTX2</td>
<td>876.5 &gt; 823.5, 876.5 &gt; 212.5</td>
<td>positive</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>PTX1</td>
<td>892.5 &gt; 839.5, 906.5 &gt; 853.5</td>
<td>positive</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>PTX6</td>
<td>894.5 &gt; 805.2</td>
<td>positive</td>
<td>40</td>
<td>25</td>
</tr>
</tbody>
</table>

Calibration curves were obtained for the following toxins: OA, YTX, PTX2, AZA1, GYM and SPX 13-desMe-C. In order to calculate concentrations of compounds for which no calibration curves were generated, it was assumed that related analogues would give a similar response to that of the parent toxins.
2.3.2. Splitting experiment

Splitting was investigated for its influence on matrix effects. The flow entering the source was split post column using a T-piece. The flow was set by adjusting the length of the peek tubing going to the waste and measuring the volume collected. The source performances were assessed for flow rates of 400, 250, 165 and 80 µl/min.

2.3.2.1. Shellfish samples for selectivity assessment

Shellfish samples with different toxin profiles were used, including one extract from Norwegian mussel HP, one from Japanese scallop (*P. yessoensis*) HP obtained from Prof. Yasumoto, as well as whole flesh mussel extracts from Ireland (*M. edulis*) and Italy (*M. galloprovincialis*). All extracts were made in 100 % methanol.

2.3.2.2. Shellfish extraction for accuracy assessment

Triple methanolic extraction was performed by weighing 2 g of sample in a 50 ml plastic centrifuge tube to which 6 ml of methanol were added. The extracts were vortex mixed for 1 min at the highest speed and centrifuged for 15 min at 6,000 rpm. The supernatant was transferred to a 20 ml volumetric flask and the pellet was re-extracted in the same way. The third extraction was carried out by adding an additional 6 ml of methanol and blending the extract at high speed (ultraturrax) at 11,000 rpm for 1 min. The extract was centrifuged and the supernatant transferred to the volumetric flask with the two previous extracts. The volume was then completed to the mark. The solutions were filtered using 0.2 µm filters prior to dispensing in LC vials for analysis.
2.3.3. Results

2.3.3.1. Selectivity

Figure 2.10 shows the combination of chromatograms for all compounds monitored obtained from the analysis of several shellfish extracts using their quantification trace. All 21 toxins monitored eluted within 3.8 min with a total run time of 6.6 min. The first group of toxins to elute was the cyclic imine group with GYM and SPX 13-desMeC eluting at 1.18 and 1.52 min respectively. This was followed by the YTX group with 45-hydroxy-carboxy-YTX eluting at 1.95 min, 45-hydroxy-YTX and 45-hydroxy-homo-YTX both eluting at 2.29 min, and carboxy-YTX, carboxy-homo-YTX, 1-homo-YTX and YTX eluting at 2.32, 2.38, 2.92 and 3.07 min respectively. The chromatographic conditions did not allow complete resolution of carboxy-YTX, carboxy-homo-YTX, 45-hydroxy-YTX and 45-hydroxy-homo-YTX similarly to other reported LC-MS method using the Hypersil C8 column, where carboxy-YTX and 45-hydroxy-YTX peaks overlapped (Ciminiello et al., 2003). OA, DTX1 and DTX2 eluted at 2.86, 3.43 and 3.03 min respectively. The PTX group eluted between 2.31 and 3.09 min starting with PTX1 at 2.31 min, PTX6 at 2.46 min, PTX2sa at 2.79 min, 7-epi-PTX2sa at 2.98 min and PTX2 at 3.09 min. The last group to elute was the AZAs with AZA3 at 3.22 min, AZA1 and AZA1b at 3.44 and 3.35 min and AZA2 at 3.56 min.
Figure 2.10: Combined UPLC-MS/MS chromatograms using the multi toxins method for lipophilic marine toxins.

In order to examine the efficiency of the separation of the UPLC method, the resolution of OA and DTX2 peaks was calculated and compared with the resolution obtained by the HPLC method in place at the Marine Institute (conditions B1) (Hess et al., 2005b). The resolution obtained for OA and DTX2 by UPLC was 3.81 against 2.96 obtained by HPLC. The tailing factor for a typical OA peak was 1.05 for UPLC against 0.66 by HPLC. These results show that the chromatographic separation and peak shapes obtained on the UPLC system were better than those obtained by HPLC, even though the run time was decreased by a factor of two.
2.3.3.2. Accuracy

Accuracy of the UPLC-MS determination was checked by triple methanolic extraction, as described in section 2.5, of two separate portions of the CRM-DSP-Mus-b that contained 10.1 ± 0.8 µg/g of OA. Both extracts were subsequently diluted by a factor of 20 and injected in duplicate. The concentrations obtained were 9.55 and 9.48 µg/g indicating an average of 93.7% accuracy.

2.3.3.3. Calibrations

Calibration curves were generated for OA, YTX, PTX2, AZA1, GYM, and SPX 13-desMe-C. The equations, correlation coefficients, coefficients of variation and limits of detection (LODs) obtained from standard injections are shown in Table 2.9. These results were obtained from triplicate injections. All correlation coefficients were ≥ 0.997 using a set of 10 standards for OA, YTX, AZA1 and PTX2 and a set of 6 standards for GYM and SPX-13-desMeC. The LODs ranged from 22 (SPX 13-desMeC) to 483 pg/ml (OA). Compounds analysed in positive mode showed LODs 10 times lower than those analysed in negative modes (based on lowest standards). The retention times were reproducible to ± 0.01 min over a 30 hour run sequence comprising shellfish samples and standards (data not shown).
Table 2.9: Equations obtained for the calibration of OA, PTX2, AZA1 using 10 standards, and YTX, SPX 13-desMe-C and GYM using 6 standards. Values were calculated from triplicate 10 µl injections.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Slope ± SD</th>
<th>Intercept ± SD</th>
<th>Correlation coefficient ± SD</th>
<th>LOD (^a) (pg/ml)</th>
<th>LOQ (^a) (pg/ml)</th>
<th>Range of standards (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>80.7 ± 1.2</td>
<td>270.5 ± 39</td>
<td>0.9970 ± 0.0012</td>
<td>483.1</td>
<td>1 610</td>
<td>1.5 - 232</td>
</tr>
<tr>
<td>PTX2</td>
<td>769.9 ± 7.0</td>
<td>188.5 ± 0.1</td>
<td>0.9996 ± 0.0007</td>
<td>47.7</td>
<td>160</td>
<td>0.5 - 96</td>
</tr>
<tr>
<td>AZA1</td>
<td>877.0 ± 10.7</td>
<td>-139.3 ± 188.1</td>
<td>0.9996 ± 0.0004</td>
<td>32.8</td>
<td>110</td>
<td>0.4 - 77</td>
</tr>
<tr>
<td>YTX</td>
<td>31.9 ± 0.2</td>
<td>22.8 ± 7.5</td>
<td>0.9969 ± 0.0009</td>
<td>336.3</td>
<td>1 120</td>
<td>2.8 - 56</td>
</tr>
<tr>
<td>GYM</td>
<td>1548.6 ± 1.18</td>
<td>2478.2 ± 512.4</td>
<td>0.9974 ± 0.0014</td>
<td>60</td>
<td>200</td>
<td>1.5 - 111</td>
</tr>
<tr>
<td>SPX 13-desMe-C</td>
<td>1563.3 ± 59.5</td>
<td>-318.85 ± 1086.3</td>
<td>0.9966 ± 0.0037</td>
<td>22</td>
<td>70</td>
<td>1 - 80</td>
</tr>
</tbody>
</table>

\(^a\) LOD and LOQ were calculated from the lowest standard at S/N = 3 and S/N = 10 respectively

2.3.3.4. Splitting results

An extract of a laboratory reference material prepared from mussels was used to assess the effect of splitting on toxins responses. The results shown in Figure 2.11 suggest that there is no improvement in sensitivity for OA, AZA1, YTX and PTX2. The AZA1 response dropped significantly when the flow rate entering the source was reduced.
Figure 2.11: Effects of splitting on signal intensity of OA, PTX2, AZA1 and YTX

2.3.4. Discussion

The UPLC-MS/MS method described in this work allows the detection of 21 lipophilic toxins in a single run, which is the most comprehensive method reported to our knowledge. All 21 toxins monitored eluted within 3.8 min with a total run time of 6.6 min demonstrating the speed of the method. Despite the fact that all toxins were not chromatographically resolved, the recent advances in instrumentation (latest generation triple quadrupole MS) allows the monitoring of several transitions at the same time without loss in sensitivity. The method presented here covers the analysis of all the lipophilic toxins currently required under EU legislation apart from the OA and DTXs ester derivatives (DTX3), which are quantified by difference in the concentration of the parent compounds before and after hydrolysis. The Commission Decision 2074/2005 (Anonymous, 2005a) specifies that the MBA is the reference test for determination of lipophilic marine biotoxins in official control of food stuffs. However, for ethical reasons, laboratories are encouraged to use non-animal test methods, such as the LC-MS method proposed here. Some of the non-regulated, bioactive
compounds described here (SPX 13-desMe-C, GYM) may lead to false positive results by the MBA, and therefore to discrepancies with physicochemical methods, focusing on the determination of regulated toxins from the OA, AZA, YTX and PTX groups. Thus, the simultaneous determination of these bioactive compounds, in our method, may be useful in the interpretation of positive MBA results, where the regulated toxins are absent or present in low amounts.

The rapidity of the method is a strong advantage for monitoring laboratories, as it would allow a faster turnaround time of sample results compared to the current reference method and an increase in the number of samples that could be analysed on a routine basis.

The selectivity (expressed as elution order) obtained on the BEH C18 column (1.7 µm 2.1 x 100 mm) was qualitatively identical to the C8 BDS Hypersil (2.1 x 50 mm, 3 µm) (Quilliam et al., 2001) or the C18 ACE columns (2.1 x 30 mm, 3 µm) previously used in the author’s laboratory (unpublished data – Marine Institute).

Linear ranges and LODs were established for six compounds, OA, AZA1, YTX, SPX 13-desMeC, PTX2 and GYM. The instrument was found to be more sensitive in positive mode than in negative mode and the LOQs for OA and DTXs, PTX, YTX and AZA were found to be well below the EU regulatory levels. OA had the highest LOQ of all the tested toxins but the method was still sensitive enough to quantify 1/10 of the maximum regulatory limit (160 µg/kg). Surprisingly, the LOQ obtained for OA using the Premier XE MS was higher than the LOQ obtained on the Quattro Ultima which is an older instrument.

Kloepfer et al (Kloepfer et al., 2005) reported that matrix effects could be overcome by using a splitting device in order to reduce the number of components entering the source. The effect of splitting the flow entering the source in this study resulted in sensitivity loss, and was more
pronounced for the compounds analysed in positive ionisation mode. Further optimisation of source parameters at lower flow rates could be required to verify whether this route to eliminate matrix effects is still viable.

The method was found to be accurate, since the concentration found for the CRM-DSP-Mus-b standard extract was within the uncertainty of the certified concentration. However, it must be noted that matrix effects were minimised for this analysis since the high concentration of the material required a 1/20 dilution prior to injection.

It is important that matrix effects are checked when developing LC-MS methods for the analysis of marine toxins in shellfish since the results will strongly depend on the instrument and particularly on the source geometry.

2.4. Conclusions

Three methods were developed on the QToF instrument for the investigation of matrix effects. The different methods will allow for the comparison of different approaches for the characterisation of matrix effects e.g. standard addition, post-column infusion and post extraction addition. In addition, the influence of pH as well as the influence of elution method (gradient vs isocratic) was evaluated. It was demonstrated that the use of a less acidic pH than the commonly used 2 mM ammonium formate and 50 mM formic acid mobile phase allow for a later elution of PTX2 that can be desirable to avoid its co-elution with DTX1.

UPLC and HPLC gradient elution were compared on a triple quadrupole MS as well as two UPLC columns (C8 and C18). The selectivity of both UPLC methods was identical to the HPLC method when the Hypersil C8 column was used. The UPLC afforded higher resolution between the compounds than the HPLC method. The sensitivities of the methods were also evaluated. The UPLC methods allowed for a lower limit of detection of OA than the HPLC method. However, the LOD of AZA1 and PTX2 by UPLC was higher or the same as the LODs by HPLC. Although the UPLC methods have shown that analysis time can be
considerably reduced compared to HPLC, the development of a multi-analyte method was hampered by the MS limitation of rapid ionisation mode switching as well as the limited number of traces that can be monitored.

A UPLC-MS/MS method that allows for the detection of 21 lipophilic marine biotoxins was successfully developed. The methods proved to be a suitable and reliable for rapid turnaround of analyses. All 21 toxins monitored eluted within 3.8 min with a total run time of 6.6 min demonstrating the speed of the method. Linear ranges and LOQs were established for OA, PTX2, YTX, AZA1, SPX 13-desMe-C and GYM using the UPLC C18 BEH column.
3. **Evaluation of matrix effects in the LC-MS analysis of lipophilic marine toxins**

This chapter presents and discusses matrix effects that are encountered during the LC-MS analysis of three regulated lipophilic toxin groups (AZA, OA, PTX). The LC-MS methods used are the same as in Chapter 2.

This study investigated the degree of matrix effects associated with the LC-MS analyses of marine biotoxins. Matrix effects can be evaluated by several methods that can provide different types of information. Three different approaches were taken for the evaluation of matrix effects: standard addition (SA), post-extraction addition (PEA) and post-column infusion (PCI).

The standard addition (or matrix matched standard when the experiment is carried out in analyte-free material) is a widely used method to quantify matrix effects. In this study, SA was performed by spiking the extracts at 5 concentrations reflecting the concentration range encountered when contaminated shellfish are analysed. In addition, the same 5 levels were used to spike methanolic solutions. The slopes of the curves produced in methanol and in extracts (equivalent to response factors) were obtained from linear regression analysis. The slope obtained in a shellfish extract was then expressed as a percentage of the slope obtained in methanol, equivalent to the degree of matrix effects observed. The SA method has the advantage of providing good statistical power to the results generated since matrix effects are calculated from replicate injection of five independent levels.

The PEA method was developed in order to evaluate the influence of non-volatile material on the degree of matrix effects. Two methods were used to obtain a range of extracts that differ in their amount of non volatile material. Both methods require that the extraction of shellfish
is carried out at a low SSR (SSR 1 in this study) which was subsequently diluted either by serial or volumetric dilution. The serial dilution consisted of diluting the most concentrated extract (SSR 1) by a factor of 2, which is further diluted by a factor of 2 and so on. The second method implied that the density of the most concentrated extract is measured and that volumetric dilution is performed and weighed. The final volume is kept constant by using volumetric flasks made up to the mark with methanol. Both methods rely on the amount of dry residue that is obtained from the most concentrated extract. The amount of dry residue was determined gravimetrically by drying a known volume of extract in an oven at 105 °C and expressed in mg /ml of extract. Once a range of extracts that differ in their amount of dry residue is obtained, the same concentration (and volume) of analyte is spiked into the different extracts.

Correlation between post extraction addition and standard addition experiments required the use of a “matrix strength figure”. It was chosen to express matrix strength as the concentration of dry residue in extracts since co-eluting non-volatile compounds are often associated with signal suppression in LC-MS (King et al., 2000). Thus, PEA and SA experiments could be directly compared assuming that non-volatile compounds are largely responsible for matrix effects.

PCI is a dynamic way of assessing matrix effects and can give information on where interfering compounds leading to matrix effects occur during a chromatographic run. As the toxin being tested is introduced into the mass detector at a constant rate, a constant baseline is observed if no ionization interferences are present as a result of shellfish extract injection. On the other hand, if ionisation interferences do occur as a result of co-elution with endogenous compounds, the PCI would reveal whether signal suppression or enhancement is taking place.
Since the degree of ion suppression (or enhancement) can be dependent on the concentration of the analyte being monitored and the matrix/analyte ratio (Amnesley et al., 2003), care should be taken to ensure that the concentration of analyte that is infused and the concentration of matrix sample injected are reflecting matrix/analyte ratios encountered with typical samples.

The main advantage of the PCI is the speed and the simplicity of the experimental set up. In theory, a single chromatographic run should be sufficient. Also, it is important that the PCI method is performed under stable spray conditions which can only be ensured by using isocratic elution. However, isocratic runs are not well suited for multi-analyte methods, especially if the analyte exhibits large polarity differences, as it will result in lengthy analysis. As a consequence, step gradient methods were used in this study. Major drawbacks of the PCI include the difficulty of assessing/quantifying the degree of matrix effects and as well as the high concentration required for the infused standard solution due to dilution effect. Concentrated solutions of standard solution of marine toxins are expensive and are not available for all toxins. Another drawback of the PCI is that matrix effects that can arise from build up of non volatile material in the source or from column bleeding would not be apparent.

To the best of our knowledge there is no established rule on the degree of matrix effects that would be considered as significant, as the significance will depend on the repeatability of the analytical method used. In the case of LC-MS analysis of lipophilic marine toxins, repeatability of 5 % is considered to be achievable on most instruments. Thus, the confidence interval at 95 % implying two standard deviations equates to ca 10 % and therefore the significance of matrix effects below 10 % would be difficult to prove statistically.
Most toxin monitoring programs worldwide typically involve the extraction of raw shellfish, however, in some countries e.g. Denmark, Germany, the shellfish are mildly cooked by steaming prior to extraction (heat treatment duration has not been harmonised). Furthermore, Hess et al. (2005) reported a two-fold increase in AZA1 concentrations in cooked mussels compared to raw mussels due to the water loss occurring during the steaming process. In order to assess matrix effects related to the heat treatment process only, the water content of the shellfish that underwent heat treatment was adjusted to match the water content in raw shellfish.

3.1. Material and methods

3.1.1. LC-MS methods

The LC-MS conditions (A1-3, B1-3, C) that were used for the description of matrix effects were presented in Chapter 2.

3.1.2. Standard addition

3.1.2.1. Preparation of solutions analysed using conditions A and B

Toxin free mussels (*Mytilus edulis*) were collected from various locations around Ireland. The mussels were shucked and their flesh homogenised in a kitchen blender. All experiments described hereafter were carried out using the pooled homogenate. Heat treated flesh was obtained by immersing a closed centrifuge tube containing 2 g of homogenate in a 80 °C water bath for 10 min. This approach was taken in order to avoid water loss and the subsequent increase in matrix content concentration.

The following extraction method is the BIOTOX protocol. Mussel homogenate aliquots (2 g) were weighed into 50 ml polypropylene centrifuge tubes and 6 ml of methanol (100%) were
subsequently added. Samples were homogenised using a multitube vortex mixer for 1 min and centrifuged (Analytica Jouan, CR 422) at 4500 rpm for 5 min. The supernatant was decanted into a 20 ml volumetric flask. Then, 6 ml of methanol were added to the remaining pellet and the homogenization and centrifugation steps were repeated. The supernatant was added to the volumetric flask. An additional 6 ml of methanol were added to the pellet, blended using an Ultraturrax at 11,000 rpm for 1 min and subsequently centrifuged. The supernatant was transferred to the volumetric flask and the volume was completed to the mark with methanol. Thus, a solvent to sample ratio (SSR) of 10 ml/g was obtained.

Extracts of raw and heat-treated shellfish tissue were used to prepare the samples for the standard addition. The solutions were prepared in 10 ml volumetric flasks containing 9 ml of shellfish extract. Increasing volumes of standard stock solution were then added to the flasks and the volume was completed up to the mark with methanol. In addition, another set of solution was prepared by halving the matrix strength (4.5 ml of extract in 10 ml flask). The solutions were filtered using 0.2 µm filters (Schleicher & Schuell) into HPLC vials for the analysis. The concentrations of the standard addition solutions ranged from 4 to 33 ng/ml for OA and PTX2, and from 11 to 91 ng/ml for AZA1. Each set of solutions consisted of 5 different concentration levels and one blank (extract not spiked). Reference solutions were prepared by spiking varying volumes of standard stock solution (100, 200 and 400 µl) in methanol and quantified using calibration standards.

Standard addition was also performed with DTX1, DTX2 and compared against OA. High purity solution of DTX1 was obtained from NVI (Rundberget T., Personal communication) and high purity solution of DTX2 was obtained from the MI (Rehmann N., Personal communication). DTX1 was isolated from a phytoplankton pumping system and DTX2 was isolated from contaminated Irish mussels. Both solutions were quantified against OA calibration curve assuming equal response factors and a stock solution containing equal
concentrations (320 ng/ml) of the DSP toxins was prepared. Dilutions were carried out to obtain four diluted stock solutions (ranging from 80 to 240 ng/ml). The five stock solutions were spiked (50 µl) to methanol (450 µl) and mussel extracts (450 µl) prepared with solvent SSR of 10 and 20 (prepared following the BIOTOX protocol as above). For each matrix, five levels of concentrations ranging from 8 to 32 ng/ml were obtained and analyses of blank extracts were systematically performed.

3.1.2.2. Preparation of solutions analysed using condition C

Mussels harvested in Scotland, and oysters harvested in the Thames estuary were purchased from a local shop in Manchester, UK, February 2006. The mussels were divided into two equal portions in sufficient number to obtain pools of 100 g whole flesh. The first portion was shucked and homogenised raw using a kitchen blender. The second portion was steamed for 15 min, shucked and the flesh was homogenised using a kitchen blender. The oysters were prepared in the same way, however 45 min of steaming was necessary to open the shells with this treatment.

A triple methanolic extraction was performed by weighing 2 g of sample in a 50 ml polypropylene centrifuge tube to which 6 ml of methanol were added. The extracts were vortex mixed for 1 min at the highest speed and centrifuged for 15 min at 4000 g. The supernatant was transferred to a 20 ml volumetric flask and the pellet was re-extracted in the same way. The third extraction was carried out by adding an additional 6 ml of methanol and blending the extract at high speed (ultraturrax) at 11,000 rpm for 1 min. The extract was centrifuged and the supernatant transferred to the volumetric flask with the two previous extracts. Stock solutions of AZA1 and OA were prepared and spiked into the volumetric flasks in order to get concentrations ranging from 0.6 to 18.5 ng/ml for AZA1 and 3.5 to 25 ng/ml for OA. The volume was then completed to the mark. The solutions were filtered
using 0.2 µm filters prior to dispensing in LC vials for analysis. Thus, 6 different levels (including blank) were prepared for each toxin and each matrix.

3.1.3. Post-extraction addition (PEA) – Influence of analyte/matrix ratio

3.1.3.1. Solutions analysed using conditions A and B

The following dispersive extraction was performed in order to obtain an extract with low SSR and that will be diluted to obtain several extracts over a range of SSRs. The PEA-extraction was carried out by weighing aliquots (10 g) of mussel flesh into a 50 ml centrifuge tube and 10 ml of methanol (100%) were added. The samples were homogenised for 2 min using a multitube vortex mixer (Alpha Laboratories, V400) and were blended at high speed (11,000 rpm) with an Ultraturrax™ (IKA) for 1 min. Then, the tubes were centrifuged for 15 min at 4000 g before the supernatant was decanted. The SSR for these extracts was 1 ml/g. For further analysis 10 extracts were combined and used as pooled extract. Extracts of raw and heat-treated shellfish tissue were used to prepare the samples for the post extraction addition.

In order to reach different matrix strengths various amounts of pooled extract were transferred into a 10 ml volumetric flask. Aliquots of 0.4 ml of standard stock solution were spiked into the extracts and the volume was completed up to the mark with methanol. Aliquots (1 ml) were filtered into HPLC vials using 0.2 µm filters. The concentration in the spiked samples ranged from 16.6 to 18.3 ng/ml for OA, PTX2 and AZA1. The overall variability was assessed by adding 0.4 ml of stock solution to 9.6 ml of methanol (n=5). These solutions were quantified using calibration standards and used to calculate the recovery obtained from mussel extracts.
3.1.3.2. Solutions analysed using conditions C

Mussels samples (*Mytilus edulis*) harvested in Ballyvaughan on the West Coast of Ireland and oysters samples (*Crassostrea gigas*) harvested in Carlingford Lough on the North East coast of Ireland were used.

A 50 g portion of shellfish flesh was homogenised using a Warring® blender. Two 10 g portions of each of the shellfish homogenates were inserted in a 50 ml polypropylene centrifuge tube. One of the mussel and one of the oyster portions were cooked in closed polypropylene tubes in a 90 °C water bath for 10 minutes in order to avoid the loss of water. Methanol (10 ml) was added to 10 g of each shellfish matrix which was subsequently vortex mixed for two minutes at the highest speed. Subsequently, the solvent-tissue mixture was blended at high speed using an ultraturrax® at 11,000 rpm for one minute and centrifuged for 15 minutes at 6,000 rpm. The extracts were filtered through 0.2 μm. The initial extract was diluted in series 1:1 with methanol seven times. Aliquots (2 ml) of each extract were inserted into 5 ml glass vials. OA and AZA1 stock solutions were prepared at concentrations of 350.5 and 353.8 ng/ml respectively and 100 μl of each solution were spiked into the 2 ml extracts. In addition, five spiking replicates were done in methanol.
### SA-extraction

2 g weight of mussel homogenate into a 50 mL centrifuge tube
6 mL add 100% of methanol
1 min vortex
5 min centrifuge at 4000 g
in 20 mL volumetric flask
6 mL decant supernatant
1 min ultraturrax at 11,000 rpm
5 min centrifuge at 4500 rpm
in the same 20 mL volumetric flask
fill up to the mark with 100% methanol

10 mL/g solvent to sample ratio

### PEA-extraction

10 g
6 mL
10 min vortex
10 mL
5 min centrifuge at 4000 g
in glas vial
6 mL decant supernatant
1 min ultraturrax at 11,000 rpm
15 min centrifuge at 4500 rpm
in glas vial
6 mL decant supernatant
10 mL/g solvent to sample ratio

1 mL/g

Figure 3.1: Summary of the extraction procedures used for the preparation of the SA extracts (SSR 10) and for the preparation of the PEA extracts (SSR 1)

3.1.4. Post column infusion

Post column infusion was carried out using the step gradient conditions with Q-Tof detection (conditions A). Standard solutions of AZA1, OA and PTX2 were infused using a T piece while 5 µl of a blank mussel flesh extract (SSR 10, i.e. 2 g extracted in 20 ml) was injected.
The OA, PTX2 and AZA1 standard solution concentrations were 500, 600 and 700 ng/ml respectively.

3.1.5. Characterisation of shellfish matrices

3.1.5.1. Matrix used for conditions A and B

Two aliquots (10 ml) of extracts of raw and heat-treated mussels (SSR 1) were inserted into glass vials and dried using an oven at 105°C. After 15 hours the dry residues and moisture contents were determined gravimetrically.

The densities of the solutions obtained were measured by weighing a 5 ml extract five times.

3.1.5.2. Matrix used for condition C

The moisture content and amount of dry residue were determined gravimetrically from four 10 g portions of cooked and raw mussel and oyster tissues were placed in an oven for 13 h at 100 °C. The efficiency of the extraction towards matrix co-extractants from 10 g of flesh using 10 ml of methanol was assessed against triplicate methanolic extraction of 2 g in 20 ml (as used for the standard addition experiment) for the cooked and raw mussels and oysters.

The densities of the solutions obtained were measured by weighing a 5 ml extract five times, as above.

3.1.5.3. Lipids separation

The glycolipids, neutral and polar lipids were extracted by the Folch method (Folch et al., 1957) from a mussel homogenate according to the procedure described below and used to prepare SA solutions of OA and AZA1 in the different classes of lipids.

The classes of lipids were extracted from a mussel extract obtained using the BIOTOX protocol (SSR 10; section 3.1.2.2). Aliquots (2 x 2.5 ml) of the SSR 10 extract were separated on two silica columns to avoid overloading. For each column, a 2.5 ml aliquot of the extract
was evaporated to dryness and re-suspended in three times 500 µl of chloroform/methanol (98:2; v/v). The solution was then loaded onto an open glass micro-column packed with silica gel and eluted successively with (i) 10ml of chloroform/methanol (98:2; v/v), (ii) 10ml of acetone and (iii) 15ml of methanol. The three fractions containing respectively the neutral lipids (glycerol, fatty acids...), glycolipids, and phospholipids were collected separately and the fractions containing each class of lipid from the two columns were combined, evaporated to dryness and re-suspended in 5 ml of methanol. Six sets of SA solutions containing both OA and AZA1 were prepared in methanol, in each of the 3 lipid fractions and in mussel extracts at a SSR of 10 and 20 respectively. These SA solutions were then analysed by LC-MS, both on the Q-ToF and the QqQ (sets of conditions A and B), to assess the degree of matrix effects. In addition, recovery experiments were carried out using an extract of mussel at SSR10 spiked with AZA1 and OA and that was separated on two silica columns (2 x 2.5 ml as above). Furthermore, recoveries of evaporation step and resuspension in methanol as well as of evaporation, resuspension in chloroform followed by evaporation and resuspension in methanol were carried out in triplicate.

3.1.5.4. Matrix effects in hydrolysed extract

The hydrolysis process used to analyse the OA/DTX esters relies on the use of sodium hydroxide (NaOH) and hydrochloric acid (HCl) which leads to the formation of sodium chloride. This would be problematic if it turned out that the resulting salts were responsible for matrix effects. The objective of the experiments carried out was to investigate such effects.

A mussel homogenate extracted according to the BIOTOX protocol (SSR 10) was subsequently hydrolysed using the following procedure developed by Mountfort et al.(2001):
125µl NaOH 2.5M were added to 1ml extract. The mixture was placed in a water bath at 76°C for 40 min., allowed to cool to room temperature before adding 125µl HCl 2.5M. This “hydrolysed” bulk extract was used to prepare SA solutions of OA and AZA1 subsequently analysed using LC-MS conditions A1 and B1.

3.1.5.5. Matrix effects in salt solution

Standard addition experiments were performed in methanolic solution at 20 mg/ml of NaCl. An aqueous solution of 250 mg/ml NaCl was prepared and 400 µl of the solution introduced into each of six 5 ml volumetric flasks. The mixed standard solution (OA+DTX1+DTX2) was spiked into each flask and the volume was completed to the mark with methanol thus resulting in 5 solutions with different toxin concentrations.

The same sets of solutions were prepared by replacing the NaCl solution with water; this constituted the methanol control.

For the determination of matrix effects, a standard addition experiment was performed in real mussel extract. The pooled mussel homogenate (used for previous matrix effects experiments) was extracted following the BIOTOX procedure on six 2 g portions (SSR 10). The extracts were pooled and homogenised. Aliquots of the pooled extract (4.5 ml) were inserted into the 5 ml volumetric flasks and the mixed standard solution was spiked into the flasks. The volume was completed up to the mark with methanol in all flasks.
3.2. Results

3.2.1. Characterisation of shellfish matrices

3.2.1.1. Matrix used for conditions A (HPLC - QToF detector) and B (HPLC/UPLC - QqQ detector)

3.2.1.1.1. Moisture content

The amounts of dry residue in extracts (SSR 1) of raw and heat-treated mussels were 38.6 ± 3.1 mg/ml and 41.9 ± 0.1 mg/ml respectively. The average moisture content for the whole flesh mussel tissue was 79.0 % with a standard deviation of 0.08 %, calculation on five replicates.

3.2.1.1.2. Dry residue

Dry residues were calculated from previously determined densities. Results are shown in Table 3.1.

Table 3.1: Dry residues of raw and heat treated extracts, determination by weight of 10 ml extract after evaporation at 105°C, n=2

<table>
<thead>
<tr>
<th>Extract</th>
<th>Extraction method</th>
<th>Dry residue / mg</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>raw</td>
<td>SA</td>
<td>7.25</td>
<td>0.25</td>
<td>3.40%</td>
</tr>
<tr>
<td></td>
<td>PEA</td>
<td>41.9</td>
<td>0.1</td>
<td>0.29%</td>
</tr>
<tr>
<td>heat treated</td>
<td>SA</td>
<td>7.38</td>
<td>0.48</td>
<td>6.57%</td>
</tr>
<tr>
<td></td>
<td>PEA</td>
<td>38.6</td>
<td>3.1</td>
<td>7.98%</td>
</tr>
</tbody>
</table>

The results suggest that there is no difference in the weight of dry residue between raw and heat treated extracts. As expected a larger amount of dry residue was obtained for the extracts made by PEA-extraction than those from the SA-extraction. Ratios between PEA-extraction and SA-extraction of raw and heat treated extracts were 5.8 and 5.2, respectively.
The data for the filtered and unfiltered extracts made by PEA-extraction are given in Table 3.2.

### Table 3.2: Dry residues of filtered and unfiltered matrix extracts, determination by weight of 2 ml extract after evaporation at 105°C, n=3

<table>
<thead>
<tr>
<th>Extract</th>
<th>Treatment</th>
<th>Density mg/cm³</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>raw</td>
<td>unfiltered</td>
<td>49.9</td>
<td>0.7</td>
<td>1.42%</td>
</tr>
<tr>
<td></td>
<td>filtered</td>
<td>52.9</td>
<td>0.4</td>
<td>0.80%</td>
</tr>
<tr>
<td>heat treated</td>
<td>unfiltered</td>
<td>48.2</td>
<td>0.2</td>
<td>0.49%</td>
</tr>
<tr>
<td></td>
<td>filtered</td>
<td>49.8</td>
<td>1.3</td>
<td>2.51%</td>
</tr>
</tbody>
</table>

The filtered extracts differ from the unfiltered extracts in 6% and 3% for the raw and heat treated extracts, respectively.

3.2.1.1.3. Densities

The determined densities for the raw and heat treated extracts are shown in Table 3.3.

### Table 3.3: Gravimetric determination of densities. Average and CVs calculated from n=5

<table>
<thead>
<tr>
<th>Extract</th>
<th>Extraction method</th>
<th>Density (g/cm³)</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>raw</td>
<td>SA</td>
<td>0.815</td>
<td>0.003</td>
<td>0.36%</td>
</tr>
<tr>
<td></td>
<td>PEA</td>
<td>0.933</td>
<td>0.001</td>
<td>0.15%</td>
</tr>
<tr>
<td>heat treated</td>
<td>SA</td>
<td>0.817</td>
<td>0.002</td>
<td>0.26%</td>
</tr>
<tr>
<td></td>
<td>PEA</td>
<td>0.928</td>
<td>0.005</td>
<td>0.49%</td>
</tr>
</tbody>
</table>

The densities for the PEA-extracts were higher than those of the SA-extracts.

No difference was observed for the same extraction method between raw samples and the samples that underwent a heat treatment.

The densities of unfiltered and filtered extracts were measured to determine whether the filtration process had a noticeable influence on the density. Results are shown in Table 3.4.
Table 3.4: Gravimetric determination of densities in filtered and unfiltered PEA-extracts, Average and CVs calculated from n=5.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Treatment</th>
<th>Density (g/cm$^3$)</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>raw</td>
<td>unfiltered</td>
<td>0.9422</td>
<td>0.0076</td>
<td>0.05%</td>
</tr>
<tr>
<td></td>
<td>filtered</td>
<td>0.9500</td>
<td>0.0029</td>
<td>0.02%</td>
</tr>
<tr>
<td>heat treated</td>
<td>unfiltered</td>
<td>0.9405</td>
<td>0.0024</td>
<td>0.02%</td>
</tr>
<tr>
<td></td>
<td>filtered</td>
<td>0.9456</td>
<td>0.0025</td>
<td>0.02%</td>
</tr>
</tbody>
</table>

The densities of filtered and unfiltered extracts did not show significant differences. For the raw extract, the densities were within one standard deviation and within two standard deviations for the heat treated extract.

3.2.1.2. Matrix used for condition C (UPLC-QqQ Premier XE)

3.2.1.2.1. Moisture content

The moisture contents of the heat-treated and the raw mussel flesh were 78.1 % and 84.7 % respectively. The moisture content of the mussels that underwent heat treatment was adjusted to 84.7 % with MilliQ water. The moisture content of the heat-treated and the raw oyster flesh were 74.4 % and 83.1 % respectively. The moisture content of the oyster flesh that underwent heat treatment was adjusted to 83.1 %.

3.2.1.2.1. Dry residue

The efficiency of the extraction towards matrix co-extractants from 10 g of flesh using 10 ml of methanol was assessed against triplicate methanolic extraction of 2 g in 20 ml (as used for the standard addition experiment) for the cooked and raw mussels and oysters. Results are shown in Table 3.5.
Table 3.5: Masses of dry residue obtained using triplicate methanolic extraction on 2 g of shellfish and on single methanolic extraction using 10 g of shellfish (n=5)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>mg dry residue per ml of SA extract</th>
<th>mg dry residue per ml of PEA extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mussels Raw</td>
<td>9.02</td>
<td>45.52</td>
</tr>
<tr>
<td>Cooked</td>
<td>8.18</td>
<td>44.08</td>
</tr>
<tr>
<td>Oysters Raw</td>
<td>11.70</td>
<td>62.54</td>
</tr>
<tr>
<td>Cooked</td>
<td>11.32</td>
<td>57.76</td>
</tr>
</tbody>
</table>

3.2.1.2.2. Densities

The densities of the mussel and oyster extracts obtained using the SA and the PEA extraction method are shown in Table 3.6. There was no apparent difference in the density of the oyster and mussel extracts and no difference between the raw and cooked extracts. The extracts prepared following the PEA extraction procedure were denser than those prepared with the SA procedure.

Table 3.6: Densities of extracts obtained using triplicate methanolic extraction on 2 g of shellfish and on single methanolic extraction using 10 g of shellfish (n=5)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Densities of SA extracts</th>
<th>Densities of PEA extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mussels Raw</td>
<td>0.82</td>
<td>0.94</td>
</tr>
<tr>
<td>Cooked</td>
<td>0.83</td>
<td>0.94</td>
</tr>
<tr>
<td>Oysters Raw</td>
<td>0.83</td>
<td>0.94</td>
</tr>
<tr>
<td>Cooked</td>
<td>0.83</td>
<td>0.94</td>
</tr>
</tbody>
</table>

3.2.2. Determination of matrix effects by the standard addition method

3.2.2.1. Okadaic acid

The nature and importance of the matrix effects affecting OA were assessed for mussels (fresh with a SSR 10, fresh with a SSR 20, cooked with a SSR 10, cooked with a SSR 20) and oysters (fresh with a SSR 10, cooked with a SSR 10) using 7 different sets of conditions. The slopes, intercepts and correlation coefficients determined using LC-MS conditions A1 to A3,
B1 to B3 and C are presented in Table 3.7 to Table 3.10. The degrees of matrix effects are summarised in Table 3.11.

Table 3.7: Slopes, intercept (Int), correlation coefficients ($R^2$) and percentage of matrix effects (ME) observed for OA, AZA1 and PTX2 in extracts of raw mussel (RM) flesh and standards using HPLC-MS QToF and gradient, step gradient and neutral step gradient elutions. Analyses performed using conditions A.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Gradient $^1$ (A1)</th>
<th>Step Gradient acidic $^1$ (A2)</th>
<th>Step gradient neutral $^2$ (A3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Int.</td>
<td>$R^2$</td>
</tr>
<tr>
<td>OA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSR 10</td>
<td>0.96</td>
<td>-0.70</td>
<td>0.9982</td>
</tr>
<tr>
<td>Std</td>
<td>±0.02</td>
<td>±0.25</td>
<td>±0.0025</td>
</tr>
<tr>
<td></td>
<td>1.03</td>
<td>-5.61</td>
<td>0.9959</td>
</tr>
<tr>
<td></td>
<td>±0.03</td>
<td>±1.04</td>
<td>±0.0021</td>
</tr>
<tr>
<td>AZA1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSR 10</td>
<td>26.48</td>
<td>149.48</td>
<td>0.9979</td>
</tr>
<tr>
<td>Std</td>
<td>±2.72</td>
<td>±72.57</td>
<td>±0.0000</td>
</tr>
<tr>
<td></td>
<td>34.80</td>
<td>-12.09</td>
<td>0.9990</td>
</tr>
<tr>
<td></td>
<td>±1.29</td>
<td>±25.25</td>
<td>±0.0006</td>
</tr>
<tr>
<td>PTX2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSR 10</td>
<td>25.04</td>
<td>-19.78</td>
<td>0.9944</td>
</tr>
<tr>
<td>Std</td>
<td>±0.07</td>
<td>±0.09</td>
<td>±0.0012</td>
</tr>
<tr>
<td></td>
<td>20.45</td>
<td>-15.85</td>
<td>0.9962</td>
</tr>
<tr>
<td></td>
<td>±0.53</td>
<td>±0.76</td>
<td>±0.0020</td>
</tr>
</tbody>
</table>

$^1$ Standard slope was obtained from four replicate injections of a set of seven solutions and slopes in mussel matrices were obtained from duplicate injections of a set of five solutions.

$^2$ Standard slope was obtained from three replicate injections of a set of seven solutions and slopes in mussel matrices were obtained from duplicate injections of a set of five solutions.

Table 3.8: Slopes, correlation coefficient and intercepts obtained by five injections of spiked methanol and triplicate injection of mussel extracts SSR 10 and 20. Analyses performed using conditions A1 with a binary gradient of acidic mobile phases.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>OA</th>
<th>DTX1</th>
<th>DTX2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard curve</td>
<td>%ME</td>
<td>Standard curve</td>
</tr>
<tr>
<td>Slope MeOH</td>
<td>1.31 ± 0.1</td>
<td>0.9929 ± 0.0067</td>
<td>1.31 ± 0.09</td>
</tr>
<tr>
<td>R²</td>
<td>-3.5 ± 1.14</td>
<td></td>
<td>-3.5 ± 1.56</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.51 ± 0.01</td>
<td>0.9927 ± 0.0054</td>
<td>1.67 ± 0.04</td>
</tr>
<tr>
<td>R²</td>
<td>-4.18 ± 0.36</td>
<td></td>
<td>-4.68 ± 1.08</td>
</tr>
<tr>
<td>Slope SSR 20</td>
<td>1.69 ± 0.19</td>
<td>0.9921 ± 0.0066</td>
<td>1.46 ± 0.32</td>
</tr>
<tr>
<td>R²</td>
<td>-3.74 ± 1.72</td>
<td></td>
<td>-1.69 ± 3.65</td>
</tr>
</tbody>
</table>
Table 3.9: Slopes, intercept (Int), correlation coefficients (R²) and percentage of matrix effects (ME) observed for OA, AZA1 and PTX2 in extracts of heat-treated mussels (HM) and raw mussel (RM) flesh and standards using HPLC and UPLC equipped with BEH C8 and C18 column. Analyses performed using conditions B1, B2 and B3, respectively.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>HPLC C8 (^1) (B1)</th>
<th>UPLC C8 (^3) (B2)</th>
<th>UPLC C18 (^3) (B3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Int.</td>
<td>R²</td>
</tr>
<tr>
<td>RM SSR 10</td>
<td>116.0± 41350 0.9982</td>
<td>50.4</td>
<td>78.0</td>
</tr>
<tr>
<td>RM SSR 20</td>
<td>104.2± 33.3 0.9926</td>
<td>-35.0</td>
<td>70.5</td>
</tr>
<tr>
<td>HM SSR 10</td>
<td>115.0± 4132 0.9961</td>
<td>-49.0</td>
<td>85.3</td>
</tr>
<tr>
<td>HM SSR 20</td>
<td>99.0± 88.5 0.9973</td>
<td>28.3</td>
<td>75.7</td>
</tr>
<tr>
<td>Std 772± 137.3 0.9987</td>
<td>9.7</td>
<td>±152.1</td>
<td>±0.016</td>
</tr>
<tr>
<td></td>
<td>RM SSR 10</td>
<td>1321.1± 149.2 0.9997</td>
<td>-9.3</td>
</tr>
<tr>
<td>RM SSR 20</td>
<td>1189.5± 2838.8 0.9987±0</td>
<td>-18.4</td>
<td>692.5</td>
</tr>
<tr>
<td>HM SSR 10</td>
<td>1196.2± 49.1 0.9995±0</td>
<td>-17.9</td>
<td>712.1</td>
</tr>
<tr>
<td>HM SSR 20</td>
<td>1232.0± 1576 0.9989±0</td>
<td>-15.4</td>
<td>715.6</td>
</tr>
<tr>
<td>Std 1456.8± 1947 0.9995±0</td>
<td>±132 530</td>
<td>±0.001</td>
<td>±74.3</td>
</tr>
<tr>
<td></td>
<td>RM SSR 10</td>
<td>1119.9± 580.8 0.9989</td>
<td>45.3</td>
</tr>
<tr>
<td>RM SSR 20</td>
<td>1083.6± 423.6 0.9981</td>
<td>40.6</td>
<td>363.6</td>
</tr>
<tr>
<td>HM SSR 10</td>
<td>1188.2± 576.3 0.9993</td>
<td>54.2</td>
<td>393.1</td>
</tr>
<tr>
<td>HM SSR 20</td>
<td>1042.2± 440.0 0.9996</td>
<td>35.2</td>
<td>439.4</td>
</tr>
<tr>
<td>Std 770.7± 119.4 0.9994</td>
<td>70.8 ±404.8</td>
<td>±0.0007</td>
<td>369.7</td>
</tr>
</tbody>
</table>

\(^1\) Standard slope was obtained from six replicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from duplicate injections of a set of five solutions

\(^2\) Standard slope was obtained from four replicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from triplicate injections of a set of five solutions

\(^3\) Standard slope was obtained from triplicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from triplicate injections of a set of five solutions
OA is predominantly affected by ion enhancement to a degree that can range from 5.7 to 80.5 % in mussels and from 1.5 to 37.4 % in oysters (UPLC results). However, in 4 instances OA was associated with ion suppression ranging from -7.2 to -16.8 % in fresh mussels (SSR 10; conditions A1, A2 and C), -21.9 % in fresh oysters (SSR 10; conditions C). These examples demonstrate the variability in matrix effects when using different analytical conditions. However, this can also apply when using the same chromatographic conditions; for instance 2 experiments carried out with mussels (SSR 10) on different days using conditions A1 gave a degree of matrix effect of -7.2 and + 28.2 %, respectively. As the same mussel solutions were used, the discrepancy points towards an instrumental effect; it is reasonable to assume that these differences might be due to the state of the source and the column (degree of cleanliness).

The influence of the shellfish treatment (fresh versus cooked) on matrix effects was also investigated and the results are presented in Table 3.12. Over the 7 sets of data it turned out that in UPLC conditions using either column (C₈ or C₁₈; conditions B2 and B3 respectively),

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Matrix</th>
<th>Slope ± SD</th>
<th>Intercept ± SD</th>
<th>Correlation coefficient ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>Raw Mussels</td>
<td>72.9 ± 5.8</td>
<td>16.2 ± 13.4</td>
<td>0.9991 ± 0.0009</td>
</tr>
<tr>
<td></td>
<td>Steamed Mussels</td>
<td>94.8 ± 1.4</td>
<td>-45.1 ± 32.3</td>
<td>0.9988 ± 0.0008</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>68.4 ± 3.1</td>
<td>2.4 ± 17.4</td>
<td>0.9974 ± 0.0022</td>
</tr>
<tr>
<td></td>
<td>Raw Oysters</td>
<td>107.8 ± 2.6</td>
<td>-45.6 ± 18.4</td>
<td>0.9965 ± 0.0028</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>87.6 ± 4.1</td>
<td>83.5 ± 5.8</td>
<td>0.9956 ± 0.0025</td>
</tr>
<tr>
<td>AZA1</td>
<td>Raw Mussels</td>
<td>680.1 ± 27.0</td>
<td>39.5 ± 71.3</td>
<td>0.9996 ± 0.0005</td>
</tr>
<tr>
<td></td>
<td>Steamed Mussels</td>
<td>767.3 ± 38.3</td>
<td>94.8 ± 54.8</td>
<td>0.9996 ± 0.0006</td>
</tr>
<tr>
<td></td>
<td>Raw Oysters</td>
<td>756.0 ± 48.5</td>
<td>-35.3 ± 72.4</td>
<td>0.9998 ± 0.0002</td>
</tr>
<tr>
<td></td>
<td>Steamed Oysters</td>
<td>858.3 ± 32.6</td>
<td>-83.9 ± 76.5</td>
<td>0.9996 ± 0.0001</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>883.7 ± 23.1</td>
<td>-149.8 ± 61.7</td>
<td>0.9988 ± 0.0009</td>
</tr>
</tbody>
</table>
the matrix effects were worse in cooked mussels with an increase in the degree of matrix from 7.9 to 11.3 % at SSR 10 and from 8.1 to 11.7 % at SSR 20. In conditions B1 (gradient HPLC) cooking reduced the degree of matrix effects by 1.4 % at SSR 10 and 6.7 % at SSR 20, which are not considered as statistically different. Just like in UPLC conditions the change in matrix effect observed between fresh and cooked mussels is more important at SSR 20 than SSR 10.

Under conditions C, cooking the mussels and oysters led to a change of the nature of matrix effects at SSR 10 with ion suppression observed for fresh tissues and ion enhancement for cooked ones (Table 3.11).
Table 3.11: Summary table of the percentage of matrix effects experienced for OA, AZA1 and PTX2 for different matrices as determined by standard addition

<table>
<thead>
<tr>
<th>Matrices</th>
<th>Acids</th>
<th>HPLC Q-ToF</th>
<th>QqQ Ultima</th>
<th>QqQ Premier XE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acidic</td>
<td>Acidic</td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td>step</td>
<td>gradient</td>
<td>step (A2)</td>
<td>step (A3)</td>
</tr>
<tr>
<td>Mussel</td>
<td>Fresh</td>
<td>SSR 10</td>
<td>-7.2(^a)</td>
<td>-12.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSR 20</td>
<td>28.2(^b)</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>Cooked</td>
<td>SSR 10</td>
<td>49.0</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSR 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mussel</td>
<td>Fresh</td>
<td>SSR 10</td>
<td>-23.9</td>
<td>-22.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSR 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cooked</td>
<td>SSR 10</td>
<td>-17.9</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSR 20</td>
<td></td>
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<td></td>
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<td>SSR 10</td>
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<tr>
<td>Mussel</td>
<td>Fresh</td>
<td>SSR 10</td>
<td>22.4</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSR 20</td>
<td></td>
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<tr>
<td></td>
<td>Cooked</td>
<td>SSR 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSR 20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\): obtained as part of the comparison between conditions A1, A2 and A3 for the matrix effects affecting the analysis of OA, AZA1 and PTX2

\(^b\): obtained as part of the comparison between SSR 10 and SSR 20 for matrix effects affecting the analysis of OA, DTX1 and DTX2
Table 3.12: Comparison of matrix effects experienced for OA, AZA1 and PTX2 between fresh and cooked matrices as percentage reduction or increase in matrix effects between cooked and fresh shellfish (% cooked - % fresh)

<table>
<thead>
<tr>
<th>Matrices</th>
<th>LC-MS conditions</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B1</td>
<td>B2</td>
<td>B3</td>
</tr>
<tr>
<td>OA</td>
<td>Cooked vs fresh mussel</td>
<td>SSR 10</td>
<td>-1.4 E</td>
<td>+11.3 E</td>
</tr>
<tr>
<td></td>
<td>Cooked vs fresh oyster</td>
<td>SSR 10</td>
<td>-6.7 E</td>
<td>+8.1 E</td>
</tr>
<tr>
<td>AZA1</td>
<td>Cooked vs fresh mussel</td>
<td>SSR 10</td>
<td>-8.6 S</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Cooked vs fresh oyster</td>
<td>SSR 10</td>
<td>+3.0 S</td>
<td>*</td>
</tr>
<tr>
<td>PTX2</td>
<td>Cooked vs fresh mussel</td>
<td>SSR 10</td>
<td>+8.9 E</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Cooked vs fresh oyster</td>
<td>SSR 20</td>
<td>-5.4 E</td>
<td>+1.7 E</td>
</tr>
</tbody>
</table>

E: Enhancement  
S: Suppression  
Numbers in red indicate the cases where matrix effects were worse in cooked shellfish. (*) % reduction or increase in matrix effects between fresh and cooked shellfish was not calculated due to a change in the nature of matrix effect.

The preparation of 2 different matrix strengths (SSR 10 and 20) enabled the assessment of dilution on matrix effects. Diluting the mussel extracts by ca. a factor of 2 mostly resulted in a decrease of matrix effects for all conditions studied (A1, B1, B2 and B3) (Table 3.13). The degree of matrix effects in SSR 20 extracts decreased by 11.7 to 15.4 % for fresh mussels and by 9.8 to 20.7 % for cooked mussels.

Table 3.13: Effects of dilution on matrix effects experienced for OA, AZA1 and PTX2 in mussels % reduction or increase in matrix effects between SSR 10 and SSR 20 shellfish extracts ( % at SSR 20 - % at SSR 10).

<table>
<thead>
<tr>
<th>Matrices</th>
<th>LC-MS conditions</th>
<th>A1</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>SSR 20 vs SSR 10</td>
<td>Fresh mussel</td>
<td>-13.7 E</td>
<td>-15.4 E</td>
<td>-11.7 E</td>
</tr>
<tr>
<td></td>
<td>SSR 20 vs SSR 10</td>
<td>Cooked mussel</td>
<td>-20.7 E</td>
<td>-14.9 E</td>
<td>-9.8 E</td>
</tr>
<tr>
<td>DTX1</td>
<td>SSR 20 vs SSR 10</td>
<td>Fresh mussel</td>
<td>+15.8 E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTX2</td>
<td>SSR 20 vs SSR 10</td>
<td>Fresh mussel</td>
<td>-19.6 E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZA1</td>
<td>SSR 20 vs SSR 10</td>
<td>Fresh mussel</td>
<td>-9.1 S</td>
<td>-0.3 S</td>
<td>+7.6 S</td>
</tr>
<tr>
<td></td>
<td>SSR 20 vs SSR 10</td>
<td>Cooked mussel</td>
<td>+2.5 S</td>
<td>0</td>
<td>+6.0 S</td>
</tr>
<tr>
<td>PTX2</td>
<td>SSR 20 vs SSR 10</td>
<td>Fresh mussel</td>
<td>-4.7 E</td>
<td>*</td>
<td>-23.1 E</td>
</tr>
<tr>
<td></td>
<td>SSR 20 vs SSR 10</td>
<td>Cooked mussel</td>
<td>-19.0 E</td>
<td>+12.5 E</td>
<td>+1.1 E</td>
</tr>
</tbody>
</table>

E: Enhancement  
S: Suppression  
Numbers in red indicate the cases where matrix effects were worse in shellfish extracts of SSR 20. (*) % reduction or increase in matrix effects between SSR 10 and SSR 20 mussel extracts was not calculated due to a change in the nature of matrix effect.
3.2.2.2. DTX1

The degree of matrix effects for DTX1 was determined in fresh mussels at SSR 10 and 20 and under conditions A1. In both cases ion enhancement was observed (Table 3.11) with 11.1 and 26.9% respectively. Unlike OA for which diluting the mussel extract resulted in a 13.7 % decrease of the degree of ion enhancement, in the case of DTX1 this led to an increase of ion enhancement in the diluted extract by 15.8 % compared to the undiluted extract (Table 3.12).

3.2.2.3. DTX2

The degree of matrix effects for DTX2 was determined in fresh mussels at SSR 10 and 20 and under conditions A1. 34.0 and 14.4% ion enhancement were observed at SSR 10 and 20, respectively (Table 3.11). This indicates that dilution provided a reduction of the degree of matrix effect as was observed for OA. This reduction was determined as 19.6 % (Table 3.12) which is close to the value found for OA (13.7 %).

3.2.2.4. AZA1

The nature and importance of the matrix effects affecting AZA1 were assessed for mussels (fresh with a SSR 10 and 20, cooked with a SSR 10 and 20) and with oysters (fresh and cooked with a SSR 10) using 7 different sets of conditions. The slopes, intercepts and correlation coefficients determined in these conditions are presented in Table 3.7 to Table 3.10. The degrees of matrix effects are summarised in Table 3.11.

The nature of matrix effects affecting AZA1 is predominantly ion suppression with a range between 2.7 and 41.9% in mussels and between 2.9 and 23 % in oysters. Over all the tested conditions, AZA1 was found to be affected by ion enhancement in 1 case (cooked mussels at 20 under conditions B2).
The degree of matrix effect between fresh and cooked shellfish matrices was investigated under certain conditions (Table 3.12). It appeared that cooking mussels or oysters prior to the preparation of the SA solutions resulted, in most cases, in the reduction of the degree of matrix effects compared with fresh shellfish. This decrease ranged between 9.8 and 15.5% in cooked mussels SSR 10, between 3.0 and 13.9% in cooked mussels SSR 20; in cooked oysters SSR 10 the decrease in matrix effect was equal to 11.6% (Table 3.12). In condition B1 cooking the mussels and subsequently preparing SA solution at SSR 10 increased the degree of ion suppression by 8.6%, (Table 3.12).

Under conditions B2, the heat treatment led to the complete elimination of ion suppression but then led to ion enhancement (0.1%) that could be considered as not significant.

If dilution appeared to be the solution to minimise matrix effects affecting OA and DTX2, this was not necessarily the case for AZA1. Indeed, under conditions B1, B2 and B3 diluting the cooked mussel extract led to a decrease of ion suppression by 2.5%, 0.0% and 6.0%, respectively (Table 3.13). The decrease was 9.8% for fresh mussel SA solutions analysed under conditions B3. However in conditions B1 and B2 a dilution of the fresh mussel extract led to an increase of the ion suppression by 9.1% and 0.3%, respectively.

3.2.2.5. PTX2

The nature and importance of the matrix effects affecting PTX2 were assessed for mussels (fresh and cooked with a SSR 10 and 20) and oysters (fresh and cooked with a SSR 10), using 6 different sets of conditions. The slopes, intercepts and correlation coefficients determined in these conditions are presented in Table 3.7 to Table 3.10. The degrees of matrix effects are summarised in Table 3.11.

The nature of matrix effects affecting PTX2 is mainly ion enhancement. The degree of matrix effects in mussels ranged from 15.9 to 92.5%. Over all the tested conditions, PTX2 was found
to be affected by ion suppression in only one instance with a very low degree of matrix effect (1.5% for fresh mussels at SSR 10 under conditions B2).

The results showing the effects of cooking on the degree of matrix effects for PTX2 are presented in Table 3.12. Under conditions B3 the heat treatment resulted in a decrease of matrix effect by 15.7 % for the mussel extract at SSR 10. Similarly under conditions B1 the extract SSR 20 prepared from cooked mussels also led to a decrease of 5.4 % in the degree of ion enhancement.

In some cases the heating process had the opposite effect as it resulted in the increase of matrix effects. This was observed for the cooked mussel extract at SSR 10 under conditions B1 (+ 8.9 %), and for the cooked mussel extract at SSR 20 under conditions B2 (+ 1.7 %) and B3 (+ 8.5 %).

The heat treatment led to a change in the nature of matrix effects for the mussel extract SSR 10 analysed under B2 conditions; while 1.5% ion suppression was observed in the fresh mussel extract, and 6.4% ion enhancement was noted after heating the mussels (Table 3.11).

The effect of dilution on matrix affect was assessed for PTX2 in both fresh and cooked mussels, under conditions B1, B2 and B3. In three cases, dilution minimised matrix effects by 4.7 to 23.1 % but under conditions B2 the degree of matrix effect in the cooked mussel extract was increased by 12.5 % compared to the undiluted extract (SSR 10). The same pattern was observed under conditions B3, although to a lesser extent (1.1 % increase in the cooked mussel extract at SSR 20).
3.2.3. Determination of matrix effects by post extraction addition method

3.2.3.1. OA

The PEA plots enable the assessment of the importance of the matrix effects affecting a toxin over a range of matrix strengths, whereas in the SA experiments previously described only one or two matrix strengths were tested.

In the case of OA, in fresh mussels the accuracy is mainly affected at matrix strengths below 15 mg/ml with the degree of matrix effect decreasing with increasing matrix strengths. However it has to be highlighted that this trend is not linear and can vary slightly depending on the analytical conditions. Thus, under conditions A1 (Figure 3.2) the highest matrix effects were observed for fresh mussels at ca. 1 and 12 mg dry residue/ml with an average of ca. 20% enhancement in both cases. It must however be stated that the recovery assessed at 12 mg/ml of dry residue suffered from larger deviations than the other levels. For all the other matrix concentrations tested between 1 and 25 mg/ml the matrix effect were non significant. However after 25 mg/ml the nature of matrix effects changed from ion enhancement to ion suppression, reaching ca. -10% at around 40 mg/ml. Taking into consideration the fact that the SA solutions tested for OA had a matrix strength of ca. 7-8 mg/ml, there is no apparent correlation between the SA results in conditions A1 given above and the PEA experiment.
Figure 3.2: Post extraction addition of OA in mussel extracts using the HPLC gradient method on the Qtof (conditions A1). The error bars were calculated from duplicate injections. The two red lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.

The PEA of OA under conditions A2 is shown in Figure 3.3. Matrix effects did not seem to be significant up to 15 mg/ml. The overall trend of the PEA suggested that ion suppression is occurring as the matrix strength is increased. Also, repeatability becomes worse with increasing matrix strength. In conditions A2, the matrix effects observed when PEA of OA was performed at the matrix strength of the SA experiment correlated reasonably well with ca 10 % suppression against 12.6 % suppression, respectively.
Figure 3.3: Post extraction addition of OA in mussel extracts using the acidic step gradient method on the Qtof (conditions A2). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.

PEA was also carried out in conditions B1 (HPLC gradient method) for raw and heat treated mussels (Figure 3.4). The experiment demonstrated that signal enhancement is taking place and appeared to be directly proportional to the amount of dry residue in the extract up to 8.5 – 9 mg/ml. Then, fresh mussel extracts with higher amounts of dry residue led to a decrease in ion enhancement, while the degree of enhancement observed with the cooked mussels extracts remained unchanged. Good correlation was obtained between the SA experiments at SSR 10 and SSR 20 (7 - 8 and 3.5 – 4 mg/ml dry residue, respectively) and the PEA experiment in the conditions B1. Both experiments pointed towards the fact that dilution of the extract can be an efficient way to significantly reduce matrix effects associated with OA analysis regardless of the sample treatment.
The PEA carried out on cooked mussel extract using conditions B2 (Figure 3.5) exhibited a similar tendency as the PEA in conditions B1. However, this was not observed for the PEA in raw mussel extracts. A different profile was also observed between PEA in cooked and fresh mussel extracts in conditions B3 (Figure 3.6). In the latter conditions, PEA in the cooked mussel extracts exhibited a similar trend as in conditions B1 and B2 and the degree of signal enhancement observed was in accordance with the SA experiments. However, PEA in fresh mussel extracts using B2 and B3 conditions did not reflect the matrix effects observed in the SA experiments (compared at equal matrix strengths) carried out in the same conditions.

Figure 3.4: Post extraction addition of OA in mussel extracts using the gradient method on the with the QqQ (conditions B1). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.
Figure 3.5: OA Post extraction addition of OA in mussel extracts using the UPLC C8 method and the QqQ (conditions B2). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.

Figure 3.6: Post extraction addition of OA in mussel extracts using the UPLC C18 method and the QqQ (conditions B3). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions. Solutions were prepared by volumetric dilution.
PEA of OA were also performed using conditions C in fresh and cooked mussel extracts (Figure 3.7A) as well as in fresh and cooked oyster extracts (Figure 3.7B). Experiments performed in mussel extracts suggest that no significant matrix effects are occurring while those performed in oyster extracts show a similar tendency as the PEA performed in mussel extracts using conditions B1 (the degree of signal enhancement is proportional to the amount of dry residue in the extract). The PEA experiments in both mussel and oyster suggest that no matrix effects are observed at the matrix strength used in the standard addition experiment.
Figure 3.7: Post extraction addition of OA in cooked and raw mussel extracts (A) and cooked and raw mussel extracts (B) using the Acquity UPLC and the Premier XE (conditions C). The error bars were calculated from triplicate injections. The two bold lines represent the deviations obtained from the average recovery from four replicate injection of five spiked methanol solutions.
3.2.3.2. AZA1

The PEA of AZA1 in fresh mussel extracts performed in conditions A1, A2 and A3 suggests that when significant matrix effects are observed, ion suppression is likely to occur as shown in Figure 3.8, Figure 3.9 and Figure 3.10. Thus, when the degree of matrix effects are evaluated from the PEA plot at the same matrix strength as the SA extracts, approximately 20% ion suppression can be expected in conditions A1 and A2. Analyses using conditions A3 seem to be less affected with approximately 10% suppression. The results obtained for the PEA and the SA in A1 and A2 conditions are in agreement. However, this is not the case for conditions A3, where the PEA underestimated the degree of ion suppression compared to SA.

![Figure 3.8: Post extraction addition of AZA1 in mussel extracts using the HPLC gradient method and the Qtof (conditions A1). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.](image-url)
Figure 3.9: Post extraction addition of AZA1 in mussel extracts using the acidic step gradient method and the Qtof (conditions A2). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.

Figure 3.10: Post extraction addition of AZA1 in mussel extracts using the neutral step gradient method and the Qtof (conditions A3). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.
PEA of AZA1 analysed in conditions B1, B2 and B3 are shown in Figure 3.11, Figure 3.12 and Figure 3.13, respectively. Under conditions B1, the results suggest that no significant matrix effects should occur in extracts of cooked mussels and that moderate suppression would be observed in extracts of fresh mussels. This was not encountered during the SA experiments under the same conditions.

![Figure 3.11: Post extraction addition of AZA1 in mussel extracts using the HPLC gradient method and the QqQ (conditions B1). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions. Solutions were prepared by volumetric dilution.](image)

The PEA performed under conditions B2 led to an unusual pattern for AZA1 as signal enhancement was observed in extracts equivalent to SSR 10. Similarly to the previous experiment using conditions B1, the conclusions obtained by the SA and the PEA were not in good agreement.
PEA results obtained with conditions B3 suggest that no matrix effects are taking place at very low matrix strengths and that signal suppression will be observed when matrix effects are significant. Furthermore, Figure 3.13 shows that the degree of suppression increases as the matrix strength increases. There seem to be a quadratic relationship between the amount of dry residue and percentage of ion suppression observed. Excellent correlation coefficients were obtained for cooked and raw mussels (R² > 0.99). The equations are shown in Figure 3.13.

The SA experiments have demonstrated that dilution of 50% (SSR 10 to SSR 20) provided less than 8% reduction in the degree of suppression. However, the comparison of the slopes obtained by SA of AZA1 in extracts at SSR 10 and SSR 20 were not statistically different for both fresh and cooked mussel extracts.
Figure 3.13: AZA1 PEA on mussels using the UPLC C18 method on the Acquity/TQ (conditions B3). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions. Solutions were prepared by serial dilution.

PEA of AZA1 on mussel extract and analysed using conditions C indicated that there is no matrix effects associated with the analysis of raw mussel extracts and that analyses in cooked mussels would suffer from ion suppression when the amount of dry residue exceeds 20 mg/ml of extract (Figure 3.14A). The same experiment in cooked and fresh oyster extracts indicates that no matrix effects are occurring for low matrix strength and that significant ion suppression is taking place for extracts with dry residues higher than approximately 10 mg/ml (Figure 3.14B). SA in oyster extracts was performed at SSR 10, equivalent to 11.7 and 11.3 mg of dry residue per ml of extract of fresh and cooked oysters, respectively. SA in the latter extracts indicated that indeed, moderate signal suppression is taking place.
Figure 3.14: Post extraction addition of AZA1 in cooked and raw oysters extracts (A) and cooked and raw mussels extracts (B) using the Acquity UPLC and the Premier XE (conditions C). The error bars were calculated from triplicate injections. The two bold lines represent the deviations obtained from the average recovery from four replicate injections of five spiked methanol solutions.

3.2.3.3. PTX2

PEA of PTX2 in fresh mussel extracts was carried out and analysed using conditions A1, A2 and A3, results are shown in Figure 3.15, Figure 3.16 and Figure 3.17. The results obtained with conditions A1 and A2 have in common that signal enhancement is observed at low matrix strength (< 10 mg/ml) and that signal suppression is observed at high matrix strengths (> 15 mg/ml). PEA of PTX2 in conditions A3 also show that large signal enhancement is
taking place with a maximum of 120 % at 15 mg/ml of dry residue. In these conditions, the amount of dry residue of the extracts used for SA was 7.2 mg/ml. These observations were in agreement with the SA results as moderate signal enhancement was observed in conditions A1 and A2 (22.4 and 15.9 % respectively) while analyses of PTX2 in conditions A3 were prone to large signal enhancement (55.6 %).

Figure 3.15: Post extraction addition of PTX2 in mussel extracts using the HPLC gradient method and the Qtof (conditions A1). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.
Figure 3.16: Post extraction addition of PTX2 in mussel extracts using the HPLC acidic step gradient method and the Qtof (conditions A2). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions.

Large signal enhancements (with maximums near 100% in all conditions) were also consistently observed in conditions B regardless of sample preparation.
Although the precision of analyses using conditions B1 was poor, the PEA suggests that 20 % ion enhancement is taking place even at lowest matrix strength (0.4 mg/ml) as shown in Figure 3.18. Similar trends were observed for conditions B2 (Figure 3.19) and B3 (Figure 3.20 and Figure 3.21), suggesting that lowering matrix strength can only reduce the degree of matrix effects and not eliminate it. Figure 3.20 shows the PEA experiment where the extracts were prepared by volumetric dilution while the extracts used in Figure 3.21 were prepared by serial dilution. The PEA results obtained in conditions B2 shows that significant matrix effects could be expected from the method used (UPLC with C8 column coupled to QqQ). However, matrix effects observed during the SA carried out in the same conditions revealed that signal enhancement was considerably reduced in extracts of fresh and cooked mussels at SSR 10 and SSR 20 (to a lesser extent) compared to the other assessed chromatographic methods (B1 and B3). The degree of matrix effects observed by the PEA experiment (Figure 3.20) and the SA experiments in conditions B3 correlated very well for the extracts (fresh and cooked) at SSR 20, while matrix effects in extracts with SSR 10 seem to have been underestimated with the PEA.
Figure 3.18: Post extraction addition of PTX2 in mussel extracts using the HPLC gradient method and the QqQ (conditions B1). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions. Solutions were prepared by serial dilution.

Figure 3.19: Post extraction addition of PTX2 in mussel extracts using the UPLC C8 method and the QqQ (conditions B2). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions. Solutions were prepared by volumetric dilution.
Figure 3.20: Post extraction addition of PTX2 in mussel extracts using the UPLC C18 method and the QqQ (conditions B3). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions. Solutions were prepared by volumetric dilution.

Figure 3.21: Post extraction addition of PTX2 in mussel extracts using the UPLC C18 method and the QqQ (conditions B3). The error bars were calculated from duplicate injections. The two bold lines represent the deviations obtained from the average recovery from replicate injection of five spiked methanol solutions. Solutions were prepared by serial dilution.
3.2.4. Post-column infusion

3.2.4.1. OA

The PCI experiment carried out using the step gradient method with an acidic mobile phase (conditions A2) showed that there is a signal disturbance resulting from the injection of the blank mussel extract (Figure 3.22B). There is an initial slight increase of the baseline from 1.3 to 4.1 min and a second one, more important, from 5.7 to 7.5 min. This points towards a possible ion enhancement for OA under these conditions (acidic step gradient using a BDS Hypersil C8, 50 × 2.1mm, 3µm with guard column), as the toxin elutes at 1.9 min (Figure 3.22A). Although the chromatograms of DTX2 and DTX1 are not shown, both toxins would also be affected as they elute at 2.2 and 3.6 min, respectively. In addition, the rise in organic mobile phase (to 75 % at 5 min) led to a higher baseline from 5.6 min.

![Chromatogram of OA](image)

Figure 3.22: A- Example of chromatogram of OA; B- Post column infusion of OA standard acquired on the HPLC Acquity – QToF using the acidic step gradient (conditionsA2).

3.2.4.2. AZA1

PCI experiments were carried out for AZA1 using 2 sets of conditions: acidic (A2) and neutral step gradient (A3); in both cases a blank mussel extract (SSR 10) was injected in the
LC system. As shown in Figure 3.23B, the presence of matrix affected the AZA1 baseline with peaks eluting between 6.3 and 9.3 min; this would be typical of matrix enhancement. Smaller peaks eluted later (12.9 and 13.9 min) but no peak was observed at the retention time of AZA1 (11.1 min) under A2 conditions (acidic step gradient) as shown in Figure 3.23A.

Under neutral step gradient conditions (A3) AZA1 eluted at 4.8 min (Figure 3.24A) but under these conditions, the signal was not disturbed by the presence of matrix components as shown by the steady baseline (Figure 3.24B).

Therefore, the PCI experiments showed that under the tested A2 and A3 conditions, AZA1 would not be affected by matrix effects. The SA results contradict these observations as 22.3 and 27.7% ion suppression were observed under the same conditions (Table 3.11).

Figure 3.23: A- Example of chromatogram of AZA1; B- Post column infusion of AZA1 standard acquired using the HPLC Acquity – Qtof and the acidic step gradient (conditions A2).
3.2.4.3. PTX2

PCI experiments were carried out for PTX2 using 2 sets of conditions: acidic (A2) and neutral step gradient (A3). In both cases a blank mussel extract was injected in the LC system. As shown in Figure 3.25B, under conditions A2, the injection of blank mussel extract in the system affected the PTX2 baseline as the intensity of the signal initially dropped and rose slowly to get back to its nominal value after 3 min. However at the retention time of PTX2 under these conditions (2.42 min; Figure 3.25A) the baseline is still slightly rising indicating the likelihood of a relatively small ion suppression effect for PTX2.

Under neutral step gradient conditions (A3) PTX2 eluted at 4.6 min (Figure 3.26A) which, as shown by the PCI chromatogram (Figure 3.26B), corresponded to a time window where PTX2 would be affected by a small ion suppression.
The PCI experiments showed that under the tested conditions, PTX2 would be affected by a relatively small ion suppression effect. The SA results contradict the observations made in the PCI experiments, as 15.9 and 55.9% ion enhancement were observed in the same analytical conditions (Table 3.11).

Figure 3.25: A- Example of chromatogram of PTX2; B- Post column infusion of PTX2 standard acquired on the HPLC Acquity – Qtof using the acidic step gradient (conditions A2)
3.2.5. Influence of lipids on matrix effects

3.2.5.1. Classes of lipids

The lipids classes that were collected are as follows: neutral lipids eluted with chloroform (hydrocarbons, pigments, sterols, triglycerides, waxes, fatty acids…), glycolipids eluted with acetone and polar lipids eluted with methanol (phospholipids). The determination of the elution profile of OA and AZA1 from the silica adsorbent revealed that OA eluted in fraction 2 (acetone) and 3 (methanol) with 51.8 and 41.4% being recovered, respectively. In the case of AZA1, elution only occurred in the last fraction with 91.8% of the toxin being recovered (Table 3.14). The recovery of the successive evaporation of a methanolic solution of OA and AZA1 and resuspension step was calculated from triplicate samples and were 98 and 96 % for OA and AZA1, respectively. Furthermore, recoveries were also calculated for the consecutive evaporation of a methanolic solution, resuspension of the dry residue in chloroform followed by an additional evaporation step and resuspension in methanol (identical to sample
preparation required to perform the Folch method). The recoveries were 105 and 102 % for OA and AZA1, respectively. These results indicate that there is no apparent loss of toxins because of the successive evaporation and resuspension steps.

The complete sets of results of the SA experiments carried out using lipid fractions representative of the different classes of lipids are presented in Table 3.15.

Table 3.14: Recoveries obtained in acetone mobile polar fraction (AMPL) and polar lipids (PL) after successive evaporation and resuspension of a methanolic solution spiked with OA and AZA1 and concentrations of OA and AZA1 obtained in the different lipid fractions after separation of a spiked extract. Concentrations were obtained by Qtof analysis

<table>
<thead>
<tr>
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<th>Spiked Methanol - Control (n=3)</th>
<th>Evap. + resusp. MeOH (n=3)</th>
<th>Evap. + resusp. CHCl3 + Evap. + resusp. MeOH (n=3)</th>
<th>Conc in AMPL (n=1)</th>
<th>Conc in PL (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OA</strong></td>
<td>Conc (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.98 ± 0.51</td>
<td>19.54 ± 1.45</td>
<td>20.88 ± 0.72</td>
<td>10.34 ± 0.59</td>
<td>8.28 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>100.00 ± 2.56</td>
<td>97.78 ± 7.23</td>
<td>104.50 ± 3.62</td>
<td>51.76 ± 0.59</td>
<td>41.42 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.63 ± 0.36</td>
<td>13.06 ± 0.81</td>
<td>13.87 ± 0.12</td>
<td>&lt; LOD ± 0.48</td>
<td>12.86 ± 0.40</td>
</tr>
<tr>
<td><strong>AZA1</strong></td>
<td>Conc (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.98 ± 0.51</td>
<td>19.54 ± 1.45</td>
<td>20.88 ± 0.72</td>
<td>10.34 ± 0.59</td>
<td>8.28 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>100.00 ± 2.56</td>
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<td>41.42 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.63 ± 0.36</td>
<td>13.06 ± 0.81</td>
<td>13.87 ± 0.12</td>
<td>&lt; LOD ± 0.48</td>
<td>12.86 ± 0.40</td>
</tr>
</tbody>
</table>

\(^1\) Deviation calculated on triplicate injection
Table 3.15: Summary of the matrix effects observed for OA and AZA1 by LC-MS analysis using the QToF and the QqQ (conditions A1 and B1 respectively). The slopes, intercepts and R2 were calculated on triplicate injection of five solutions ranging from 8 to 30 ng/ml. Matrix effects percentages were calculated relative to the slope obtained for spiked methanol solutions.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>QToF</th>
<th>Triple quadrupole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Int.</td>
</tr>
<tr>
<td>Neutral lipids</td>
<td>1.12 ±0.11</td>
<td>-0.47</td>
</tr>
<tr>
<td>Glycolipids</td>
<td>1.14 ±0.13</td>
<td>±1.30</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>1.11 ±0.08</td>
<td>±1.08</td>
</tr>
<tr>
<td>OA</td>
<td>1.16 ±0.07</td>
<td>±1.37</td>
</tr>
<tr>
<td>SSR 20</td>
<td>1.60 ±0.08</td>
<td>±1.78</td>
</tr>
<tr>
<td>SSR 10</td>
<td>1.57 ±0.08</td>
<td>±1.74</td>
</tr>
<tr>
<td>Hydrolysed</td>
<td>1.11 ±0.09</td>
<td>±1.42</td>
</tr>
<tr>
<td>MeOH</td>
<td>3.21 ±0.05</td>
<td>±0.36</td>
</tr>
<tr>
<td>Glycolipids</td>
<td>±0.15</td>
<td>±1.37</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>±0.18</td>
<td>±3.80</td>
</tr>
<tr>
<td>AZA1</td>
<td>±3.34</td>
<td>±1.03</td>
</tr>
<tr>
<td>SSR 20</td>
<td>±0.37</td>
<td>±2.11</td>
</tr>
<tr>
<td>SSR 10</td>
<td>±0.14</td>
<td>±0.83</td>
</tr>
<tr>
<td>Hydrolysed</td>
<td>±0.12</td>
<td>±0.65</td>
</tr>
</tbody>
</table>

1 Calculated vs another set of MeOH solutions (data not shown). The slopes obtained for both set of MeOH were within their standard deviation.
3.2.5.2. OA

Under conditions A1 (QToF) none of the lipid fractions obtained by separation on silica column induced significant matrix effects in OA quantitation while pronounced matrix effects were observed in the SSR 10 extract (equivalent to the matrix strength in the lipid fractions) (Table 3.15). Under conditions B1 (QqQ) the glycolipids and the polar lipids showed the highest matrix effects with 13.0 and 13.8 % of signal suppression, respectively. OA analysis in the neutral class of lipids also led to signal suppression but to a lesser extent with 7.9 % suppression. Similarly to the analysis on the QToF, the matrix effects measured in all lipid fractions were lower than those observed in the extract at a SSR of 10.

3.2.5.3. AZA1

According to the data presented in Table 3.15, under conditions A1 (Qtof), fraction 3 showed the highest degree of matrix effects with 22.1% suppression, followed by fractions 1 and 2 with -13.5% and -5.4%, respectively. Under conditions B1 (QqQ) fraction 1 gave more matrix effects than fraction 3 (-22.6% against -8.6%), and once again fraction 2 gave the lowest matrix effects, although this time it was 5.5% ion enhancement.

Although AZA1 elutes in the same fraction as the phospholipids, these compounds do not seem to be the only ones responsible for matrix effects. In fact under conditions B1 glycolipids may have also played a significant role.

3.2.6. Matrix effects in hydrolysed extract

The results concerning the matrix effects in the hydrolysed mussel extract are presented in Table 3.15. It appeared that the hydrolysis process either allowed the degree of matrix effects to be unchanged or slightly increased it. Thus, in the case of OA under A1 conditions (QToF)
the degree of matrix effects was similar to that of the unhydrolysed extract (SSR 10) with 54.5 and 57.0% respectively. However, under B1 conditions the matrix effect was increased from -18.5% (unhydrolysed extract SSR 10) to -54.3%.

In the case of AZA1, the degree of matrix effects was higher under conditions A1 (15.8 % ME instead of 6.1% for the unhydrolysed extract SSR 10 and -9.9% for unhydrolysed extract SSR 20). However, the QqQ analyses carried out under conditions B1 showed no significant change in the degree of matrix effects (-40.1% against -38.3% for the unhydrolysed extract SSR 10).

As the changes in matrix effects could be related to the changes in matrix composition resulting from the hydrolysis process but not necessarily to the salts present, a further experiment was performed to investigate this. This was assessed by using a salt solution spiked with OA, DTX1 and DTX2. This time round AZA1 was not included because it would not be analysed in a hydrolysed extract since this toxin would be degraded during the process.

3.2.7. Influence of salts on matrix effects

The influence of salts and NaCl in particular on matrix effects was assessed by preparing SA solutions of OA, DTX1 and DTX2 in methanol and in NaCl solution. The NaCl solution was at a concentration of 20.0 g/l, which would be greater than the NaCl concentration would be after hydrolysis (14.6 g/l).

The results obtained for the different toxins spiked in methanol/water and in methanol/aqueous NaCl solution are presented in Figure 3.27. Whatever the toxin considered (OA, DTX1 or DTX2), the salt did not seem to have any effect on the instrument response as the SA solutions in methanol and NaCl overlapped perfectly. This rules out the salt as being responsible for the matrix effects reported for hydrolysed extracts in the previous paragraph (3.2.6).
Figure 3.27: (A) OA, (B) DTX1 and (C) DTX2 SA in methanol/water and methanol/aqueous NaCl salt solution under conditions A1.
3.2.8. Memory effect

One OA standard (230 ng/ml) was injected 10 times before and after the injection of 10 LRM (made of mussel tissue) using the LC-MS conditions B1. After the injection of the 10 matrix solutions the response increased by 10%. The results are shown in Figure 3.28.

![Figure 3.28: Illustration of column memory effect when analysing 10 standards (Std) of OA (230 ng/ml) before and after injection of 10 mussel matrix (LRMs) using conditions B1](image)

3.3. Discussion

3.3.1. Standard addition

3.3.1.1. Effect of chromatography

Three types of chromatography were assessed for their influence on matrix effects: a gradient elution, an isocratic elution with an acidic mobile phases and an isocratic elution with a neutral mobile phase. In the three cases the detection was performed with the QTof instrument. Matrix effects that affected the analysis of OA were variable when the gradient method was used and large retention time shifts occurred when the isocratic method with neutral mobile phase was used. This did not allow for the establishment of a relationship
between the matrix effects affecting the analysis of OA and the type of chromatography used. In the case of AZA1 a signal suppression of 22 to 28% was observed in the three conditions demonstrating that the matrix effects that affect the analysis of AZA1 do not depend on the type of chromatography. In the case of PTX2, signal enhancement was observed in the three types of chromatography but the degrees of ion enhancement were not equal. The isocratic elution with an acidic mobile phase was the condition that was the less prone to matrix effects (16% signal enhancement) while the isocratic elution with the neutral mobile phase was the condition that exhibited the most matrix effects (56% signal enhancement). This observation suggests that the pH of the mobile phase plays a role in the matrix effects affecting the analysis of PTX2.

3.3.1.2. Effect of stationary phase

Three conditions were used for the investigation on the effect of the stationary phase and included a C₈ phase with 3 µm particle size (HPLC), a C₈ phase with 1.7 µm particle size (UPLC) and a C₁₈ phase with 1.7 µm particle size (UPLC). The three conditions were performed using gradient elution with the same mobile phases. The flow rate and gradient speed were higher for the conditions that used the 1.7 µm particle size (UPLC).

The use of UPLC always reduced matrix effects associated with OA analysis when compared to HPLC. In addition, it was observed that the UPLC stationary phase seems to play a role in the matrix effects associated with AZA1 and PTX2. The use of UPLC with a C₁₈ column for their analyses led to a higher degree of matrix effects than those observed in HPLC. This may be due to the higher capacity of retention of disturbing lipophilic compounds by the C₁₈ columns which can be released from the stationary phase during subsequent runs. The use of UPLC with a C₈ column proved to be the most suitable method for the quantification of OA, AZA1 and PTX2 in mussels.
3.3.1.3. Effect of instruments

One of the HPLC condition and one of the UPLC conditions were used on two different instruments. The QqQ Ultima and the QTof were used for the detection of OA, AZA1 and PTX2 in extracts of raw mussels with the gradient HPLC conditions (conditions A1 and B1). The QqQ Ultima and the latest generation QqQ Premier XE were used to detect OA and AZA1 in extracts of cooked and raw mussels with a UPLC separation carried out on a C18 column (conditions B3 and C).

The matrix effects affecting the analysis of OA seemed to be highly variable and instrument dependent. For example the analysis of raw mussels in conditions C led to signal suppression while the same type of matrix led to signal enhancement in conditions B3. Furthermore, the degree of ion enhancements that were observed for OA in raw mussels (SSR 10 and SSR 20) were 20 to 22 % higher for the QqQ Ultima (condition B1) than when the QToF was used (condition A1).

The type and the degree of matrix effects that were observed for the analysis of AZA1 were similar regardless of the instrument that was used. An 11 and 6 % difference in the degree of ion suppression was obtained between extracts of fresh and cooked mussels analysed in UPLC conditions (B3 and C). The difference was larger (15 %) for the matrix effects observed in fresh mussels using HPLC (conditions A1 and B1) although both conditions suffered from ion suppression.

Ion enhancement was observed for both HPLC conditions that were used to investigate the matrix effects associated with PTX2 with a difference of 23 %. In this case the instrument seems to have limited effect on matrix effects.

The differences of matrix effects observed on the different instruments may be attributed to the source design especially between the QqQ Premier and the QqQ Ultima. The QTof and the QqQ Ultima were equipped with the same ESI source (z-spray) but this does not mean
that the differences in matrix effects cannot be attributed to the source. Attempts were made to exchange the sources of both instruments in order to investigate whether matrix effects are dependent on the ESI spray position. This experiment was not successful because the MS inlets of the two instruments are placed at different positions and the absence of graduation for the X, Y, Z, position of the spray restricted further investigation. As the analysis of PTX2 was always compromised by ion enhancement and as the AZA1 analysis was almost always compromised by ion suppression, it is not possible to attribute a particular direction of matrix effects to an ionization mode, e.g. positive or negative. Similarly, matrix effects were not quantitatively correlated with the sensitivity towards a particular analyte.

3.3.1.4. Effect of species

Limited data are available to discuss the effect of species. Matrix effects were assessed for OA and AZA1 in mussels and oysters extracts using the condition UPLC-Premier XE. In these conditions, analyses of fresh shellfish extracts were affected by ion suppression with only 5 and 8 % difference, between mussels and oysters for OA and AZA1, respectively. A larger difference was observed between cooked mussels and cooked oysters. The type of matrix effects affecting the analysis of OA changed to ion enhancement for both cooked shellfish with a difference of 15 % between mussels and oysters. Matrix effects affecting the analysis of AZA1 in cooked shellfish remained a signal suppression effect and a 10 % difference between oysters and mussels was observed. Shellfish species do not seem to play a major role on the type and the degree of matrix effects that are observed. However, further species and conditions should be investigated in order to confirm the above observations.
3.3.1.5. Effect of heat treatment

Standard addition of OA and AZA1 in extracts of cooked shellfish generally led to an increased response. Thus, in conditions B (1, 2 and 3) at SSR 10 and 20 the matrix effects that affected the analysis of OA were always ion enhancement. Out of these 6 cases, heat treatment increased the degree of ion enhancement in four cases (conditions B2 and B3). When the UPLC-Premier XE method (condition C) was used the SA of OA in heat treated shellfish neither improved nor worsened the matrix effects observed in the fresh oyster extracts as 23% of signal enhancement was observed in heat-treated flesh against 22% of signal suppression in raw flesh.

Therefore, in most cases the cooking of shellfish flesh would result in more pronounced matrix effects in the analysis of OA especially if the LC-MS system is prone to ion enhancement when OA is analysed in raw shellfish extract.

Since heat treatment generally increased the analytical response and the nature of matrix effects that affect the analyses of AZA1 was always ion suppression, heat treatment of shellfish generally reduced matrix effects associated with the analysis of AZA1 for both mussels and oysters (6 cases out of 7).

There is no apparent effect of heat treatment for the analysis of PTX2. In most cases when a pronounced ion enhancement was observed in the raw shellfish extract, similar strong ion enhancement occurred in the heat treated shellfish extract (e.g. conditions B1 and B3). When weak matrix effects were observed (conditions B2) heat treatment of the shellfish extracts also resulted in weak matrix effects.

Thus, the introduction of a heat treatment step in the routine analytical method could be beneficial for AZA1, as the sample treatment led to a reduction of the signal disturbances. In addition, heating treatments would speed up the shucking process and in the case of shellfish
that are typically eaten cooked such as mussels, the reported results would be closer to the amount of toxins that consumers are exposed to.

3.3.1.6. Effect of dilution

Diluting the extract proved to be an efficient way of reducing matrix effects for OA. The influence of dilution was investigated in 7 LC-MS conditions all of which showed significant reduction of matrix effects when OA analysis was carried out in diluted extracts. This behaviour was also observed for DTX2 in condition A1 but was not observed for DTX1.

Dilution did not appear to reduce matrix effects in a systematic manner for the analysis of AZA1 and PTX2.

Despite the fact that diluting the extract may not appear to reduce matrix effects for all toxins that were considered in this study, limiting the amount of dry residue entering the instrument is desirable because other types of matrix effects can occur. This particular aspect is discussed in details in the PEA section below. It must however be considered that dilution will increase the LOD of the method and may therefore not be applicable to routine monitoring for all types of instruments.

3.3.1.7. Summary

The influence of all parameters and treatments discussed above are summarised in Table 3.16.

Table 3.16: Summary of the effect of chromatography, stationary phase, instrument, shellfish species, shellfish heat treatment and extract dilution on the matrix effects affecting the analysis of OA, AZA1 and PTX2.

<table>
<thead>
<tr>
<th></th>
<th>LC</th>
<th>Stationary Phase</th>
<th>Instrument</th>
<th>Species</th>
<th>Heat Treatment</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>n/a</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AZA1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PTX2</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ : observed effect; - : no observed effect; +/- : limited observed effect; n/a: not available
Generally, ion enhancement was observed in the analysis of OA in shellfish extracts. The matrix effects seemed to be dependent on the MS instrument that was used but not on the type of stationary phase. Heat treatment increased the degree of ion enhancement while dilution significantly reduced it.

Matrix effects that affected the analysis of AZA1 were always the ion suppression type and did not appear to be dependent on the type of chromatography or the instrument used but on the type of the stationary phase \((C_8 \text{ or } C_{18})\). The results obtained for mussels and oysters suggest that there is a limited effect of the shellfish species on the matrix effects for AZA1 analysis and that dilution does not reduce the degree of matrix effects observed.

When matrix effects were encountered in the analysis of PTX2, ion enhancement was always observed. Both chromatography and stationary phase affected the degree of matrix effects observed while only limited effect was observed when different instruments were used. Heat treatment of the shellfish flesh and dilution of the shellfish extract did not seem to affect the type and the degree of matrix effects.

3.3.1.8. Lipids/silica effect

Contribution of lipids to matrix effects has been demonstrated in plasma analysis where phospholipids (polar lipids) were identified as the main cause of matrix effects (Chambers et al., 2005; Bennett and Liang, 2004). For the present study it was chosen to assess the influence of the different classes (polar, non-polar and acetone mobile lipids) on matrix effects. It was anticipated that if one particular class of lipid can be found responsible for matrix effects, adequate clean-up methods could be developed to avoid the presence of the interfering compounds.

The methodology consisted of performing SA of OA and AZA1 in different lipidic fractions. The three lipid classes (polar, acetone mobile and non-polar lipids) were obtained by the
Folch method (Folch et al., 1957). The lipid fractions were obtained at a SSR of 10 and therefore, if a particular lipid class is causing matrix effects, similar matrix effects as those observed with an extract with a SSR of 10 were expected. When SA of OA in a mussel extract at SSR 10 was carried out and in conditions A1, 57% signal suppression was observed while no significant matrix effects were observed for an extract at SSR 20. In conditions B1 the SA of OA in extract at SSR of 10 demonstrated that signal suppression is taking place and similarly to the results obtained in conditions A1, no significant matrix effects were observed at SSR of 20. The spiked extracts were analysed using two different systems (conditions A1 and B1). Analyses of OA in conditions A1 did not demonstrate that more pronounced matrix effects were taking place with any particular class of lipids suggesting that the separation on silica column may have acted as a clean-up step. The analyses of OA that were performed in conditions B1 showed that signal suppression (< 14%) occurred in the fraction corresponding to glycolipids (acetone mobile lipid fraction) and polar lipids. The matrix effects that were observed in each lipid class were lower than those observed in the extract at SSR of 10 suggesting that overall the use of silica proved successful in reducing matrix effects for OA.

Analyses of AZA1 in conditions A1 have shown that neutral lipids and polar lipids are contributing towards matrix effects, while in conditions B1 only neutral lipids were found to contribute to matrix effects. However, the analysis of WF extracts at a SSR of 10 in conditions A1 did not result in significant matrix effects. The neutral lipid fraction that includes sterols, triglycerides and fatty acids was found to cause signal suppression in both analytical conditions. These results suggest that the presence of neutral lipids can be responsible for signal suppression in AZA1 analyses. This route should be further investigated with extracts that cause more matrix effects and with material that has been fully characterised for their lipid profile.
3.3.1.9. Hydrolysis/salt effect

Compounds referred to as DTX3 include several ester derivatives resulting from the reaction of OA, DTX1 and DTX2 with a wide range of fatty acids. It is a well accepted approach to quantifying these ester derivatives by differences of concentration of the parent DSP toxins in hydrolysed and non-hydrolysed extracts. In this study we investigated if additional matrix effects would occur as a result of the hydrolysis procedure. Blank mussel extracts (SSR 10) were hydrolysed, neutralised and subsequently spiked with OA and AZA1. It is expected that the hydrolysis procedure would yield a number of free fatty acids as a result of triglycerides break down. The results obtained show that similar matrix effects were taking place after hydrolysis (when compared to SSR of 10) for AZA1 in both analytical conditions and for OA in conditions A1. The same analyses carried out for OA in conditions B1 showed that significantly more pronounced signal suppression took place with the hydrolysed extract. The lack of reproducibility in matrix effects between the two analytical methods prevented us from producing a general statement on the influence of hydrolysis on matrix effects.

In addition to the change in the chemical nature of the compounds present in a sample before and after hydrolysis, the influence of salt on OA, DTX1 and DTX2 analyses was also investigated. Salt is produced as a result of the reaction between sodium hydroxide (used for hydrolysis) and hydrochloric acid (used for neutralisation). The solutions that were spiked contained the double of the concentration of NaCl that is generated by the “well accepted” hydrolysis procedure (Yasumoto et al., 1985; Mountfort et al., 2001). None of the DSP toxins were found to be affected by the presence of salts. This is not in contradiction with the fact that salt can be responsible for matrix effects in LC-MS analyses (cf introduction). Salts are usually present in mobile phases as pH buffers and therefore are continuously competing with analytes ionisations.
3.3.2. Post extraction addition

In almost all cases the PEA of OA provided information that was in agreement with the SA experiments. Thus, when signal enhancement was encountered for example in conditions B1 to B3, this was also observed at equivalent SSRs. Furthermore, the reduction of matrix effects through sample dilution was clearly illustrated in most PEA experiments dilution. The signal suppression that was observed in conditions A3 using the SA method was also encountered when PEA was carried out. Regardless of the type of matrix effects observed (signal enhancement or signal suppression) it was always observed that signal suppression occurred when the amount of dry residue in the extract is increased.

The signal suppression that was observed for AZA1 was confirmed by the PEA in most conditions. More severe suppression was usually observed when the amount of dry residue in the extract was increased.

For PTX2 signal enhancement was always confirmed. Similarly to AZA1 and OA signal suppression started to occur once relatively high concentration of dry residue was reached, typically after 10 to 15 mg/ml for PTX2 analysis. According to the SA experiments, dilution of the extract was not successful in reducing matrix effects associated with PTX2 analysis.

The PEA experiments suggest that dilution can reduce matrix effects although a higher dilution factor than in the SA may be required to observe a significant improvement.

This method provided evidences that non-volatile material can have a major impact on matrix effects for some toxin/chromatographic method combinations. However, high content of non volatile material did not always result in more pronounced matrix effects. One hypothesis would be that two types of matrix effects are occurring. The first type of matrix effects is dependent on the nature of the interference and can thus lead to suppression or enhancement whereas the second type always leads to suppression and is caused by non volatile material. The amount of dry residue and the degree of matrix effects do not have a linear relationship.
Relationship of a quadratic nature has been observed in only in one condition / analyte combination (AZA1 - condition B3 - Figure 3.13).

3.3.3. Post column infusion

The results of the PCI did not lead to new findings regarding the cause of matrix effects. However, one observation was that for all toxins, strong signal suppression can occur during the first 1 – 1.5 min of the chromatographic run. Therefore, care should be take when developing new methods that the analyte of interest is not eluting too closely to the solvent front. This phenomenon is believed to be due to early eluting compounds such as salts that would compete for the charges during the ionisation process. For all toxins that were considered, no co-eluents leading to either ion enhancement or ion suppression were observed.

3.3.4. Other effect/memory effects

In addition to the parameters that are affecting matrix effects presented above, another effect was observed. Injections of 10 OA standards before and after the injection of mussel matrix have shown that the response was increased after the injection of mussel matrix. The type of matrix effects observed is consistent with the SA and PEA experiments as signal enhancement was observed for OA. The cause of this “memory effect” is not well understood and can be due to the column because of late eluting compounds or to the cleanliness of the source.

3.3.5. Context / general discussion

When significant matrix effects occurred during AZA1 analyses signal suppression was always the observed effect. Large signal enhancement was usually observed in the analysis of PTX2 in shellfish. Similarly to AZA1, dilution did not prevent or reduce matrix effects. Heat treatment of the shellfish did not seem to have a particular effect. Ion enhancement was
consistently encountered in the analysis of OA when significant matrix effects were observed by the standard addition method. The experiments carried out with DTX2 have shown very similar matrix effects as for OA. However, the matrix effects that were observed for DTX1 followed a different trend.

Ion enhancement in the analysis of OA in shellfish has been reported in previous studies (Stobo et al., 2005). There was 80-130% ion enhancement for OA in the assessed matrices (mussels, cockles, oysters, scallops). McNabb et al. (2005) also reported matrix effects when OA was analysed in Greenshell mussel (*Perna canaliculus*), Pacific oyster (*Crassostrea gigas*), NZ cockle (*Austrovenus stutchburyi*) and scallop roe (*Pecten novaezelandiae*). Less than 10% enhancement was observed for OA in all matrices. Analysis of OA and DTX1 in scallops using a sonic-spray interface led to signal suppression (up to 33% for OA) despite a liquid/liquid partitioning procedure with chloroform (Ito and Tsukada, 2002). As outlined above the nature of matrix effects observed in the analysis of OA is probably instrument/source dependent.

Signal suppression of 27 to 48% was observed when AZA1 was analysed in mussels, cockles, oysters, scallops (Stobo et al., 2005). The nature of the matrix effects is in agreement with our findings as signal suppression was observed in the 7 analytical conditions that were used.

The analysis of PTX2 in mussels, oysters, cockles and scallops induced a 10 to 12% signal enhancement (McNabb et al., 2005). The nature of matrix effects is in agreement with the matrix effects observed for PTX2 in this work (signal enhancement observed in 7 analytical conditions).

As outlined in Chapter 1, efforts are currently being made by the several research groups in the attempt to offer one or several methods for the replacement of animal assays currently in
place in the EU for the detection of marine biotoxins in shellfish. The replacement method, especially if it implies LC-MS technique, should preferably be validated in order to demonstrate that the chosen method is fit for the purpose. Validation generally implies two phases: single laboratory validation (SLV) and interlaboratory validation (ILV). Guidelines for SLV methods of analysis have been published by the International Union for Pure and Applied Chemists (IUPAC) (Thompson et al., 2002).

As outlined in the IUPAC guidelines, the performance characteristics of the analytical methods are: applicability, selectivity, calibration (that includes assessment of matrix effects), trueness, precision, recovery, operating range, LOQ, LOD, sensitivity, and ruggedness. To these can be added measurement uncertainty, fitness-for-purpose and matrix variation.

ILV, also known as collaborative studies, requirements have been summarised in the IUPAC/AOAC protocol (Horwitz, 1995). Collaborative studies involve 10 to 12 participants and in the analysis of five materials consisting of sample matrices containing a representative concentration of analyte, usually in duplicate. The materials should be representative of the matrices and the levels of analytes typically encountered. This is a challenging task in the field of marine toxins due to the wide range of shellfish species and toxins that are analysed by regulatory laboratories worldwide. Variations of matrix types are additional source of variation due to within-class matrix effects. The IUPAC guidelines for SLV emphasised that: “Matrix variation is, in many sectors, one of the most important but least acknowledged sources of error in analytical measurements” (Thompson et al., 2002). The present chapter has demonstrated that matrix effects encountered in the analysis of lipophilic marine toxins in shellfish can be instrument dependant and that several other parameters such as species, stationary phase or matrix strength of a particular extract may play a major role in the degree of matrix effects observed. Within the limited number of laboratories that conduct shellfish analysis for marine biotoxins numerous LC-MS systems are being used (different
manufacturers, models, detectors, source designs) which can have some impact on the nature and on the degree of matrix effects occurring. Since there are many additional parameters that will equally affect matrix effects (analyte considered, species…) the influence of the different LC-MS configurations is difficult to anticipate. Furthermore, it must also be noted that representative matrix i.e. particular analyte/shellfish species combination, is dependent on the geographical site considered. Thus, ILV for the analysis of lipophilic marine biotoxins would be very challenging and difficult to achieve. One alternative is to carry out SLV for each group of toxin separately (e.g. OA, AZA and YTX group) with a defined protocol for extraction procedure (e.g. type of solvent, SSR) and defined parameters for the MS detection of the toxins (e.g. ionisation mode, choice of fragment ions). The type of chromatography and analytical column would be chosen by the laboratory after careful investigation of the robustness of the method (e.g. accuracy, precision, matrix effects). This approach would allow the laboratories to focus on the validation of methods that are suitable for the analysis of particular toxin that are occurring particular locations. For example, a validated method for the analysis of YTX would be important in laboratories that monitor shellfish from Italy but not in laboratories that monitor shellfish from Ireland as YTX does not seem to be produced in Irish waters. Conversely, a validated method for the analysis of AZAs in shellfish would be highly desirable in Ireland and of limited use in Italy.

Focusing on one group of toxin will also facilitate the development of clean-up procedures since a particular procedure should prove to be suitable for the analysis of a smaller number of analytes than when all toxin groups are included in the same method. Furthermore, the toxins that belong to the same toxin groups are structurally related to each other and hence have similar chemical and physical properties. The approach of considering each toxin group separately would therefore allow for a higher specificity in sample extraction and sample clean-up than in a universal method for the analysis of lipophilic toxin.
3.4. Conclusions

This work provided new information regarding matrix effects occurring in the LC-MS analysis of lipophilic marine toxins. Matrix effects are highly variable and depend on the analyte considered, the stationary phase used, the MS instrument, the shellfish species and eventual heat treatment as well as the matrix strength at which the extract is analysed.

This work identified one method that was not affected by matrix effects which consisted of a UPLC separation on a BEH C8 column and triple quadrupole detection. These findings need to be further investigated by assessing matrix effects with other shellfish species and MS instruments. Dilution of shellfish extracts can also reduce or even eliminate matrix effects associated with OA and DTX2, as well as reducing the overall build up of dirt in the source and reducing the amount of non volatile material.

Some memory effect has been shown to play a role in the degree of matrix effects observed. This can be due to build up of dirt in the source or to column bleeding.

Our results were also in agreement with other studies that suggested that the presence of non volatile compounds can result in matrix effects (King et al., 2000). However, it is clear from our studies that this was not the only cause of matrix effects and the attempt to detect possible co-eluents by PCI was not successful. Lipids have been shown to play a role in matrix effects but no particular trend was observed because of major matrix effects differences between instruments.

In addition to the above recommendations, efforts should continue towards the development of clean-up methods that can provide analyses in cleaner extracts. Furthermore, the
development of internal standards (preferentially isotopically labelled internal standard) for lipophilic marine toxins should be strongly encouraged as it would enable the introduction of correction factors to compensate for signal suppression/enhancement and thus improve the overall accuracy and precision of the analytical procedure.

3.5. Future work - Methods arising from UPLC-MS methods

The findings regarding the ability of the UPLC-QqQ method with the C8 column to reduce or eliminate matrix effects led to the development of new methods for the eventual replacement of the LC-MS method currently in place as part of the national monitoring program at the MI (condition B1). As outlined in Chapter 2 the Quattro Ultima QqQ does not have the capability of rapid mode switching and this prevents the monitoring of the co-eluting toxins DTX1 and AZA3 in a single analytical run. Therefore it was chosen to develop two isocratic methods, one for the analysis of OA, DTX1 and DTX2 and the second method for the analysis of AZA1, -2 and -3. A total run time of 10 min is necessary to perform both methods while the gradient routine method requires 12 min. When the analysis of the DSP ester derivatives is required the isocratic approach requires 10 min to run hydrolysed and non-hydrolysed samples against 24 min for the routine method.

Both methods are currently under in-house validation. The two analytical conditions are given in 3.5.1 and in 3.5.2 and examples of chromatograms are shown in Figure 3.29 and Figure 3.30.

3.5.1. LC conditions for DSP toxins analysis

An Acquity UPLC BEH C8 column (100 × 2.1 mm, 1.7 µm particle size, in-line filter 0.2 µm) was used for the separation of OA, DTX1 and DTX2. An Isocratic elution consisting of 55% B was used at a flow rate of 400 µl/min. The injection volume was set at 10 µl and the total run time was 5 min.
3.5.2. LC conditions for AZA toxins analysis

An Acquity UPLC BEH C₈ column (100 × 2.1 mm, 1.7 μm particle size, in-line filter 0.2 μm) was used for the separation of AZA1, AZA2 and AZA3. Isocratic elution consisting of 60% B was used at a flow rate of 400 μl/min. The injection volume was set at 10 μl and the total run time was 5 min.
Figure 3.30: Example of chromatogram of AZA1, AZA2 and AZA3 obtained using the UPLC-QqQ isocratic method, C8 column, run time = 5 min
4. **Solid Phase Adsorption Toxin Tracking technique: Adsorption and Desorption considerations for several polymeric resins**

4.1. Introduction

The recent development of passive samplers, referred to as solid phase adsorption toxin tracking (SPATT) for the detection of lipophilic marine toxins, offers a new tool to researchers to study lipophilic phycotoxins (MacKenzie et al., 2004). The use of passive samplers could be very useful in toxin distribution studies due to their ability to accumulate toxins onto the receiving phase *in-situ*, without need of energy and most importantly, avoiding bio-transformation occurring in living organisms. Beyond the wide range of applications that SPATT could offer to research purposes, their use in toxin monitoring programs could also be beneficial. Indeed, passive samplers have the advantage of providing a spatially and temporally integrated response, thereby reducing the number of analyses for long time monitoring.

The SPATT bags described by (MacKenzie et al., 2004) consist of sachets made of 95 µm mesh that contain the Diaion® HP-20 resin, a styrene divinylbenzene (DVB) copolymer. MacKenzie et al. found that, out of three different structure-based polymeric resins i.e. brominated styrene-divinylbenzene resin, acrylic-type polymer and styrene divinyl benzene copolymer, the latter was found to be the most suited receiving phase for SPATT bags (MacKenzie et al., 2004). This type of macroreticular polymer is widely used for water treatment applications such as removal of phenolic compounds (Li et al., 2002), pesticides
(Kyriakopoulos et al., 2005), salicylic acid (Otero et al., 2005) and phenylhydrazine derivatives (Zhai et al., 2003), as it offers a number of advantages over other adsorbents e.g. silica gels and alumina, such as ease of regeneration, high chemical stability and product availability for a wide range of pore sizes, densities and surface areas. These properties are expected to have a great influence on adsorption rate, equilibration rate and adsorbent capacities.

The potential use of the SPATT technology in monitoring programmes requires the development of a rapid and efficient method for toxin desorption from the receiving phase. The extraction method described by MacKenzie et al. (2004) is not practical for the extraction of large numbers of SPATTs as large volumes of methanol are required for the extraction and its subsequent evaporation is quite time consuming. Therefore, an alternative method for toxin desorption is required.

Conventional approaches of loading a known amount of analyte onto the adsorbent for extraction recoveries calculation was not applicable because of the chemical affinities of toxins for both methanol and the polymeric resin. Furthermore, spiking concentrated methanolic solutions of toxins into water for subsequent accumulation in an immersed SPATT disc was not successful and rapidly abandoned due to artefacts formation such as hydrolysis of PTX2, and toxin adsorption on glassware (data not shown – Marine Institute). As a consequence, toxin accumulations on SPATT receiving phases were investigated by immersing the resins in cultures of Prorocentrum lima. P. lima is a well-known producer of OA and DTX1 as well as DTX4, a sulphated water soluble toxin (Hu et al., 1995b) and several OA diol ester derivatives (Hu et al., 1995a). P. lima cultures have been extensively studied and have the advantages of being robust and easy to cultivate. Therefore, the latter culture organism was chosen to load toxins on the SPATT for extraction method development as well as to compare the accumulation rate of OA and DTX1 in five types of styrene-DVB
when used as receiving phases. In addition, several naturally contaminated SPATT discs were used for desorption method development and for resin comparison.

4.2. Material and methods

4.2.1. Solvents and Reagents

Acetonitrile (ACN) used for liquid chromatography coupled to mass spectrometry (LC-MS) mobile phase in Ifremer and methanol were purchased as HPLC grade from Baker, Deventer, Holland. ACN used for LC-MS mobile phases in the Marine Institute was purchased as pestican grade from Labscan Ltd., Dublin, Ireland. Dichloromethane (DCM), hydrochloric acid (HCl, 37%) and sodium hydroxide (NaOH) were obtained as analytical grade from Merck, Darmstadt Germany. The 95µm nylon mesh was purchased from John Staniar & Co., Whitefield Manchester, UK. The embroidery hoops used to hold the mesh and the resin were purchased from Singer Sewing Centre, Galway, Ireland. The polymeric resins Diaion® HP-20, Sepabeads® SP850, Sepabeads® SP825L, Amberlite® XAD4 and Dowex® Optipore® L-493 were purchased from Sigma Aldrich, UK. Formic acid and ammonium formate were obtained from Sigma–Aldrich, UK (F-0507 and F-2004, respectively). Water was obtained from MilliQ water dispenser. Standards of OA and PTX2 were purchased as certified calibration solutions from the National Research Council (NRC), Halifax, Canada. Standards of Azaspiracid-1 (AZA1) were prepared from AZA1 isolated in 2001 in Tohoku University, Sandai – Japan, from naturally contaminated mussels from the South West of Ireland (Hess et al., 2007).

4.2.2. Polymeric resin properties and SPATTs preparation

Five macroporous styrene-divinylbenzene copolymer resins were selected to evaluate a range of densities, pore sizes, pore volumes and surface areas (Table 4.1).
Table 4.1: Properties of selected polymeric resins

<table>
<thead>
<tr>
<th></th>
<th>HP-20</th>
<th>SP825</th>
<th>SP850</th>
<th>L-493</th>
<th>XAD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density [g/L]</td>
<td>680</td>
<td>690</td>
<td>670</td>
<td>680</td>
<td>1020</td>
</tr>
<tr>
<td>Pore volume [mL/g]</td>
<td>1.3</td>
<td>1.4</td>
<td>1.2</td>
<td>1.16</td>
<td>0.98</td>
</tr>
<tr>
<td>Surface Area [m²/g]</td>
<td>500</td>
<td>1000</td>
<td>1000</td>
<td>1100</td>
<td>725</td>
</tr>
<tr>
<td>Pore radius [Å]</td>
<td>200</td>
<td>57</td>
<td>38</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>Particle size [µm]</td>
<td>297 - 840</td>
<td>250 - 840</td>
<td>250 - 840</td>
<td>297 - 840</td>
<td>250 - 840</td>
</tr>
</tbody>
</table>

Figure 4.1: Pictures of A) SPATT bag made of HP-20, B) SPATT disc made of HP-20, C) SPATT disc made of SP-850, D) SPATT disc made of L-493 and E) SPATT disc made of XAD-4.

Two types of SPATT designs were prepared: SPATT bags and SPATT discs (Figure 4.1). The SPATT bag design is well suited for studies in artificial media, as the bags can easily be inserted and retrieved from the carboys and culture flasks. The sachets were sewed in the nylon mesh and filled with resin (3.00 ± 0.05 g). The resins were activated by immersing 30 sachets in methanol (1 l) for 48 h. The sachets were then immersed in water and sonicated a few minutes. Alternatively, experiments at sea were carried out using a SPATT disc design which is more robust, rapidly assembled and avoids sewing step. This design has been adapted from Sandvik and Rundberget - Norwegian Veterinary Institute (Personal communication, 2005). Portions of resins (3.00 ± 0.05 g) were weighed and methanol (100 ml) was subsequently added. Activation was performed by leaving the resins in contact
with the methanol overnight, prior to a filtration on 95 µm mesh (≈21 x 12 cm). The resin was wrapped with the mesh and clipped in an embroidery frame (diameter 8.8 cm) allowing exposure on both sides of the frame. Methanol residues were removed by a 10 min sonication step in 500 ml water. The SPATT discs were stored in MilliQ water at 4 - 6 °C until deployment.

4.2.3. *P. lima* culture and resins exposure

A mixed strain of *P. lima* (PL 4V and PL 2V) isolated in Galicia (Spain) was used to inoculate four batches in 10 l experimental units each containing 6 l of culture. This approach was chosen in order to have a sufficient quantity of inoculum and hence to reduce the time to obtain a large biomass. Both strains were grown in F/2 medium (Guillard and Ryther, 1962; Guillard, 1975) at 36 ‰ salinity under 12/12 light/dark cycle and exposed to 50 µE.m⁻².s⁻¹ irradiance at 16 °C.

After 4 weeks of incubation the concentration reached 15 000 ± 3 000 cells/ml (n=4) determined on Nageotte counting chamber. Three 6 l batches were pooled together and allowed to grow for another 4 weeks while the remaining batch was lysed by the “freeze-thaw” method (Quilliam et al., 1996). After defrosting, the culture was sonicated for 10 minutes and dispensed in two 2 l experimental units containing 1.8 l of lysed culture. Five sachets, each containing one of the selected polymeric resins, were placed in each experimental unit for four days.

After 80 days of growth, the pooled culture media reached a density of 30 000 ± 2 000 cells/ml (n=3). Two culture aliquots (100 ml) were passed through a GF/C filter (Whatman 1.2 µm). The cells were rinsed with distilled water to remove the salts and the wet residue was dried in an oven at 60 °C until constant weight was reached, resulting in dry residues of 16.1 and 17.3 mg. The pooled media (18 l) were homogenised for 30 minutes using magnetic stirring and 250 ml aliquots were dispensed into 60 carboys while maintaining
agitation. A 100 ml sample was put aside and preserved in Lugol iodine for cell counts. One filled resin sachet was immersed in each carboy and was placed in the culture chamber until the end of the experiment. Carboys were shaken manually in the mornings and evenings. Three SPATT bags made of each resin were collected at 4 time points, 12, 24, 48 and 72 h, and were kept at 6°C and extracted within 48 h of the exposure end.

Figure 4.2: SPATT bag in carboy

Figure 4.3: Experimental mesocosm set up for the comparison of OA and DTX1 adsorption rate in several styrene-DVB resins
4.2.4. Toxin desorption from SPATT portions

4.2.4.1. Extraction method development

The five resins that were immersed in one of the experimental media (1.8 l lysed culture) were used to determine the elution profile of OA and DTX1 using preparative chromatography equipment. In addition, a naturally contaminated SPATT disc that was placed at five meters depth in Mc Swynes Bay - Bruckless on the North West Coast of Ireland, for one week ending on the 15th of November 2005, was also used for desorption profile determination as above. The resins contained in the sachets were rinsed twice in 500 ml of MilliQ water and vigorously shaken to remove salts. A preparative chromatography system Shimadzu 10Avp (Kyoto, Japan) that includes a binary LC-8A Shimadzu pump and an automatic fraction collector Shimadzu FRC-10 was used for the determination of the toxin desorption profile. A preparative low pressure analytical glass column (15.0 x 1.0 cm) was used to pack the polymeric resins (3 g). Methanol at 1 ml/min was used as mobile phase and 5 ml fractions were collected over 60 min. Each fraction was weighed for volume corrections and the extracts were transferred into LC vials and analysed on a Hybrid Quadrupole Time of Flight (QTOF) MS using an isocratic chromatographic method.

4.2.4.2. Extraction protocol for polymeric resins

SPATTs were rinsed twice in 500 ml of MilliQ water to remove salts and vigorously shaken. The contaminated resins were removed from the mesh and inserted into empty solid phase extraction (SPE) glass cartridges placed on a manifold. Vacuum was applied in order to remove the remaining water. A 23 ml methanol portion was used to elute the resin at ca 1 ml/min flow rate. The extracts were transferred into 25 ml volumetric flasks and an additional 2 ml was used for rinsing and to complete up
to the mark. Extract aliquots were taken from the volumetric flask, inserted into LC vials and injected in a LC-MS system.

Figure 4.4: SPATT extraction. SPE glass cartridge filled with HP-20 resin and surmounted with syringe

4.2.5. Determination of toxin contents of cultures

The *P. lima* culture was homogenised slowly using a magnetic stirrer for one hour. The culture was constantly stirred, to avoid sedimentation of the cells, and 13 aliquots (40 ml) were taken using a pipette and dispensed into polypropylene centrifuge tubes. One 40 ml aliquot was put aside and preserved in Lugol iodine for cell counts under optical microscope.

Method A: Three solutions were placed in a water bath at 100 °C for 10 minutes, allowed to cool at room temperature and subsequently hydrolysed by adding 5 ml of 2.5 M NaOH solution and placed for 20 minutes in a water bath at 76 °C. The solutions were allowed to cool at room temperature and were neutralised by addition of 5 ml of 2.5 M HCl solution. The hydrolysed solutions were partitioned by liquid/liquid extraction (LLE) using DCM (40 ml); this was repeated three times. The DCM fractions were combined and evaporated to dryness using a speed vac at 50 °C.
The dry residue was resuspended in 5 ml of methanol, filtered on 0.2 µm filters and analysed by LC-MS.

Method B: Three solutions were placed in a water bath at 100 °C for 10 minutes, allowed to cool at room temperature and subsequently partitioned using DCM (40 ml); this was repeated three times. The DCM fractions were combined and evaporated to dryness using a speed vac at 50 °C. The remaining aqueous phase was hydrolysed following the procedure described in method A. The hydrolysed solution was partitioned three times using DCM as described above. The dry residue obtained from the three portioning steps was reconstituted in 5 ml methanol, filtered on 0.2 µm cartridges and analysed by LC-MS.

Method C: Three solutions were placed in a water bath at 100 °C for 10 minutes, allowed to cool at room temperature and stored at -20 °C. An additional three solutions were frozen at -20 °C without heat treatment. The six frozen samples were allowed to defrost at room temperature for 24 hours and placed in a sonicating bath containing ice for 10 minutes. The solutions were centrifuged at 3000 rpm for 20 minutes at 0°C and extracted on C18 SPE cartridges (VWR; 500 mg; 3 ml). SPE conditions were optimised on yield, extraction efficiency and reproducibility from P. lima culture samples. The different steps of the SPE procedure consisted in drying and compressing the C18 stationary phase with nitrogen for 4 min. The cartridge was then equilibrated with 100 % methanol (20 ml) and conditioned with water (20 ml) at 8 ml/min. The culture sample (5 ml) was loaded on the SPE at 2.5 ml/min and subsequently washed with water (5 ml) at 3 ml/min. Then, the toxins were eluted with 100 % methanol (5 ml) at 3 ml/min and the stationary phase was dried with pulse air (6 ml). The effluent (5 ml) was evaporated to dryness using a speed vac. The dry
residue was reconstituted in 100 % methanol (1 ml) and inserted in a LC vial for LC-MS analysis using the LCQ ion trap MS.

4.2.6. Naturally contaminated SPATTs

4.2.6.1. Bruckless Bay

A SPATT disc made of Diaion HP-20 resin was deployed in Mc Swynes Bay - Bruckless, NW Ireland [54°36’47.98”N, 8°23’31.99”W]. The SPATT disc was immersed from the 8th until the 15th of November 2005 at 5 meters depth.

4.2.6.2. Galway Bay

SPATT discs were placed at a single location in outer Galway Bay [53°16’10.10”N, 8°56’4.33’’ W] from the 17th until the 31st of August 2006. The sampling system consisted of three replicates of SPATT made of the five resin types (15 SPATTs) at two depths, one and five meters. The SPATTs were replaced after one-week exposure.

Figure 4.5: System used for the comparison of the different styrene-DVB resins in the field

4.2.7. LC-MS systems

Three LC-MS systems were used for quantitation of toxins. Analyses performed in Ifremer were carried out on an ion trap LCQ instrument. Analyses performed at the Marine Institute were carried out on a Q-TOF Ultima for samples that contain OA and
DTX1 and on a triple quadrupole (QqQ) Quattro Ultima for samples additionally containing toxins other than OA and DTX1.

4.2.7.1. Analyses using QTOF

Analyses for the study of the elution profiles of OA and DTX1 from the polymeric resins were performed using a HPLC Acquity system (Waters Corp., Manchester, UK) coupled to a Q-TOF Ultima (Micromass Ltd., Manchester, UK) equipped with a z-spray ESI source. Separation was achieved by isocratic elution for 8 min with 53% phase A (100 % aqueous) and 47 % phase B (95 % aqueous ACN), both containing 2 mM ammonium formate. The flow rate was set at 0.25 ml/min and 5 µl of samples were injected on a C8 BDS Hypersil (50 x 2 mm, 3 µm particle size, guard column, 10 x 2 mm, 3 µm) column maintained at 25 °C. The Q-TOF was used in TOF-MS/MS mode, where the molecular ion is isolated in the quadrupole and where after collision in the collision cell, the whole fragmentation spectrum is obtained in the TOF. DTX1 was quantified using OA calibration as no DTX1 certified calibration solution was commercially available at the time of the study.

4.2.7.2. Analyses using Quattro Ultima

The resins used in SPATT discs deployed in Bruckless Bay and Galway Bay as well as the extracts of the SPATTs exposed to the P. lima culture for adsorption rate determination were analysed using a 2695 Waters HPLC coupled to a QqQ Quattro Ultima (Micromass Ltd., Manchester, UK) also equipped with a z-spray ESI source. Separation was achieved using a gradient elution using binary mobile phases at 0.25 ml/min on a C8 BDS Hypersil column (50 x 2 mm, 3 µm particle size, guard column, 10 x 2 mm, 3 µm). Mobile phase A consisted of 100 % aqueous while B was 95 % ACN. Both mobile phases contained 2 mM ammonium formate and 50 mM
formic acid. The gradient started with 30% B at time zero linearly rising to 90% B at 8 min. Then, 90% B was held for 0.5 min, decreased to 30% B over 0.5 min which was held again for 5 min until the next run. The flow was diverted to the waste for the first 5 min of the analysis. The column temperature was set at 25 °C and the injection volume at 5 µl. The Quattro Ultima was operated in multiple reaction-monitoring (MRM) mode, analysing two fragment ions per compound. Monitored transitions were as follows (precursor > fragment): OA 803.5>255.5 and 803.5>803.5; DTX2 803.5>255.5 and 803.5>803.5; DTX1 817.5>255.5 and 817.5>817.5;; PTX2 876.5>823.5; AZA1 842.5>654.4 and 842.5>672.4; AZA2 856.5>654.4 and 856.5>672.4; AZA3 828.5>640.4 and 828.5>658.4. OA and DTXs were analysed in negative ionisation mode while PTX2 and AZAs were analysed in positive ionization mode. AZA2 and AZA3 were quantified using AZA1 calibration as no certified calibration solutions of these compounds were commercially available at the time of the study.

4.2.7.3. Analyses using LCQ

Culture extracts obtained by method C were analysed on a LCQ ion trap MS (Finnigan MAT, San Jose, US) with an ESI source. Separation was achieved with a Spectra system HPLC equipped with a Kromasil C18 column (250 x 2.0 mm, 5 µm particle size) at 40 °C. Isocratic elution was carried out using a mobile phase ACN/water (75:25) containing 0.1 % TFA. The flow rate was set at 0.2 ml/min and the injection volume was 5 µl. Multiple tandem MS produced collision-induced dissociation (CID) spectra and were obtained using the protonated molecule for each toxin which fragmented similarly giving major ions due to the sequential loss of water molecules. OA and DTX1 were determined in positive ionisation mode and using
MS\textsuperscript{3} methods with the following target precursor and fragment ion combinations in the mass spectrometer: MS\textsuperscript{3} method OA (\textit{m/z} 787.0 > 769.1 > 751.0); MS\textsuperscript{3} method DTX1 (\textit{m/z} 801.0 > 783.1 > 765.1). DTX1 was quantified using OA calibration as no DTX1 certified calibration solution was commercially available at the time of the study.

4.2.8. Statistical evaluation

Statistical calculations were carried out using Sigmastat 3.0. The significance test used to discriminate between the toxin adsorption capabilities of the polymeric resins was the two-way analysis of variance Holm-Sidak test.

4.3. Results

4.3.1. Toxin desorption from naturally and artificially contaminated resins

The elution profiles of OA and DTX1 determined for each polymeric resin using methanol at 1 ml/min are shown in Figure 4.6A and Figure 4.6B respectively. The elution of OA and DTX1 from the HP-20, SP825L and XAD4 resins have a similar profile with a Gaussian type peak where the desorption reaches a maximum after elution with 5 – 7 ml of methanol. The desorptions of OA and DTX1 from the SP850 and the L-493 did not follow that trend, as the first fraction was in both cases the most concentrated one. Excellent recoveries were obtained for the five resins using an elution with 23 ml of methanol, ranging from 96 to 99 % for both OA and DTX1 (calculated relatively to the amount of toxin recovered using 60 ml of methanol), as shown in Table 4.2.
Table 4.2: Recoveries and concentrations of OA and DTX1 found in lysed *P. lima* culture.

<table>
<thead>
<tr>
<th></th>
<th>Total amount of OA [µg]</th>
<th>Recovery at 23 ml</th>
<th>Total amount of DTX [µg]</th>
<th>Recovery at 23 ml</th>
<th>Amount of OA by elution [µg]</th>
<th>Amount of DTX1 by elution [µg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-20</td>
<td>8.70</td>
<td>99 %</td>
<td>6.73</td>
<td>99 %</td>
<td>10.04</td>
<td>5.38</td>
</tr>
<tr>
<td>L-493</td>
<td>7.79</td>
<td>96 %</td>
<td>5.90</td>
<td>96 %</td>
<td>10.74</td>
<td>2.56</td>
</tr>
<tr>
<td>SP825L</td>
<td>11.54</td>
<td>99 %</td>
<td>8.70</td>
<td>98 %</td>
<td>18.18</td>
<td>9.25</td>
</tr>
<tr>
<td>SP850</td>
<td>11.38</td>
<td>97 %</td>
<td>8.84</td>
<td>97 %</td>
<td>16.38</td>
<td>8.27</td>
</tr>
<tr>
<td>XAD4</td>
<td>7.28</td>
<td>98 %</td>
<td>5.36</td>
<td>98 %</td>
<td>9.31</td>
<td>4.65</td>
</tr>
</tbody>
</table>

* 5 ml fractions (+ 3 ml dead volume) were collected and summed after individual analyses
* one single fraction of 23 ml was collected and analysed

Similarly, the same experiment has been carried out using the HP-20 resin from a naturally contaminated SPATT that was deployed in Bruckless for one week. OA, DTX2, PTX2, AZA1, -2 and -3 were found at quantifiable levels. The recoveries obtained using a 23 ml elution with methanol (calculated relatively to the total amount recovered using 60 ml) ranged from 85 to 100 % recoveries. The lowest recovery was obtained for AZA3 while the highest was obtained for DTX2 as shown in Table 4.3.

Table 4.3: Recoveries obtained from SPATT discs, naturally contaminated in Bruckless, North West of Ireland, October 2005.

<table>
<thead>
<tr>
<th></th>
<th>Total Amount of toxin / ng</th>
<th>Recovery at 23 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>874.4</td>
<td>98 %</td>
</tr>
<tr>
<td>DTX2</td>
<td>268.7</td>
<td>100 %</td>
</tr>
<tr>
<td>AZA1</td>
<td>740.5</td>
<td>93 %</td>
</tr>
<tr>
<td>AZA2</td>
<td>219.9</td>
<td>91 %</td>
</tr>
<tr>
<td>AZA3</td>
<td>52.3</td>
<td>85 %</td>
</tr>
<tr>
<td>PTX2</td>
<td>738.1</td>
<td>98 %</td>
</tr>
</tbody>
</table>

The elution profile obtained for these toxins is shown in Figure 4.6C, where the Y-axis was normalised to the highest OA concentration found. Figure 4.6D shows the
OA desorption profiles obtained using the naturally contaminated HP-20 resin exposed in Bruckless and the artificially contaminated HP20 resin immersed in lysed *P. lima* culture for four days. The elution profile from the naturally contaminated HP-20 shows that after elution with 23 ml of methanol, OA could still be quantified in the extracts but only accounted for 2% of the total amount that was recovered using 60 ml.
Figure 4.6: A) Elution profile of OA from five polymeric resins immersed in a lysate P. lima culture. Quantitation was performed by LC-MS Q-TOF. B) Elution profile of DTX1 from five polymeric resins immersed in a lysate P. lima culture. Quantitation was performed by LC-MS Q-TOF. C) Elution profile of OA, DTX1, PTX2, AZA3, -2 and -1 from naturally contaminated SPATT disc. Quantitation was performed by LC-MS QqQ. D) Comparison of OA elution profile from naturally contaminated SPATT disc and SPATT bag contaminated in P. lima culture. Quantitation was performed by LC-MS Q-TOF.
4.3.2. Toxicity *P. lima* culture

The toxicities of the *P. lima* cultures were determined from the sum of intracellular and extracellular toxin content; the results are shown in Table 4.4.

**Table 4.4: Sum of intracellular and extracellular toxins determined by LLE / basic hydrolysis and by SPE / freeze-thaw technique in the *P. lima* culture.**

<table>
<thead>
<tr>
<th></th>
<th>OA ng/ml of culture</th>
<th>DTX1 ng/ml of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LLE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free toxin</td>
<td>79.1 ± 1.3</td>
<td>126.5 ± 4.6</td>
</tr>
<tr>
<td>Hydrophilic toxin derivatives</td>
<td>18.0 ± 0.8</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Free toxin + hydrophilic derivatives</td>
<td>125.2 ± 9.6</td>
<td>101.6 ± 8.2</td>
</tr>
<tr>
<td>Free toxin</td>
<td>63.8 ± 4.9</td>
<td>99.7 ± 11.6</td>
</tr>
<tr>
<td><strong>SPE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophilic toxin derivatives</td>
<td>37.5 ± 6.3 *</td>
<td>&lt; LOD *</td>
</tr>
<tr>
<td>Free toxin + hydrophilic derivatives</td>
<td>101.3 ± 1.4</td>
<td>93.1 ± 2.6</td>
</tr>
</tbody>
</table>

* Calculated by difference between hydrolysed and non-hydrolysed extract

The concentration of free OA determined by LLE was 79.1 ± 1.3 ng/ml (Method B) while the concentration obtained by SPE was 63.8 ± 4.9 ng/ml (Method C). The quantification of the sum of free OA and OA ester derivatives determined by LLE combined to hydrolysis using NaOH (Method A) resulted in concentration of 125.2 ± 9.6 ng/ml against 101.3 ± 1.4 ng/ml determined by the SPE method and freeze-thaw technique (Method C). These results were in good agreement since both the hydrolysis procedure and the LC-MS methods and instruments were different. Furthermore, the concentration of free OA accounted for 63.2 % and 63.0 % of the sum of the concentrations of free OA and OA ester derivatives using LLE and SPE, respectively. The concentration of free DTX1 determined by LLE was 126.5 ± 4.6 ng/ml and 99.7 ± 11.6 ng/ml by SPE. DTX1 ester derivatives were not detected in any extract. The sum of the concentrations of free DTX1 and DTX1 ester derivatives
obtained by method A was lower than the free DTX1 concentration obtained by method B. This suggests either DTX1 degradation during the hydrolysis process or signal suppression in the ESI source due to the presence of sodium chloride in the extract, as salts may cause signal suppression in LC-MS analysis. Sodium chloride is present as HCl is added to neutralise the extract hydrolysed by NaOH. The concentrations obtained for free DTX1 and the sum of free DTX1 and DTX1 ester derivatives using method C were within the margin of errors.

4.3.3. Toxin accumulation in polymeric resins from \textit{P. lima} culture

The average amount, calculated from three replicates, of OA and DTX1 accumulated on each resin at 12, 24, 48 and 72 hours is shown in Table 4.5 and Table 4.6, respectively.

\textbf{Table 4.5:} Average OA concentration (n=3) in ng/g of the five polymeric resins after immersion in \textit{P. lima} culture for 12, 24, 48 and 72 h.

<table>
<thead>
<tr>
<th></th>
<th>L-493</th>
<th>HP-20</th>
<th>SP 825</th>
<th>SP 850</th>
<th>XAD-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
<td>48 h</td>
<td>72 h</td>
<td>12 h</td>
</tr>
<tr>
<td>L-493</td>
<td>267.4 ± 33.2</td>
<td>498.8 ± 139.6</td>
<td>779.3 ± 106.8</td>
<td>973.8 ± 114.5</td>
<td></td>
</tr>
<tr>
<td>HP-20</td>
<td>418.5 ± 59.6</td>
<td>585.5 ± 127.0</td>
<td>982.2 ± 43.9</td>
<td>1607.2 ± 238.3</td>
<td></td>
</tr>
<tr>
<td>SP 825</td>
<td>455.3 ± 42.0</td>
<td>749.66 ± 8.5</td>
<td>1275.9 ± 81.2</td>
<td>1222.5 ± 124.8</td>
<td></td>
</tr>
<tr>
<td>SP 850</td>
<td>515.3 ± 89.6</td>
<td>723.4 ± 255.1</td>
<td>1072.5 ± 145.0</td>
<td>1149.7 ± 134.0</td>
<td></td>
</tr>
<tr>
<td>XAD-4</td>
<td>452.8 ± 9.9</td>
<td>437.5 ± 98.8</td>
<td>867.8 ± 158.0</td>
<td>1061.7 ± 72.9</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6: Average DTX1 concentration (n=3) in ng/g of the five polymeric resins after immersion in *P. lima* culture for 12, 24, 48 and 72 h.

<table>
<thead>
<tr>
<th></th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-493</td>
<td>199.5 ±</td>
<td>463.0 ±</td>
<td>594.60 ±</td>
<td>856.70 ±</td>
</tr>
<tr>
<td></td>
<td>31.8</td>
<td>151.6</td>
<td>112.4</td>
<td>203.4</td>
</tr>
<tr>
<td>HP-20</td>
<td>311.3 ±</td>
<td>458.8 ±</td>
<td>845.5 ±</td>
<td>1291.3 ±</td>
</tr>
<tr>
<td></td>
<td>60.9</td>
<td>61.5</td>
<td>62.3</td>
<td>185.4</td>
</tr>
<tr>
<td>SP 825</td>
<td>355.9 ±</td>
<td>695.3 ±</td>
<td>996.6 ±</td>
<td>964.70 ±</td>
</tr>
<tr>
<td></td>
<td>49.3</td>
<td>161.2</td>
<td>35.9</td>
<td>114.4</td>
</tr>
<tr>
<td>SP 850</td>
<td>431.9 ±</td>
<td>526.8 ±</td>
<td>832.3 ±</td>
<td>982.9 ±</td>
</tr>
<tr>
<td></td>
<td>74.1</td>
<td>176.3</td>
<td>134.0</td>
<td>136.9</td>
</tr>
<tr>
<td>XAD-4</td>
<td>460.3 ±</td>
<td>398.5 ±</td>
<td>804.8 ±</td>
<td>968.6 ±</td>
</tr>
<tr>
<td></td>
<td>48.1</td>
<td>87.0</td>
<td>150.6</td>
<td>66.5</td>
</tr>
</tbody>
</table>

All resins were able to accumulate OA and DTX1 following a 12 hours exposure to the *P. lima* culture. The ratios of the amount of OA and DTX1 adsorbed on each resin were all between 1.20 and 1.25 apart for XAD4 where the ratio found was 1.05. The adsorption profiles of OA and DTX1 onto each resin is shown in Figure 4.7, A and B respectively.
The percentage of adsorption was calculated from the amount of OA and DTX1 found in the SPATTs and the amount of free OA and DTX1 in the 250 ml culture medium equivalent to 19.8 and 31.6 µg of OA and DTX1, respectively (Table 4.4). SP825L and SP850 were found to be the resins that adsorbed OA and DTX1 the most rapidly up to 48 hours. The HP-20 was the only resin that accumulated OA and DTX1
linearly up to 72 h, correlation coefficients obtained for OA and DTX1 were 0.9819
and 0.9955 respectively. After 72 hours exposure, the HP-20 resin accumulated the
largest amount of OA and DTX1 compared to the other resins assessed, reaching 24
and 12 % of the free OA and DTX1 contained in the cells and in the culture media.
However, the Holm-Sidak two way analysis of variance using contact time and
polymeric resin type as the variable factors showed that there are no statistical
differences between the OA adsorption rate obtained with the HP-20, the SP825L and
the SP850 resins, and with the L-493 and XAD4 resins. Furthermore, the same
statistical test showed that L-493, SP825L, SP850 and XAD4 resins adsorbed
equivalent amounts of OA following a 48 hours contact time and following a 72 hours
contact time, suggesting that equilibrium was reached. The results obtained for DTX1
adsorption (Figure 2A) were in accordance with the OA results except for XAD4 that
demonstrated similar adsorption rate towards DTX1 with the HP-20, the SP825L and
the SP850 resins. No statistical differences were observed between HP-20, SP825L,
SP850 and XAD4 resin types for DTX1 adsorption rate and similarly to the OA
results, the SP825L, SP850 and XAD4 resins adsorbed equivalent amounts of DTX1
following 48 and 72 hours contact time.

4.3.4. Toxin accumulation in polymeric resins from West Coast of Ireland

The concentrations of OA obtained from the naturally contaminated SPATT discs
made containing the five selected polymeric resins are shown in Figure 4.8.
Figure 4.8: OA concentrations found in five polymeric resins immersed in Galway bay. Error bars represent the standard deviation obtained on three SPATT replicates. Quantitation was performed by triplicate injection on LC-MS QqQ.

It was found that HP-20 always accumulated a significantly greater amount of OA than the other resins. No significant differences were observed in the amount of OA adsorbed onto the SP825L and SP850. The amount of OA accumulated on the L-493 resin was lower than the previously mentioned resins. Finally, the XAD4 had extremely poor performances in the natural media as OA was barely quantifiable in the extracts. In addition to OA, the resins also accumulated quantifiable amounts of DTX2 as shown in Table 4.7.
Table 4.7: DTX2 concentration (ng/g) ± SD accumulated onto the five polymeric resins in Galway Bay – West Coast of Ireland

<table>
<thead>
<tr>
<th>Retrieval Date</th>
<th>24/08/06</th>
<th>31/08/06</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling Depth</td>
<td>1 m</td>
<td>5 m</td>
</tr>
<tr>
<td>L-493 [ng/g]</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>HP-20 [ng/g]</td>
<td>46.4 ± 8.3</td>
<td>39.4 ± 6.0</td>
</tr>
<tr>
<td>SP 825 [ng/g]</td>
<td>33.3 ± 6.1</td>
<td>31.6 ± 4.2</td>
</tr>
<tr>
<td>SP 850 [ng/g]</td>
<td>30.5 ± 11.5</td>
<td>20.2 ± 1.6</td>
</tr>
<tr>
<td>XAD-4 [ng/g]</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
</tbody>
</table>

DTX2 was only quantifiable using the HP-20, the SP825L and the SP850.

Furthermore, all resins also accumulated small amounts of AZA1 as well as relatively high amounts of PTX2 which were not quantified (data not shown).

4.4. Discussion

The SPATT technique is an attractive tool for the study of the distribution of lipophilic toxins at sea, as it presents a number of advantages over the current monitoring tools. The accumulation of the parent toxins avoiding bio-transformation that occur in shellfish such as the esterification of okadaic acid into 7-O-acyl Okadaic ester (Suzuki et al., 1999), the conversion of PTX2 into PTX2 seco-acid (Suzuki et al., 2001b; Miles et al., 2004a) and PTX2 seco-acid ester derivatives (Wilkins et al., 2006). Similarly, the oxidation of YTX into 45-hydroxy-YTX and carboxy-YTX (Aasen et al., 2005) can be avoided, thereby reducing the number of analytes to be considered in the analytical procedure. Moreover, it is often difficult to evaluate the toxicity of harmful algae bloom by cell identification and quantification in the water column. This is especially true for *P. lima* due to its epiphytic behaviour (Foden et al., 2005) and to the high variation in cell toxicities depending on their strains (Bravo et al., 2001). The SPATT technique is well suited to link cell populations and their
densities to qualitative and quantitative data on their toxin production. In addition, the SPATT technique could be beneficial for the monitoring of AZAs as the organism responsible for the production of these toxins, initially reported as *Protoperidinium crassipes* (James et al., 2003), has still not been unambiguously identified. (Hess et al., 2005b).

The comparison of toxin desorption behaviours from the five polymeric resins was carried out by exposing the SPATTs to a lysed culture of *P. lima* where the resins were competing with each other for the accumulation of OA and DTX1. This approach was initially taken as an artificial contamination step, in order to load similar amounts of toxins on the five resins for subsequent desorption profile determination. The latter was achieved using preparative chromatography equipment in order to ensure good reproducibility in the methanol flow rate and in the volumes of fractions collected. The desorption profiles shown in Figure 4.6A and Figure 4.6B demonstrate the applicability of the elution method for the desorption of OA and DTX1 toxins from all resins selected for assessment. Furthermore, when the desorption profile relative to the most concentrated fraction in OA obtained from naturally contaminated SPATT (made of HP-20) is plotted with the desorption profile from SPATT (also made of HP-20) contaminated in artificial *P. lima* culture (Figure 4.6D) it is clear that both contamination processes led to a similar desorption profile.

The use of naturally contaminated SPATT from Bruckless, Ireland, allowed determination of the desorption profiles for AZA1, -2, -3, PTX2, OA and DTX2 as shown in Figure 4.6C. The data shown in Table 3 demonstrate the efficiency of the elution method for lipophilic toxin analysis as the recoveries obtained for OA, DTX2 and PTX2 were 98, 100 and 96 % respectively. Lower recoveries were obtained for AZA3, -2 and -1 with 85, 91 and 93 % respectively, probably due to the higher
lipophilicity of these compounds. It must be emphasised that, from previous experiments carried out in Bruckless using the SPATT samplers for several weeks, it was observed that AZA3 is usually found in low concentrations compared to AZA1 and -2 (Fux et al., manuscript in preparation).

Applications of styrene DVB copolymer resin in aqueous environment requires a pre-wetting step because of the high hydrophobicity of the resins due to their highly aromatic surfaces. The wetting or activation process consists of the immersion of the resin in a water-soluble solvent such as methanol or acetone to displace the air from the pores, followed by immersion in water to displace the solvent by water molecules. Hence, care must be taken to ensure that the resins are kept moist until deployment at sea. Recent research in polymeric materials have shown that the wetting step of the polymeric resin XAD4 can be avoided by carrying out a Friedel Crafts acylation on the styrene DVB resin structure (Li et al., 2001). The introduction of a similar acylation step on the resins used for the preparation of SPATTs could offer more flexibility in deployment and storage.

Previous studies demonstrated that *P. lima* (PL2V) has a growth rate of 0.1 to 0.3 divisions/day, which would allow cultures to be in the late log phase after 30 days (Rausch de Traubenberg and Morlaix, 1995). Thus, the comparison of the five polymeric resins for their ability to accumulate OA and DTX1 from *P. lima* culture was carried out in a 2-month-old culture in order to ensure that significant variation in toxin concentration in the culture medium would not occur over the 72 h study period. Adsorption of OA and DTX1 on the HP-20 resin rose linearly up to 72 h while the other four resins seem to have reached saturation more rapidly. The results obtained for the HP-20 are in agreement with field trials carried out by MacKenzie et al. (2004) where the resin has been shown to accumulate toxins linearly up to at least 5 days.
This may be due to the larger pore size of the HP-20 and could be explained when looking at the diffusion pathways. Two types of diffusion must be considered, film diffusion and internal diffusion, which are due to the migration of molecules from the solution to the surface of the particles and to the migration from the surface to the interior of the particles, respectively. A simplified model of the adsorption mechanism is proposed in Figure 4.9.

![Simplified model proposed for diffusion processes in macroreticular polymer particles. Film diffusion is shown by the migration of analyte from the solution to the surface of the particles. Internal diffusion is shown by migration of analyte from the surface to the interior of the particle.](image)

Both phenomena are affected by the particle sizes, since the use of smaller particle size increases the surface area for film diffusion, and reduces the internal volume through which the molecule must diffuse. Therefore, the larger the particle sizes, the longer the time to reach equilibrium. Nevertheless, differences in adsorption rate were observed in this study despite the use of polymeric resin with similar particle size ranges given by the manufacturer (Table 4.1). The HP-20 was the only resin to adsorb a significantly higher amount of toxin between 48 and 72 hours when exposed to live culture and adsorbed the greater amount of toxins during the field trials suggesting that the pore size of the resin may govern the capacity and equilibration rate of toxin adsorption. Since the HP-20 had the smallest surface area and yet showed good
performances for toxin adsorption, the adsorption rate does not seem to be dependent on the surface area.

The HP-20 adsorbed 24% of OA and 12% of DTX1 from the culture. The difference in concentration adsorbed may be related to their different excretion pathways and the different amounts of free toxin available for adsorption from the aqueous media. Studies on the production of OA and DTX1 in \textit{P. lima} cultures over a 90 day period showed that OA is either solely or partially produced from enzymatic hydrolysis of DTX4 (Quilliam et al., 1996). This was suggested following the discovery of high intracellular DTX4 concentration with only low concentrations of OA and DTX1 in boiled extracts, where enzymatic activity was stopped. Since OA was found to be the predominant toxin in the culture medium, followed by DTX1 and DTX4 in trace amounts, it was suggested that OA could be produced in the cell in low amount similar to DTX1, and that the majority of OA found in the medium is the result of a very fast enzymatic hydrolysis into OA diol ester derivatives, which are further hydrolysed at a lower rate to yield OA. Studies on \textit{P. lima} cell cycle and their related toxin production showed that DTX4 and OA/DTX1 are produced at different phases of the cell cycle (Pan et al., 1999). The same study reported that DTX4, OA, DTX1 and OA diol esters accounted for 54, 30, 12 and 4% of the total intracellular toxin content respectively, while only OA and trace amounts of DTX1 were detected in the culture medium. However, this study did not provide any evidence of OA being directly produced by the cell and as of yet, there is no study to the author’s knowledge that demonstrated the potential production of free OA from \textit{P. lima} cells. DTX1 production is not believed to occur through the same mechanism as there is no evidence of DTX1 derivatives being produced in \textit{P. lima} cultures (Quilliam et al., 1996; Bravo et al., 2001; Nascimento et al., 2005).
The results obtained from the SPATTs immersed in Galway Bay are consistent with the above findings. The HP-20 resin has shown the ability to accumulate a significantly larger amount of OA than the other evaluated resins. The data from this study show that L-493 and XAD4 resins are not suitable to be used as receiving phase in passive samplers.

4.5. Conclusions

This study reported an efficient extraction method suitable for OA, DTX1, DTX2, AZA1, -2, -3 and PTX2 desorption from styrene-DVB polymeric resin. The comparison of several polymeric resins for toxin adsorption demonstrated that the use of the initially reported HP-20 resin as SPATT receiving phase remains the resin of choice for this application since field trial results showed a significantly greater amount of toxin being adsorbed on the HP-20 resin. Furthermore, this work presented evidences of significant toxin adsorption taking place on the assessed polymeric resins within 12 h of exposure in *P. lima* culture. This study re-iterates the potential application of the SPATT technology to rapidly obtain lipophilic toxin profiles and overcome some of the problems associated with the numerous shellfish metabolites.

4.6. Future work

A prototype device that allows the measurement of the flow and of the temperature was developed in order to study the adsorption behaviour of the lipophilic toxins. One SPATT disc can be placed in the upper cylinder of the device (shown in Figure 4.10 and Figure 4.11). This system allows for the determination of the volume of water that passes through the SPATT.
The flow is calculated using a sensor that measures the speed of the water. The working range of the sensor is 0.1 knot to 30 knots (corresponding to 0.0514 m/s to 15.43 m/s) and has a precision of 0.01 knots (0.00514 m/s). The device is equipped with four batteries that allow measurement to be made for two consecutive months. The electronics and the memory are connected to a micro-processor which allow for the measurement frequency to be changed. The data can be recovered with a connection type “plug and play” using a laptop.

The prototype device is also equipped with fins that allow free rotation with the flow which avoid the use of an expensive flow meter measuring water speed in two or more directions.

The system has been tried in the field (Ros a' Mhíl – Ireland) and did perform well (free rotation, water proof, recording data at 10 m depth). However, further development work still has to be done on the calibration. Ideally the sensor can be calibrated versus oceanographic equipment (Acoustic Doppler Current Profiler) designed for accurate water flow measurement from the sea bed.

In addition to the equipment, software that enables quick and easy transfer to excel was also developed.
Figure 4.10: A) Photograph of prototype B) Photograph of SPATT disc fitting in the upper cylinder of the prototype

Figure 4.11: Sketch of the prototype developed

The work described in this section (4.6) has been carried out in collaboration with Thibaut Fux and Sacha Bernet studying electronics at the Engineer School of Mulhouse – Alsace South (France).
5. Comparative accumulation and composition of lipophilic marine biotoxins in passive samplers and in mussels (*M. edulis*) on the West Coast of Ireland

5.1. Introduction

Under EU Regulation (EC) 854/2004 classified production areas must be periodically monitored to check for the presence of biotoxins in live bivalve molluscs and of toxin-producing plankton in production and relaying waters (Anonymous, 2004b). Although phytoplankton monitoring has increased our understanding of toxic events it only provides a snapshot of the phytoplankton present at the time and location of sampling and it is often difficult to establish a clear correlation between the presence of toxic phytoplankton and shellfish toxicity. Additionally, phytoplankton sampling and subsequent cell identification and enumeration is time consuming, labour intensive and requires specialist taxonomic skills.

Shellfish testing continues to be the best tool for determining the fitness of product for human consumption, provided appropriate detection methods are employed. Adequate detection methods should provide high selectivity and sensitivity towards one or several classes of toxins as well as offering a quantitative response. LC-MS is a well accepted fully quantitative multi-analyte method that provides high sensitivity and high selectivity, especially when MS/MS is used. This method is perceived as a method of choice for toxin quantitation in shellfish and is currently being evaluated as a replacement to the reference animal tests currently in place in the legislation (Anonymous, 2005a). Although shellfish flesh testing remains the most appropriate approach for food safety purposes, the use of shellfish for monitoring the distribution
of toxins in the marine environment has several disadvantages. These include difficulties in sample collection, handling and transport to the laboratory, which may be some distance from the growing area; species specific differences in toxin uptake and depuration; bio-transformation in the shellfish as well as analytical interferences due to matrix effects (Chapter 3).

The use of passive samplers for monitoring the distribution of marine toxins could overcome some of these issues as the technique offers a spatially and temporally integrated response. The use of passive sampling also avoids the problem of bio-transformation, as well as providing a cleaner extract for LC-MS compared to shellfish extracts. Although, quantification of toxins in shellfish is essential for food safety purposes because of toxicity differences in the toxin metabolites compared to the parent toxins, analysis of toxins in passive samplers can be convenient for screening purposes. Mackenzie et al. (2004) have identified a passive sampler suitable for the accumulation of lipophilic toxins, referred to as SPATT and we have also shown the applicability of such SPATTs in mesocosm and field experiments for a wide range of lipophilic toxins (Chapter 4).

The present work describes the use of the SPATT approach in three locations on the West Coast of Ireland during the summer 2005. The toxin profiles and concentrations of the regulated toxins belonging to the OA, AZA, PTX and YTX groups, as well as the unregulated SPX group obtained on the SPATT samples were compared to the toxins accumulated in both indigenous and transplanted mussel samples. The indigenous mussel samples were commercially cultivated on site while the transplanted mussels originated from areas shown to be free of toxins at the time of study. The transplanted bivalves were immersed next to the SPATT samples for the
same period of time. Toxin quantitation in all matrices was performed by either traditional LC-MS/MS or ultra-performance UPLC-MS/MS.

5.2. Materials and methods

5.2.1. Solvents and Reagents

Certified standard solutions of OA, PTX2, YTX, GYM and SPX-13-desMe-C were obtained from the National Research Council in Halifax, Canada. AZA1, obtained from mussel isolation, was a generous gift from Satake and Ofuji (Tohoku University, Japan) to the Marine Institute. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich, Ireland. Methanol and acetonitrile (ACN) used for LC-MS mobile phases were purchased as pestiscan grade from Labscan Ltd., Dublin, Ireland. Formic acid (98 %) and ammonium formate (97 %) used for LC-MS mobile phases were obtained from Sigma–Aldrich, UK. MilliQ water was obtained from a reversed osmosis system. For UPLC analysis, water and ACN were purchased as HPLC grade from Fisher Scientific, Loughborough, UK. Ammonium formate (97 %) was purchased from Sigma-Aldrich, Steinheim, Germany and formic acid (98 %) from BDH laboratory, Poole, UK.

5.2.2. SPATT design and handling

The design of the SPATTs was as reported in Chapter 4. The frames (diameter 8.8 cm) were embroidery hoops, purchased from Singer Sewing Centre, Galway, Ireland. The 95µm nylon mesh was purchased from John Staniar & Co., Whitefield Manchester, UK. The Diaion ® HP-20 resin was purchased from Sigma Aldrich, UK. The SPATT discs were prepared as follows: Diaion ® HP-20 resin was weighed (3.00 ± 0.05 g) and methanol (100 mL) was subsequently added. The resin was activated by
shaking for 40 min in a multitube vortexer prior to filtration on 95 μm mesh (≈ 21 x 12 cm). The resin was wrapped with the mesh and clipped in the frame allowing exposure on both sides of the frame. Methanol residues were removed by sonicating for 10 min in 500 mL water. The SPATTs were stored in MilliQ water until deployment.

5.2.3. Sampling

5.2.3.1. SPATT and mussel sampling

Three SPATT discs were fixed to PVC tubes (ca. 30 cm length) attached to a 15-m polypropylene rope at three different depths: just below the surface, 5 and 10 m as shown in Figure 5.1. Nets containing 300g of uncontaminated, transplanted mussels (*Mytilus edulis*) were placed at the same depth as the SPATT discs.

![Figure 5.1: System used for SPATT discs and transplanted mussels deployment](image)

Three locations on the West Coast of Ireland, Bantry Bay, Killary harbour and McSwynes Bay (Figure 5.2) were selected for the study, based on the occurrence of
biotoxins in these areas in the past number of years. In McSwynes Bay and Killary Harbour SPATT discs and transplanted mussels were deployed in shellfish production areas (station depths were 15 and 17 m, respectively) and indigenous mussels collected from the same areas as part of the Irish National Biotoxin Monitoring programme. For logistic reasons, in Bantry Bay, SPATT discs and transplanted mussels were deployed at a salmon farm (15 m depth), and indigenous mussels collected from the closest shellfish farm (Whiddy Point), some 6 km to the east.

Figure 5.2: Sampling locations along the West Coast of Ireland

The SPATTs and the transplanted mussels were typically replaced on a weekly basis. In some cases, however, bad weather conditions did not allow retrieval and the SPATT discs and mussels were immersed for two consecutive weeks. Indigenous mussels were collected as part of the national monitoring programme (integrated
sampling), usually with a one-week frequency. Sampling dates and duration are summarised in Table 5.1.

Table 5.1: Sampling dates and durations using the SPATT discs and the transplanted mussels (TM).

<table>
<thead>
<tr>
<th>Locations</th>
<th>Beginning of study</th>
<th>End of Study</th>
<th>Weeks of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TM: 10/08/2008</td>
<td></td>
<td>TM: 5</td>
</tr>
<tr>
<td>McSwynes Bay</td>
<td>SPATT: 15/08/2005</td>
<td>SPATT + TM: 20/10/2005</td>
<td>SPATT: 9</td>
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<tr>
<td></td>
<td>TM: 25/08/2005</td>
<td></td>
<td>TM: 7</td>
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</table>

The transplanted mussels (*Mytilus edulis*) originated from various locations in Ireland that were not affected by the presence of toxic phytoplankton at the time of study. LC-MS analyses of the transplanted mussels were systematically carried out prior to their deployment using the hybrid quadrupole time of flight (QTof) MS method described later.

5.2.3.2. Phytoplankton sampling / cell counting

Weekly phytoplankton sampling was carried out in the shellfish production areas (at the SPATT discs locations for Killary Harbour and McSwynes Bay and in the same location than the indigenous mussels in Whiddy Point) using a 15 m Lund tube. Cell counting was carried out according to the Utermöhl method (Hallegraeff et al., 2003). The collected water sample was introduced in a 25 ml Utermöhl sedimentation chamber and left to settle overnight before cell identification and quantification.
5.2.4. SPATT extraction method

The SPATT extraction method consisted of a slow elution using 23 ml of methanol as described in Chapter 4. Briefly, the SPATTs were rinsed twice in 500 mL of MilliQ water to remove salts, and vigorously shaken to remove the excess water. The contaminated resins were removed from the mesh and inserted into empty SPE glass cartridges placed on a manifold. Vacuum was applied in order to remove the remaining water. A methanol portion (23 mL) was used to elute the resin at ca 1 mL.min\(^{-1}\) flow rate. The extracts were transferred into 25 mL volumetric flasks and an additional 2 mL was used for rinsing and made up to the mark. Aliquots were taken from the volumetric flask, filtered on 0.2 µm and inserted into LC vials for LC-MS analysis. Occasionally the concentrations of AZA1 and OA were above the linearity range, and further dilution by weight was carried out on these samples. Procedural blanks were systematically carried out using non-contaminated resin.

5.2.5. Mussel extraction

On receipt, the transplanted mussels were steamed until the shell opened in order to stop enzymatic activity. The flesh was removed and homogenised using a kitchen blender and stored frozen until extraction. The indigenous mussels were analysed fresh as part of the national statutory Marine Biotoxin Monitoring Programme. A portion of shellfish flesh (100 g) was homogenised in a kitchen blender before extraction.

For both transplanted and indigenous mussels, the extraction procedure consisted of 2 g of whole flesh homogenate extracted with 9 ml of methanol (100 %), followed by 1 min homogenisation using a Vortex mixer. After centrifugation (4000 g, 5 min), the supernatant was decanted in a 25 ml flask and the pellet re-extracted using 9 ml of
methanol and homogenised for 1 min using an ultra-turrax at high speed. After centrifugation (4000 g, 5 min), the supernatant was decanted in the 25 ml volumetric flask and the volume made up to the mark with methanol (100 %). The extracts were filtered (0.2 µm) before LC-MS injection.

5.2.6. Hydrolysis

Alkaline hydrolysis was performed on all mussel samples as well as on some selected SPATT extracts by adding 125 µl of 2.5 M NaOH solution to 1 ml of extract. The solution was then placed for 20 min in a water bath at 76 °C. The solutions were allowed to cool to room temperature and were neutralised by addition of 125 µl of 2.5 M HCl solution.

5.2.7. Instrumentation

Quantitative LC–MS analysis was carried out, using a binary mobile phase with A (100% aqueous) and B (95% ACN) both containing 2 mM ammonium formate and 50 mM formic acid (Quilliam et al., 2001). A signal-to-noise ratio (S/N) of 3 was regarded as the limit of detection (LOD) and a S/N of 10 as the limit of quantification (LOQ). Three different instruments were used including a 2695 Waters HPLC coupled to a Micromass Quattro Ultima triple quadrupole (TQ), a 2795 Waters HPLC coupled to a Micromass Q-TOF Ultima and an Acquity UPLC system coupled to a Waters-Micromass Quattro premier XE mass spectrometer. The LODs obtained for the SPATT and the mussel extracts using the three systems are summarised in Table 5.2.
Table 5.2: Limit of detection (S/N = 3) obtained for the SPATT using the UPLC-TQ, the LC-QToF and the LC-TQ methods (ng/g of resin or mussel)

<table>
<thead>
<tr>
<th></th>
<th>UPLC-TQ</th>
<th>LC QToF</th>
<th>LC-TQ</th>
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<td></td>
<td>SPATT</td>
<td>Mussels</td>
<td>SPATT</td>
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<tr>
<td>OA</td>
<td>4.0</td>
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</tr>
<tr>
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<td>4.2</td>
<td>12.0</td>
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<tr>
<td>AZA1</td>
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<td>0.5</td>
<td>6.0</td>
</tr>
<tr>
<td>PTX2</td>
<td>0.4</td>
<td>0.6</td>
<td>7.5</td>
</tr>
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<td>GYM</td>
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<tr>
<td>SPX 13-desMeC</td>
<td>0.2</td>
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5.2.7.1. Analysis of indigenous mussels

The indigenous mussel samples were analysed using 2695 Waters HPLC coupled to a Micromass Quattro Ultima triple quadrupole (TQ) equipped with a z-spray electrospray (ESI) source. The Quattro Ultima was operated in multiple reaction-monitoring (MRM) mode, analysing two fragment ions per compound. The samples were analysed for OA, Dinophysistoxin-1 (DTX1), Dinophysistoxin-2 (DTX2), AZA1, -2 and -3. Monitored transitions were reported elsewhere (Fux et al., 2007). A C8 BDS Hypersil (50 x 2 mm, 3 µm particle size, guard column, 10 x 2 mm, 3 µm) was used with a gradient elution, starting with 30% B at time zero linearly rising to 90% B at 8 min. Then, 90% B was held for 0.5 min, decreased to 30% B over 0.5 min which was held again for 3 min until the next run. The flow rate was set at 0.25 ml/min and single injection of 5 µl of sample was performed.

5.2.7.2. SPATT discs and transplanted mussels from Killary Harbour

The SPATT discs and the transplanted mussels from Killary were analysed using a 2795 Waters HPLC equipped with a C18 ACE (30 x 2.1 mm, 5 µm) column coupled to a Micromass Q-ToF Ultima (quadrupole-time-of-flight hybrid) also equipped with a z-spray ESI source. The Q-ToF was used in ToF-MS/MS mode, where the
molecular ion is isolated in the quadrupole and where after collision in the collision cell, the whole fragmentation spectrum is obtained in the ToF. AZA1, -2, -3 and PTX2 were analysed in positive ionisation mode with an isocratic run of 60% B for 7 min and OA, DTX1, DTX2 and YTX were analysed in negative ionisation mode with an isocratic run of 55% B for 6.5 min. The flow rate was set at 0.25 ml/min and 5 µl of extract was injected. In addition, the SPATTs and the transplanted mussels from Killary were analysed qualitatively using the system described in the next section.

5.2.7.3. SPATT discs and transplanted mussels from McSwynes Bay and Bantry Bay

SPATT discs and the transplanted mussels from Bantry Bay and McSwynes Bay were analysed using the recently developed UPLC-MS/MS method which allows the analysis of more than 20 lipophilic toxins in one run (Chapter 2). The system consisted of an Acquity UPLC system coupled to a Quattro premier XE mass spectrometer (Waters-Micromass, Manchester, UK) equipped with a z-spray ESI source. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) equipped with an in-line 0.2 µm Acquity filter. A gradient from 30 % B rising to 90 % B was run over 3 min and then held for 1.5 min. At 4.5 min the gradient was set back to the initial composition and equilibrated for 2 min. The flow rate was set at 0.4 ml/min and 10 µl of each sample (maintained at 5°C) was injected onto the column at 30 °C. The ESI source was operated simultaneously in both positive and negative mode by rapid switching. Duplicate injection was performed for the SPATT discs and the transplanted mussels.
Certified standard solutions were not available for all toxins of interest. Therefore, DTX2 and AZA2, -3 quantitations were performed assuming that they have the same response factors as OA and AZA1, respectively. Although a standard solution of PTX2sa was commercialised by the NRC, it was not available in our laboratory and therefore its quantitation was performed against PTX2.

5.3. Results

5.3.1. Killary Harbour

SPATT discs were deployed for a 7 week period from the 24th July to 14th September 2005. *D. acuminata* was present in the water on the 4th of July (prior to the beginning of the experiment) at concentrations of 120 cells/L. *D. acuminata* concentrations declined until the 18th of July and the organism was not detected from the 25th of July (Figure 5.3). *D. acuta* was detected on the 4th of July at a concentration of 40 cells/L and was not detected in the subsequent samples obtained in July.

During the first three weeks of sampling starting on the 24th July *D. acuta* and *D. acuminata* were not detected and the SPATT discs indicated a constant level of OA (1.2 to 1.4 µg/g), low amounts of PTX2 (12 to 25 ng/g) and no DTX2. During this period, only one SPATT from each depth was analysed while the other SPATTs were used for method development trials. On the 22nd of August *D. acuta* reached a concentration of 160 cells/L, and on 24th August there was an increase of OA concentration in the transplanted mussels and an increase of OA and PTX2 concentrations in the SPATT samplers. Furthermore, the first detectable levels of DTX2 were observed in the transplanted mussels and in the SPATT discs (Figure 5.3A). Significant differences were also observed in the levels of OA, DTX2 and
PTX2 in the SPATT discs deployed at different depths. The SPATTs immersed at 10 meters typically accumulated the highest level of all 3 toxins. During the course of the experiment, OA was clearly the predominant toxin reaching a maximum of 2640 ng/g OA at 10 m on the 24th August, followed by DTX2 which reached a maximum of 617 ng/g at 10 m on 31st August and PTX2 which reached a maximum of 220 ng/g at 10 m on 31st August (Figure 5.3A). High amounts of OA and DTX2 were only found in the transplanted mussels that were immersed during the occurrence of *D. acuta* (samples retrieved on the 24th of August), with maximum concentrations of 365 and 141 ng/g of OA and DTX2, respectively (Figure 5.3B). In addition, a large proportion of their ester derivatives were also detected after alkaline hydrolysis. Highest concentrations of OA, DTX2 and their ester derivatives were obtained at the surface for the transplanted mussels in contrast to the SPATT discs which accumulated the highest amount of toxin at 10 meters. The extracts of the SPATT discs immersed from the 17th until the 24th of August were also hydrolysed and quantitation on hydrolysed and non-hydrolysed extracts did not show any evidence of OA or DTX2 ester derivatives being accumulated. Similar to the SPATT discs, DTX2 was found in the indigenous mussels during the three weeks that followed the *D. acuta* event (Figure 5.3C). The indigenous mussels also accumulated higher concentration (32 ng/g) of free OA than the week prior to the event (< LOD) as shown in Figure 5.3C. When OA and DTX2 were detected in the indigenous mussels after the 22nd of August, the concentration of DTX2 was higher than the concentration of OA. Relative amounts of OA ranged from 0 to 35 % of the sum of OA and DTX2. The levels of OA and DTX2 ester derivatives also enabled quantification in most indigenous mussel samples (Figure 5.3C).

Figure 5.4 shows the comparison of the sum of free OA and DTX2 concentrations (ester derivatives are not accounted for in the plot) found in the three samplers. The
average concentrations of the sum of OA and DTX2 found in the SPATT discs from the three sampled depths were constant for three weeks prior to the *D. acuta* event (1.3 – 1.4 µg/g) (Figure 5.4). Then, the concentrations rose by a factor two (2.6 µg/g) and declined for the following three weeks after the event with 2.2, 2.0 and 1.5 µg/g.
Figure 5.3: OA, DTX2 and PTX2 toxins and phytoplankton results in Killary Harbour. A) Concentrations of OA, DTX2 in SPATT discs (n=1 from 24/7/05 to 17/8/05; n=3 from 17/8/05 to 14/9/05); B) Concentrations of OA, DTX2 and their ester derivatives in transplanted mussels (n=1). Samples retrieved on 14/9/05 are not shown and were below LOD. C) Concentrations of OA, DTX2 and their ester derivatives in indigenous mussels (n=1). * indicates that the concentrations were below LOD.
Figure 5.4: Comparison of the sum of the concentrations of OA and DTX2 accumulated in transplanted mussels (n=3), indigenous mussels (n=1) and in the SPATT discs (n=3) from 2/8/05 to 17/8/05; (n=9) from 17/8/05 to 14/9/05 immersed in Killary Harbour. For the SPATT discs and the transplanted mussels each data point represents the average of the three depths.

5.3.2. McSwynes Bay

The field sampling period in McSwynes Bay was carried out from the 15th of August until the 24th of October 2005. Weekly replacement of the SPATT discs and transplanted mussels was attempted, however, due to bad weather conditions, samplers retrieval were sometimes post-poned. Numerous lipophilic toxins were detected in the SPATT discs and included OA, DTX2, YTX, PTX2, AZA1, AZA2 and AZA3 (Table 5.4 and Table 5.5). An example of chromatogram of one SPATT retrieved on the 15th of September is shown in Figure 5.5.
Figure 5.5: Normalised toxin profile from one SPATT disc immersed at 5 meters in McSwynes Bay from the 8th of September until the 15th of September determined by UPLC-MS/MS. Retention times are given in italics and areas in brackets.

The trend observed in the accumulation of AZA1 and -2 were similar, although AZA1 concentrations were usually more than 10 fold higher than AZA2 (Table 5.4). AZA3 concentrations did not follow the patterns observed for AZA1 and -2. The highest AZA concentrations, up to 6.8 µg of AZA1 and 621 ng of AZA2 per g of resin, were found in the SPATTs immersed close to the surface from the 25th to the 31st of August. During this period, the concentrations of AZA1 and -2 in SPATT discs immersed at 10 meters were 33 % and 42 % lower than those exposed close to the surface.

Similarly to the SPATT discs, the transplanted mussels accumulated the highest amounts of AZAs from the 25th to the 31st of August and a decline in the sum of the concentrations of AZA1, -2 and -3 was observed as the sampling depth was increased.
with 642, 598 and 568 ng/g at 0, 5 and 10 meters, respectively (Table 5.4). Nevertheless, the total concentration of AZAs accumulated in the transplanted mussels was considerably lower than in the SPATT discs and AZA1 was no longer the predominant analogue. AZA3 clearly dominated the AZAs distribution (45 – 65 % of the total AZAs), followed by AZA1 and -2.

The AZA distribution in the indigenous mussel analysed from raw flesh extracts was dominated by AZA1 (60 – 70 % of the total AZAs) followed by AZA2 and -3 (Table 5.4). AZA3 accounted for a higher percentage of the total AZA composition (3 – 10 %) than in the SPATT discs (< 1 %). The highest concentrations of AZAs were found on the 29th of August and on the 5th of September (7.4 µg/g), at the same period than the highest concentrations observed in the SPATT discs and the transplanted mussels. The levels of AZAs found in indigenous mussels and in the SPATT discs (average of concentrations found at three depths) were similar (Figure 5.4). Furthermore, the concentration in AZAs in both samplers increased until end of August/ beginning of September prior to a decline. The accumulation of AZAs in the SPATT discs decreased linearly until the 13th of October.
YTX was detected above quantification limit in all SPATT discs. Low levels were observed for the first three weeks of sampling (23 - 98 ng/g) followed by a significant increase to 190 - 270 ng/g in the SPATT immersed in the water from the 8th until the 15th of September (Table 5.5). The highest YTX concentration observed was 347 ng/g in SPATTs retrieved on the 22nd of September from 10 meters. The variability observed between replicate SPATT discs immersed at the same depths was higher for YTX than for OA, PTX2 and AZAs. The appearance or significant increase in concentration of phytoplankton species that coincided with the raise in YTX concentration were *Heterocapsa sp*, *Prorocentrum balticum/minimum* and *Prorocentrum dentatum* (Table 5.3). To our knowledge, none of these species have been reported to produce YTX. YTX was also detected at quantifiable levels in the transplanted mussels that were retrieved on the 15th of September.
At the beginning of the experiment (15/08/05) *D.acuminata* was the only *Dinophysis* species that was detected in the water (120 cells/L). The same organism was subsequently detected in the two water samples collected in August (22/08/05 and 29/08/05) and one collected in September (12/09/05). In this experiment, OA, DTX2 and PTX2 were always detected in the SPATT discs. Highest amounts of OA, DTX2 and PTX2 (25/08/05) were detected following the first week of sampling where at 10 meters depth, OA, PTX2 and DTX2 concentrations reached 5.6, 1.4 and 0.5 µg/g of resin respectively (Table 5.5). OA was always the predominant toxin, followed by PTX2 and DTX2.
Table 5.3: Phytoplankton detected in McSwynes Bay during the sampling period. Concentrations are expressed in number of cells/L

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<tr>
<th>Date</th>
<th>Alexandrium sp.</th>
<th>Ceratium horridum</th>
<th>Ceratium lineatum</th>
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<th>Ceratium tripos</th>
<th>Chaetoceros (Phaeoceros) sp</th>
<th>Chaetoceros danicus</th>
<th>Dinophysis acuminata</th>
<th>Dinophysis acuta</th>
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<th>Heterocapsa sp</th>
<th>Karenia mikimotoi</th>
<th>Leptocylindrus danicus</th>
<th>Minuscula bipes</th>
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<th>Oxytoxum sp</th>
<th>Prorocentrum balticum/minimum</th>
<th>Prorocentrum dentatum</th>
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<th>Protoperidinium sp</th>
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<th>Pseudo-nitzschia delicatissma group</th>
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</table>
The sampling depth had an influence on OA, DTX2 and PTX2 concentrations in the SPATT samples collected in August (25/8/05 and 31/8/05) and increased as the sampling depths increased with for example OA concentrations of 1.7, 2.0 and 2.4 µg/g at 0, 5 and 10 meters in the SPATTs retrieved on the 31\textsuperscript{st} of August (Table 5.5). On the 12\textsuperscript{th} of September, \textit{D. acuminata} concentration rose by more than a factor of four compared to the previous water sample taken on the 29\textsuperscript{th} of August. This was associated with a higher accumulation of OA and OA ester derivatives in the transplanted mussels, however, no increase in OA concentration was observed in the SPATT discs and the indigenous mussels (Table 5.5).
Table 5.4: Concentrations of OA, DTX2 and their respective ester derivatives accumulated in SPATTs and in indigenous and transplanted mussels from McSwynes Bay (ng/g). When available, standard deviations (SD) are given in brackets. SPATT analysis was carried out on two SPATT discs, each injected in duplicate and transplanted mussel’s concentrations were calculated from duplicate injection.

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</tr>
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<td>533</td>
</tr>
<tr>
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</tr>
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<td>1693</td>
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<td>139</td>
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<td>&lt;LOD</td>
<td>0</td>
<td>&lt;LOD</td>
<td>&lt;LOQ</td>
</tr>
<tr>
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<td>177</td>
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<td>11</td>
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<td>&lt;LOQ</td>
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<td>&lt;LOD</td>
<td>&lt;LOQ</td>
</tr>
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<td>2408</td>
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<td>220</td>
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<td>(5)</td>
<td>25 (35)</td>
<td>&lt;LOD</td>
</tr>
<tr>
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</tr>
<tr>
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<td>&lt;LOD</td>
<td>&lt;LOQ</td>
</tr>
<tr>
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<td>115</td>
</tr>
<tr>
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<td>243 (55)</td>
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<td>(136)</td>
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<td>47 (10)</td>
</tr>
<tr>
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<td>0</td>
<td>414</td>
<td>(60)</td>
<td>319 (79)</td>
<td>&lt;LOD</td>
</tr>
<tr>
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<td>1540</td>
<td>(136)</td>
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<td>55 (3)</td>
</tr>
<tr>
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<td>234</td>
<td>(4)</td>
<td>228 (61)</td>
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</tr>
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<td>1210</td>
<td>(74)</td>
<td>na</td>
<td>59 (17)</td>
</tr>
<tr>
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<td>237</td>
<td>(21)</td>
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<td>&lt;LOD</td>
</tr>
<tr>
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<td>582</td>
<td>504</td>
<td>60</td>
<td>36</td>
</tr>
<tr>
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<td>1295</td>
<td>(112)</td>
<td>na</td>
<td>68 (16)</td>
</tr>
<tr>
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<td>18 (1)</td>
<td>&lt;LOD</td>
<td>&lt;LOQ</td>
</tr>
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<td>1045</td>
<td>(428)</td>
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<td>66 (31)</td>
</tr>
<tr>
<td>22-Sep</td>
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<td>1274</td>
<td>(260)</td>
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<td>77 (18)</td>
</tr>
<tr>
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<td>22</td>
<td>(15)</td>
<td>52 (32)</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>26-Sep</td>
<td>Indig.</td>
<td>na</td>
<td>520</td>
<td>253</td>
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<td>31</td>
</tr>
<tr>
<td>7-Oct</td>
<td>SPATT</td>
<td>0</td>
<td>1749</td>
<td>(140)</td>
<td>na</td>
<td>131 (28)</td>
</tr>
<tr>
<td>7-Oct</td>
<td>Transpl.</td>
<td>5</td>
<td>&lt;LOD</td>
<td>25 (23)</td>
<td>&lt;LOD</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>7-Oct</td>
<td>Transpl.</td>
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<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>10-Oct</td>
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<td>252</td>
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</tr>
<tr>
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<td>Transpl.</td>
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<td>&lt;LOD</td>
<td>1172 (203)</td>
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<td>&lt;LOQ</td>
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<tr>
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<td>Transpl.</td>
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<td>&lt;LOQ</td>
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<td>&lt;LOQ</td>
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<tr>
<td>13-Oct</td>
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<td>31 (37)</td>
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<td>17-Oct</td>
<td>Indig.</td>
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<td>&lt;LOD</td>
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<td>24-Oct</td>
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<td>0</td>
<td>397</td>
<td>(47)</td>
<td>na</td>
<td>73 (15)</td>
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<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>24-Oct</td>
<td>SPATT</td>
<td>5</td>
<td>452</td>
<td>(49)</td>
<td>na</td>
<td>96 (10)</td>
</tr>
<tr>
<td>24-Oct</td>
<td>Transpl.</td>
<td>5</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOQ</td>
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<td>&lt;LOD</td>
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<td>253</td>
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<td>&lt;LOD</td>
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* indicates that only one SPATT was available for analysis
Table 5.5: Concentration of PTX2, AZA1, -2, -3 and YTX obtained in SPATTs and in indigenous and transplanted mussels from McSwynes Bay (ng/g). When available, standard deviations (SD) are given in brackets. SPATT analysis was carried out on two SPATT discs, each injected in duplicate and transplanted mussel’s concentrations were calculated from duplicate injection.

<table>
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<th>AZA1</th>
<th>AZA2</th>
<th>AZA3</th>
<th>YTX</th>
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<td>357</td>
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<td>193</td>
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<tr>
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</table>

* indicates that only one SPATT was available for analysis

5.3.3. Bantry Bay

The sampling period in Bantry Bay started on the 31st of August and ended on the 23rd of October 2005. The SPATT discs always accumulated OA, DTX2 and PTX2.
despite the fact that *Dinophysis* species were not detected during the sampling period. OA concentration in SPATTs were found to remain constant during the first month of sampling with concentrations in the range 1000 – 1200 ng/g (Table 5.6). The maximum concentrations of DTX2 and PTX2 accumulated by the SPATT discs were 1.1 and 1.0 µg/g, respectively, and were reached during the first week of sampling. Then, both concentrations declined slowly. In samples retrieved after the 5th of October, OA, DTX2 and PTX2 concentrations dropped significantly down to concentrations below 400 ng/g. No clear pattern in the distribution of the toxins along the water column was observed during the course of the sampling experiment at this location. Several toxins belonging to the PTX group were also found in the SPATT discs at concentrations far below those reported for OA, DTX2 and PTX2 (Table 5.6). After PTX2, PTX2 seco-acid (PTX2sa) was the predominant PTX with concentrations up to 60 ng/g, followed by two unknown compounds of m/z 892.5 and 890.5 as well as 7-epi-PTX2 seco-acid (7-epi-PTX2sa). The compound detected at m/z 890.5 (marked X on the chromatogram Figure 5.9) corresponds to the mass of PTX3. However, PTX3 was not available when the UPLC method was developed and therefore, its retention time is not known. The compound detected at m/z 892.5 eluted at 2.52 min and was detected because it eluted slightly after PTX1 (2.38 min). This suggests that the PTX1 isomer is less polar than PTX1. Known isomers of PTX1 are PTX4, PTX8, PTX11 and PTX13. Figure 5.7 shows the concentration of the sum of OA and DTX2 accumulated in the indigenous mussels from the 27th of July and in the transplanted mussels and the SPATT discs from the 31st of August. *D. acuminata* and *D. acuta* were detected on the 2nd of August at 520 and 640 cells/L, respectively and on the 8th of August at 160 and 240 cells/L. This was associated with OA and DTX2 concentrations of 660 ng/g in the indigenous mussels on the 15th of August. OA,
DTX2 and PTX2 were not detected in the transplanted mussels. The SPATT discs accumulated OA and DTX2 to concentrations 10 fold higher than in the indigenous mussels. The disappearance of toxins from the water and the depuration of the toxins from the indigenous mussels seemed to occur at similar rates.

In addition to OA, DTX2 and PTX toxins, the SPATTs and the mussels accumulated significant amount of AZAs (up to 289 ng/g of AZA1 on SPATT samples retrieved on the 5th of October). The SPATTs mainly accumulated AZA1 and AZA2 (some very low amounts of AZA3 were occasionally observed), while the mussels accumulated AZA1, -2 and -3 (Table 5.7). The transplanted mussels were found to contain a higher proportion of AZA3 than the indigenous samples (Table 5.7).
**Table 5.6: Concentration of DSP and PTXs toxin obtained in SPATTs and in indigenous and transplanted mussels from Bantry Bay. When available, standard deviations (SD) are given in brackets. SPATT analysis was carried out on two SPATT discs, each injected in duplicate and transplanted mussel's concentrations were calculated from duplicate injection.**

<table>
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<th>Date</th>
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<th>DTX2</th>
<th>DTX2 esters</th>
<th>PTX2</th>
<th>PTX2sa</th>
<th>7-epi-PTX2sa</th>
<th>PTX1 isomer</th>
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* indicates that only one SPATT was available for analysis
Table 5.7: Concentrations of AZAs and SPX-13-desMec-C obtained in SPATTs and in indigenous and transplanted mussels from Bantry Bay. When available, standard deviations (SD) are given in brackets. SPATT analysis was carried out on two SPATT discs, each injected in duplicate and transplanted mussel’s concentrations were calculated from duplicate injection.

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<tr>
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<td>(1)</td>
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<td>&lt;LOQ</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
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<tr>
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<td>(3)</td>
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<td>(1)</td>
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<tr>
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<td>(5)</td>
<td>46</td>
<td>(5)</td>
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<tr>
<td>14-Sep</td>
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<td>5*</td>
<td>42</td>
<td>(1)</td>
<td>7</td>
<td>(1)</td>
</tr>
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<td>&lt;LOQ</td>
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<tr>
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<td>(7)</td>
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<td>(3)</td>
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<tr>
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<td>(10)</td>
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<tr>
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<td>109</td>
<td>(11)</td>
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<td>(2)</td>
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<tr>
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<tr>
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<td>(7)</td>
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<td>(17)</td>
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<td>(8)</td>
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The trend of AZAs accumulation in the SPATT was very similar to the one obtained for the indigenous and the transplanted mussels (Figure 5.8). The transplanted mussels and the SPATTs accumulated AZAs prior to the indigenous mussels and reached their maximum AZAs concentrations at the same period as the indigenous mussels (5th - 10th of October).
The transplanted mussels and the SPATTs accumulated AZAs prior to the indigenous mussels and reached their maximum AZAs concentrations at the same period as the indigenous mussels (5th - 10th of October).

Furthermore, SPX-13-DesMe-C was also detected in all SPATT deployed in Bantry Bay but not in the transplanted mussels. It must however be emphasised that the observed levels in the SPATTs were far below those observed for OA or AZAs (Table 5.6).

![Figure 5.7: Comparison of the sum of the average concentration of OA and DTX2 accumulated in transplanted mussels (n=3), indigenous mussels (n=1) and in SPATT discs (n=6) immersed in Bantry Bay. For the SPATT discs and the transplanted mussels each data point represents the average of the three depths.](image-url)
Figure 5.8: AZA1, -2 and -3 accumulation in transplanted mussels (n=3), indigenous mussels (n=1) and in the SPATT discs (n=6) from Bantry Bay. For the SPATT discs and the transplanted mussels each data point represents the average of the three depths.

Figure 5.9: Toxin profile from one SPATT disc immersed at 10 meters in Bantry Bay from the 14th of September until the 21st of September determined by UPLC-MS/MS. Retention times are given in italics and peak areas in brackets.
5.4. Discussion

This study reports the monitoring of OA, PTX, YTX and AZA group toxins from July to October 2005 in three locations on the West Coast of Ireland using passive samplers (SPATT discs), indigenous and transplanted mussels. This approach allowed i) the comparison of toxin accumulation in SPATTs with those in mussels ii) the comparison of toxin levels accumulated in indigenous with those accumulated in transplanted mussels iii) the comparison of toxin profiles in SPATT and in live bivalve samplers and iv) the correlation of phytoplankton appearance with the toxin present in the water and subsequent accumulation in passive samplers and in bivalves. This is discussed by toxin class.

5.4.1. OA group

OA is known to be produced by several *Dinophysis* species, such as *D. fortii* (Lee et al., 1989), *D. acuminata* (Lee et al., 1989; MacKenzie et al., 2005) and *D. acuta* (Lee et al., 1989; Suzuki et al., 2004; Fernandez - Puente et al., 2004; Miles et al., 2004a) (MacKenzie et al., 2005). In addition to OA, its isomer DTX2 was also found to be produced by European strains of *D. acuta* from Ireland (James et al., 1997a; Draisci et al., 1998a; James et al., 1999a; Fernandez - Puente et al., 2004) and Portugal (Vale, 2004a) but was not detected in *D. acuta* strains from Norway (Miles et al., 2004b) and from New Zealand (MacKenzie et al., 2005). Amongst the different strains of the benthic species *Prorocentrum lima*, some have been shown to produce OA and/or DTX1 (Morton and Tindall, 1995; Bravo et al., 2001) as well as, in some cases, traces of DTX2 (Bravo et al., 2001).

In this study, the levels of OA always allowed for quantitation even when no *Dinophysis* species were detected in the water, similarl to the results obtained with the SPATT samplers in New Zealand (MacKenzie et al., 2004). Ongoing statutory
monitoring of the toxins had shown that, in all three areas, algae producing OA-group toxins had already occurred and accumulated in the indigenous mussels before the start of this study. Consequently, indigenous mussels generally contained OA, DTX2, or both, as well as their respective ester derivatives. Occurrence of *D. acuta* (160 cells/L) in Killary Harbour was followed by a substantial increase of OA and to the appearance of DTX2 in the indigenous mussels. This increase coincided with the accumulation of relatively large amounts of OA group toxins in transplanted mussels. Similarly, the occurrence of *D. acuta* and *D. acuminata* in McSwynes was also followed by the contamination of the transplanted mussels. *Dinophysis spp* cells were detected in all water samples collected from July to September 12th when *D. acuminata* reached its highest concentration. The transplanted mussels accumulated relatively large amounts of OA and derivatives on the 8th of September and the highest concentration was obtained in mussels retrieved from the water on the 15th of September. The transplanted mussels deployed in Bantry did not accumulate OA group toxins during the entire sampling period despite their relatively high concentrations in the SPATTs in September (2 µg of OA and DTX2 per g of resin). Contamination of the transplanted mussels in both Killary Harbour and McSwynes Bay occur when *Dinophysis spp* were present in the water during the sampling period. Conversely, water samples that were obtained during the sampling period in Bantry did not contain *Dinophysis spp*. This suggests that bivalve mussels cannot accumulate dissolved or suspended DSP toxins and that feeding on the toxin producing organism is required for contamination to occur.

The appearance of *D. acuta* in Killary was associated with toxin accumulation in both transplanted and indigenous mussels, but not to the same extent. The transplanted mussels accumulated higher OA and DTX2 levels than the indigenous mussels by a
factor of four. At least two reasons could explain the differences observed i) since the transplanted mussels were cooked when returned in the lab, the loss of water resulted in a concentration step of the lipophilic toxins (Hess et al., 2005b; McCarron et al., 2008); or ii) the transplanted mussels were purchased after storage in clean water tanks that did not contain food and spent 1-2 days in a starvation period. Therefore, once back in the water, the bivalves may have fed intensively which, in the case of feeding on toxic phytoplankton, would result in a greater accumulation of toxins.

When OA or DTX2 were accumulated in the transplanted mussels esterification occurred in less than one week. The transplanted mussels immersed in Killary contained a higher content of ester derivatives than the parent toxins OA and DTX2. The rapidity of the OA analogues esterification process in bivalves has been demonstrated in the past with mussels and scallops. When Japanese scallops (Patinopecten yessoensis) were fed twice a day with D. fortii that produced exclusively DTX1, it was demonstrated that after 5 days the esters of DTX1 accounted for at least 75 to 90 % of the total DTX1 (Suzuki et al., 1999). The kinetics of the esterification of DTX1 (produced by D. fortii in Japan as above) in scallops (P. yessoensis) and mussels (Mytilus galloprovincialis) were compared over 12 days (Suzuki and Mitsuya, 2001). On day 1 of the experiment DTX1 and DTX1 esters were not detected in any of the shellfish species while esterified DTX1 accounted for more than 91 % and 94 % of the total DTX1 in scallops on day 8 and 12 respectively. The esterification rate was slower in mussels as esters of DTX1 accounted for 17 % and 34 % of the total DTX1 on day 8 and 12 respectively. In the present study we observed that when transplanted mussels were immersed for one week in water containing Dinophysis spp., OA esters accounted for 75 to 100 % of the total OA concentration in Killary and 19 to 100 % in McSwynes Bay, while DTX2 esters
accounted for 27 to 66% of the total DTX2 concentration (Killary). This difference in the extent of esterification between OA and DTX2 is in agreement with the observations made in Portugal (Vale, 2007).

Following the occurrence of *D. acuta*, the amounts of OA, DTX2 and PTX2 that accumulated on SPATT discs were significantly different at different water depths for two weeks after the toxic event. *Dinophysis* *spp* are known to occur in thin-layers (Marcaillou et al., 2001; Lunven et al., 2005) and a larger population of cells at a given depth in the water column would release more toxins resulting in higher concentration in the SPATT discs. Another explanation is that daily vertical migration of *Dinophysis* *spp* that has been previously reported could result in higher concentration of toxins at specific depths (Reguera et al., 1993)

The levels of OA accumulated by the SPATT discs in Killary and in Bantry were more than one order of magnitude higher compared to the indigenous mussels. In Bantry, the rate of disappearance from the water was similar to the rate of depuration of the indigenous mussels for OA and DTX2.

Data was obtained during three weeks before *D. acuta* appeared in Killary Harbour where a constant OA background was observed and no DTX2 was detected (Figure 3A). No DTX2 was observed in the SPATTs prior to the occurrence of *D. acuta* and DTX2 appeared in the SPATT samplers at the same time as in the mussels. Therefore the ability of the SPATT to forecast shellfish contamination that was previously reported (MacKenzie et al., 2004) was not observed in the present study. A higher sampling frequency may have permitted the detection of an increase of toxins concentrations in the SPATT prior to the accumulation in the mussels. This would suggest that a 2-3 days early warning might be obtained using the SPATTs, which would be of very limited use to the shellfish industry. Ideally, an early warning should
allow shellfish farmers to harvest a quantity of shellfish that can overcome the product loss generated by a closure, i.e. the early warning should at least be more than one week. Furthermore, the possible 2-3 days early warning that may be obtained does not take into account the time spent in shipment and the analysis time.

5.4.2. PTX group

PTX2 was found in the SPATT discs but not in the transplanted mussels as a result of rapid hydrolyses to PTX2sa which consequently epimerize to the thermodynamically more stable 7-epi-PTX2sa (Suzuki et al., 2001b). Furthermore, low concentrations of PTX2sa were detected in the SPATT discs from Bantry. PTX2-sa was also present in the initial study on the SPATT in New Zealand (MacKenzie et al., 2004). It was suggested that the dissolved PTX2sa present in bloom- extract is produced by enzymatic reaction, as a result of enzyme leakage from damaged cells (MacKenzie et al., 2002). Healthy hand-picked cells of *Dinophysis* did not contain significant amounts of PTX2-sa (Vale and Sampayo, 2002b; Fernandez - Puente et al., 2004; Miles et al., 2004a; MacKenzie et al., 2005). The SPATT discs immersed in Bantry Bay also accumulated several toxins such as PTX2, PTX2sa and 7-epi PTX2sa as well as one isomer of PTX1 and one isomer of PTX3. PTX11 is an isomer of PTX1 that has been reported in *D. acuta* from New Zealand (Suzuki et al., 2003). Other PTX1 isomers such as PTX4, PTX8 were also reported to occur as a result of acid catalysed interconversion of PTX1 (Sasaki et al., 1998). Due to the low concentrations of the compounds detected at m/z 890.5 and 892.5 and the non-availability of their semi purified solutions in our laboratory, it is difficult to assign the identity of these compounds. Also, because of the low concentrations observed, these compounds may be artefacts of the sampling and sample treatment procedures.
5.4.3. YTX group

The levels of YTX accumulated in the SPATT discs from McSwynes Bay were above the LOQ. The accumulations occurred in the apparent absence of known YTX producing organisms such as *Protoceratium reticulatum* (Satake et al., 1997), *Lingulodinium polyedrum* (Tubaro et al., 1998) and *Gonyaulax spinifera* (Rhodes et al., 2006). These organisms are known to occur occasionally in Irish waters (Silke, 2008 – Personal Communication) and analysis of cells of *Lingulodinium polyedrum* from Ireland by ELISA detected low amounts of YTX in the past (Clarke, 2001 – Personal Communication). Phytoplankton sampling indicated that the numbers of *Heterocapsa sp*, *Prorocentrum balticum/minimum* and *Prorocentrum dentatum* (Table 4) rose at the same period as YTX in the SPATTs. The origin of YTX in the present study needs further investigation.

5.4.4. SPX group

Spirolides are a non-regulated group of toxins that are not believed to be toxic to humans (Munday et al., 2004). However, they are fast acting toxins that, when injected intraperitoneally into mice result in rapid death with neurological symptoms (Munday et al., 2004). Therefore, the monitoring of these toxins offers the advantage of providing an explanation for eventual discrepancies between the mouse bio-assay and chemical methods or enzymatic assays due to the presence of fast acting toxins. SPX was found to be produced by *Alexandrium ostenfeldii* (Cembella et al., 1999). SPX-13-desMe-C was detected at quantifiable concentrations in all SPATTs that were deployed in Bantry Bay. The concentrations were two orders of magnitude lower than those obtained for OA and one order of magnitude less than for AZAs. SPX 13-DesMe-C was not detected in the transplanted mussels, possibly as a result of the low
toxin concentration or because of metabolites that may be formed in mussels. Further studies are required to clarify whether spirolides occur at levels above background in Irish shellfish and whether they are indeed contributing to unexplained mouse bioassay results.

5.4.5. AZA group

Consumption in the Netherlands of contaminated Irish mussels harvested in Killary Harbour led to the discovery of AZA1 (McMahon and Silke, 1996; Satake et al., 1998; McMahon and Silke, 1998). Further isolation in mussel samples led to the discovery of AZA2 and AZA3 (Ofuji et al., 1999b) as well as of hydroxyl derivatives AZA4-5 (Ofuji et al., 2001), AZA7-11 and of AZA6 (James et al., 2003), an isomer of AZA1. The organism that produces AZA had not been unambiguously identified at the time of the study (Twiner et al., 2008).

In the present study, AZA contaminations occurred in Bantry and McSwynes Bay. In the indigenous mussels from the latter location toxins reached levels of AZAs that were ca. 50 times greater than the regulatory limit at the peak of the contamination. The AZA profiles in the SPATT discs from both locations were dominated by AZA1 and AZA2. AZA2 accounted for 20% of the concentration of AZAs in Bantry and less than 10% in McSwynes Bay. AZA1 and AZA2 were found to follow the same time trends while AZA3 which was only detected in low amounts in SPATT samples from McSwynes Bay (< 1% of the total AZAs) did not follow the same trend. These concentration and time trend differences between AZA1 and -2 on one hand and between AZA3 on the other hand suggest that AZA1 and AZA2 are toxins produced by the causative organism and that AZA3 is more likely to be a metabolite. AZA3 was the predominant form of AZAs in the transplanted mussels while it only accounted for less than 10% of the total AZAs in the indigenous mussels. A separate
study conducted in the authors’ laboratory will explain the influence of heat treatment on AZA profiles.

Similar to the results obtained for the OA, DTX2 and PTX2 toxins, when contamination reached its maximum, concentrations of AZAs varied with depth. Accumulation of AZAs in the SPATTs followed the accumulation pattern that was observed in the indigenous mussels as the maximum concentration of AZA in the SPATT coincided with the maximum concentration in the indigenous mussels in both locations. The transplanted mussels accumulated AZAs for 7 consecutive weeks in the two locations. This prolonged accumulation contrasts markedly with the results obtained for the OA group toxins and could be explained by different hypotheses:

1) different factors control the occurrence of the AZA producing organism than those controlling the occurrence of the OA/DTX2 producing *Dinophysis* spp (*Dinophysis* is often associated with wind driven advective events) and the organism that produces AZAs remained in the water for longer periods that *Dinophysis*

2) mussels were able to accumulate AZAs directly from water or suspended matter

3) the AZA-producing organism is subject to predation, and trophic transfer of AZAs to different phytoplankton which are subsequently accumulated in mussels plays a major role or

4) a combination of the above.

The levels of AZAs that were obtained in the transplanted mussels from McSwynes Bay were lower than in the indigenous mussels by about one order of magnitude while in Bantry Bay levels of contaminations were similar in both mussel types. This difference may be due to different algae being present resulting in different trophic transfer mechanisms or due to different levels of contamination as one week exposure may not be sufficient for the mussels to accumulate the microgram amounts that were
accumulated by the indigenous mussels in McSwynes Bay. In Bantry Bay, the SPATT and the transplanted mussels accumulated AZAs before the indigenous mussels. This lag may be due to the fact that the SPATTs and the transplanted mussels were placed a few miles from the production area where the indigenous mussels were sampled and that the AZA event may have reached the SPATTs and transplanted mussels first. Studies have shown that, within this coastal zone, meteorological conditions are important in the occurrence of dinoflagellate and other phytoplankton (Raine and McMahon, 1998).

5.4.6. Early warning

The use of SPATT did not provide an early warning of shellfish contamination as the concentration of toxins rose in SPATT discs and in mussels at the same time. Nevertheless, the passive samplers have shown the ability of responding rapidly to the presence of harmful phytoplankton (Fux et al., 2008b). Previous experiments have shown that significant amounts of OA and DTX1 accumulate in the SPATT after only 12 h exposure. Thanks to these rapid accumulation times, early warning may be possible if the duration of transportation of toxic algae along ocean currents and tides are significantly longer than the duration of toxin adsorption on the SPATT. In such a scenario, SPATT samples would need to be obtained at high frequency from sites that are upstream of the shellfish aquaculture sites. Thus, the early warning obtained would be similar to early warning obtained from phytoplankton with the added advantage of obtaining a spatially and time integrated response.
5.5. Conclusions

This work has shown that the SPATT discs were very sensitive detectors of lipophilic marine toxins. The SPATT responded to the appearance of phytoplankton by accumulating concentrations of toxins above the background levels. The results we obtained in this study suggest that toxins belonging to the OA group only accumulate in shellfish if the latter had been feeding on toxin-producing phytoplankton. The presence of OA in the water without the presence of toxic phytoplankton did not induce shellfish contamination. In the two sampling locations where AZAs outbreaks occurred, the toxic event resulted in accumulation of AZA in the transplanted mussels for seven consecutive weeks. The AZA profile obtained in the SPATT consisted essentially of AZA1 and AZA2 suggesting that the two toxins are biosynthesized by the same organism and that AZA3 is a shellfish metabolite. The high sensitivity of SPATT discs and their subsequent selective analysis using UPLC-MS/MS allowed for the detection of YTX and SPX-13-DesMe-C that, to our knowledge, have not been reported before in Irish waters. The possibility of obtaining an early warning through the use of passive samplers was not observed in our study as the concentration of toxins in the SPATT and in the mussels placed next to them rose at the same time.
6. **Dinophysis acuta** and its toxin at low depth in the Celtic Sea

6.1. Introduction

A ten day phytoplankton survey was carried out in July 2007 in the Celtic Sea on the Marine Institute’s vessel, the Celtic Explorer (Figure 6.1).

![Figure 6.1: Photograph of the Celtic Explorer](image)

During the course of the survey a population of *Dinophysis acuta* cells were identified. The present study describes the toxin profile in harvested cells and obtained by LC-MS. Interesting differences and similarities with other strains of *D. acuta* are discussed. Furthermore, SPATT discs were deployed in a location close to the position where the phytoplankton occurred and thus accumulated toxins from the same *Dinophysis* population.
6.2. Material and methods

6.2.1.1. Solvents and reagents

Acetonitrile was purchased as pestiscan grade from Labscan Ltd., Dublin, Ireland. Dichloromethane (DCM), hydrochloric acid (HCl, 37%) and sodium hydroxide (NaOH) were obtained as analytical grade from Merck, Darmstadt Germany. Formic acid and ammonium formate were obtained from Sigma–Aldrich, UK (F-0507 and F-2004, respectively). Water was obtained from a MilliQ water purifier. Standards of OA and PTX2 were purchased as certified calibration solutions from the National Research Council (NRC), Halifax, Canada. OA-D8 was provided by Dr. Michael Quilliam and PTX11 by Dr Chris Miles.

6.2.1.2. SPATT design and handling

The SPATT discs were prepared as previously reported (Fux et al., 2008). A circular frame was used as resin holder instead of the resin filled sachets described by MacKenzie et al. (2004).

The 95µm nylon mesh that contains the resin was purchased from John Staniar & Co., Whitefield Manchester, UK. The frames (diameter 8.8 cm) used to hold the mesh and the resin were embroidery hoops and were purchased from Singer Sewing Centre, Galway, Ireland. Diaion HP-20 resin was purchased from Sigma Aldrich, UK.

The SPATT discs were prepared as follows: HP-20 Diaion resin was weighed (3.00 ± 0.05 g) and methanol (100 mL) was subsequently added. The resin was activated by a 40 min shaking step in a multitube vortexer prior to filtration on 95 µm mesh (≈21 x 12 cm). The resin was wrapped with the mesh and clipped in the frame allowing exposure on both sides of the frame. Methanol residues were removed by a 10 min
sonication step in 500 mL water. The SPATTs were stored in MilliQ water at 6 °C until deployment.

6.2.1.3. *Dinophysis acuta* cells collection

6.2.1.3.1. Pumping at specific depths

Both concentrated samples and net hauls were taken on the 25th of July 2007.

Location: Samples were taken on the South West of Ireland (Lat. 08°57.03 W and Long. 51°27.26 N) (Figure 6.2).

Water was pumped from the profiler (Ifremer) and filtered through 200 µm and 20 µm mesh. The cells collected on the 20 µm mesh were scooped in a jug, concentrated in 150 ml and the homogenous solution divided into 3 x 50 ml centrifuge tubes. One sample was preserved in lugol for cell counts and another 50 ml sample was used for toxin profile determination.

<table>
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<tr>
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<td>1H55</td>
<td>469</td>
<td>26</td>
</tr>
<tr>
<td>4H10</td>
<td>416</td>
<td>27-29</td>
</tr>
<tr>
<td>7H20</td>
<td>408</td>
<td>27-29</td>
</tr>
</tbody>
</table>

6.2.1.3.2. Deep *D. acuta* sample

An additional sample was collected from 81 meters on the 27th of July by concentrating 138 l into 50 ml. (Lat 7°34.91W; Lon 51° 28.02 N) (Figure 6.2).
6.2.1.4. SPATT sampling

SPATT discs were deployed on location: [Lat 9°11.35 W; Lon 51°17.27N] (Figure 6.2) from the 23rd of July at 17h55 GMT to the 29th of July at 13H00 GMT. At this location the depth was 98 m. The SPATT discs were deployed using a 135 m rope attached to a floating buoy and ballasted to the bottom using concrete blocks. A 35 m slack was given to the rope in order to prevent the buoy lifting the concrete blocks at high tide. Two SPATT discs were deployed at each depth (30, 50, 70 and 110 m).

6.2.1.5. Extraction of toxins from *D. acuta* cells

Extraction of phytoplankton was performed by filtering aliquots (50 ml) of phytoplankton concentrates on GF/C filter. After the aqueous phase had passed through the filter the vacuum was stopped and the cells on the filter were rinsed twice with 2 x 5 ml methanol. The combined aqueous/methanol filtrate was partitioned three
times against DCM (1:1). The DCM was reduced to ca 1 ml in a TurboVap ® 500 at 38 °C, and transferred into test tubes. The glassware used for evaporation was rinsed twice with small portions of DCM (< 5 ml) and the rinses were combined in the test tube. The DCM was subsequently evaporated to dryness under nitrogen in a heating block at 35°C. The residue was resuspended in methanol and transferred into a volumetric flask (5 ml). The test tubes were subsequently rinsed with methanol and the rinsing solutions were combined in the volumetric flask. The volume was made up to the mark with methanol and the solution was filtered on 0.2 µm filter before LC-MS injection.

6.2.1.6. Hydrolysis of *D. acuta* extracts

NaOH 2.5 M (125 µL) was added to a 1 ml aliquot of *D. acuta* extract and the solution heated at 76°C for 40 min then cooled at room temperature and neutralised by addition of 125 µL of 2.5 M HCl (Mountfort et al., 2001).

6.2.1.7. Extraction of toxins from SPATT discs

The SPATT extraction method consisted in a slow elution using 23 ml of methanol as reported elsewhere (Fux et al., 2008). This method achieved excellent recoveries for OA, DTX2, PTX2 and AZA1, -2. Briefly, the SPATTs were rinsed twice in 500 mL of MilliQ water to remove salts and vigorously shaken to remove the excess of water. The contaminated resins were removed from the mesh and inserted into empty SPE glass cartridges placed on a manifold. Vacuum was applied in order to remove the remaining water. A 23 mL methanol portion was used to elute the resin at ca 1 mL.min⁻¹ flow rate. The extracts were transferred into 25 mL volumetric flasks and an additional 2 mL was used for rinsing and to complete up to the mark. Aliquots
were taken from the volumetric flask, filtered on 0.2 µm and inserted into LC vials for LC-MS analysis.

6.2.1.8. Instrumentation

LC-MS/MS analyses were performed on an Acquity system (Waters Corp., Manchester, UK) coupled to a hybrid quadrupole - time of flight mass spectrometer Q-TOF (Micromass Ltd., Manchester, UK). Separation was achieved on a BEH C8 column (150 x 2 mm ID; 1.7 µm particle size) maintained at 30 °C by gradient elution. Mobile phase A was 100 % water and mobile phase B was 95 % acetonitrile, both containing 2 mM ammonium formate and 50 mM formic acid (Quilliam et al., 2001). The gradient started with 30 % B linearly rising to 100 % in 35 min. The 100 % B was held for 5 min and reduced to 30 % for 3 min equilibration before the next injection. The flow rate was set at 0.2 ml/min and 5 µl of samples maintained at 5 °C were injected in the system.

The Q-TOF was used in TOF-MS/MS mode, where the precursor ion is isolated in the quadrupole and where after collision in the collision cell, the whole fragmentation spectrum is obtained in the TOF. Precursor ions were either the molecular ion or the ammonium ion. The masses and precursor ions were as follows: OA and DTX2: [M - H]⁻, 803.5; PTX2: [M + NH₄]⁺, 876.5; PTX11: [M + NH₄]⁺ : 892.5 PTX2sa: [M + NH₄]⁺ : 894.5; OA-D8 [M + NH₄]⁺ : 946.5. Quantitation of toxin was performed using a six level calibration curve for each OA and PTX2. PTX2sa and PTX11 were quantified using PTX2 standards and DTX2 using OA standards.
6.2.1.9. Phytoplankton counts

Phytoplankton cell counts were carried out by Dr Gonzalez-Gil from the oceanography center in Vigo. One mL aliquots of the concentrated lugol-fixed samples were placed in Sedgewick Rafter sedimentation chambers (Graticulates Limited, Tonbridge, England) and count under an inverted microscope (Nikon Eclipse TE2000-S). The volume of water pumped (L) calculated in section 2.2 (Table 6.1) was employed to estimate the number of *Dinophysis acuta*, *D. acuminata*, *D. ovum*, *D. dens* and *D. tripos* in cells L\(^{-1}\) in the non concentrated original water samples.

6.2.1.10. Rate of toxin production

The rate of toxin production was calculated following Equation 6.1 as reported previously (Pizarro et al., 2008).

\[
\mu_i = \frac{\ln Q_{t2} - \ln Q_{t1}}{(t_2 - t_1)}
\]

*Equation 6.1: Rate of toxin production from Pizarro et al., 2008*

Where \(Q_{t2}\) = concentration of toxin per cell at hour \(t_2\); \(Q_{t1}\) = concentration of toxin per cell at hour \(t_1\); \(\mu_i\) = toxin production rate (h\(^{-1}\)).
6.3. Results

6.3.1.1. Cell counts

<table>
<thead>
<tr>
<th>Date</th>
<th>Time (GMT)</th>
<th>Vol conc /L</th>
<th><em>D. acuta</em> cells/L*</th>
<th><em>D dens</em> cells/L*</th>
<th><em>D.acuminata</em> cells/L*</th>
<th>Total <em>Dinophysis</em> cells/L</th>
<th><em>D. acuta</em> %</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-Jul</td>
<td>17:20:00</td>
<td>467</td>
<td>1913</td>
<td>41.43</td>
<td>2.89</td>
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</tr>
<tr>
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<td>927</td>
<td>4.76</td>
<td>8.82</td>
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</tr>
<tr>
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<td>1086</td>
<td>0</td>
<td>7.13</td>
<td>1093</td>
<td>99%</td>
</tr>
<tr>
<td>26-Jul</td>
<td>01:55:00</td>
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<td>245</td>
<td>1.92</td>
<td>0</td>
<td>247</td>
<td>99%</td>
</tr>
<tr>
<td>26-Jul</td>
<td>04:10:00</td>
<td>416</td>
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<td>2.162</td>
<td>1.09</td>
<td>389</td>
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</tr>
<tr>
<td>26-Jul</td>
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<td>289</td>
<td>0</td>
<td>3.92</td>
<td>293</td>
<td>99%</td>
</tr>
</tbody>
</table>

* concentration in original sample

Six phytoplankton samples were collected over a 14 h period by concentrating between 408 to 663 l of water pumped from specific depths (Table 6.2). *D. acuta* accounted for more than 98 % of *Dinophysis* spp in all samples. Some samples contained low amounts of *D. acuminata* and *D. dens*. Cell counts were also performed from one sample at 81 m and 40 cell/L of *D.acuta* were found. Concentration of *D. acuta* of *Dinophysis* spp from specific depths ranged from 245 to 1913 cells/L.

6.3.1.2. Toxin profiles

OA, DTX2, PTX2, and PTX2sa were detected in the *D. acuta* samples. In addition, an isomer of PTX11 that eluted 0.5 min later than PTX11 was observed. The chromatographic separation of these compounds is shown in Figure 6.3. The mass spectra obtained for PTX2 and the PTX11 isomer are shown in Figure 6.4. The fragmentation pattern of the PTX11 isomer matched exactly the fragmentation of the PTX11 standard (Figure 6.4). The fragmentation pattern of PTX11 and its
isomer have similarities with the fragmentation pattern of PTX2 with up to 9 losses of water from the ammonium adduct as well as the specific fragments 551, 533, 515 and 213. The proposed fragmentation pattern of PTX2 and PTX11 is shown in Figure 6.5.

The OA-D8 was also monitored and was not detected in any of the *D. acuta*. A sample of *P.lima* extract obtained as a kind gift from Prof. M. Quilliam was injected during the sequence to demonstrate the system capability of detecting the compound (Figure 6.3).

![Figure 6.3: Example of chromatogram obtained using a BEH C8 column by LC-MS QTof. All compounds were obtained from *D. acuta* sample except for OA D8 which was obtained from *P. lima* extract as reference compound](image)
Figure 6.4: MS/MS product ion spectra obtained from PTX2 (m/z 876), PTX11 isomer form D. acuta and PTX11 standard (m/z 892) using a 35 eV collision energy. Product ions were monitored from 100 to 1000 amu.
Figure 6.5: Proposed MS fragmentation pattern of PTX2 and PTX11

Figure 6.6A shows the OA, DTX2 and PTX2 cell content. OA concentration ranged from 8 to 15 pg/cell, DTX2 from 12 to 28 pg/cell and PTX2 from 15 to 22 pg/cell. DTX2 and PTX2 concentrations were higher than OA in all samples analysed. The ratios of the concentrations of OA/PTX2; DTX2/PTX2 and OA/DTX obtained in the *D. acuta* samples collected over 14 hours are shown in Figure 6.8A. Large variations were observed for the DTX2/PTX2 ratios which ranged from 0.61 to 1.26, with the highest ratio obtained in the sample collected at 7h20. The OA/PTX2 ratios exhibited a similar trend to the DTX2/PTX2 ratios and ranged from 0.45 to 0.74. The OA/DTX2 ratios were more constant with an average of 0.66 ± 0.05 (Figure 6.9).

The cells sampled at 81 m depth contained 16.3, 26.4 and 39.0 pg/cell of OA, DTX2 and PTX2. The ratios of OA/PTX2, DTX2/PTX2 and OA/DTX2 were 0.42, 0.68 and 0.62, respectively (Figure 6.9).
Figure 6.6: Average concentration of A) OA, DTX2 and PTX2 and B) PTX2sa and PTX11 obtained in *D. acuta* cells by LC-MS. Average and deviations were calculated from triplicate injection.

The average concentration (expressed in ng of toxin per gram of resin used as receiving phase) of OA, DTX2 and PTX2 obtained by LC-MS from SPATT discs is shown in Figure 6.7A. The concentrations ranged from 0.8 to 2.9 µg/g of OA, from 0.9 to 3.7 µg/g of DTX2 and from 1.1 to 4.5 µg/g of PTX2. PTX2 was the predominant toxin followed by DTX2 and OA in all passive samplers. PTX2sa was also detected in all passive samplers at levels ranging from 79 to 113 ng/g of resin (Figure 6.7B). The ratios of the concentrations of OA/PTX2; DTX2/PTX2 and OA/DTX obtained in the SPATT discs deployed at 30, 50, 70
and 110 m depths were constant (< 5% variation) (Figure 6.8B). The average ratios were 0.69 ± 0.03 for OA/PTX2, 0.84 ± 0.04 for DTX2/PTX2 and 0.82 ± 0.03 for OA/DTX2 (Figure 6.9).

Figure 6.7: Average concentration of A) OA, DTX2 and PTX2 and B) PTX2sa obtained by LC-MS from SPATT discs deployed at 30, 50, 70 and 110 m. Average and deviations were calculated from triplicate injection.
Figure 6.8: A) Ratios of the concentrations of OA/PTX2; DTX2/PTX2; PTX2sa/PTX11 and OA/DTX obtained in the D. acuta samples collected over 14 hours and B) Ratios of the concentrations of OA/PTX2; DTX2/PTX2 and OA/DTX obtained in the SPATT discs deployed at 30, 50, 70 and 110 m. depths
Figure 6.9: Comparison of the average ratios of OA/DTX2, DTX2/PTX2 and OA/PTX2 obtained in the *D. acuta* cells (n=6), in the SPATT discs (n=4) and in the sample of *D. acuta* obtained from 81 m (n=1).

The toxin productions of OA, DTX2 and PTX2 calculated from Equation 6.1 are shown in Figure 6.10. The production rate curves of OA and DTX2 are almost identical suggesting that both toxins are produced simultaneously.

PTX2 does not follow this trend and seem to have a slower toxin production cycle.

Figure 6.10: Accumulation rate of toxins in *D. acuta* harvested over a 14 h period $\mu$ (h$^{-1}$)
6.4. Discussion

Toxin profiles of *D. acuta* from Ireland have been established in the past, and the occurrence of OA, DTX2, PTX2 and PTX2sa have been previously shown (James et al., 1997a; Draisci et al., 1998a; James et al., 1999b; Fernandez-Puente et al., 2004). The levels of OA and DTX2 that are reported in this study (8-15 and 12 – 28 pg/cell, respectively) are lower than the 60 and 80 pg/cell measured by James et al. and the 85 and 77 pg/cell reported by Fernandez-Puente et al. The latter study also reported concentrations of PTX2 ranging from 7 to 14 pg/cell, while the concentrations reported in this work ranged from 15 to 22 pg/cell. PTX2sa was detected, however it is not believed to be produced by the cells but rather believed to result from the hydrolysis of PTX2 by enzymes released by stressed cells (MacKenzie et al., 2002).

PTX11 is an oxidised form of PTX2 and has been detected in *D. acuta* from New Zealand (MacKenzie et al., 2002; Suzuki et al., 2003; Miles et al., 2004a; MacKenzie et al., 2005; Suzuki et al., 2006). Low amounts (0.4 – 2 pg/cell) of PTX11 were also detected in *D. acuminata* from New Zealand (MacKenzie et al., 2005) and were below LOD in *D. acuminata* from Norway (Miles et al., 2004b). The PTX11 isomer detected in this study is more than likely a hydroxylated PTX2. According to the MS spectra obtained this compound is different to PTX1 and has the exact same fragmentation pattern as PTX11. However, the PTX11 isomer elutes earlier than PTX11 suggesting either that *D. acuta* from Ireland produces a different hydroxylated PTX2 than *D. acuta* from New Zealand or that the isomer of PTX11 is an artefact product. Furthermore, a novel PTX analogue was detected in all *D. acuta* samples and will be reported separately.

Recently, OA diol esters (OA-D8) have been detected in *D. acuta* from New Zealand (Suzuki et al., 2004; Suzuki et al., 2004; Miles et al., 2004a; Miles et al., 2006a) and from Spain (Pizarro et al., 2008). Similarly to the study by Fernandez Puente et al., OA-D8 was not
detected in our *D. acuta* samples from the Celtic Sea. It was demonstrated that the LC-MS parameters that were applied were capable of detecting OA-D8 from *P. lima*. Furthermore, an alternative extraction method successfully employed for the detection of OA D8 from *D. acuta* samples from New Zealand (Suzuki et al., 2004) was carried out on a sample obtained using a phytoplankton net. The latter extraction procedure consisted of a filtration of condensed phytoplankton sample through GF/C filter on which the cells were subsequently rinsed with methanol. The aqueous filtrate and methanol were combined and transferred to a C18-silica solid-phase cartridge extraction. This procedure allowed for the detection of OA, DTX2 and PTX2 but similarly to the extract obtained by LLE extraction, no OA D8 was detected. The extract obtained by LLE was hydrolysed using the procedure as described by Mountfort et al. (Mountfort et al., 2001) and the concentrations of OA and DTX2 in hydrolysed extracts remained unchanged when compared to the non-hydrolysed extracts (data not shown). This suggests that OA-D8 is not produced by the *D. acuta* strain that was collected as part of this study. Therefore, the production of OA and DTX2 does not require the presence of OA-D8 which is in accordance with the conclusions from previous experiments (Pizarro et al., 2008).

Analyses for OA-D8 in the SPATT disc extracts were also below the LOD. This would have been expected to have accumulated, if present in the water, due to their lipophilic character, as observed in SPATT immersed in *P. lima* cultures (unpublished data). This provides another indication that OA-D8 does not seem to be produced by the Irish strain of *D. acuta*.

The amounts of toxin per cell measured in the *D. acuta* sample collected from 81 m were very similar to those obtained during the investigation of toxin production over the 14 h period. However, the concentration of PTX2 in the sample from 81 m was higher (up to a factor 2) than in the samples collected at approximately 30 m. Furthermore, the ratio of OA and DTX2 from the low depth sample (0.62) was within the deviation obtained from the average of the
ratio of OA/DTX2 over the 14 h period (0.66 ± 0.05) (Figure 6.9) demonstrating that the amount and the ratio of the DSP toxins are not modified by the low light environment.

The toxin accumulation rate obtained over the 14 h period suggests that the biosynthetic route for OA and DTX2 production is different than for the production of PTX2 and therefore that it is likely that different polyketide synthesases are involved in their production.

The use of passive sampling in this study has provided the advantage of a spatially and temporarily integrated response as well as the capability of obtaining toxin profiles at very low depths (110 m). The SPATT discs accumulated large amounts of OA, DTX2 and PTX2 as well as smaller amounts of PTX2sa and the novel PTX related compound mentioned above. The PTX11 isomer was the only compound that was present in the phytoplankton cells and was not detected in the SPATT. This could be due to a poor stability of the compound in sea water or as mentioned above, the PTX11 isomer may be an artefact product produced during the toxin extraction.

The results have shown a difference in the amount of toxin that accumulated in the SPATT from different depths with a maximum of OA, DTX2 and PTX2 at 50 m depth. The amounts of toxin that accumulated in the SPATT deployed at 30 and 70 m were very similar while the passive samplers exposed at 110 m accumulated the lowest amount of toxin compared to the other depths. The levels of PTX2sa that accumulated at the different depths were not significantly different again confirming that PTX2sa is not likely to be biosynthetically but ubiquitously distributed throughout the water column. Trophic transfer to copepods of PTX2 and DTX1 have been suggested in other studies (Kuuppo et al., 2006). The ratios of DTX1 and PTX2 in faeces of copepods collected in the sediments and the ratios observed in the phytoplankton were 10 fold higher in the sediments than in the phytoplankton and no Dinophysis cells were observed in the sediments. In our study, the ratios of toxin (OA/DTX2, OA/DTX2, DTX2/PTX2) were constant along the entire water column suggesting that
accumulation of toxin at low depths are not a consequence of trophic transfer but most likely due to the presence of *D. acuta*.

The ratios of toxins observed in the SPATT, in the *D. acuta* cells collected during the 14 h period and the cells collected at 81 m were compared (Figure 6.9). The ratio of OA and DTX2 was slightly higher in the SPATT than in the cells while the ratios of OA/PTX2 and DTX2/PTX2 were similar in the SPATTs and in the cells collected over the 14 h period. Although small variations could have occurred in the toxin production of the phytoplankton over the 6 days when the SPATTs were immersed, these results demonstrated that passive samplers are capable of providing a toxin profile that correlates well with the one in the phytoplankton cells.

Dinophysis species, in particular *D. acuminata* have been found at low depths (< 75 m) in the Baltic sea providing evidences that this organism can occur in darkness (Setala et al., 2005). Incubation experiments carried out as part of the latter study have suggested that *D. acuminata* could persist in low light environments for several weeks. Photosynthetically active radiation measurements have indicated that a 90 % attenuation of the incident light is occurring at 15 to 17 m and that the euphotic zone (> 1% of incident light) is above 30 – 35 m depth. Therefore, results presented in this study have demonstrated that not only *D. acuta* cells were present at low depth and therefore in complete darkness, but also that they contained toxins.

6.5. Conclusions

The toxin profiles obtained in the phytoplankton and in the SPATT were very similar and demonstrated that different abundances of toxin that are produced by the cells can be estimated from SPATT samplers exposed to these cells.

Examination of toxin production over a 14 h period has shown that no particular toxin is produced at a given time of the day and that the toxin production cycle of OA and DTX2 is different than for PTX2. The OA diol ester (OA-D8) does not seem to be produced by strains
of *D. acuta*. Interesting PTX analogues were observed and included an isomer of PTX11 with the same fragmentation spectrum as PTX11 standard and a different retention time. Furthermore, a compound structurally related to PTX was also observed and its structure determination is ongoing.

The toxin ratios in SPATT discs were constant throughout the water column. The comparison of the toxins in *D. acuta* cells collected in complete darkness, in SPATT exposed to low depths and in *D. acuta* cells collected in the thin layer over a 14 h period suggested that phytoplankton can produce toxins in the absence of light.
7. Evaluation of lipophilicity and acidity constant of seven regulated lipophilic marine biotoxins

7.1. Introduction

Lipophilicity expressed as logP\textsubscript{ow} is an important physico chemical parameter used in quantitative structure/activity relationship, drug and pesticide design and toxicology studies. Lipophilicity measurement is required by the EU regulation for any new chemical available commercially. Two methods are described in the Organisation for Economic Co-operation and Developments (OECD) guidelines for the measurement of logP\textsubscript{ow}:

1) the shake flask method (OECD, 1995) and 2) chromatographic methods (OECD, 2004).

NMR spectroscopy can also be used for the determination of the pK\textsubscript{a}. This technique was used for the determination of the pK\textsubscript{a} of the ASP toxin DA by investigating the $^1$H NMR and $^{13}$C NMR spectra as a function of pH and pD (Walter et al., 1992).

The motivation behind the investigation of the lipophilicity, and ultimately the acidity constant (pK\textsubscript{a}), of lipophilic marine toxin was two fold:

1) Future correlation between toxin accumulation in passive sampling and lipophilicity as well as bioaccumulation in shellfish.

2) The in-depth investigation of pH dependency and retention time (governed by the pK\textsubscript{a}) on lipophilic toxin separation which are valuable information to the analytical chemist.
7.1.1. Definitions

- Lipophilic: ‘Having an affinity for fat and high lipid solubility: a physico-chemical property which describes a partitioning equilibrium of solute molecules between water and an immiscible organic solvent, favouring the latter, and which correlates with bioaccumulation’ (IUPAC, 1994)

- Hydrophobic: ‘Describing the character of a molecule or atomic group which is insoluble in water, or resistant to wetting or hydration (IUPAC, 1994)

7.1.2. The shake flask method

7.1.2.1. Principle of the method: Partition and distribution coefficients

\[
\text{Octanol} \quad \text{HA} \\
\downarrow \quad \downarrow \\
\text{Water} \quad \text{HA} \leftrightarrow \text{H}^+ + \text{A}^-
\]

Figure 7.1: Illustration of a solvent water system and the species partition for a weak acid (Reproduced from Gocan et al., 2006).

The partition and distribution of a weak acid in immiscible aqueous and organic phases is shown in Figure 7.1. The neutral species, represented as HA, partitions between the organic and aqueous phase, whereas the ionised species remains in the aqueous phase. The partition coefficient can be expressed as shown in Equation 7.1, and the distribution coefficient can be expressed as shown in Equation 7.2.
\[ P_{\text{ow}} = \frac{[\text{unionised species}] \text{ octanol}}{[\text{unionised species}] \text{ water}} \]

Equation 7.1 : The partition coefficient of octanol-water.

LogD is the logarithm of the distribution coefficient, D. As demonstrated in Equation 7.2, D is the ratio between the concentrations of the unionised and ionised species of a solute dissolved in two immiscible phases at equilibrium.

\[ D = \frac{[\text{unionised + ionised species}] \text{ octanol}}{[\text{unionised + ionised species}] \text{ water}} \]

Equation 7.2: The distribution coefficient is equal to the total concentration of the molecule in octanol divided by the total concentration of the molecule in water.

7.1.2.2. Shake Flask method experiment (OECD, 1995)

A stock solution of known concentration of the test substance in n-octanol, pre-saturated with water, is prepared. Duplicate vessels containing accurately measured amounts of the two solvents and stock solution are used. The test vessels are placed in a mechanical shaker or are shaken by hand. After phase separation, the total quantity of substance present in both phases should be calculated and compared with the quantity originally introduced. A \( \log P_{\text{ow}} \) value is calculated from the data of each run. Altogether six values are obtained since the test conditions comprise three duplicate runs with different solvent ratios, and possibly also different quantities of test substance.
7.1.2.3. Limitations and drawbacks of the shake flask method

The shake flask method can be used to determine logP<sub>ow</sub> values in the range of -2 to 4 (occasionally up to 5) (OECD, 1995).

Limitations and drawbacks of the method include:

- Stable emulsions can be produced, preventing measurement of the analyte concentration in either the aqueous or organic phase.
- Non-applicability of the method to surface-active materials.
- Relatively large amounts of analyte of high purity are required.
- Some compounds can adhere to the surface of the vessel, particularly lipophilic compounds.
- Some compounds can form micelles in the aqueous phase, act as surfactants that concentrate at the interface between the two liquids, or form foams.

7.1.3. Chromatographic methods

7.1.3.1. Principle of the chromatographic methods

An alternative to the shake flask method for the evaluation of lipophilicity can be achieved using liquid chromatography (OECD, 2004). The retention of a compound in reversed-phase high performance liquid chromatography is governed by its lipophilicity/hydrophobicity, and thus shows correlation with an octanol-water partition coefficient (Valko, 2004).

The chemicals are retained in proportion to their hydrocarbon-water partition coefficient. The retention time is described by the capacity factor k which can be calculated from the retention time and the void time of the system. The void time is the time taken for an unretained substance to elute. The chromatographic method relies on the production of a calibration
curve with reference compounds of known lipophilicity that are chosen to cover a range of different lipophilicities.

7.1.3.2. Limitations and drawbacks of the chromatographic methods

Measurements can be performed on ionisable substances in their non-ionised form (free acid or free base) only by using an appropriate buffer with a pH below the pKₐ for a free acid or above the pKₐ for a free base (OECD, 2004). Therefore, the investigation of the acidity constants of the toxins considered was also carried out.

There are many advantages in using HPLC methods to determine the lipophilicity and the pKₐ.

- It requires a much smaller amount of sample.
- It is relatively insensitive to impurities.
- Practical problems encountered with the shake-flask technique, such as formation of stable emulsions, are no longer an issue.
- It can be used for a larger lipophilicity scale than is practically possible in shake-flask experiments with logP₂₀ ranging from 0 to 6 (OECD, 2004).

The main difficulty in the application of this method to marine toxins relies in the detection system used. Within the toxins considered in this work, only PTX2 is suitable for LC-UV analysis and LC-MS detection is therefore commonly used for the lipophilic toxins detection. The use of LC-MS for the lipophilicity measurement implied that the reference compounds chosen are ionisable in an ESI source and that their subsequent MS detection can be performed. This chapter investigates the use of LC-MS for the determination of the acidity constants and the lipophilicity of marine toxins belonging to the DSP, AZP, YTX and PTX groups.
7.2. Material

7.2.1. Chemicals and solvents

Deionised water was obtained from a reverse-osmosis purification system (Barnstead, Ireland). The list of chemicals and solvents used in this study is given in Table 7.1.

Table 7.1: List of chemicals and manufacturers

<table>
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<th>Name</th>
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<th>Purchased from:</th>
<th>Location</th>
<th>Manufacturer</th>
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</thead>
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<td>Pestiscan</td>
<td>Lab-scan analytical sciences</td>
<td>Ireland</td>
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<tr>
<td>Methanol</td>
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<td>HPLC grade water</td>
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<td>Ammonium formate</td>
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<td>99.4%</td>
<td></td>
<td></td>
<td>Switzerland</td>
</tr>
<tr>
<td>pH buffer calibration solutions</td>
<td>pH 4.01 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 7.00 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9.21 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium Carbonate</td>
<td>30-33%</td>
<td>Sigma-Aldrich</td>
<td>Germany</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>98%</td>
<td></td>
<td></td>
<td>Analytical,</td>
</tr>
<tr>
<td>Tylosin Tartrate</td>
<td>95%</td>
<td></td>
<td></td>
<td>Fluka</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>95%</td>
<td></td>
<td></td>
<td>Biochemika,</td>
</tr>
<tr>
<td>Ammonium Bicarbonate</td>
<td>99%</td>
<td></td>
<td></td>
<td>Fluka</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>99%</td>
<td></td>
<td></td>
<td>Biochemika,</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>98%</td>
<td>Sigma</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td></td>
<td>Sigma</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td></td>
<td>Sigma</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td></td>
<td>Sigma</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td></td>
<td>Sigma</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Potassium Bromide</td>
<td>99%</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>99%</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Ammonium Acetate</td>
<td>97%</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Ammonium Hydroxide</td>
<td></td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>37-38%</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>99%</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Leucine Enkephalin acetate hydrate</td>
<td>95%</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>OA</td>
<td></td>
<td>National Research Council (NRC)</td>
<td>Canada</td>
<td></td>
</tr>
<tr>
<td>AZA1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTX2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTX1</td>
<td></td>
<td>Norwegian Veterinary Institute (NVI), Thomas Runberget.</td>
<td>Norway</td>
<td></td>
</tr>
<tr>
<td>DTX2</td>
<td></td>
<td>Isolated at the MI, Oranmore</td>
<td>Ireland</td>
<td></td>
</tr>
<tr>
<td>AZA2</td>
<td></td>
<td>Passive sampler</td>
<td>Ireland</td>
<td></td>
</tr>
<tr>
<td>YTX</td>
<td></td>
<td>Isolated from <em>Protoceratium reticulatum</em>, by Chris Miles</td>
<td>NZ</td>
<td></td>
</tr>
</tbody>
</table>
7.2.1. Preparation of solutions

The reference compounds and toxin solutions were prepared in methanol at the concentrations indicated in Table 7.2.

7.2.2. HPLC analysis

HPLC analysis was performed using a HPLC 2795 Separations Module (Waters Ireland ltd, Ireland).

A binary mobile phase was used, with phase A (100 % aqueous) and phase B (95 % acetonitrile), both containing 5 % buffer (aqueous). Buffer type varied depending on the desired pH.

HPLC was performed using a Gemini C18 column (50 x 2.0 mm, 3 μm particle size) (Phenomenex, UK). The flow rate was set at 0.25 ml/min and the injection volume at 5 μl. The column temperature was set at 30 °C and the sample temperature was 11 °C. All samples were run under isocratic conditions. When the mobile phase composition was changed, the column was allowed to re-equilibrate for 15 minutes before the next condition was used.

7.2.3. MS detection

The MS detection system used for analysis was a hybrid Q-ToF Ultima (Micromass Ltd, UK) equipped with a z-spray ESI source. The acquisition mode used was fragment ion scan, where the precursor ion was isolated by the first quadrupole, fragmented in the collision cell, and the final fragmentation spectrum obtained by the ToF.

When recording the retention time of an analyte, the mass of the most abundant fragment was always extracted and smoothed if necessary.

Both positive and negative ionisations were investigated for the detection of the reference compounds. Different cone voltages were also used in an attempt to improve the sensitivity. When visible the main fragment ion observed in another study was extracted for the
determination of the retention time (Castiglioni et al., 2005). The ionisation mode, cone voltage, collision energies, precursor and fragment ions that were used data summarised in Table 7.2.

**Table 7.2: Reference compounds and toxins analysed, concentration, MS settings and ions visible. Precursor ions of the reference compounds were obtained from Castiglioni et al., 2005.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng/ml)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion I (m/z)</th>
<th>Product ion II (m/z)</th>
<th>Mode</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bezafibrate</td>
<td>2300</td>
<td>361.8</td>
<td>139.0</td>
<td>121.0</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>2520/5040</td>
<td>205.1</td>
<td>191.0</td>
<td>161.1</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>2400</td>
<td>237.3</td>
<td>194.0</td>
<td>193.0</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2140</td>
<td>332.3</td>
<td>231.0</td>
<td>314.0</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>2580</td>
<td>361.4</td>
<td>261.0</td>
<td>262.0</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Tylosin Tartrate</td>
<td>2060</td>
<td>916.6</td>
<td>772.5</td>
<td>174.1</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2120</td>
<td>734.0</td>
<td>158.0</td>
<td>576.3</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>4240</td>
<td>253.3</td>
<td>108.0</td>
<td>92.0</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>2040</td>
<td>461.2</td>
<td>426.2</td>
<td>350.1</td>
<td>Positive</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>AZA1</td>
<td>77.4</td>
<td>842.5</td>
<td>654.5</td>
<td>362.0</td>
<td>Positive</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>AZA2</td>
<td>856.5</td>
<td>838.4</td>
<td>654.3</td>
<td>Positive</td>
<td>40</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>PTX2</td>
<td>218.6</td>
<td>876.5</td>
<td>823.5</td>
<td>212.5</td>
<td>Positive</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>OA</td>
<td>266.0</td>
<td>803.5</td>
<td>255.2</td>
<td>113.0</td>
<td>Negative</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>DTX1</td>
<td>250.0</td>
<td>817.5</td>
<td>255.5</td>
<td>113.0</td>
<td>Negative</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>DTX2</td>
<td>2080.0</td>
<td>803.5</td>
<td>255.2</td>
<td>113.0</td>
<td>Negative</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>YTX</td>
<td>1.97</td>
<td>1141.5</td>
<td>1061.5</td>
<td>924.5</td>
<td>Negative</td>
<td>35</td>
<td>50</td>
</tr>
</tbody>
</table>

7.2.4. Preparation of mobile phases

The mobile phases consisting of 5 % buffer in water and in ACN were filtered before use and stored at ~ 6 °C. The mobile phase solutions were sonicated for 15 minutes before use. The buffers used to reach the desired pHs are summarised in Table 7.3
Table 7.3: Buffer concentrations and pHs used for the mobile phases. The pH was measured in 100 % aqueous phase

<table>
<thead>
<tr>
<th>Buffers and concentrations</th>
<th>pH of buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic Acid (5M)</td>
<td>1.25</td>
</tr>
<tr>
<td>Formic Acid (3M)</td>
<td>1.57</td>
</tr>
<tr>
<td>Formic Acid (1M)</td>
<td>2</td>
</tr>
<tr>
<td>Formic Acid (1 M)/ Ammonium Formate (40 mM) *</td>
<td>2.52</td>
</tr>
<tr>
<td>Acetic acid (50 mM) / Ammonium Acetate (5mM)*</td>
<td>3.68</td>
</tr>
<tr>
<td>Acetic acid (50 mM)/ Ammonium Acetate (50mM)*</td>
<td>4.71</td>
</tr>
<tr>
<td>Acetic acid (50 mM)/ Ammonium Acetate (0.5 M)*</td>
<td>5.79</td>
</tr>
<tr>
<td>Ammonium Formate (40 mM)</td>
<td>6.16</td>
</tr>
<tr>
<td>Ammonium Acetate (40 mM)</td>
<td>6.92</td>
</tr>
<tr>
<td>Ammonium Bicarbonate (100 mM) /Hydrocloric Acid (1 M)*</td>
<td>7.42</td>
</tr>
<tr>
<td>Ammonium Bicarbonate (2 mM)</td>
<td>7.92</td>
</tr>
<tr>
<td>Ammonium Carbonate (50 mM)</td>
<td>9.18</td>
</tr>
<tr>
<td>Ammonium Bicarbonate (2 M)/ Ammonium Hydroxide (1M)*</td>
<td>9.5</td>
</tr>
<tr>
<td>Ammonium Hydroxide (0.1 M)*</td>
<td>10.75</td>
</tr>
</tbody>
</table>

The couples marked * were used as buffer solutions while the other compounds were used a single component pH modifier.

7.2.5. pH measurement

All pH (and electrode potential, mV) measurements were made using a pH meter Thermo Orion (UK), model 420A plus, equipped with an automatic temperature compensation probe. The pH meter was systematically calibrated before use and occasionally when many pH measurements were performed to ensure no drift had occurred. Three certified aqueous buffer solutions (pH 4.01 ± 0.02, pH 7.00 ± 0.02 and pH 9.21 ± 0.02) were used for the calibration. When pH adjustments of a “buffer solution” were carried out (e.g. acidification of 0.1 M Ammonium Bicarbonate with 1 M Hydrochloric Acid) a magnetic stirrer was used, with a sheet of cardboard placed in between the stirring mantle and the vessel, in order to avoid excess mechanical friction and thus heat production.
7.3. Method

7.3.1. The acidity constant

Acidity constant, $K_a$, is also known as acid dissociation constant. “The term $K_a$ is the ionisation constant of a chemical reaction in solution in which a molecule accepts or loses a hydrogen ion, in response to change in pH”. Equation 7.3 and Equation 7.4 show the ionisation of an acid and the simplified calculation of the dissociation constant of an acid.

$$HA \leftrightarrow H^+ + A^-$$

Equation 7.3: Chemical equilibrium and of an acid.

$$K_a = \frac{[H^+][A^-]}{[HA]} \leftrightarrow pK_a = -\log_{10} K_a$$

Equation 7.4: Simplified dissociation constant and $pK_a$ of an acid.

Since values for $K_a$ vary over a large magnitude, it is common to take the negative logarithm to base ten of the value, $pK_a$ as shown in Equation 7.4.

The simplified equation was used for the dissociation constant in order to avoid complications involved in using activity. The ionic strength of the medium is therefore not taken into account in the calculation of the $pK_a$.

7.3.2. Determination of $pK_a$ and lipophilicity by chromatography

The determination of the lipophilicity relies on the relationship shown in Equation 7.5. The constants $a$ and $b$ are determined by injection of reference compounds of known $\log P_{ow}$. 

$$[H^+][A^-]$$
\[ \log P = a \log k_w + b \]

Equation 7.5: Relationship between \( \log P \) and \( \log k_w \), where \( a \) and \( b \) are the slope and intercept, respectively.

Where \( \log k_w \) represents the retention time of a compound in a chromatographic environment where only buffered water is used as a mobile phase. The value of \( \log k_w \) can not be measured directly and need to be calculated by extrapolation of the Equation 7.6 expressing the capacity factor \( k \) as a function of the fraction of organic modifier \( \varphi \).

\[ \log k = c + d \varphi + e \varphi^2 \]

Equation 7.6: Quadratic relationship between \( \log k \) and the fraction of organic modifier in the aqueous phase (\( \varphi \)) (Gocan et al., 2006).

The capacity factor (\( k \)), is determined from the retention time (\( t_r \)) and the dead time (\( t_0 \)).

The retention time (\( t_r \)), also known as peak elution time, is the time (min) it takes for a compound to elute and is usually the peak maximum.

The dead time (\( t_0 \)), also known as hold-up time or void time, is the time that is required to elute a component that is not retained by the stationary phase. The dead time includes any volumes contributed by the sample injector, the detector and connectors. The dead time (min) can be calculated by dividing the dead volume (ml) by the flow rate (ml/min).

The adjusted retention time (\( t'_r \)) is shown in Equation 7.7.

\[ t'_r = t_r - t_0 \]

Equation 7.7: Adjusted retention time is equal to the retention time minus the dead time.

The capacity factor (\( k \)) is a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase. As shown in Equation 7.8 the capacity factor can be calculated by dividing the adjusted retention time by the void time.
\[ k = \frac{t_r - t_0}{t_0} \]

Equation 7.8: Capacity factor is equal to the adjusted retention time divided by the void time.

The capacity factor, \( k \), is established from the retention time of each compound analysed (at each mobile phase composition, in each buffered mobile phase). The percentage of organic phase present in the mobile phase is plotted (x-axis) against the logarithm of the capacity factor (logk) (y-axis). At 0% organic phase, log \( k = \log k_w \) and Equation 7.6 reduces to log \( k_w = c \).

7.3.3. Relationship between distribution coefficient, lipophilicity and acidity constant

The distribution coefficient has the expression:

\[ \log D = \log P + \log[H^+] - \log K_a \]

Equation 7.9: The logarithm of the distribution coefficient as a function of the logarithm of the partition coefficient, the logarithm of the concentration of H\(^+\) in solution and the logarithm of the dissociation constant.

This may also be written as:

\[ \log D = \log P + pK_a - pH \]

Equation 7.10: Relationship between the logD, log P, pK\(_a\) and pH.

Equation 7.9 and Equation 7.10 both display the mathematic relationship between logD, logP, pK\(_a\) and pH. As shown in Equation 7.10, logD is dependant on pH while logP\(_{ow}\) is not. Therefore, where a value for logD is stated, a pH value must also be given. Figure 7.2 illustrates the relationships described above for Equation 7.9 / Equation 7.10.
Figure 7.2: Illustration of the relationship between log D, log P, pK\textsubscript{a} and pH. Reproduced from (Gocan et al., 2006)

Figure 7.2 shows the plot for an acid where a negative slope is obtained. Bases yield positive slopes (mirror image). The end of the plateau just before the slope decreases indicates the point where the pK\textsubscript{a} of the compound is equal to the pH. The whole area of the plateau gives a value for the distribution coefficient, which at this pH is unionised and is therefore equal to the partition coefficient.

7.3.1. pH determination and convention

The pH nomenclature used in the following sections was chosen according to the IUPAC definition (IUPAC, 1997). The \textit{w} pH scale refers to pH standardisation with aqueous reference buffers and pH measurement in water while \textit{s} pH refers the pH scale refers to pH standardization with aqueous reference buffers and pH measurement in the organic solvent. The pH of a buffer solution can be calculated from Equation 7.11, where [A\textsuperscript{-}] and [HA] are the concentrations of the basic and acid components of the buffer.
\[ pH = pK_a + \log \frac{[A^-]}{[HA]} \]

**Equation 7.11 : Relationship between pH, pK\textsubscript{a} and concentration of buffer components**

By definition the buffering capacity of a buffer solution is limited to ratios of the concentration [Base]/[Acid] from 0.1 to 10. Therefore, an acid/base couple solution is considered as a buffer if the pH is within 1 pH unit of the pK\textsubscript{a}.

7.3.2. The reference compounds

The OECD guidelines require that a calibration graph using at least 6 points has to be established in order to correlate the measured capacity factor of a substance with its logP\textsubscript{ow}. It is up to the user to select the appropriate reference substances. The reference substances should normally have logP\textsubscript{ow} values which encompass the logP\textsubscript{ow} of the test substances. Extrapolation should only be used in exceptional cases. LogP\textsubscript{ow} values of the reference substances used for the calibration should be based on reliable experimental data. It is recommended that the reference substances are structurally related to the test substances (OECD, 2004).

The compounds chosen for this study are further discussed in the results section 7.4.5.1.

7.3.3. Determination of lipophilicity and acidity constant using computer models

Software programmes are available for the calculation of logP, logD and pK\textsubscript{a} from the chemical structures using mathematical models and predictions. Free access computer softwares that were used for the pK\textsubscript{a} and logP\textsubscript{ow} calculations included Advanced Chemistry Development (ACD) (ACD, 2008) and ALOGPS 2.1 program from the Virtual
Computational Chemistry Laboratory website (VCCLab, 2008). The latter program calculates an average of $\log P_{ow}$ obtained from 10 software prediction.

The conversions of chemical structures to SMILES format necessary to perform computational predictions were obtained with ChemDraw ® Ultra.

### 7.4. Results and Discussion

#### 7.4.1. Determination of the void time

Since the capacity factor is dependent on the void time care must be taken to ensure that the most accurate void time is measured. The different methods that can be used for the determination of the void volume by HPLC-UV were reviewed (Rimmer et al., 2002). Most of the described methods were not applicable to MS detection.

In order to measure the void time of a LC-MS system the time taken for unretained species to elute must be monitored (also known as the solvent front). Accurate determination implies that a good peak shape and signal can be obtained from the injection of an unretained species such as salt or solvent. Salts can be detected by MS assuming that their molecular masses are sufficiently high for the detector. Aqueous solutions of Leucine Eukephalin acetate and potassium bromide were prepared and used as marker for the void time determination.

The second approach used was to inject ACN, methanol or methanol/water (1:1) mixture and to monitor a contaminant of the solvent.

Figure 7.3 shows the chromatograms obtained when determining the void time in 55 % organic mobile phase at pH 4.34. Both concentrations of potassium bromide, 3.3 ng/ ml and 9.9 ng/ ml, had similar sensitivity when the most abundant ion was extracted (m/z 99). Injection of methanol provided a greater sensitivity when an ion from an unknown contaminant present in the solvent was extracted (m/z 257). The void time that was
established by injection of the lower concentration of potassium bromide (3.3 ng/ml) was 0.61 min, whereas the higher concentration of potassium bromide (9.9 ng/ml) and the methanol resulted a void time of 0.60 min. The use of solvent (methanol or ACN) was preferred as the injection of salts sometimes led to poor peak shapes (Figure 7.3).

Injection of methanol or ACN always allowed for the unambiguous determination of the void time. As expected, the pH or the percentage of organic mobile phase did not influence the void time. However, two columns were used to perform this study and higher void times were generally observed for the second column.

Examples of void times that were recorded at different pHs and using the two different columns are shown in Table 7.1 The analytical column used had a greater effect on the void volume of the overall system than the different pH conditions that were used.
Table 7.4: Void times of the various \( w_pH \) buffered solutions (60 % organic mobile phase)

<table>
<thead>
<tr>
<th>( w_pH ) of aqueous mobile phase</th>
<th>Void time (min)</th>
<th>Retention time based on the peak of:</th>
<th>Gemini column</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.96</td>
<td>0.57</td>
<td>methanol</td>
<td>#1</td>
</tr>
<tr>
<td>9.16</td>
<td>0.62</td>
<td>methanol</td>
<td>#2</td>
</tr>
<tr>
<td>8.81</td>
<td>0.56</td>
<td>methanol</td>
<td>#1</td>
</tr>
<tr>
<td>7.72</td>
<td>0.57</td>
<td>methanol</td>
<td>#1</td>
</tr>
<tr>
<td>7.39</td>
<td>0.61</td>
<td>methanol</td>
<td>#2</td>
</tr>
<tr>
<td>6.17</td>
<td>0.61</td>
<td>methanol</td>
<td>#2</td>
</tr>
<tr>
<td>6.11</td>
<td>0.62</td>
<td>methanol</td>
<td>#2</td>
</tr>
<tr>
<td>5.53</td>
<td>0.54</td>
<td>methanol</td>
<td>#1</td>
</tr>
<tr>
<td>4.34</td>
<td>0.59</td>
<td>acetonitrile</td>
<td>#1</td>
</tr>
<tr>
<td>3.62</td>
<td>0.54</td>
<td>methanol</td>
<td>#1</td>
</tr>
<tr>
<td>2.83</td>
<td>0.56</td>
<td>methanol</td>
<td>#1</td>
</tr>
<tr>
<td>2.45</td>
<td>0.54</td>
<td>methanol</td>
<td>#1</td>
</tr>
<tr>
<td>1.97</td>
<td>0.60</td>
<td>methanol</td>
<td>#2</td>
</tr>
<tr>
<td>1.85</td>
<td>0.61</td>
<td>methanol</td>
<td>#2</td>
</tr>
</tbody>
</table>

7.4.2. pH and mobile phase buffers considerations

The use of LC-MS for the determination of \( \log P_{ow} \) and \( pK_a \) implied that the set of pH modifiers are used to cover a wide pH range. Buffer solutions are commonly used as pH modifiers in LC-MS mobile phases. LC-MS instruments equipped with ESI source require that buffers/pH modifiers are volatile salts and therefore sodium and phosphate salts must be avoided. Suitable buffers for LC-MS systems are shown in Table 7.5. Considering that the use of TFA was avoided due to strong ion-suppressing effect in the negative mode (Quilliam et al., 2001), the buffers that were available did not cover the range of pH required and therefore single component pH modifiers were used instead of the buffer couple. The pH of solutions used as mobile phases were measured to ensure that the use of a single acidic or basic compound did not result in poorer performances than the conventional LC-MS buffers. Furthermore, other studies observed a good correlation when the \( \log k_{50} \) measured at 50 % organic for the determination of the \( \log P_{ow} \) of a series of lipophilic pyrazines and monosubstituted pyrazines (Yamagami, 2001). According to the IUPAC, this implies that the pH is measured after mixing the organic and aqueous phase when the chromatographic
retention of ionisable compounds needs to be rigorously related to the pH of the mobile phase (IUPAC, 1997). Relationships between \( p\text{H} \), \( p\text{H}^+ \) and \( p\text{H}^- \) are buffer dependent and therefore it is not possible to create a general interconversion between pH values measured before mixing aqueous buffer and organic phases and pH value measured after mixing (Roses and Rosch, 2002).

Table 7.5: Volatile buffers suitable for use with LC-MS (Source Waters).

<table>
<thead>
<tr>
<th>Additive/Buffer</th>
<th>pK\textsubscript{a}</th>
<th>Buffer range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifluoroacetic acid (TFA)</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>4.76</td>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
<td>3.75</td>
<td></td>
</tr>
<tr>
<td>Acetate (NH\textsubscript{4}CH\textsubscript{2}COOH)</td>
<td>4.76</td>
<td>3.76 - 5.76</td>
</tr>
<tr>
<td>Formate (NH\textsubscript{4}COOH)</td>
<td>3.75</td>
<td>2.75 - 4.75</td>
</tr>
<tr>
<td>4-Methylmorpholine</td>
<td>~8.4</td>
<td>7.4 - 9.4</td>
</tr>
<tr>
<td>Ammonia (NH\textsubscript{4}OH)</td>
<td>9.2</td>
<td>8.2 - 10.2</td>
</tr>
<tr>
<td>Ammonium bicarbonate (NH\textsubscript{4}B\textsubscript{3}O)</td>
<td>10.3</td>
<td>8.2 - 11.3</td>
</tr>
<tr>
<td>Ammonium (acetate)</td>
<td>9.2</td>
<td>8.2 - 10.2</td>
</tr>
<tr>
<td>Ammonium (formate)</td>
<td>9.2</td>
<td>8.2 - 10.2</td>
</tr>
<tr>
<td>1-Methylpiperidine</td>
<td>10.2</td>
<td>9.3 - 11.3</td>
</tr>
<tr>
<td>Triethylamine (as acetate salt)</td>
<td>10.7</td>
<td>9.7 - 11.7</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>11.3</td>
<td>10.3 - 12.3</td>
</tr>
</tbody>
</table>

Figure 7.4 and Table 7.6 compare the buffering capacity as well as the influence of the percentage of organic phase in buffered solutions of acetic acid (0.1 M) prepared by addition of strong base (sodium hydroxide), weak base (ammonium hydroxide) and of the conjugate base of acetic acid (ammonium acetate).

As shown in Figure 7.4, the three methods for the preparation of the buffer solutions appeared to be equivalent. A 1/20 dilution of the buffers resulted in little change in pH with differences ranging from 0.01 to 0.43 units between the buffer and the diluted solution (Table 7.6). Surprisingly, the largest difference between the buffer and the diluted solution were observed when the pH equals to the pK\textsubscript{a} and therefore when the buffering capacity is optimal. This observation was consistent for the three methods used for the preparation of the buffer.
solutions (Table 7.6). The percentage of organic phase had a pronounced effect on the measured pH. The difference between the diluted solution made of 100 % aqueous and the diluted solution (90 % organic) ranged from 2.32 to 2.82 pH units (Table 7.6). The three buffer solutions were affected to the same extent regardless of the method of preparation. The same trend was observed with the formic acid (1M) /ammonium formate buffer (40 mM) (Figure 7.5).

Figure 7.4: Comparison of the buffering capacity at pH = pK\textsubscript{a} + 1; pH = pK\textsubscript{a} and pH = pK\textsubscript{a} – 1, of the couple acetic acid and sodium hydroxide prepared by neutralization of 0.1 M acetic acid with a 1 M sodium hydroxide solution, of the couple 0.1 M acetic acid and ammonium hydroxide prepared by neutralization of 0.1 M acetic acid with ammonium hydroxide, and of the couple acetic acid and ammonium acetate prepared by weight. The pK\textsubscript{a} for acetic acid and ammonium acetate buffer couple is 4.76.
Figure 7.5: Illustration of the buffering capacity of 5% of the standard laboratory buffer (40 mM ammonium formate / 1 M formic acid) in various percentages of organic phases.

The effect of the dilution and the percentage of organic mobile phase were investigated for all pH modifiers that were used (Table 7.7). Generally dilution had a greater effect on the pH when a single component solution was used as pH modifier than when a buffer solution was used. An increase in the fraction of organic solvent in the medium in single component pH modifiers did not result in pH differences as large as those observed for the buffer solutions. A more pronounced effect was observed when the pH difference of the 100% aqueous solution was compared with the 90% ACN (Figure 7.6 A to J). The pH of a buffer solution consisting of the conjugated buffer couple seemed to be more affected by the increase in organic fraction than when the individual components of the buffer were used individually. A pH difference between solutions of 0% and 90% ACN containing 5% formic acid (1M) of 0.61 was observed and of 1.15 when 5% ammonium formate (40 mM) was used (Table 7.7). A higher difference was obtained when a buffer solution of formic acid (1 M) together with ammonium formate (40 mM) was assessed.
This phenomenon has been observed in previous studies (Espinosa et al., 2002) where hydrochloric acid was tested as a function of the percentage of acetonitrile and a difference of 0.14 pH units was observed between 0 – 60 % acetonitrile. When the buffer 2,3-dichloropropionic acid / potassium dichloropropanoate was tested in the same conditions a difference of 2 pH units was observed.

It has been suggested that pH measurements in aqueous and organic phase differ in the primary medium effect for the transfer of the H⁺ ion from water to the solvent (Espinosa et al., 2000).

Correlation between logk₅₀ measured at 50 % organic that was used for the determination of the logPₜₜ of a series of lipophilic pyrazines (Yamagami, 2001) was attempted in this experiment by plotting the logk₅₀ of the reference compounds and the pH. Low correlation coefficients were obtained (data not shown).
Table 7.6: pH effect of dilution and percentage of ACN in buffers prepared by three different methods

<table>
<thead>
<tr>
<th>Buffer solution</th>
<th>pH = pKₐ - 1</th>
<th>pH = pKₐ</th>
<th>pH = pKₐ + 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COOH/NaOH</td>
<td>3.90</td>
<td>3.79</td>
<td>5.67</td>
</tr>
<tr>
<td>CH₃COOH/NH₃</td>
<td>3.87</td>
<td>3.71</td>
<td>5.68</td>
</tr>
<tr>
<td>CH₃COOH/CH₃COO-</td>
<td>3.68</td>
<td>3.62</td>
<td>5.79</td>
</tr>
</tbody>
</table>

Table 7.7: pH effect of dilution and percentage of ACN in the buffers used for the pKₐ measurement

<table>
<thead>
<tr>
<th>Buffer solution</th>
<th>pH = pKₐ - 1</th>
<th>pH = pKₐ</th>
<th>pH = pKₐ + 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COOH/NaOH</td>
<td>3.90</td>
<td>3.79</td>
<td>5.67</td>
</tr>
<tr>
<td>CH₃COOH/NH₃</td>
<td>3.87</td>
<td>3.71</td>
<td>5.68</td>
</tr>
<tr>
<td>CH₃COOH/CH₃COO-</td>
<td>3.68</td>
<td>3.62</td>
<td>5.79</td>
</tr>
</tbody>
</table>

Formic acid (5M) | 1.25 | 1.85 | 0.6 | 2.43 | 0.58
Formic acid (3M) | 1.57 | 1.97 | 0.4 | 2.67 | 0.7
Formic acid (1M) | 2.0 | 2.45 | 0.45 | 3.06 | 0.61
Ammonium formate (40 mM) / formic acid (1 M) | 2.52 | 2.83 | 0.31 | 4.73 | 1.9
Ammonium formate (40 mM) | 6.16 | 6.11 | 0.05 | 7.26 | 1.15
Ammonium acetate (40 mM) | 6.92 | 6.17 | 0.75 | 7.87 | 1.70
Ammonium bicarbonate (100 mM) / Hydrochloric acid (1 M) | 7.42 | 7.72 | 0.3 | 8.93 | 1.21
Ammonium bicarbonate (2 mM) | 7.92 | 7.39 | 0.53 | 8.60 | 1.21
Ammonium carbonate (50 mM) | 9.18 | 8.81 | 0.37 | 9.24 | -0.43
Ammonium bicarbonate (2 M) / Ammonium hydroxide (1M) | 9.5 | 9.16 | 0.34 | 9.09 | -0.07
Ammonium hydroxide (0.1 M) | 10.75 | 9.96 | 0.79 | 9.80 | 0.16

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Figure 7.6: A) 5% 5 M formic acid, B) 5% 3 M formic acid, C) 5% 1 M formic acid, D) 5% 40 mM ammonium formate, E) 5% 40 mM ammonium acetate, F) 5% 0.1 M ammonium bicarbonate, acidified with 1 M hydrochloric acid to pH 7.42, G) 5% 2 mM ammonium bicarbonate, H) 5% 0.05 M ammonium carbonate, I) 5% 2 mM ammonium bicarbonate, J) 5% 0.1 M ammonium hydroxide.
7.4.3. Examples of toxin separation

A Gemini C18 column was chosen for its stability over a wide range of pHs (1 – 12). Modified C18 silica columns are commonly used for the determination of lipophilicities and the Gemini column was specifically evaluated for the determination of lipophilicity of weak acids and weak bases. The chromatographic method provided good correlations with literature values (Benhaim and Grushka, 2008).

The determination of lipophilicity by chromatography typically involves the use of methanol as the mobile phase (Gocan et al., 2006). However, since ACN is the organic phase of choice in the LC-MS analysis of lipophilic toxins it was chosen to conduct the experiment with the same solvent.

The elution of OA, DTX1, DTX2, AZA1, AZA2 and PTX2 using 80 % ACN and the acetic acid/ammonium acetate buffer (\( \text{pH} \ 5.72 \)) is shown in Figure 7.7.

The elution of OA at various pH using an isocratic elution of 70 and 50 % ACN is shown in Figure 7.8 A and B, respectively. In general, the more acidic the pH is, the longer the retention time. This is observed in both A and B of Figure 7.8. As the percentage organic mobile phase decreases the retention time increases.

The elution of PTX2 at various pH using an isocratic elution of 70 and 50 % ACN is shown in Figure 7.9 A and B, respectively. There is no apparent trend in retention times as the pH becomes more acidic. When the percentage organic mobile phase decreases the retention time increases.
Figure 7.7: Elution of OA, DTX2, YTX, DTX1, AZA1, PTX2, AZA2 in 80% ACN at pH 5.79 using the C18 Gemini column
Figure 7.8: A) Chromatograms of OA at 70% ACN at various pHs B) Chromatograms of OA at 50% ACN at various pHs. pHs are indicated as "pH".
Figure 7.9: A) Chromatograms of PTX2 at 70% ACN at various pHs B) Chromatograms of PTX2 at 50% ACN at various pHs. pHs are indicated as $\text{pH}$.  

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7.4.4. Determination of pKₐ using chromatography

As indicated in Equation 7.10 the logD is dependent on the pH and, in the case of a weak acid, will drop significantly once the pH is greater than the pKₐ (Figure 7.2). In the following experiment the logkₐ was used instead of the logD for the Y-axis. Since both parameters are proportional to the logPₐ (Equation 7.5 and Equation 7.10) a significant drop in the logkₐ will also occur at pHs exceeding the pKₐ of the analytes.

The capacity factors (k) of all analytes were determined using Equation 7.8 plotted (y-axis) against the percentage of organic phase (x-axis). The quadratic equation obtained from regression analysis allowed for the extrapolation of the capacity factor to 0 % organic phase (log kₐ). Examples of the the curves obtained for the extrapolation of logkₐ of AZA1 at the various pHs are shown in Figure 7.10. The logkₐ obtained for the references compounds are summarised in Table 7.9 and those obtained for the toxins in Table 7.10.
Figure 7.10: Example of the plots obtained for the percentage of organic phase (X-axis) vs. logk (Y-axis) at various pHs for AZA1. The logk is predicted from the quadratic equation shown on the top of the plots when x=0 (0 % organic phase). pHs are indicated as pHw.
7.4.4.1. Determination of pK$_{a}$ values for AZA1 and AZA2

The extrapolated log$k_{w}$ values obtained for AZA1 and AZA2 (Table 7.9) were plotted against the $^w$pH (x-axis) (Figure 7.11). The log$k_{w}$ of both AZA1 and AZA2 were constant up to $^w$pH of 5.5 before their decrease. The graphical determination of the pK$_{a}$ of AZA1 and AZA2 from Figure 7.11 suggests that both compounds have a pK$_{a}$ of approximately 5.8 ± 0.2. AZA1 and AZA2 possess two ionisable functions, one carboxylic acid and one cyclic amine. The second pK$_{a}$ was not readily observed from the set of results shown in Figure 7.11.

![Figure 7.11: Plot of $^w$pH vs. the predicted log$k_{w}$ values for AZA1 and AZA2](image-url)
7.4.4.2. Determination of $pK_a$ values for OA, DTX1 and DTX2

Figure 7.12 displays a plot of $pH$ vs. the extrapolated $\log k_w$ values for OA, DTX1 and DTX2 (Table 7.9). The $\log k_w$ values for OA, DTX1 and DTX2 decreased from $pH$ 4.5 indicating that the compounds have similar $pK_a$. Graphical determination of OA, DTX1 and DTX2 from Figure 7.12 indicates a $pK_a$ value of approximately 4.9 ± 0.5. This suggests that OA, DTX1 and DTX2 are more acidic than AZA1 and AZA2. Since AZAs are zwitterions it is natural that their $pK_a$ is higher than for the OA group toxins which only have a carboxylic acid group without any other functional group in the molecule possibly interacting with the carboxy-group.

![Graphical determination of OA, DTX1 and DTX2 from Figure 7.12 indicates a $pK_a$ value of approximately 4.9 ± 0.5. This suggests that OA, DTX1 and DTX2 are more acidic than AZA1 and AZA2. Since AZAs are zwitterions it is natural that their $pK_a$ is higher than for the OA group toxins which only have a carboxylic acid group without any other functional group in the molecule possibly interacting with the carboxy-group.](image)

Figure 7.12 : Plot of $pH$ vs. the predicted $\log k_w$ values of OA, DTX1 and DTX2
7.4.4.3. Determination of pKₐ value for PTX2

PTX2 appeared as a neutral molecule (without strongly basic or acidic functional groups) and the results obtained in this experiment were consistent with the behaviour predicted from the structure. Figure 7.13 displays a plot of \( \text{pH}_{w} \) vs. the predicted \( \log k_w \) values for PTX2 (Table 7.9). The data plotted in Figure 7.13 were obtained from 14 curves of the PTX2 capacity factor versus the percentage of organic phase (8 compositions ranging from 45 to 80 % organic phase). The average of the extrapolated \( \log k_w \) plotted in Figure 7.13 was 4.06 and the CV was 6 % (Table 7.10).

![Figure 7.13: Plot of \( \text{pH}_{w} \) vs. the predicted \( \log k_w \) values of PTX2](image)

Figure 7.13 : Plot of \( \text{pH}_{w} \) vs. the predicted \( \log k_w \) values of PTX2
7.4.4.4. Determination of pKₐ value for YTX

Figure 7.14 displays a plot of pH vs. the extrapolated logKₐ values for YTX. There are four, possibly five data points indicating a plateau before the logKₐ values begin to decrease. It is questionable as to whether the fifth data point is a member of the plateau or not. The graphical estimation of the pKₐ of YTX from Figure 7.14 is approximately 6.9 ± 0.5.

YTX possesses two acidic sulphate groups that therefore YTX is expected to have two pKₐ’s. It was anticipated that the first pKₐ is very low since sulphuric acid is a strong acid. The pKₐ of a methyl sulphate ester (CH₃OSO₂OCH₃) was estimated to be – 3.4 while the pKₐ of sulphuric acid was estimated to be – 2.8 (Guthrie, 1978). Such low values of pKₐ would not be readily visible when the pKₐ is determined by a chromatographic method. Also, it appeared that the second pKₐ was much higher than initially expected. The high pKₐ can be due to the interaction of the 2 sulphate groups and the lability of one hydrogen to move from one sulphate group to the other.
7.4.5. Determination of logP<sub>ow</sub> by chromatography

7.4.5.1. The choice of reference compounds

The use of LC-MS did not allow for the detection of the compounds recommended by the OECD (2004). Instead it was chosen to select pharmaceutical compounds for which logP<sub>ow</sub> have been established by several methods and compiled (Beause, 2004). The experimental logP<sub>ow</sub> and pK<sub>a</sub> are summarised in Table 7.8 as well as the predicted logP<sub>ow</sub> and pK<sub>a</sub> obtained by computational methods. Ideally, the reference compounds should have a similar structure to the analyte (OECD, 2004), however, due to the complexity of the chemical structure of the marine toxins this is difficult to achieve. The reference compounds were chosen to reflect one or several functional groups contained in structures of the toxins studied (Carboxylic acids, cyclic polyether; amine…). The structures of the reference compounds are shown in Figure 7.15 to Figure 7.23 (Babic et al., 2007). The choice of the reference compounds also took into account the price of the substances.
Table 7.8: Reference compounds selected for calibration curve.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Mass (g/mol)</th>
<th>pK_a</th>
<th>logP&lt;sub&gt;ow&lt;/sub&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bezafibrate</td>
<td>361.82</td>
<td>3.6</td>
<td>4.25</td>
<td>(Beausse, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.00</td>
<td>3.72</td>
<td>(VCCLab, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.4</td>
<td>3.46</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>206.28</td>
<td>4.42</td>
<td>3.97</td>
<td>(Beausse, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.4</td>
<td>3.5</td>
<td>(Kaliszan et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.51</td>
<td>3.49</td>
<td>(VCCLab, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.30</td>
<td>3.72</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>236.27</td>
<td>13.9</td>
<td>2.45, 2.25</td>
<td>(Beausse, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(VCCLab, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>331.34</td>
<td>5.86</td>
<td>0.4</td>
<td>(Beausse, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.38</td>
<td>0.32</td>
<td>(VCCLab, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.10</td>
<td>0.65</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>361.37</td>
<td>5.97</td>
<td>0.35</td>
<td>(Beausse, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.97</td>
<td>0.55</td>
<td>(VCCLab, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.10</td>
<td>0.84</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td>Tylosin tartrate</td>
<td>1066.2</td>
<td>3.31</td>
<td>3.5</td>
<td>(Beausse, 2004)</td>
</tr>
<tr>
<td>Tylosin</td>
<td>916.3</td>
<td>7.1</td>
<td>1.87</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>8.00</td>
<td>3.27</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>733.93</td>
<td>8.90</td>
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<td></td>
<td></td>
<td>8.9</td>
<td>2.33</td>
<td>(VCCLab, 2008)</td>
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<td></td>
<td></td>
<td>8.60</td>
<td>2.83</td>
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<tr>
<td>Sulphamethoxazole</td>
<td>253.18</td>
<td>1.8</td>
<td>0.89</td>
<td>(Beausse, 2004)</td>
</tr>
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<td></td>
<td></td>
<td>5.7</td>
<td>0.78</td>
<td>(VCCLab, 2008)</td>
</tr>
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<td></td>
<td></td>
<td>1.80</td>
<td>0.89</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.70</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>496.5</td>
<td>3.22</td>
<td>-1.22</td>
<td>(Beausse, 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.27</td>
<td>-1.42</td>
<td>(VCCLab, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.30</td>
<td>1.50</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.20</td>
<td>0.57</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-0.13</td>
<td>(ACD, 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-3.23</td>
<td>(ACD, 2008)</td>
</tr>
</tbody>
</table>
Figure 7.15: Chemical structure of Bezafibrate – used as a lipid regulator drug.

Figure 7.16: Chemical structure of Ibuprofen – used as an anti-inflammatory drug.

Figure 7.17: Chemical structure of Carbamazepine – used as a central nervous system drug (anti-epileptic).

Figure 7.18: Chemical structure of Ciprofloxacin – used as an antibiotic for human use (quinolone class).

Figure 7.19: Chemical structure of Ofloxacin – used as an antibiotic for human use (quinolone class).

Figure 7.20: Chemical structure of Tylosin – used as an antibiotic for veterinary use (macrolide class).

Figure 7.21: Chemical structure of Erythromycin – used as an antibiotic for human use (macrolide class).

Figure 7.22: Chemical structure of Sulphamethoxazole – used as an antibiotic for human use (sulfamide class).

Figure 7.23: Chemical structure of Oxytetracycline – used as an antibiotic for veterinary use (tetracycline class).
The extrapolated log\(k_w\) of the different reference compounds, their logP\(_{ow}\) (obtained from the literature) and the slopes obtained from the plot of log\(k_w\) versus logP\(_{ow}\) at different pHs are shown in Table 7.9.

Some of the log\(k_w\) obtained were considered as outliers and removed. Outliers included the log\(k_w\) values of bezafibrate at \(w\)\(pH\ 9.92, 9.16, 8.81, 7.72\) and 6.17 as well as the log\(k_w\) of erythromycin at \(w\)\(pH\ 6.11\) and of tylosin. The log\(k\) values obtained for erythromycin at \(w\)\(pH\)s ranging from 1.85 to 3.62 were also removed. The correlation coefficients ranged from 0.05 to 0.99 and were obtained at pHs 5.53 and 3.62, respectively. Since no correlation of logP\(_{ow}\) and log\(k_w\) was obtained for the reference compounds at pH 5.53, this condition was not used in the determination of the lipophilicity of the toxins.

According to the acidity constants of the toxins considered determined in the previous section, the correlation coefficients of the curves used to evaluate the lipophilicity of AZA1, -2 and OA, DTX1, -2 ranged from 0.69 to 0.99 (pH 1.85 to 4.34). The equations were obtained from 5 data points but the linearities were sometimes questionable.

### 7.4.5.2. Lipophilicity of marine toxins

The logP\(_{ow}\) of the toxins ranged from 5.05 to 9.66 with OA < DTX2 < DTX1 < PTX2 < AZA1 < AZA2 < YTX. The log\(k_w\) and the calculated logP\(_{ow}\) of the toxins are shown in Table 7.10. The logP\(_{ow}\) of AZA1 and AZA2 determined were 7.54 and 8.18. The experimental logP\(_{ow}\) values of OA, DTX1 and DTX2 were 5.05, 6.88 and 5.61, respectively. The logP\(_{ow}\) of PTX2 was between DTX1 and DTX2 with a value of 6.47 and YTX of 9.66.

Since PTX2 appeared to be a neutral molecule, all pHs were used for the determination of its logP\(_{ow}\). For YTX, only two conditions were used for the determination of logP\(_{ow}\) (\(w\)\(pH\ 3.34\) and 6.11) which make conclusions doubtful.
The high logP_{ow} obtained for YTX is questionable because there are several pieces of evidence that demonstrate that YTX is less lipophilic than OA.

- YTX elutes before OA when analysed using an acidic mobile phase in reverse phase HPLC
- When YTX is allowed to partition in a mixture of 50% aqueous methanol and dichloromethane, the YTX is found to be present in the aqueous methanol layer (Yasumoto, 2001).
- When YTX is fed to mice, it passes through the digestive system without being absorbed into the bloodstream/body (Aune et al., 2002).

The variability of the logk_{ow} of the toxins was remarkably low considering the number of measurements with CVs ranging from 4 to 7% (except for YTX) with n = 5 to 13. The CVs of the logP_{ow} were considerably higher with values of 13 to 21% with the same number of measurement than for the logk_{ow} calculation. This suggests that the calculation of the logP_{ow} using the calibration curves obtained from the reference compounds introduced high variability. The non-availability of reference compound(s) with logP_{ow} above those found for the toxins (logP_{ow} > 5) implied the extrapolation of curves with logP_{ow} without evidences of linearity above logP_{ow} of 4.2. Therefore, we can not conclude whether a chromatographic method is suitable for the determination of the logP_{ow} in the range of the results obtained.
Table 7.9: Literature logP<sub>ow</sub> and calculated logk<sub>w</sub> values for the reference compounds. At each pH, the logk<sub>w</sub> values of the reference compounds were plotted (x-axis) against logP (y-axis). The r<sup>2</sup>, slope and intercept of the calibration curve produced are shown. The presence of the symbol * in a data cell means that no retention times were available to obtain a logk<sub>w</sub> value. The presence of the symbol ^ in a data cell means that data was available but excluded from the calibration curve in order to achieve a better r<sup>2</sup>.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bezafibrate</th>
<th>Carbamazepine</th>
<th>Ciprofloxacin</th>
<th>Ofloxacin</th>
<th>Tylosin Tartrate</th>
<th>Erythromycin</th>
<th>Sulphamethoxazole</th>
<th>r&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>logk&lt;sub&gt;w&lt;/sub&gt;</td>
<td>logk&lt;sub&gt;w&lt;/sub&gt;</td>
<td>logk&lt;sub&gt;w&lt;/sub&gt;</td>
<td>logk&lt;sub&gt;w&lt;/sub&gt;</td>
<td>logk&lt;sub&gt;w&lt;/sub&gt;</td>
<td>logk&lt;sub&gt;w&lt;/sub&gt;</td>
<td>logk&lt;sub&gt;w&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.96</td>
<td>^</td>
<td>1.1141</td>
<td>*</td>
<td>*</td>
<td>3.2348</td>
<td>3.3014</td>
<td>-0.7479</td>
<td>0.9333</td>
<td>0.5691</td>
<td>1.4929</td>
</tr>
<tr>
<td>9.16</td>
<td>^</td>
<td>0.8532</td>
<td>*</td>
<td>*</td>
<td>1.3351</td>
<td>0.9378</td>
<td>-0.5306</td>
<td>0.9650</td>
<td>1.3771</td>
<td>1.5814</td>
</tr>
<tr>
<td>8.81</td>
<td>^</td>
<td>1.367</td>
<td>*</td>
<td>*</td>
<td>2.7091</td>
<td>1.9534</td>
<td>0.136</td>
<td>0.9744</td>
<td>1.037</td>
<td>0.8766</td>
</tr>
<tr>
<td>7.72</td>
<td>^</td>
<td>1.3465</td>
<td>*</td>
<td>*</td>
<td>2.4065</td>
<td>1.6893</td>
<td>-0.4735</td>
<td>0.9880</td>
<td>0.925</td>
<td>1.326</td>
</tr>
<tr>
<td>7.39</td>
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<td>1.0778</td>
<td>*</td>
<td>*</td>
<td>0.8042</td>
<td>0.4169</td>
<td>0.308</td>
<td>0.0174</td>
<td>0.514</td>
<td>2.5193</td>
</tr>
<tr>
<td>6.17</td>
<td>^</td>
<td>0.9504</td>
<td>*</td>
<td>*</td>
<td>1.3181</td>
<td>1.2584</td>
<td>0.7976</td>
<td>0.8838</td>
<td>4.3189</td>
<td>-2.1943</td>
</tr>
<tr>
<td>6.11</td>
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<td>1.1693</td>
<td>*</td>
<td>*</td>
<td>0.5916</td>
<td>^</td>
<td>0.6647</td>
<td>0.1481</td>
<td>1.7888</td>
<td>1.1658</td>
</tr>
<tr>
<td>5.53</td>
<td>0.7197</td>
<td>1.3469</td>
<td>*</td>
<td>*</td>
<td>1.1786</td>
<td>0.8975</td>
<td>0.5817</td>
<td>0.0554</td>
<td>0.9418</td>
<td>1.9401</td>
</tr>
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<td>4.34</td>
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<td>-0.6294</td>
<td>-0.3581</td>
<td>1.4924</td>
<td>0.7934</td>
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<td>1.4575</td>
<td>1.0074</td>
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<td>^</td>
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<td>0.9928</td>
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<td>1.2255</td>
<td>-1.575</td>
<td>-0.5176</td>
<td>^</td>
<td>^</td>
<td>0.7226</td>
<td>0.8272</td>
<td>0.9467</td>
<td>1.2014</td>
</tr>
<tr>
<td>2.45</td>
<td>2.7259</td>
<td>1.3748</td>
<td>0.639</td>
<td>0.412</td>
<td>^</td>
<td>^</td>
<td>0.5697</td>
<td>0.9666</td>
<td>1.7182</td>
<td>-0.2982</td>
</tr>
<tr>
<td>1.97</td>
<td>2.6015</td>
<td>1.0178</td>
<td>-1.2722</td>
<td>-0.2861</td>
<td>^</td>
<td>^</td>
<td>0.6196</td>
<td>0.8576</td>
<td>1.0675</td>
<td>1.0957</td>
</tr>
<tr>
<td>1.85</td>
<td>2.3044</td>
<td>1.2394</td>
<td>0.9801</td>
<td>-0.9329</td>
<td>^</td>
<td>^</td>
<td>0.2352</td>
<td>0.6935</td>
<td>1.1582</td>
<td>0.7817</td>
</tr>
</tbody>
</table>

logP<sub>ow</sub> | 4.25 | 2.45 | 0.40 | 0.35 | 3.50 | 3.06 | 0.89 |
Table 7.10: Extrapolated log$k_w$ values and calculated log$P_{ow}$ of the toxins at pH’s ranging from 1.85 to 9.96. The presence of the symbol * in a data cell means no retention times were available to obtain a log$k_w$ value. The presence of the symbol ^ in a data cell means that data was available but excluded when processing the results.

<table>
<thead>
<tr>
<th>$pH$</th>
<th>AZA1</th>
<th>AZA2</th>
<th>PTX2</th>
<th>OA</th>
<th>DTX1</th>
<th>DTX2</th>
<th>YTX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log$k_w$</td>
<td>calc. log$P_{ow}$</td>
<td>log$k_w$</td>
<td>calc. log$P_{ow}$</td>
<td>log$k_w$</td>
<td>calc. log$P_{ow}$</td>
<td>log$k_w$</td>
</tr>
<tr>
<td>9.96</td>
<td>2.2451</td>
<td>2.0698</td>
<td>4.1746</td>
<td>3.87</td>
<td>0.4405</td>
<td>0.2523</td>
<td>-0.1949</td>
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<tr>
<td>9.16</td>
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<td>2.1847</td>
<td>4.0522</td>
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<td>0.6015</td>
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<tr>
<td>8.81</td>
<td>^</td>
<td>^</td>
<td>3.7215</td>
<td>4.74</td>
<td>0.7357</td>
<td>1.9162</td>
<td>1.0213</td>
</tr>
<tr>
<td>7.72</td>
<td>4.3926</td>
<td>4.5285</td>
<td>4.3752</td>
<td>5.37</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>7.39</td>
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<td>3.7165</td>
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<td>4.61</td>
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<td>2.4952</td>
</tr>
<tr>
<td>5.53</td>
<td>^</td>
<td>^</td>
<td>^</td>
<td>^</td>
<td>^</td>
<td>^</td>
<td>^</td>
</tr>
<tr>
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<td>5.0174</td>
<td><strong>8.32</strong></td>
<td>5.5867</td>
<td><strong>9.15</strong></td>
<td>4.2702</td>
<td><strong>7.23</strong></td>
<td>2.9096</td>
</tr>
<tr>
<td>3.62</td>
<td>4.9851</td>
<td><strong>8.51</strong></td>
<td>^</td>
<td>4.1023</td>
<td><strong>7.07</strong></td>
<td>3.4129</td>
<td><strong>5.94</strong></td>
</tr>
<tr>
<td>2.83</td>
<td>5.135</td>
<td><strong>6.06</strong></td>
<td>5.6812</td>
<td><strong>6.58</strong></td>
<td>4.1194</td>
<td><strong>5.10</strong></td>
<td>3.142</td>
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<tr>
<td>2.45</td>
<td>5.0347</td>
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<td>5.8741</td>
<td><strong>9.79</strong></td>
<td>4.3755</td>
<td><strong>7.22</strong></td>
<td>3.5537</td>
</tr>
<tr>
<td>1.97</td>
<td>5.275</td>
<td><strong>6.73</strong></td>
<td>6.2569</td>
<td><strong>7.77</strong></td>
<td>3.8232</td>
<td><strong>5.18</strong></td>
<td>3.269</td>
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<tr>
<td>1.85</td>
<td>5.5885</td>
<td><strong>7.25</strong></td>
<td>5.9084</td>
<td><strong>7.62</strong></td>
<td>3.9054</td>
<td><strong>5.30</strong></td>
<td>3.27</td>
</tr>
<tr>
<td>average</td>
<td>5.17</td>
<td><strong>7.54</strong></td>
<td>5.86</td>
<td><strong>8.18</strong></td>
<td>4.03</td>
<td><strong>6.47</strong></td>
<td>3.2595</td>
</tr>
<tr>
<td>std dev</td>
<td>0.23</td>
<td><strong>1.01</strong></td>
<td>0.26</td>
<td><strong>1.28</strong></td>
<td>0.25</td>
<td><strong>2.37</strong></td>
<td>0.2220</td>
</tr>
<tr>
<td>cv</td>
<td>4%</td>
<td><strong>13%</strong></td>
<td>4%</td>
<td><strong>16%</strong></td>
<td>6%</td>
<td><strong>37%</strong></td>
<td>7%</td>
</tr>
</tbody>
</table>
7.4.5.3. Comparison of software prediction, literature data and experimental results

The predicted (VCCLab, 2008) and the experimental pK\textsubscript{a} are summarised in Table 7.11 and the predicted (VCCLab, 2008) and the experimental logP\textsubscript{ow} are summarised in Table 7.12.

YTX was not recognised by the software. It is likely that errors in the conversion of YTX structure into SMILES format occurred. The pK\textsubscript{a} obtained experimentally were systematically higher than the predicted values. Two pK\textsubscript{a} were predicted for AZA due to the carboxylic acid and the cyclic amine functions. Only one pK\textsubscript{a} was readily apparent experimentally. The relative acidity of the toxins with DSP toxins more acidic than AZAs was consistent in the two set of results.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Predicted pK\textsubscript{a}</th>
<th>Experimental pK\textsubscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZA1</td>
<td>4.90, 9.20</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>AZA2</td>
<td>4.90, 9.20</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>OA</td>
<td>3.80</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>DTX1</td>
<td>3.80</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>DTX2</td>
<td>3.80</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>PTX2</td>
<td>No acidic function</td>
<td>No apparent pK\textsubscript{a}</td>
</tr>
<tr>
<td></td>
<td>na</td>
<td>observed</td>
</tr>
<tr>
<td>YTX</td>
<td>na</td>
<td>6.9 ± 0.5</td>
</tr>
</tbody>
</table>

The results obtained using the predicting software were associated with a relatively large error. The program (VCCLab, 2008) provided an average value of the logP\textsubscript{ow} from 10 prediction softwares (details are available on-line). The high standard deviation was a consequence of the wide range of results obtained by the different methods. For example, the logP\textsubscript{ow} of OA ranged from 1.98 to 9.56 (obtained with MLOGP and and KOWWIN softwares, respectively).
Table 7.12: Predicted and experimental logP values (± SD) for AZA1, AZA2, OA, DTX1, DTX2 and PTX2.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Predicted logP&lt;sub&gt;ow&lt;/sub&gt;</th>
<th>Experimental logP&lt;sub&gt;ow&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZA1</td>
<td>4.06 ± 2.20</td>
<td>7.54 ± 1.01</td>
</tr>
<tr>
<td>AZA2</td>
<td>4.32 ± 2.33</td>
<td>8.18 ± 1.28</td>
</tr>
<tr>
<td>OA</td>
<td>4.45 ± 2.60</td>
<td>5.05 ± 0.72</td>
</tr>
<tr>
<td>DTX1</td>
<td>4.79 ± 2.67</td>
<td>6.88 ± 1.41</td>
</tr>
<tr>
<td>DTX2</td>
<td>4.46 ± 2.55</td>
<td>5.61 ± 0.81</td>
</tr>
<tr>
<td>PTX2</td>
<td>4.45 ± 1.99</td>
<td>6.47 ± 2.37</td>
</tr>
</tbody>
</table>

The experimental logP<sub>ow</sub> values of AZA1 and AZA2 were 7.54 and 8.18, respectively. This is a 7.8 % difference between the experimental logP<sub>ow</sub> values of AZA1 and AZA2. Similarly the difference between the logP<sub>ow</sub> of AZA1 and AZA2 from the prediction software was 6 %. The values obtained for the experimental logP<sub>ow</sub> were higher than those obtained by the prediction software (4.06 and 4.32 for AZA1 and AZA2, respectively).

The experimental logP<sub>ow</sub> values of OA, DTX1 and DTX2 were 5.05, 6.88 and 5.61, respectively. The predicted logP<sub>ow</sub> values of OA, DTX1 and DTX2 were 4.45, 4.79 and 4.46 respectively corresponding to a 7 % difference between the logP<sub>ow</sub> of OA and DTX1, while there is only a 0.2 % difference between the logP<sub>ow</sub> of OA and DTX2. The experimental data obtained were equivalent to a 27 % difference between the logP<sub>ow</sub> of OA and DTX1 and a 10 % difference between the logP<sub>ow</sub> of OA and DTX2.

Two studies have reported a logP<sub>ow</sub> value for OA. A chromatographic method where the retention time of OA was compared against compounds of known logP<sub>ow</sub> assigned a logP<sub>ow</sub> of 3.4 to OA (Takahashi et al., 2008). No details on the method, pH of measurement and nature or number of reference compounds were given. A second article assigned a logP<sub>ow</sub> of 5.89 to OA and was obtained by multiple minimisation calculations which allowed an estimate of the partition coefficient using the program MacroModel 8.5 (Paz et al., 2007). The authors used the results of each minimisation
to evaluate the free energy difference between octanol and water. The minimum energy conformer for each toxin was obtained after a Monte Carlo multiple minimum conformational search of 5000 steps.

The experimental logP$_{ow}$ of PTX2 determined in this work was 6.47 against 4.45 as determined using the prediction software. Similarly to the other toxins studied, the experimental result was higher than the predicted one. One logP$_{ow}$ value for PTX2 was available in the literature and determined by the same chromatographic method as for OA. The authors assigned a logP$_{ow}$ of 3.7 to PTX2 (Takahashi et al., 2008).

7.4.6. General discussion

No data on the pK$_a$ of the toxins considered in this work were available from the literature and led us to their determination by LC-MS. The evaluation of the acidity constant has shown that, as one would expect, OA, DTX1 and DTX2 have a similar pK$_a$ and that the pK$_a$ of AZA1 was similar to AZA2. Since all retention times of the toxins were established in solutions containing only one analyte a good confidence can be attributed in the data obtained. Identical pK$_a$’s were expected for the different toxin analogues since their difference in chemical structures are not affecting the lability of the hydrogen of the carboxylic groups.

The selection of tylosin and erythromycin as reference compounds was not judicious. Both compounds behaved as weak bases and therefore were not used in the production of the calibration curves which were produced at acidic pH. Ibuprofen was also initially selected as reference compound and was not ionisable under our conditions. The difficulty in finding suitable reference compounds for LC-MS lay with the ability to ionise non-polar compounds. As a result the calibration curves produced for the determination of the logP$_{ow}$ of the toxins was restricted to 5 points.
with a maximum logP_{ow} of 4.2 for bezafibrate (Table 7.9). The lipophilicities of all compounds were above the upper limit of the curve. The logP_{ow} obtained from the toxins were surprisingly high and consistently higher than the predicted values. Although the lipophilicities of the toxins relative to each other should reflect the reality (except for YTX) the values obtained were surprisingly high compared with the lipophilicity of compounds encountered in environmental analysis (polyaromatic hydrocarbons, polychlorinated biphenyls…). The use of additional reference compounds would have allowed us to gain more confidence in the results.

The use of the reference compounds recommended by the OECD would allow for the production of calibration curve up to logP_{ow} of 6.5 with Dichloro-Diphenyl-Trichloroethane (DDT). This experiment would imply the use of: 1) a single injection to a HPLC where the column outlet is split to a UV detector for the detection of the reference compounds and to a MS detector for the detection of the toxins, or 2) since UV is a non-destructive method, a single injection to a HPLC, with UV and MS detectors in series.

**7.5. Conclusions**

This study investigated the pK_{a} and lipophilicity of some lipophilic marine toxins. This implied that LC-MS was used instead of LC-UV that is generally employed for this type of experiment. This led to the investigation of the influence of buffers and organic solvent on pH measurements.

The results obtained allowed for the evaluation of the pK_{a} of some of the marine toxins. OA, DTX1 and DTX2 were found to have the same pK_{a}’s (4.9 ± 0.5) and were more acidic than AZA1 and AZA2 which also were found to have the same pK_{a} (5.8
PTX2 appeared as a neutral molecule. The determination of the pK\textsubscript{a} of YTX was attempted and only a limited number of measurements allowed for the calculation of a pK\textsubscript{a} value.

The evaluation of the lipophilicities of the toxins was calculated from calibration curves obtained from reference compounds of known logP\textsubscript{ow} obtained at pHs below the pK\textsubscript{a} of the toxins (apart for PTX2 for which all pH conditions were used in the calculation). The results obtained were surprisingly high (logP\textsubscript{ow} > 5 for OA, DTX1, DTX2, PTX2 and logP\textsubscript{ow} > 6 for AZA1 and AZA2). Limited confidence can be attributed to the results due to the lack of reference compounds with logP\textsubscript{ow} > 5. The predictions of the logP\textsubscript{ow} using computational predictions were systematically lower than those obtained experimentally (logP\textsubscript{ow} ranging from 4.06 to 4.79) but were associated with large variability.

The limited calibration range with reference compounds not exceeding logP\textsubscript{ow} of 4.5 led to a relatively large uncertainty in the logP\textsubscript{ow} values established. Thus, the determination of the lipophilicity by LC-MS proved to be a challenging task and further development is required to establish if chromatographic method are suitable for the determination of the logP\textsubscript{ow} of lipophilic marine toxins.
8. General Conclusions

8.1. Research overview

The primary objective of this thesis was to evaluate the usefulness of passive sampling as an early warning system to forecast shellfish contamination. The occurrence of toxic phytoplankton and the level of accumulation in shellfish are highly variable and the first challenge of the work was to choose the appropriate time and location for the study of harmful events. Sampling experiments were therefore carried out over several weeks and in different locations to increase the probability of observing a toxic event. The results presented in Chapter 5 have been obtained in 2005 in which shellfish contamination occurred at a remarkably high frequency (Figure 1.3). The toxic events that coincided with the sampling duration included a severe AZA outbreak and the appearance of *D. acuta* after several weeks of sampling. In our experiments the passive sampling approach did not provide an early warning of shellfish contamination since the toxins were detected in the SPATT and in the mussels at the same time. Accumulation of toxins in the SPATT reflected well the trend observed with the contamination of shellfish (contamination or depuration phase). In addition to the comparison of shellfish collected from production areas as part of the national monitoring programme, it was chosen to place mussels that did not contain marine toxins next to the SPATT discs. These additional samplers were used in order to have an indicator that will reflect the occurrence or disappearance of toxins in shellfish without prior contamination. It was anticipated that weekly replacement of mussels can facilitate the interpretation of the results in the case of consecutive toxic events. This approach led to interesting findings regarding the bioaccumulation of lipophilic marine toxins by shellfish. It was observed that contamination of shellfish
with DSP toxins can not occur through the filtration of water containing the DSP toxins but that feeding of phytoplankton is necessary for toxin accumulation to occur. On the other hand, the AZA events that were studied appeared to contaminate shellfish for several consecutive weeks. The producer organism was not known at the time of the experiment which did not allow us to conclude whether contamination of shellfish by AZAs is different from that by DSP toxins or that the organism that produced AZA remained in the water for a longer period.

Although the use of SPATT as an early warning system was not successful when placed at the same location than the mussels, forecasting shellfish contamination may however be possible if the samplers are placed in a location where the phytoplankton can be found prior to the contamination in the shellfish production area (sometimes referred to as sentinel sites). This approach would allow for the detection of toxins in the SPATT at the same time than when the toxic phytoplankton is observed in the water (assuming that the producing organism is known). The mesocosm experiment presented in Chapter 4 has shown that adsorption of toxins by PSD is a very fast process and that high levels of toxins are accumulated within 12 h implying that low levels of toxin can be found with very short contact times.

The contamination of SPATT with OA and DTX1 from a culture of phytoplankton enabled the comparison of the potential of several polymeric adsorbents to be made in SPATT devices as well as to study the adsorption of the toxins. The results have shown that the HP-20 resin was the resin with the best performances and that the toxins were accumulated linearly up to 72 h of immersion. Contamination of the SPATT in the laboratory also allowed for the investigation of the desorption process. The extraction method developed in this work achieved excellent recoveries for the
toxin encountered in Ireland and was used for the determination of the levels of toxins accumulated in the SPATT in all experiments described in this thesis.

The encouraging results obtained in the mesocosm experiments led us to compare the toxin profiles obtained in *D. acuta* cells harvested in the Celtic Sea with SPATT discs that were placed in a nearby location. This study described in Chapter 6 showed that the toxin profiles that were obtained on the SPATT were very similar to the one obtained in the cells collected in the thin layer. In addition, the SPATT were used to determine the toxin profile at various depths and down to 110 m. The toxin ratios obtained in the SPATT immersed at the different depths and in the *D. acuta* cells collected at 80 m were very similar to the cells that were collected in the thin-layer. Not only had this demonstrated that the SPATTs are good indicator of the toxins produced by the phytoplankton cells, but also that phytoplankton can produce toxins in complete darkness.

The different experiments that were carried out with the SPATT technique have shown that the SPATT devices are very sensitive detectors of marine toxins. Complex toxic profiles were observed in the samplers and allowed for the first reports of the occurrence of YTX and SPX in Irish waters (Chapter 5).

The sensitivity of the SPATT samplers and the large number of mussels and SPATT discs that were retrieved for the assessment of the early warning system led to the development of a high throughput LC-MS method with the capability of monitoring numerous toxins in a single run. The UPLC method presented in Chapter 2 allowed for the detection of 21 lipophilic marine toxins with high selectivity as two traces per compound were monitored in most cases. UPLC separation was used with a latest generation MS instrument as well as with older generation MS and was compared
with conventional HPLC. The use of a sub 2 µm stationary phase provided a better separation of the toxins although co-elution of toxins occurred. The monitoring of co-eluting compounds that are analysed in two different modes was successful with the latest generation MS but is beyond the capabilities of many conventional instruments as acquisition must take place with rapid positive/negative switching.

The LODs and the linearities of the commercially available standards and AZA1 were established. The UPLC method was used for the determination of the levels of toxin that accumulated in the SPATT as well as in the transplanted mussels that were replaced weekly from Chapter 5. The method showed good performances over several thousand injections.

LC-MS analyses of marine toxins are almost exclusively carried out in shellfish and therefore the performance of the method should also be assessed in shellfish.

The MBA currently in place for the detection of lipophilic marine toxins is becoming unacceptable and one or several replacement methods are desirable. Among them, chemical analysis using LC-MS techniques is perceived as well suited to the quantitation of multi-analyte in biological samples. However, LC-MS quantitation in biological samples is challenging because of matrix effects.

Chapter 3 presented the types and severity of matrix effects affecting the analysis of marine toxins in shellfish encountered in the UPLC and HPLC separations combined with different MS instruments. The matrix effects observed were highly variable and analyte dependent. Three approaches were employed to investigate matrix effects. The post-column infusion did not suggest that the methods suffered from matrix effects while both standard addition and post-extraction addition revealed that signal suppression or enhancement was taking place.
The results obtained using one UPLC method where separation was achieved on a C8 column suggested that matrix effects affecting AZA1 and PTX2 are eliminated. The matrix effects that affected the analysis of OA were systematically reduced when the analyses were carried out in diluted samples equivalent to SSR of 20. Reduction of matrix effects affecting the analysis of OA can therefore be achieved through dilution but at the price of sensitivity.

This work demonstrated the severity of matrix effects as well as their high variability. Several parameters that affected the analysis of a particular toxin were evaluated for their influence on matrix effects. These parameters included the effect of instrument, stationary phase, chromatography, heat treatment, shellfish species and dilution. Inter-laboratory validation often involves participants with a range of different detectors and instruments from several different manufacturers. Therefore, it is important that matrix effects and their consequences on a particular system are understood.

Monitoring laboratories perform analyses in a number of shellfish species that mainly depend on their geographical locations. Consequently, the investigation of matrix effects arising from the analysis of particular analyte/species combination is important in internal laboratory validation.

The development and use of one or several internal standard would allow for many issues to be overcome through the establishment of correction factor on particular instrument and in particular shellfish species. Alternatively, clean-up methods should also be further investigated and may compensate for the differences of matrix effects that would be observed between different species.

The evaluation of the lipophilicity, and ultimately the acidity constant, of marine toxins was investigated as it is an important parameter in bioaccumulation processes,
passive sampling and also governs chromatographic separation. The lipophilicity influences the partition coefficient between the passive sampling receiving phase/media and the mobile/stationary phases of chromatographic systems. Lipophilicity and acidity constant are also very valuable parameters for the isolation of toxins from shellfish and from phytoplankton. The latter isolation method consists of pumping phytoplankton through a HP-20 resin (2 kg) and to extract the toxins accumulated in the resin (Rundberget et al., 2007).

Furthermore, lipophilicity is widely used in drug development to make estimates for membrane penetration and permeability including gastrointestinal absorption and blood-brain barrier crossing (Van de Waterbeemd, 2003). The permeability of toxins has a great impact on toxicity and their determinations are therefore very useful in risk assessment. Figure 8.1 summarises the different applications of the lipophilicity and the acidity constant.

![Figure 8.1: Summary of applications of lipophilicity and acidity constant](image)
The determination of logP\textsubscript{ow} and pK\textsubscript{a} by chromatographic method is an alternative to the shake flask method (OECD, 2004). UV detectors are generally used for the chromatographic method. Chapter 7 investigated the use of LC-MS for the evaluation of the pK\textsubscript{a} and logP\textsubscript{ow} of lipophilic marine toxins. The capacity factors (that depend on retention times) were investigated over a wide range of pHs and organic fractions in the mobile phase. The results allowed for the estimation of the pK\textsubscript{a}'s of OA, DTX1, DTX2, AZA1 and AZA2. This provided important information on the influence of pH on the chromatographic selectivity for the separation of lipophilic toxins.

A calibration curve with pharmaceutical compounds of known logP\textsubscript{ow} was produced in an attempt to evaluate the logP\textsubscript{ow} of the toxins as a function of the capacity factor. The logP\textsubscript{ow} of the toxins ranged from 5 to 8 which exceeded the linearity range of the method and good correlation with the reference compounds and their logP\textsubscript{ow} were not always obtained. The determination of the lipophilicity of marine toxins by LC-MS proved to be a challenging task and further work is required to establish the logP\textsubscript{ow} of the toxins that were considered in this work.

**8.2. Future work**

Passive sampling is widely used for environmental monitoring of pollutants and PSD that work in kinetic and equilibrium regimes are distinguished. The kinetic exchange between PSD and water phase rely on both uptake and offload rate constants of the analyte. Since the sampling rate is dependent on the volume of water that the samplers have been in contact with, the prototype instrument for flow rate / volume measurement discussed in Chapter 4 would provide additional information on the toxin adsorption dynamics. Furthermore, the prototype instrument could be used to
evaluate the performance reference compounds used or developed for passive sampling applications other than marine toxins.

Following the results obtained in this thesis regarding the occurrence of matrix effects, an isocratic UPLC C8 column is currently being evaluated as a replacement method of the HPLC gradient method used in parallel with the MBA in Ireland. Matrix effects in different species will also be further investigated using this method.
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